ABSTRACT

PURPOSE: To compare stromal riboflavin absorption after 20% alcohol application and partial or complete epithelial removal by analyzing light transmission properties of porcine corneas after riboflavin/ultraviolet A (UVA) corneal collagen cross-linking.

METHODS: Riboflavin 0.13% eye drops were applied to 18 porcine eyes (6 in which 20% alcohol solution had been applied for 40 seconds, 6 eyes with a grid pattern of full-thickness epithelial trauma, and 6 with the central epithelium fully removed) at 5-minute intervals for 35 minutes. In all eyes, the corneal surface was exposed to UVA light for 30 minutes during riboflavin administration. The light transmission spectra of the corneas were analyzed with a spectrophotometer and compared to those of 9 untreated controls (4 corneas with epithelium and 5 without) and to the spectra of riboflavin 0.13% solution.

RESULTS: The spectra of riboflavin-treated corneas in the alcohol group were similar to controls. Those with grid-pattern epithelial trauma showed a dip in light transmission between 400 and 490 nm, but this was significantly less than that in eyes for which epithelial removal was complete, where the spectrum was similar to that of riboflavin 0.13% solution.

CONCLUSIONS: Complete removal of the corneal epithelium appears to be necessary to allow sufficient riboflavin absorption into the stroma to alter the normal light transmission properties of the porcine cornea. Although partial grid-pattern epithelial removal allows some riboflavin penetration, uptake is limited and non-homogeneous, which may affect the efficacy of the cross-linking process. [J Refract Surg. 2009;25:771-775.] doi:10.3928/1081597X-20090813-03

Keratoconus is a degenerative, noninflammatory disorder of the cornea in which stromal thinning and conical ectasia result in irregular astigmatism and associated visual loss.1 Although the use of rigid contact lenses provides visual rehabilitation in the majority of keratoconic patients, corneal transplantation may be necessary in advanced disease due to contact lens intolerance, progressive ectasia, and corneal scarring.2-5

Riboflavin (vitamin B2)/ultraviolet A (UVA) (370 nm) corneal collagen cross-linking is a new therapeutic intervention aimed at stabilizing the keratoconic process.6 In laboratory studies, it has been shown to increase the stress-strain measurements of the corneal stromal tissue7,8 and its resistance to enzymatic digestion.9 It has been postulated that this is achieved by inducing cross-linking between the stromal collagen molecules. Riboflavin is essential to this process, having the dual function of acting as a photosensitizer for the production of oxygen free radicals, which is thought to induce physical cross-linking of collagen via the lysyl oxidase pathway10 as well as concentrating and absorbing the UVA irradiation, thus preventing damage to deeper ocular structures.11,12 In laboratory and clinical studies, the procedure has been shown to be safe with no loss of corneal transparency, no endothelial damage (provided the stroma is thicker than 400 µm), and no damage to either the lens or retina.11,13,14

From the Department of Ophthalmology, St Thomas’ Hospital, London (Samaras, O’Brart, Marshall); Kings College London, The Rayne Institute, St Thomas’ Hospital, London (Samaras, O’Brart, Marshall); and School of Optometry and Vision Sciences, Cardiff University, Cardiff, United Kingdom (Doutch, Hayes, Meek).

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Correspondence: David P. O’Brart, MD, FRCS, FRCOphth, Dept of Ophthalmology, St Thomas’ Hospital, Lambeth Palace Rd, London SE1 7EH, United Kingdom. Tel: 44 20718 84331; Fax: 44 20718 81611; E-mail: davidobrart@aol.com

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In an initial pre-clinical study, the necessity for complete epithelial removal was demonstrated by a lack of alteration of biomechanical properties of corneal tissue where the procedure had been performed with the epithelium intact. On this basis, the epithelium was removed prior to treatment in the first published clinical studies. Despite this recommendation, a number of clinicians have elected to perform the technique with the epithelium intact to reduce the postoperative discomfort experienced by patients. They advocate the use of multiple applications of the topical anesthetic tetracaine 1% in an attempt to loosen the epithelial tight junctions. In a recently published paper, we demonstrated that an intact basal epithelial layer appears to act as a barrier to riboflavin absorption, which is not sufficiently altered either by superficial epithelial trauma or tetracaine eye drops. Other clinicians have advocated limited full-thickness epithelial debridement in a grid-like pattern, with islands of intact epithelium to facilitate more rapid postoperative epithelial healing (Dan Z. Reinstein, personal communication, 2007). In the present study, to investigate techniques that may facilitate more rapid postoperative epithelial healing, we attempted to assist the entry of riboflavin into the stroma by either loosening the epithelial tight junctions with an application of 20% alcohol solution for 40 seconds or by removing the epithelium in a grid pattern rather than complete debridement. These techniques were compared to complete central epithelial removal and control corneas with no riboflavin administration. To assess riboflavin stromal absorption, we measured the light transmission spectra of porcine corneas after riboflavin eye drop administration and UVA exposure.

**MATERIALS AND METHODS**

Thirty-five porcine eyes were transported on ice from a local abattoir within 24 hours of sacrifice. Gross examination of each specimen for the presence of corneal scarring or opacity resulted in 8 eyes being excluded from the study. The remaining 27 eyes were stored overnight in a sealed bag at 4°C. These eyes were divided into the following treatment groups.

**Alcohol Group.** In six eyes, 20% alcohol solution was applied to the central corneal epithelium with a 9-mm laser epithelial keratomileusis well for 40 seconds. Following alcohol administration, no attempt was made to remove the epithelium. Riboflavin eye drops (10 mg riboflavin-5’-phosphate in a 10 mL dextran T-500 20% solution) were then administered to the anterior corneal surface. After waiting 5 minutes, the corneas were exposed to 3 mW/cm² UVA (370 nm) at a distance of 1 cm from the anterior surface of the cornea for 30 minutes. During exposure time, further riboflavin drops were applied at 5-minute intervals.

**Grid Pattern Epithelial Debridement.** Following a grid pattern, full-thickness epithelial trauma of the central cornea consisting of 30 to 40 separate abrasions placed within a minum of 7×7-mm sized area, riboflavin drops were applied to six eyes and UVA cross-linking treatment was performed in the same manner as described above (Fig 1).

**Complete Central Epithelial Removal.** Following complete removal of the corneal epithelium, riboflavin drops were applied to six eyes and UVA cross-linking treatment was performed in the same manner as described above (see Fig 1).
Control Group. The epithelium was completely removed from five corneas and left intact on four corneas. No riboflavin or UVA were applied to these eyes.

Immediately following treatment, each cornea with a 3-mm scleral rim was dissected from the globe and placed into a specially designed sample holder. The natural curvature of the cornea was maintained by clamping the scleral rim within the sample holder and injecting silicon oil (Dow Corning 200/5cS; BDH Laboratory Supplies, Poole, United Kingdom) into the chamber behind it. Silicon oil was also injected into the front chamber of the holder to maintain a uniform refractive index and reduce light scatter. The sample holder was then positioned into the spectrophotometer (Unicam SP8-100 UV/VIS; Pye Unicam Ltd, Cambridge, United Kingdom) in a way so that light passed through the center of the cornea in the anteroposterior direction. The optics and aperture of the unit were set to give a slit no larger than $\frac{1}{10} \text{mm}$ on the surface of the cornea (ie, at the point where the center of the cell lies), and it was ensured that the cell lay in the path length so that the beam was always directed on the center. A transmission spectrum was measured for each cornea at 10-nm intervals within the range of 400 to 700 nm. Although this spectrum is within the visible spectrum, it is outside the treatment wavelength of 350 to 380 nm. However, it includes one of the peak absorption spectra of riboflavin at 400 to 490 nm and is therefore able to detect changes in light transmission due to stromal absorption of riboflavin. Using the method detailed by Kostyuk et al., the transmission spectrum for each sample was normalized against a baseline transmission spectrum of the chamber filled with silicon oil. A further transmission spectrum over the same wavelength range (400 to 700 nm) was obtained for riboflavin solution alone.

Student $t$ tests were used to compare transmission values. Results $P<.05$ were considered statistically significant.

RESULTS

Removal of the epithelium had no significant effect on the transmission spectra of control corneas. In each case, a gradual increase in light transmission occurred between 400 and 700 nm. Based on this finding, the spectra of all control corneas (with or without epithelium) were averaged for comparison with the other groups. The transmission spectrum of corneas with application of 20% alcohol solution applied for 40 seconds and treated with riboflavin and UVA were similar to controls (Fig 2), with no significant differences among groups for wavelengths between 400 and 510 nm, corresponding to one of the absorption peaks of riboflavin (Fig 3). The transmission spectrum of corneas with grid-pattern epithelial trauma treated with riboflavin and UVA (see Fig 2) showed a dip in transmission between 400 and 490 nm ($P<.03$) compared to controls, which may be attributed to the presence of riboflavin in the stroma. This dip in transmission was less than that seen in the complete epithelial debridement group (see Fig 2), with significant differences noted in these two groups between 400 and 490 nm ($P<.001$). This is attributable to increased stromal uptake of riboflavin with complete epithelial debridement. Figure 1A shows a cornea with grid-pattern epithelial trauma following riboflavin/UVA treatment, and although areas of yellow discoloration in a grid pattern can be seen beneath the areas of full-thick-
ness epithelial trauma, there is lack of homogeneous absorption compared to full epithelial debridement as seen in Figure 1B.

DISCUSSION

Riboflavin/UVA corneal collagen cross-linking is the first therapeutic modality that may halt the progression of the ectatic process in keratoconus and ectasia after keratorefractive surgery. Riboflavin is a key component of the photochemical cross-linking treatment in that it increases corneal absorption of UVA to approximately 95% and protects the deeper ocular structures, especially the endothelium, from UVA damage.

To date, published clinical and laboratory studies of corneal collagen cross-linking therapy have advocated the complete removal of the epithelium to allow penetration of the riboflavin into the corneal stroma. However, in an attempt to reduce the early postoperative discomfort experienced by the patient (caused as a result of epithelial removal), some clinicians have elected to perform the procedure with the epithelium intact. They postulate that topical anesthetic drops can loosen epithelial tight junctions allowing penetration of riboflavin into the corneal stroma. In our previous study, however, it was suggested that the presence of an intact basal epithelial layer appears to act as an effective barrier to riboflavin absorption by the corneal stroma, which is not sufficiently altered either by superficial epithelial trauma or tetracaine eye drops. In this study, we used spectrophotometry to investigate the importance of total epithelial removal by assessing the ability of riboflavin to penetrate the stroma of corneas on which 20% alcohol solution was applied for 40 seconds or for which the epithelium was debrided in a grid pattern instead of being fully removed.

We previously demonstrated that in the immediate postoperative period, the light transmission spectrum of fully de-epithelialized porcine corneas treated with riboflavin eye drops was altered by the presence of riboflavin within the stroma, and the subsequent exposure of the cornea to UVA light did not produce any further changes to the transmission spectrum. As riboflavin decomposes in the presence of light at wavelengths below 500 nm, the acute changes in light transmission due to riboflavin absorption are short-lived and in the clinical setting, the yellow discoloration of the cornea due to riboflavin, which is clearly visible following the treatment, is clear 24 hours later.

With 20% alcohol solution applied for 40 seconds, there were no differences in transmission spectra between 400 and 510 nm compared to non-treated control corneas. This suggests that an application of 20% alcohol in the presence of an intact epithelium is not sufficient to allow adequate riboflavin penetration into the corneal stromal to alter the normal light transmission spectrum.

A grid pattern of full-thickness epithelial debridement appears to allow some riboflavin stromal penetration, indicated by a significant dip in the transmission spectrum between 400 and 490 nm compared to controls. This dip in transmission, however, was significantly less compared to that seen after complete central epithelial removal (see Fig 2). This suggests that although partial full-thickness epithelial debridement allows riboflavin stromal absorption, it
is less than with complete removal. Indeed, examination of the corneas immediately following treatment revealed that although riboflavin had penetrated the stroma immediately beneath the areas of epithelial debridement, the areas beneath intact epithelium had not been stained yellow, with the uptake of riboflavin being heterogeneous (see Fig 1). This is in contrast to corneas where the epithelium had been completely removed and the yellow discoloration was homogeneous (see Fig 1). Such considerations are important, as failure to achieve adequate stromal absorption of riboflavin is likely to limit the cross-linking process.11,14

It is important to note, however, that spectrophotometric analysis does not provide direct assessment of riboflavin stromal uptake and whereas the absence of any significant alteration of light transmission spectra suggests limited riboflavin absorption, it does not preclude small amounts of riboflavin uptake. Further studies directly measuring stromal riboflavin levels are indicated. It may also be of interest to investigate the UVA range from 350 to 380 nm where the treatment wavelength is situated, although our analysis of the visible spectrum does include one of the peak absorption spectra of riboflavin at 400 to 490 nm. It is also important to remember that in eyes with advanced keratoconus the basal epithelial layer is often broken and may behave differently in terms of its barrier functions in comparison to a normal healthy cornea,22 although riboflavin/UVA corneal collagen cross-linking is typically only performed on eyes with mild to moderate keratoconus with central corneal thickness ≥400 μm in which the basal layer is usually intact.

Analysis of the light transmission spectra of porcine corneas following riboflavin/UVA corneal cross-linking treatment suggests the need to completely remove the epithelium to allow adequate homogeneous penetration of riboflavin into the stroma.

AUTHOR CONTRIBUTIONS

Study concept and design (K.S., D.P.O., S.H., J.M., K.M.M.); data collection (K.S., D.P.O., J.D.); interpretation and analysis of data (K.S., D.P.O., J.D., J.M., K.M.M.); drafting of the manuscript (K.S., D.P.O., J.M.); critical revision of the manuscript (K.S., D.P.O., J.D., S.H., J.M., K.M.M.); statistical expertise (D.P.O.); obtained funding (D.P.O., K.M.M.); administrative, technical, or material support (K.S., D.P.O., S.H., J.M.); supervision (J.M.)

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