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Conservative treatment of keratoconus by riboflavin-UVA-induced cross-linking of corneal collagen: Qualitative investigation of corneal epithelium and subepithelial nerve plexus regeneration by *in vivo* HRT II system confocal microscopy in humans

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PURPOSE. *To assess corneal tissue modifications after riboflavin-UVA-induced cross-linking of corneal collagen in patients with progressive keratoconus as well as regeneration of epithelium and subepithelial nerve plexus by* in vivo *HRT II system confocal microscopy in humans.*
METHODS. *Ten patients with progressive keratoconus were treated by riboflavin-UVA-induced cross-linking of corneal collagen, involving assessment of ultrastructural modifications of the corneal epithelium and subepithelial nerve plexus by HRT II system confocal microscopy. Treatment included instillation of 0.1% riboflavin–20% dextrane solution 5 minutes before UVA irradiation and every 5 minutes for a total of 30 minutes. Radiant energy was 3 mW/cm^2 or 5.4 Joule/cm^2 and the source was dual UVA (370 nm) light-emitting led. The protocol included the operation followed by antibiotic medication and eye dressing with a soft therapeutic contact lens. Changes in epithelium and subepithelial and stromal nerve plexus were assessed by HRT II system confocal microscopy* in vivo.
RESULTS. *After 5 days of soft contact lens wearing corneal epithelium has a regular morphology and density. Disappearance of subepithelial stromal nerve fibers was observed in the central irradiated area where, 1 month after the operation, initial reinnervation was microscopically observed. No changes in nerve fibers were observed in the peripheral untreated with a clear lateral transition between the two areas. Six months after the operation, the anterior subepithelial stroma was recolonized by nerve fibers with restoration of corneal sensitivity.*
CONCLUSIONS. *HRT II system confocal microscopy confirms corneal epithelium restore and re-innervation after riboflavin-UVA-induced collagen cross-linking directly* in vivo *in humans. (Eur J Ophthalmol 2006; 16: 530-5)*

KEY WORDS. *Crosslinking, Corneal epithelium, HRT II confocal microscopy, Keratoconus, Nerve plexus, Riboflavin-UVA*

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INTRODUCTION

Keratoconus is a degenerative, non inflammatory disease of the cornea with onset generally at puberty. It is progressive in 20% of cases and can be treated by lamellar or perforating keratoplasty. Its incidence in the general population is reported at about 1/2000 (1, 2). Changes in corneal collagen structure (3, 4), organization (5) and intercellular matrix (6), as well as apoptosis (7) and necrosis of keratocytes (8) (Fig. 1), prevalently or exclusively involving the central anterior stroma and the Bowmann lamina, are documented in the literature (9). These findings are evidence of structurally weakened corneal tissue.

The method of corneal collagen cross-linking consists in photopolymerization of stromal fibers by the combined action of a photosensitizing substance (riboflavin or vitamin B2) and ultraviolet light from a solid state UVA source. Photopolymerization increases the rigidity of corneal collagen and its resistance to keratectasia.

The first studies in photobiology began in the early 1990s, with attempts to identify biological glues that could be activated by heat or light to increase the resistance of stromal collagen (10). It was discovered that the gluing effect was mediated by an oxidative mechanism associated with hydroxyl radical release. A similar mechanism of hardening and thickening of collagen fibers has been demonstrated in corneal aging (11), related to active glycosylation of age-dependent collagen molecules.

The biomechanical resistance of the cornea is only half the normal value in keratoconus patients (1). The biomechanical properties of the cornea depend on the characteristics of collagen fibers (3, 4), their spatial-structural disposition (5) and inter-fibril bonds (6). The technique of corneal collagen cross-linking has been used experimentally (Dresden, Zurich, Siena) to at least temporarily block progression of keratoconus. Collagen turnover is about 2 to 3 years. Cross-linking “freezes” stromal collagen, increasing the biomechanical stability of the cornea. The pilot study (12, 13) was conducted in Dresden in 1998 and the results were so encouraging that we repeated it for the first time in Italy at our department. We are currently conducting further treatments and validating preclinical studies (14) using for the first time at international level (phase III Study) the HRT II system confocal microscopy directly *in vivo* in humans.

METHODS

Unanimous approval of the research “Eye Cross Siena Project 2004” was obtained from the local Ethical Committee under the Helsinki declaration. The “Eye Cross Siena Project” was awarded by the Italian Society of Ophthalmology (SOI) as best research project of the year 2004 in Opthalmology in Italy.

Riboflavin-UVA-induced corneal cross-linking was performed in 10 progressive keratoconus patients in stage II (second phase, prospective, nonrandomized, open study). The day surgery protocol included aseptic conditions and topical anesthesia (4% lidocaine eyedrops) for a period of 30 minutes. After applying the blepharostat, we used a 9 mm diameter marker, removing the corneal epithelium in a central circle by means of a thin spatula. Photosensitizing solution 0.1%

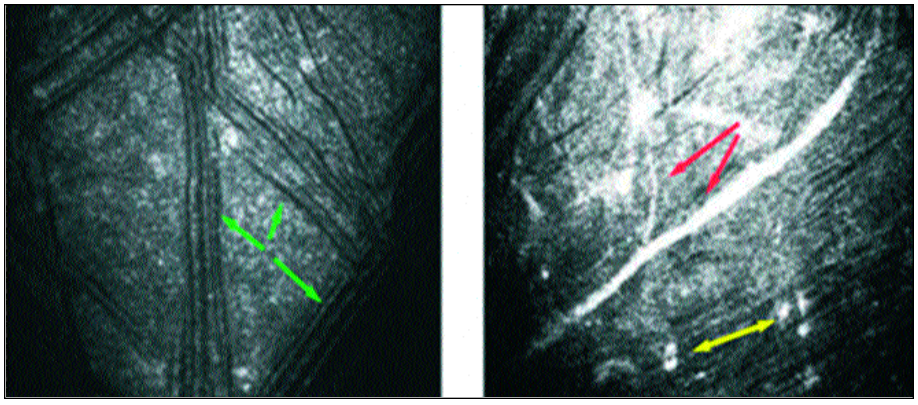

Fig. 1 - *Dark microstriae (green arrows), activated keratocytes (yellow arrows), and stretching of deeper nervous fibers (red arrows) in the anterior stroma of a patient with progressive keratoconus (HRT II system confocal microscopy, Department of Ophthalmology, Siena University).*

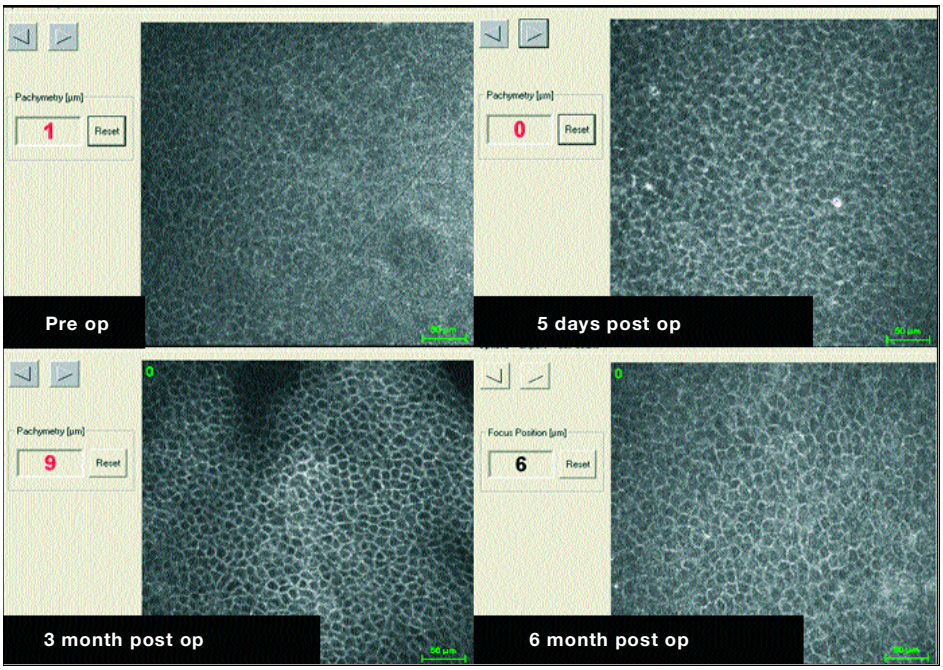

Fig. 2 - *Corneal epithelium regrowth after riboflavin UVA collagen cross-linking is normal after 5 days of soft contact lens bondage. Cell mosaic and morphology appear to be very regular at 3 and 6 months after the treatment (Department of Ophthalmology, Siena University).*

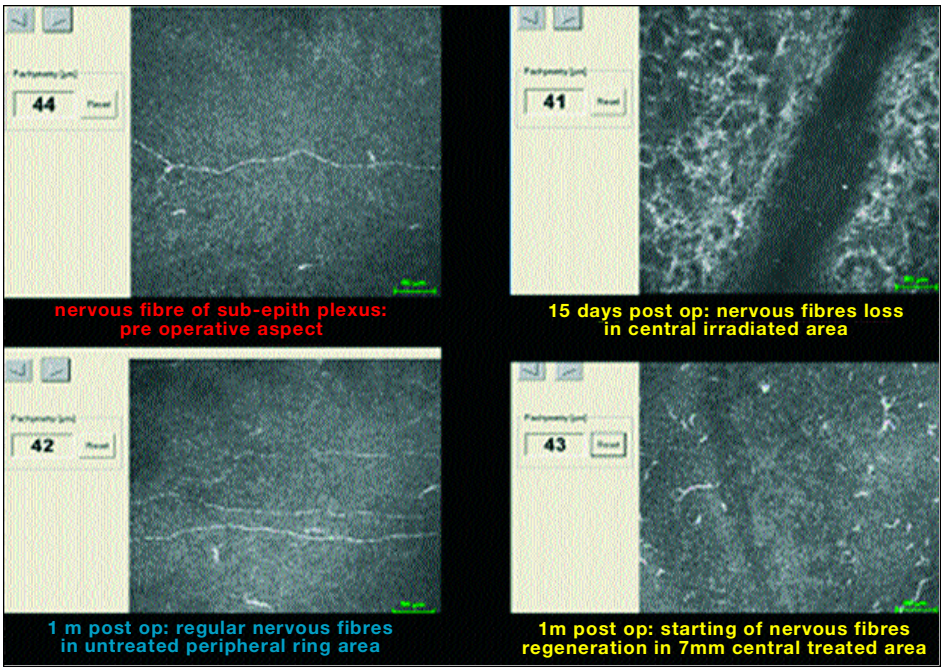

Fig. 3 - *After 15 days subepithelial nervous fibers have disappeared and anterior stroma appears with lacunar edema. In the peripheral untreated area no changes in subepithelial fibers are detectable microscopically (HRT II system). Nerve regeneration began 1 month after riboflavin-UVA-induced corneal collagen cross-linking (Department of Ophthalmology, Siena University).*

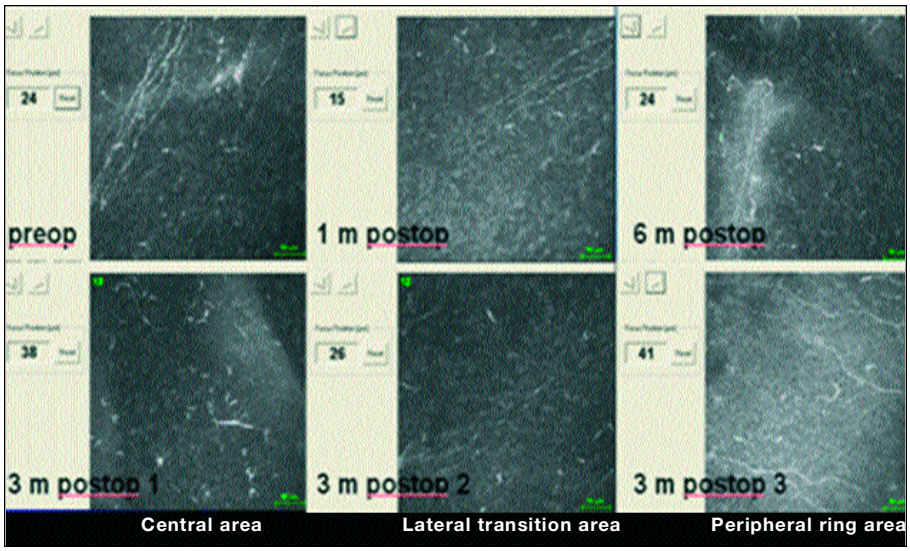

Fig. 4 - *Images of subepithelial plexus modifications after corneal collagen cross-linking: normal preoperative plexus (preop) and nervous fiber regeneration in central treated area after 1 month (1 m postop), 3 months (3 m postop 1), and 6 months (6 m postop). Normal subepithelial plexus appeared in lateral transition area (3 m postop 2) and untreated peripheral ring area (3 m postop 3). After 6 months regeneration of nerve fibers appears to be completed (6 m postop) (HRT II system confocal microscopy, Department of Ophthalmology, Siena University).*

riboflavin, 20% dextran T 500 (Sooft Italia) was instilled 5 minutes before beginning irradiation and every 5 minutes thereafter for 30 minutes. The UVA source was a solid state device (Siena CBM Caporossi, Baiocchi, Mazzotta X LInker, CSO Ophthalmics, Florence, Italy) consisting of UVA LED array (370-10, CSO Ophthalmics, Florence, Italy)) with a potentiometric voltage regulator (CSO Ophthalmics controller). Irradiated energy was checked by a UVA power meter (Lasermate Q-Coherent). Wavelength was 370 nm at a power of 3 mW/cm^2 or 5.4 joule/cm^2; distance was 1.5 cm from the cornea. After treatment patients were medicated four times with topical antibiotic (ofloxacin drops) and twice with mydriatic (cyclopentolate) and the eye was dressed with a soft therapeutic contact lens (Shalkcon) for 4 days. The lens was removed an average of 4 days (3-5 days) after treatment. Postoperative follow-up was performed as well as *in vivo* ultrastructural analysis of the subepithelial nerve plexus by HRT II system confocal microscopy (Rostock Cornea Module, Heidelberg, Germany) at 1, 3 and 6 months. Confocal microscopy was also performed preoperatively after topical application of corneal anesthesia (oxybuprocaine chlorhydrate drops) and corneal gel (methylcellulose).

RESULTS

Confocal microscopy showed normal regeneration and morphological structure of corneal epithelium after treatment (Fig. 2).

Confocal analysis showed a complete absence of nerve fibers 15 to 30 days after treatment (Fig. 3). Riboflavin concentrates 95% of the UVA energy at subepithelial and anterior stromal level in a 400 µm thick cornea, causing necrosis of nerve fibers in the 7 mm central irradiated circle. Subepithelial nerve regeneration was detected microscopically and clinically starting at 1 months after the operation (Figs. 3 and 4), with initial restore of corneal sensitivity.

No structural modifications of nerves were observed in the non irradiated peripheral ring surrounding the 9 mm scraped area (Fig. 3). A lateral transition zone was evident in all patients (Fig. 4).

Regeneration of nerve fibers was characterized by appearance of subepithelial neuritic flocculation in the central irradiated area (Fig. 3). It continued throughout the postoperative period, subsequently becoming complete and restoring a normal corneal sensitivity after 6 months (Fig. 4).

DISCUSSION

Riboflavin-UVA-induced corneal cross-linking destroyed subepithelial nerve fibers in the central treated area by a combined process. The mechanisms of temporary sub-basal, subepithelial and anterior stromal nerve fibers disappearance are not still completely clear. In our opinion, nerve disappearance could be related to a combined effect of epithelial mechanical abrasion and corneal exposure to riboflavin-UVA. Corneal epithelial scraping in the central area 9 mm in diameter was the prevalent factor in the disappearance of sub-basal intra-epithelial nerve fibers and in the slight damage to subepithelial nerve fibers in the de-epithelized ring area between 7 and 9 mm, whereas a combined effect of mechanical abrasion and riboflavin-UVA exposure (inducing photo-necrosis and apoptosis) seems more in keeping with subepithelial and anterior stromal nerve fibers loss in the 7 mm-diameter central irradiated area. Corneal epithelium regenerates normally after 5 days of soft contact lens wearing. No epithelial defects were observed during the follow-up. The nerve regeneration was not visible in the subepithelial layer in a depth of 40 µm until a month after the operation. Nerve fibers begin to regenerate centripetally from the untreated peripheral area then from the deeper layers reaching the central subepithelial layer during the first month. Nerve fibers regeneration and restoration of corneal sensitivity is very rapid after corneal collagen cross-linking treatment comparing with other surgical procedures. For example in laser *in situ* keratomileusis (LASIK) the reinnervation is reduced for 1 year (15) and after penetrating keratoplasty (PK) reinnervation of the central basal epithelium was found at 2 years after (16).

The cytotoxic effects of cross-linking were concentrated in the anterior part of the cornea. Absorption of UVA by riboflavin prevented it from affecting deeper layers. Nerve fibers in the untreated peripheral area were spared and a transition zone was detectable by confocal microscopy. One month after treatment, reinnervation and restoration of corneal sensitivity became evident and continued throughout follow-up, becoming complete about 6 months after the operation when corneal tactile and pain sensitivity measured by kerato-esthesiometry (Cochet-Bonnet instrument) returned to normal. There was no evidence of altered corneal transparency or trophism due to deinnervation-related stromal neurodystrophic mechanisms. HRT II system confocal microscopy enabled precise assessment of corneal ultra-structural changes after cross-linking.

Collagen cross-linking could become a standard treatment for progressive keratoconus. Long-term studies are needed to identify any complications and to determine the duration of the stabilizing effect of treatment on the disease. This method would reduce the need for donors and keratoplasty, with many ethical and social security implications. It is economical and therefore lends itself to application in developing countries where donors and eye banks are not available. Collagen cross-linking seems to stabilize keratoconus without clinical side effects, however further clinical and diagnostic research, including ultrastructural studies, are needed to detect subclinical effects that may emerge in time.

Study of epithelium, sub-basal and subepithelial nerve regeneration by confocal microscopy, performed for the first time in Italy in our department, enables assessment of these processes *in vivo* in humans, and can be used to monitor the efficacy and safety of this promising treatment.

The method of corneal cross-linking using riboflavin and UVA is technically simple and less invasive than all other therapies proposed for keratoconus, and unlike other "mini-invasive" methods, such as intrastromal rings (INTACS) and excimer laser surgery, which do not block keratectasia but treat the refractive effects of the disease, treats and prevents some of the underlying pathophysiological mechanism.

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