Abstract: Purpose: evaluation of riboflavin stromal concentrations by HPLC assay to ensure the efficacy and safety of corneal cross-linking by the standard and trans-epithelial procedures.

Methods: 14 keratoconic patients enrolled for penetrating keratoplasty were selected as in vivo samples donors and 16 warm-stored ex vivo sclerocorneal rings unsuitable for transplant were used. In vivo samples were immediately exposed to sterile 0.1% riboflavin solution. 7 of the 14 specimens were debrided and the other 7 were left with the epithelium in situ. One of the latter and one of the debrided samples were not exposed with riboflavin (control groups). In 7 sclerocorneal rings epithelium was removed and in 9 was left in situ. Debrided and not debrided samples were soaked with 0.1% riboflavin solution, instilled every 2 minutes for 5 min, 15 min and 30 min in both in vivo and ex vivo groups. Riboflavin concentrations were determined by HPLC.

Results: Control samples did not show any riboflavin emission peak. In samples exposed with epithelium, riboflavin concentrations were 2.54 μg/g after 5 min and 3.88 μg/g after 15 min of exposure, whereas in the debrided samples they were 96.28 μg/g after 5 min and 98.92 μg/g after 15 min exposure.

Conclusion: HPLC quantitative study shows that stromal concentrations of riboflavin increase with exposure time only if the epithelium is removed. A safe and effective riboflavin concentration (>15 μg/g tissue) can be obtained for UVA-induced corneal cross-linking only removing the epithelium and after at least 10 min exposure, applying riboflavin every 2 minutes.
Introduction

Corneal tissue affected by keratoconus shows fewer links between and within collagen fibrils than normal corneal tissue (Andreasson et al). With advances in photopolymerisation made in the 1990s at Dresden University (Germany), research was undertaken into the efficacy of methods of polymerisation in the treatment of corneas affected by keratoconus. Basic studies revealed the potential of photo-polymerisation induced by irradiation with UVA (wavelength 360 nm) after application in disepithelialized cornea of 0.1% riboflavin to increase links between and within fibrils of corneal collagen, while maintaining structural integrity and corneal transparency and preserving corneal and ocular anatomical and histological structures (endothelium, lens and retina). The parameters emerging from the Dresden research, subsequently confirmed in other studies, envisaged debridement of corneal epithelium in order to obtain cross-linking to a depth of at least 250-300 micron into the corneal stroma, using an energy of 3 mW/cm² 360 nm wavelength for a total exposure time of 30 minutes. Experimental data on the penetration of UVA radiation and its effects demonstrated ex vivo were confirmed in vivo in humans by our confocal microscopy studies in patients with keratoconus treated by the standardised procedure. Subsequent experiences by Boxer-Wachler et al., obtained results for corneal cross-linking administering 0.1% riboflavin solution without epithelial debridement, reporting an efficacy similar to the standard method involving epithelial debridement.

The absence of experimental assessment and studies in vitro, ex vivo and in vivo in animal models confirming the efficacy and safety of the procedure of trans-epithelial cross-linking, considered inefficacious according to basic and recent studies by Hayes et al., prompted us to evaluate the concentration of riboflavin that can be obtained in stroma and corneal epithelium (when present) by administration of riboflavin to corneas with and without epithelium in situ. The aim of the study was to evaluate intrastromal concentrations of riboflavin with and without epithelium to ensure the efficacy and safety of corneal cross-linking by the standard and transepithelial procedures. The assay method we used was high precision liquid chromatography (HPLC).

Methods

At the Department of Ophthalmological Sciences, Siena University, we selected 14 patients with keratoconus in Krumenich stage 4 (without stromal scarring) enrolled for penetrating keratoplasty, as donors of in vivo samples. At the Tuscany Eye Bank (Lucca, Italy) we requested and received 16 warm-stored ex vivo sclerocorneal rings unsuitable for transplant for serological reasons.

Treatment of central corneal buttons in vivo

After removal by means of a Hannà trephine (diameter 8.25 mm in all cases), in vivo samples were immediately exposed to sterile 0.1% riboflavin solution (Ricrolin® SOOFT, Italy) after fixing the buttons on the drill blade using Cyanacrilate glue (Attak®) to avoid penetration of riboflavin into the stroma via the lateral wall (Fig. 1). Seven of the 14 specimens were debrided with a blunt metal spatula (ASICO) before fixing to the blade and the other seven were left with the epithelium in situ. One of the latter and one of the debrided samples (controls) were not treated with riboflavin. Six samples of each type were mounted on the blade as described previously and a drop of riboflavin solution was instilled on the surface every 2 minutes for 5 min (2 samples per group), 15 min (2 samples per group) and 30 min (2 samples per group). The anterior surface of samples was then washed with 10 cc BSS on to remove riboflavin that had not bound to corneal tissue. Then they were removed from the blade. Samples instilled with the epithelium in situ were then debrided with the spatula. All samples were sealed in sterile screw-capped histology containers, shielded from the light with aluminium foil and stored at –80°C.

Figure 1. Central corneal button fixed to trephine blade before (a) and during (b) instillation of sterile 0.1% riboflavin solution. The sides in contact with the blade were sealed with a drop of cyanoacrylic glue to avoid penetration of riboflavin via the lateral walls.

Treatment of sclerocorneal rings

The rings were removed from the conservation liquid and washed in BSS. Seven specimens were debrided centrally (diameter 8.5 mm) with the blunt spatula (ASICO) after marking with a 8.5 mm Thornton marker (Storz). The epithelium of the other nine specimens was left in situ. Samples were placed convex side up on a silicone block and six debrided and eight with epithelium were impregnated with 0.1% riboflavin solution by instilling a drop every 2 min (Fig. 2).

Figure 2. Sclerocorneal ring with epithelium in situ mounted on silicone block for impregnation with sterile 0.1% riboflavin solution. Syringe with Ricrolin® is visible in upper part. Impregnation time was 5 min for two samples without epithelium (Fig. 3) and two samples with epithelium in situ, 15 min for two samples without epithelium and two samples with epithelium in situ, and 30 min for two samples of each group. One sample per group (controls) was not impregnated. After exposure to riboflavin, the anterior surface of the specimens was washed with 10 cc BSS to remove riboflavin excess. Specimens were placed endothelial side up on silicone blocks and punched with a 8.25 mm diameter blade.
(Storz). Two samples for each exposure time with epithelium in situ were debrided with the blunt metal spatula (Fig. 4). Samples were placed in histological screw-capped containers, shielded from the light with aluminium foil and stored at −80°C. The two remaining samples with epithelium exposed for 15 min. were divided into equal parts after punching. One part was debrided and the other was left with epithelium in situ. These samples were then placed in containers and frozen as above.

Figure 3. Central corneal button of sclerocorneal ring after epithelial debridement and soaking with 0.1% riboflavin solution (Ricrolin®) for 5 min. Note yellow staining of stroma.

Figure 4. Mechanical debridement of epithelium of punched sclerocorneal ring after exposure to riboflavin with epithelium in situ.

**Preparation of cornea samples and analysis of riboflavin by HPLC**

Samples of cornea were homogenized in K+-phosphate buffer, pH 7.4 at 0°C. One ml of homogenate was used for the riboflavin determination according the method described by Capo-chichi et al20. (2000) with minor modifications.

One ml of cornea sample was spiked with 1 ml 15 mM magnesium acetate solution and the mixture was incubated at 65°C for 15 min. Proteins were precipitated by adding 0.5 ml 10% trichloroacetic acid and centrifuging at 3200 g for 10 min at 4°C. The supernatant was kept and the pellet rinsed once with 1 ml 15 mM magnesium acetate solution and centrifuged. The two supernatants were mixed and injected in a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) previously activated with 2 ml methanol and 2 ml of a solution containing (v/v) methanol - 10 mM potassium dihydrogen phosphate and 15 mM magnesium acetate (pH 3.4 with orthophosphoric acid)(solution A). The Sep-Pak cartridge was rinsed with 2 ml of solution A prior to elution of riboflavin in 2 ml of methanol. Samples were dried under nitrogen stream. The extracts were spiked with 50 µl methanol and 20 µl was used for the HPLC run. The HPLC system was composed of a 600 E System Controller (Waters, Milford, MA, USA), a 1046 A programmable fluorescence detector connected to an 3395 integrator (both from Agilent Technologies, Santa Clara, CA, USA). HPLC analysis of riboflavin was carried out on an Ultrasphere C18 column (5 µm, 250 mm x 4 mm, Beckman). The mobile phase (15% acetonitrile in solution A) was used under isocratic conditions at a flow-rate of 1 ml/min. The spectrophotometer was set at 445 nm excitation and 530 nm emission wavelengths. The detection limit of riboflavin was of 0.5 nM. Calibration curves were obtained by plotting riboflavin (R) peak area as a function of concentration.

**Results**

After thawing, samples were weighed with a balance having a precision of 0.01 mg. Buttons from donor material showed homogeneous values in the range 29.08-31.44 mg, mean 30.32 mg. Samples from PKP patients showed higher variability, weighing 13.45-26.12 mg, mean 21.84 mg. The greater dispersion and lower weight depended on stage of keratoconus. After homogenisation and dilution of homogenate, HPLC assay was performed. Control samples of debrided cornea not exposed to riboflavin did not show any emission peak at 553 nm, the wavelength of riboflavin. Samples treated with riboflavin with epithelium in situ and subsequently debrided showed weak peaks of 80-100 ng/g tissue without any differences between samples with viable integral epithelium from patients undergoing PKP impregnated with riboflavin before trephining and riboflavin- treated samples with non viable or altered epithelium (ex vivo sclerocorneal buttons from unsuitable donors).

**Analysis of samples with epithelium in situ**

After 5 min exposure to riboflavin solution, a mean concentration of 91.56 ng/g tissue was recorded in the two corneas of the first group (in vivo samples) and a mean concentration of 91.88 ng/g in corneas of the second group (ex vivo samples). After 15 min exposure to riboflavin, mean concentrations were 95.66 ng/g in the two corneas of the first group (in vivo samples) and 94.92 ng/g in corneas of the second group (ex vivo samples). After 30 min exposure, similar mean intrastromal concentrations were recorded: 90.43 ng/g in corneas of the first group (in vivo samples) and 89.89 ng/g in corneas of the second group (ex vivo samples).

**Analysis of debrided samples**

Samples exposed to riboflavin after epithelial debridement showed much higher stromal concentrations of the order of tens of µg/g tissue which increased with increasing exposure time. Again, no differences were observed between PKP and ex vivo eye bank samples. After 5 min of exposure to riboflavin, tissue concentrations were 14.42 µg/g in the in vivo group and 14.88 µg/g in the ex vivo group. After 15 minutes of exposure the corresponding concentrations were 20.92 and 21.08 µg/g, respectively. After 30 minutes of exposure they were 24.06 and 24.10 µg/g, respectively (Fig. 6). A significant correlation between riboflavin concentration in tissue and exposure time was
Figure 6. Concentrations of riboflavin in stroma of samples exposed after debridement of epithelium. Riboflavin concentrations were correlated with exposure time and showed a time-dependent linear increase typical of passive diffusion driven by concentration gradient between 0.1% Ricrolin® and stroma.

Figure 7. Linear regression of riboflavin concentration in corneas exposed after debridement. Values of x and y show a correlation close to one (R² = 0.991; R = 0.98021).

When riboflavin concentrations in samples instilled with and without epithelium were plotted on a logarithmic scale, those of debrided samples were two orders of magnitude greater than those of samples exposed with epithelium in situ. This showed that the epithelium is a barrier against diffusion of the riboflavin molecule into the corneal stroma (Fig. 8).

Figure 8. Stromal concentrations of riboflavin in corneas exposed after debridement and with epithelium in situ plotted on a logarithmic scale. The gap of two orders of magnitude on the log scale indicates a 1:100 ratio in favour of debrided corneas. The difference in concentrations was even more evident when plotted on a numerical scale (Figure 9).

Figure 9. The same data as Fig. 8 plotted on a numerical scale showed that concentrations of riboflavin were negligible in stroma of corneas exposed with epithelium in situ.

In the two samples exposed with epithelium in situ and cut in half, one of which was then debrided and the other not, we found two different patterns: in samples with epithelium, riboflavin concentrations were 2.54 µg/g after 5 min and 3.88 µg/g after 15 min of exposure, whereas in the debrided halves they were 96.28 µg/g after 5 min and 98.92 µg/g after 15 min exposure. Since the weight of epithelium was 1/20 the weight of the stroma (1.18-1.23 mg), epithelial concentrations were 39.66 µg/g after 5 min and 43.23 µg/g after 15 min, which was more than double that encountered in stroma exposed without epithelium (Fig. 10).

Figure 10. Results of samples exposed to riboflavin with and without epithelium for 5 and 15 minutes: stromal concentrations of riboflavin (exposure with and without epithelium and cornea exposed without epithelium) and whole cornea concentrations (exposure with epithelium in situ). Epithelial concentrations of riboflavin were calculated by subtracting the stromal concentration of the cornea exposed with epithelium in situ from that of the sample exposed and weighted with epithelium in situ, expressing the difference in relation to the total weight of the cornea with epithelium.

Discussion

HPLC is an extremely sensitive, standard method of quantifying chemical compounds. Its only limit is that it cannot separate substances with overlapping absorption and emission peaks. Reference corneal chromatograms were constructed using control samples, namely two in vivo cornea samples of keratoconus patients undergoing PKP and two ex vivo donor samples from the eye bank, not exposed to riboflavin and assayed with and without epithelium in situ. These control corneas did not show any absorption or emission peak in the 360-370 nm and 530-550 nm bands of riboflavin, respectively. This confirms the sensitivity and reliability of the method.

The results obtained confirmed that normal corneas and corneas with keratoconus do not contain quantifiable traces of riboflavin in the stroma, endothelium or epithelium. This observation and the finding of blood levels of riboflavin in the range 13-29 ng/ml, the maximum concentration of which is saturable (29 ng/ml is saturation) as well as the absence of reports of accumulation, suggests that oral or parenteral administration of riboflavin to keratoconus patients would be ineffective if not toxic. Regarding the results of exposure of samples to 0.1% riboflavin solution, the sharp difference (1:100) in stromal concentrations of the vitamin B2 observed between samples treated with the epithelial barrier in situ and those treated after debridement, suggests that two different aspects of cross-linking should be considered: the safety of the procedure with epithelium in situ and the efficacy of the procedure with such low stromal concentrations of riboflavin. Stromal concentrations of 10-100 ppm (90 ng/g tissue) indicate passage of riboflavin through the corneal epithelium, which is impermeable to compounds of MW > 100 Da, though riboflavin has a MW of 338 Da. This suggests the existence of an ATP-dependent carrier. The fact that concentrations did not increase with exposure time in samples with epithelium in situ depends on the saturability of the system and inhibition by structural analogues of riboflavin. Contrary to results in the animal model, the quantity of energy available or epithelial viability seem to have little effect on the activity of this kinase, since we found similar concentrations in the in vivo and ex vivo models. These observations are in line with and give a more precise quantitative assessment than the qualitative results of Hayes et al.18 on the inefficacy of pretreatment with tetracaine (epithelium toxic-lytic), benzalconium (bond-lytic) and mechanical damage to the superficial epithelium (partial scraping) in increasing passage of riboflavin across the epithelium, demonstrating the inefficacy of the transepithelial treatment. As far as safety was concerned, stromal concentrations of riboflavin were well below the absorption threshold postulated by Spoerl4,9, making endothelial and intraocular damage a possibility for UVA treatment (370 nm) at the recommended dose of 3 mW/cm². The observations of Hayes et al18 also confirm that this wavelength is transmitted in materials.
having absorption spectra on the whole similar to those of corneal tissue not exposed to riboflavin, with absorption coefficients one quarter those shown by corneal tissue without epithelium exposed to riboflavin. This parameter is actually compensated by the property of epithelium to concentrate riboflavin, as observed in our differential quantitative evaluation: indeed, human corneal epithelium complete with basement membrane naturally absorbs 30-33% of UVA radiation (400-350 nm) and with substantial concentrations of riboflavin in the epithelium it seems to block about 85% of the dose of UVA administered. This high epithelial absorption reduces the risk of endothelial toxicity of UV but at the same time gives rise to doubts about the efficacy of transepithelial passage. Stromal concentrations of riboflavin one hundredth those recorded in debrided corneal stroma would greatly reduce production of singlet oxygen and limit cross-linking. When this insufficient concentration of “activatable” oxidant (riboflavin) is combined with a dose of activator (UVA), already at the cytotoxic threshold of 0.5 mW/cm² at the Bowman lamina, though reduced by an epithelium high in riboflavin, it is evident that transepithelial cross-linking would not comply with the parameters established by the Dresden school2,3,6,7. In conclusion, the results of our published laboratory and clinical studies oblige us to underline that none of the efficacy and safety parameters are met by the transepithelial cross-linking procedure. This method should not be applied to humans because there is evidence that it is not effective; besides, there is a total lack of preclinical and clinical evidence of the safety of the procedure and its effectiveness beyond 6 months. In vivo confocal microscopy performed in patient after a transepithelial crosslinking also shows the lack of induced keratocytes apoptosis in the anterior-mid stroma (Seiler T. ASCRS, Chicago 2008) that is essential for cells replacement and new collagen structure and lamellar interconnections observed after the standardised crosslinking procedure. This aspect is considered the possible key of recorded long term stability demonstrated by Wollensak recent electrophoretic analysis21 and Mazzotta late postoperative confocal investigations19. The present quantitative study stresses the importance of exposure time, showing that stromal concentrations of riboflavin increase with exposure time only if the epithelium is removed, and that a safe and effective concentration (>15 µg/g tissue) can only be obtained for UVA-induced corneal cross-linking without the epithelium and after at least 10 min exposure, applying riboflavin every 2 minutes.

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