ABSTRACT

PURPOSE: to assess the effectiveness of riboflavin-UVA-induced cross-linking of corneal collagen in reducing progression of keratoconus and in improving visual acuity in patients with progressive keratoconus.

SETTING: Department of Ophthalmology, Siena University. Second phase prospective non randomised open study.

METHODS: Starting in September 2004, ten eyes of ten patients (31.4 years m a) with bilateral keratoconus treated by combined Riboflavin - UVA collagen cross-linking. Radiant energy was 3 Mw/cm2 or 5.4 joule/cm2 for a 30 min exposure at 1 cm distance from corneal apex. A complete ophthalmological examination (UCVA, SSCVA, BSCVA) was performed. Patients underwent corneal computerized topographic examination, linear scan optical tomography, endothelial cell count, US pachometry, IOP evaluation and HRT II system confocal microscopy at 1, 2, 3 and 6 months. After treatment eyes were medicated and dressed with a soft contact lens.

RESULTS: Comparative pre and post operative results showed increases of 3.6 lines for UCVA (p=0.0000112), 1.85 lines for SSCVA (p=0.00065) and 1.66 lines for BSCVA (p=0.00071). Topographic analysis showed a mean K reduction of 2.1 ± 0.13 D in the central 3 mm. Statistical analysis of IOP and endothelial cell count don't showed statistically significant differences. Topo-aberrometric analysis of corneal symmetry showed a trend of increasing corneal symmetry with a major reduction in asymmetry between vertical hemimeridians.
DISCUSSION: Refractive results showed a reduction of about 2.5 D in the mean spherical equivalent, topographically confirmed by the reduction in mean K. Surface aberrometric analysis demonstrate the improvement in morphological symmetry with a significant reduction in comatic aberrations.

Key words: collagen cross-linking, coma, refractive outcomes, hemimeridians, topo- aberrometric analysis
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Parasurgical therapy of keratoconus by riboflavin-UVA-induced cross-linking of corneal collagen: preliminary refractive results in Italian study.

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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 to be about 1/2000 (1). Incidences of 1/600-1/420 seem more in keeping with current diagnostic capacity. Changes in corneal collagen structure (2, 3), organisation (4) and intercellular matrix (5), as well as apoptosis (6) and necrosis of keratinocytes (7)(Fig. 1), prevalently or exclusively involving the central anterior stroma and the Bowmann lamina, are documented in the literature (4-8). These findings are evidence of structurally weakened corneal tissue typical of keratoconus.

The technique of corneal collagen cross-linking consists in photopolymerisation of stromal fibres by the combined action of a photosensitising substance (riboflavin or vitamin B2) and ultraviolet light from a solid state UVA source (9). Photopolymerisation increases the rigidity of corneal collagen and its resistance to keratectasia (10).

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 (11). 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 fibres has been demonstrated in corneal aging (12), related to active glycosylation of age-dependent tropocollagen molecules.

The idea to use this conservative approach to treat keratoconus was conceived in Germany in the 1990s by a research group at Dresden Technical University (9). The aim was to slow or arrest progression so as to delay or avoid recourse to perforating keratoplasty. The basis for its use finds clinical and scientific support in
the fact that young diabetic patients never have keratoconus, and in the few exceptions it predated the onset of diabetes and did not progress due to the natural cross-linking effect of glucose, which increases corneal resistance in these patients.

The biomechanical properties of the cornea depend on the characteristics of collagen fibres (2, 3), interfibril bonds (5) and their spatial-structural disposition (4). The biomechanical resistance of the cornea of keratoconus patients is half the normal value. The technique of corneal collagen cross-linking has been used experimentally (Dresden, Zurich, Siena) to at least temporarily block progression of keratoconus in the refractive phase. Collagen turnover is about 2-3 years. Cross-linking “freezes” stromal collagen, increasing the biomechanical stability of the cornea.

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 merely treat the refractive effects of the disease, it treats and prevents the underlying pathophysiological mechanism (9).

**Purpose**

The main aim of the study was to assess the effectiveness and safety of riboflavin-UVA-induced cross-linking of corneal collagen in reducing progression of keratoconus and in improving visual acuity. This is the first Italian second phase prospective non randomised open study. The subjects were ten patients with progressive keratoconus.

**Methods**

The study was approved by the ethics committee of Siena University under the principles of the Helsinki declaration; informed specific consent was previously (minimum 10 days) obtained from all participants. Beginning in September 2004, we selected and treated ten eyes (6 right, 4 left) of ten patients (2 women, 8 men) with a mean age of 31.4 years (21-39 years). In two patients, the fellow eye (left)
had undergone perforating keratoplasty (2 and 3 years previously) and was not used as control. In the other 8 cases the fellow eye was used as control. All patients had bilateral keratoconus without subepithelial scarring and could not tolerate contact lenses. Vogt striae were detected by biomicroscopic examination in 5 cases and were absent in the other five. Five eyes were in Krumeich stage 2 and five in stage 3. In the eight fellow control eyes no striae or scars were observed; four were in stage 2 and four in stage 1.

Before treatment, all patients underwent biomicroscope examination, assessment of uncorrected visual acuity (UCVA), best spectacle corrected visual acuity (BSCVA) and intraocular pressure (IOP; Tono-pen II XL mentor), those with myopic ametropia also sphere corrected VA (SpCSVA). They also underwent corneal computerized topographic examination (Eye Top CSO V 7.1.1), linear scan optical tomography (Orbscan II B & L), endothelial cell count by non contact endothelial microscope (Konan Keeler Non Con Robo) and ultrasound pachymetry (DGH Pachette 2). Patients also underwent laser confocal microscope examination (Heidelberg HRT II - Germany ). All examinations were repeated 1, 2, 3 and 6 months after cross-linking treatment. The surgical procedure (Figure 2) consisted of topical anaesthesia (instillation of 4% lidocaine 3 times in 15 min), placing the patient under the operating microscope and inserting a lid speculum with closed valves and screw regulation (S.I.R. Ophthalmic 812T ). A 7 mm circle was traced on the epithelium with a Thornton corneal marker (ASICO AE2710) in which the epithelium was removed with a blunt spatula (ASICO AE2766). This was followed by instillation of 2-4 drops of a solution containing 0.1% riboflavin (Streuli Pharma, Switzerland) and 20% Dextran (500,000 Da, Dextran T500, Amersham Biosciences, Belgium) prepared immediately before the operation and placed in a 1 ml syringe which was left in place for 5 min before beginning UVA exposure. After instilling another 2-4 drops the UVA lamp was turned on. The UVA source was a solid state experimental device (Exerion-Sas, Prato, Italy) consisting of two UVA LEDs (370-10 TO 46 ball lens, 750 microwatt, Roithner Lasertechnic, Wien) with a potentiometric voltage regulator (Figure 3). Irradiated energy was controlled by a UVA power meter (Lasermate Q-Coherent). The source was focused on the apex of the cornea, distance 10-12 mm, to obtain a radiant energy of 3 MW/cm² or 5.4
joule/cm² for 5 min. The lamp was then turned off and the riboflavin-dextran solution was again instilled and the 5-min exposure repeated five times (total exposure time 25 min, total time for treatment 30 min). After treatment, the eye surface was washed with 20 cc BSS (Alcon Laboratories), medicated with 2-4 drops of ofloxacin (Exocin, Allergan) and 2-4 drops of cyclopentolate (Ciclolux, Allergan) and dressed with a soft contact lens (Schalkon SofClear +0.5 D). Patients received paracetamol-codeine (500 mg – 30 mg; Co-Efferalgan UPSA Pharma) every 8 h and the post-operative medication was repeated four times/day until removal of the contact lens on day 5. Biomicroscopic examination was then performed to assess epithelial repair. Topical therapy with flurbiprofen eye-drops (Ocufen Allergan 4 times/day) and ofloxacin eye-drops (Exocin Allergan 4 times/day) was continued for a further 20 days.

Topographic assessment was performed with axial (step 0.75/1D) and tangential (step 1.5 D) algorithms. Altimetric evaluation was also performed using an aspherotoric reference surface located tangentially to the geometric centre of the cornea, scale 5 µm, reducing dioptric power of the reference sphere by 10 D so as to highlight the localisation and elevation of the apical region. After removal of the topographic revealed pupil we performed aberrometric analysis of the corneal wavefront (Seidel) with a 5 mm simulated entrance pupil (Figure 4). Orbscan examination was only used for anterior elevation parameters since posterior elevations and pachymetry were impeded by post-operative sub-edema. The numerical data extrapolated from all procedures was entered in a data sheet (MS Excel XP) and analysed with GraphPad Prism 3.0.

**Results**

Mean refraction for the best visual acuity before treatment was −2.85 D spherical (+1.00/-8.00) and −4.7 D cylindrical (-2.50/-6.50) with a mean spherical equivalent of −4.7 D (-0.75/-10.75) (Figure 5). Mean UCVA was 1.3/10 (20/154), range 1/30 - 3/10 (20/600-20/66) and SSCVA was 2.87/10 (20/70), range 1/10 - 5/10 (20/200-20/40). Mean best spectacle corrected visual acuity (BSCVA) was 4.1/10 (20/48), range 2/10 - 6/10 (20/100-20/33) (Figure 6).
Mean central corneal thickness (CCT) before treatment measured by ultrasound pachymetry was 431.5 µm (406-468), showing a small increment after treatment, presumably due to sub-edema (mean 463.3 µm, range 418-487) that dropped to 450.6 µm (416-480) at 3 month follow-up as edema cleared. Student’s test for paired data did not reveal any significant difference between these values. Statistical analysis of IOP and endothelial cell count didn’t showed statistically significant differences between pre- and post-operative data at all follow-up times. Subjective refraction shows a slow and continuous reduction in the follow-up: sphere value starting from a mean of -2.85 D (SD ±3.68) preoperatively, at 1 month was -1.95 (SD ±2.83), at 2nd month was -1.875 D (SD ±2.56) and at 3rd month results meanly -1.425 D (SD ±2.54). Cylindric error have a similar course: starting from a preoperative mean of -4.7 D (SD ±1.27) decrease at the first month at -4.175 D (SD ±1.29), -3.65 D (SD ±1.35) at the second month and -3.5 D (SD ±1.25) at the end of the follow-up. Spherical equivalent value shows a decreasing starting from -4.7 (SD ±3.35) preoperatively, to -4.0375 D (SD ±3.24) at the first month, to -3.7 D (SD ±3.18) and ending to -2.495 D (SD ±3.06) at third month follow-up. Functional results included increases in UCVA and BCVA, preoperative BCVA identical to UCVA at 3 months and increases of 3 Snellen lines in UCVA and 1.2 lines in BSCVA between pre-operative and 1-month postoperative examinations, with statistical significance of p=0.00002 for UCVA and p=0.00093 for BSCVA (Mann-Whitney U-test). Comparison of preoperative and 3-month follow-up data showed increases of 3.6 lines for UCVA (p=0.0000112), 1.85 lines for SSCVA (p=0.00065) and 1.66 lines for BSCVA (p=0.00071). Topographic analysis with the axial refractive algorithm showed a mean (± SD) reduction in dioptic power of 2.1 ± 0.13 D in the central 3 mm, with a reduction of 2.4 ± 0.16 D in the minimum value and 1.9 ± 0.08 D in the maximum value at 3 months, in line with the pilot study (Figure 7). Analysis of corneal symmetry based on automatic calculation of symmetry index by our topographer showed a trend of increasing corneal symmetry (Figure 8) with a reduction in mean value from 6.263 before treatment to 4.258 at 3 months and a similar trend in all eyes analysed from the second month. A similar result emerged from analysis of hemimeridians, with a major reduction in asymmetry between vertical hemimeridians, leading to a statistically significant
reduction between preoperative and second month values (paired t-test) and increasing significance from p=0.029 at 2 months (significant with 95% confidence interval) to p=0.001 at 3 months (99% confidence interval). The reduction in difference between horizontal hemimeridians was also significant (p=0.033) at 2 months, becoming highly significant at 3 months p=0.0001 (Figure 9). The reduction in asymmetry of horizontal and vertical hemimeridians was not significant at 1 month (p=0.11 and 0.39, respectively). Anterior apical elevation, evaluated by corneal topography and Orbscan II, using as reference both the tangent at the corneal geometric centre and best fit sphere floating, showed a significant reduction that reproduced a similar proportional reduction in anterior elevation, though with different numbers (Figure 10). Statistical analysis of elevation parameters showed significant differences at 2 months with respect to before treatment (p<0.001), maintained at 3 months with both analyses.

Corneal wavefront surface aberrometry according to Seidel (Figure 11) showed a major reduction in RMS which was significant at 2 and 3 months and borderline significant at 1 month with respect to before the operation. Spherical and higher order aberrations did not show statistically significant variations in the follow-up period, whereas the coma component showed a very significant reduction at 1 month with respect to before the operation and persisted throughout the follow-up period (Figure 12). In the fellow eye we have observed a progression of keratoconus in 37.5% of the cases (3/8 eyes) with increased corneal curvature, elevation and comatic aberrations and decreased UCVA, SpCSVA, BSCVA and pachometric values. In other 5 eyes (62.5%) we don’t observe topographic, aberrometric and functional impairment.

DISCUSSION

Although the present study was limited in terms of follow-up and number of patients, it confirms the encouraging results of the pilot study on riboflavin-UVA-induced corneal collagen cross-linking, as far as safety and effectiveness are concerned. The procedure has few side-effects for the cornea (no numerical or morphological modifications of the endothelium) or for the posterior segment, where OCT examination conducted preoperatively and 7 days and 3 months after
treatment showed identical retinal macular and perimacular thicknesses. These two results, together with confocal microscope evidence of almost complete disappearance of keratinocytes in the anterior 350 µm of the stroma and their presence at preoperative levels in areas immediately adjacent, confirm that UVA emitted by the LEDs is perfectly calibrated in energy density to produce apoptosis and hence necrosis of “unhealthy” activated keratinocytes, besides being completely absorbed by riboflavin beyond the programmed dose and necessary thicknesses. Confocal microscope evidence of subclinical corneal edema only in areas devoid of keratinocytes indicates the significant degenerative effect of the radiation and the lack of inflammatory phenomena, confirmed by the absence of inflammatory cells in treated and adjacent areas. Refractive results were similar to those obtained by the pilot study in Dresden, namely a reduction of about 2.5 D in the mean spherical equivalent, as confirmed by the reduction in mean K detected topographically. This finding is significant and no worse than the results of much more invasive procedures with complication rates of 20-30%, but only partly explains the improved functional performance obtained in these ten patients. It seems logical that reduction in refractive defect be associated with a significant increase in UCVA (+3.6 Snellen lines) but the increase in SpCSVA and especially BSCVA (2 lines) is not easy to explain. Topographic and surface aberrometric analysis together with Orbscan, however, provide an insight: the improvement in morphological symmetry reflected a significant reduction in comatic aberrations at one month and a decrease in anisorefractance of the corneal hemimeridians, which means greater, necessarily symmetrical coherence between defect and correcting lens. Although our medium period refractive and functional results were in line with those of the pilot study, in our series improvement was already evident at the first postoperative examination with significant differences in evolution of corneal K readings and particularly corneal form descriptors, against a latency of about 3 months in the pilot study. This difference could be due to instrumentation and therapy. Indeed, the instrument used by us (Exerion S.A.S., Prato, Italy) is powered by an electronic controller that stabilised the energy during UVA treatment, eliminating the initial emission peak and the tail at the end of treatment, typical of battery-powered devices. The constant voltage and energy
of the supply system ensures constant intensity of emission by the LED over the whole exposure time. The other source of difference is the practice of dressing the cornea with a soft contact lens. This has positive effects on epithelial regrowth and on the initial stage of stromal rearrangement, that cannot be obtained by simply bonding the eye. Our preliminary data shows that Riboflavin-UVA crosslinking procedure is very safe and effective to reduce the progression of keratoconus overall in early stages of the disease (first and second Krumeich stage) especially in young patients without Vogt striae. The optical and visual performances improvement seems to be particularly related to corneal symmetry increasing induced by a restored corneal rigidity.
References

FIGURES LEGENDS

Figure 1: left image (A) shows “dark” micro-striae (green arrows) and activated keratocytes (yellow arrows) in posterior stroma 350 µ in progressive keratoconus. Right image (B) shows stretching and granular (necklace pearls) aspect of nervous fibres (red arrows) in progressive keratoconus, anterior subepithelial stroma (45 µ), in vivo HRT II confocal microscopy. (Department of Ophthalmology, Siena University, 2005)

Figure 2: Summary of surgical procedure and timing of UVA (violet) and Riboflavin-Dextran administration

Figure 3: Left image (A) shows the Dresden modified Siena Tip with double 370/10 UVA-Leds (Exerion Sas). In right image (B) the experimental electronic device for UVA leds alimentation (Exerion Sas).

Figure 4: CSO EyeTop Corneal Wavefront Seidel Analysis 5 mm pupil in left keratoconic eye. A) total anterior OPD, B) astigmatism deviation, C) spherical aberration, D) coma deviation, E) high order (over fourth) aberration, the F barplotting of the single Zernike coefficients.

Figure 5: Preoperative refraction in the ten treated eyes. Cylindric component was expressed in negative value.

Figure 6: Preoperative visual acuity of the ten treated eyes (decimal fraction).

UCVA= UnCorrected Visual Acuity
SSCVA= Spectacle Sphere Corrected Visual Acuity
BSCVA= Best Spectacle Corrected Visual Acuity

Figure 7: Topographic measured minimum, maximum and mean K readings at 3 mm. The lines over the bars indicates calculated linear regression.

Figure 8: Topographic calculated Simmetry Index in the follow-up. The bars was the mean value and the lines indicates the single cases.
Figure 9: Vertical (A) and Horizontal (B) hemimeridian difference. Lines was the single cases and bar was the mean. A larger amount of difference and a higher reduction in difference in vertical hemimeridian is evident; this significative reduction explain a significative reduction in Simmetry Index. The horizontal hemimeridians don’t have the same evolution.

Figure 10: Anterior surface elevation with tangent aspherotoric surface reference 10D reduced measured by CSO EyeTop corneal topography (A) and anterior surface elevation with floating best fit sphere measured by Orbscan linear scanion tomography (B): the absolute value was different but the same percentage of reduction was measured.

Figure 11: CSO EyeTop topographer Seidel corneal wavefront analysis with 5 mm simulated pupil, centration on geometric corneal centre. Linear regression shows a significative, good correlation in HO RMS and coma RMS reduction between preoperative and postoperative data; the linear regression for Spherical aberration data shows a not significative reduction in the follow-up.

Figure 12: Percentage variations in analyzed kerato-topographic parameters; red arrows indicates (paired t-test) difference with preoperative data statistically significant p<0.01.
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