Endothelial cell damage after riboflavin–ultraviolet-A treatment in the rabbit

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Purpose: To evaluate the possible cytotoxic effect of combined riboflavin–ultraviolet-A (UVA) treatment on the corneal endothelium.

Setting: Department of Ophthalmology, Technical University of Dresden, Dresden, Germany.

Methods: The right eyes of 34 New Zealand White rabbits were treated with riboflavin and various endothelial UVA doses ranging from 0.16 to 0.9 J/cm^2 (0.09 to 0.5 mW/cm^2, 370 nm) and postoperative enucleation times of 4 hours and 24 hours. The endothelial cells were evaluated in histological sections. The terminal deoxynulceotidyl transferase deoxy-UTP-nick-end labeling (TUNEL) technique and transmission electron microscopy were used to detect apoptosis.

Results: There was no endothelial damage in the 6 rabbit eyes enucleated at 4 hours. In those enucleated at 24 hours, there was significant necrosis and apoptosis of endothelial cells in the corneas treated with an endothelial dose of $\geq$0.65 J/cm^2 (0.36 mW/cm^2), which is about twice the endothelial UVA dose used in the treatment of keratoconus patients.

Conclusions: In rabbit corneas with a corneal thickness less than 400 μm, the endothelial UVA dose reached a cytotoxic level of $\geq$0.65 J/cm^2 (0.36 mW/cm^2) using the standard surface UVA dose of 5.4 J/cm^2 (3 mW/cm^2). Pachymetry should be routinely performed before riboflavin–UVA treatment; in thinner corneas, irradiation should not be done because of the cytotoxic risk to the endothelium.

J Cataract Refract Surg 2003; 29:1786–1790 © 2003 ASCRS and ESCRS

Collagen cross-linking in the cornea using combined riboflavin–ultraviolet-A (UVA) treatment leads to a significant increase in mechanical stiffness, as shown in stress-strain measurements[1–3] and increased resistance to collagenases.[4] The treatment's main clinical use is in progressive keratoconus,[5] corneal ulcers,[6] and before laser in situ keratomileusis in eyes with high myopia to reduce the risk for postoperative myopic regression or iatrogenic keratectasia.

Preservation of the endothelium is crucial for every treatment involving the cornea; 400 to 800 endothelial cells/mm^2 is the minimum endothelial cell count for a clear cornea.[7] However, the risk involved in the new method of cross-linking is unknown and a major concern that must be clarified before the technique can be applied on a larger scale. In this experimental study, we evaluated the cytotoxic damage to the corneal endothelium after riboflavin–UVA treatment.

Materials and Methods

Rabbit Eyes

In the experiments, 36 right eyes of 36 female New Zealand White rabbits weighing 2.0 to 2.5 kg were used.

Accepted for publication February 13, 2003.

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None of the authors has a financial interest in any product mentioned.

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0886-3350/03/$–see front matter
doi:10.1016/S0886-3350(03)00343-2

The animals were divided into 2 groups: 7 rabbits killed 4 hours postoperatively and 29 rabbits killed 24 hours postoperatively.

In the first group, 2 rabbits were irradiated with an endothelial UVA dose of 0.65 J/cm^2 (irradiance 0.36 mW/cm^2); 1 with 0.9 J/cm^2 (0.5 mW/cm^2); 2 with 0.33 J/cm^2 (0.18 mW/cm^2), which was equivalent to the endothelial UVA intensity used in humans; and 1 with 0.16 J/cm^2 (0.09 mW/cm^2) (Table 1). These animals (n = 6) were killed 4 hours postoperatively. One additional rabbit received a corneal abrasion only and was also killed at 4 hours.

In the second group, 28 rabbits treated with riboflavin and irradiated with an endothelial UVA dose ranging from 0.16 to 0.9 J/cm^2 (irradiance 0.09 to 0.5 mW/cm^2) (ie, surface epithelial doses of 1.35, 2.7, 3.4, 4.0, 4.7, 5.4, and 7.2 J/cm^2 [irradiance 0.75, 1.5, 1.88, 2.25, 2.62, 3.0, and 4.0 mW/cm^2]) (Table 1) were killed 24 hours postoperatively. One additional rabbit received a corneal abrasion only and was also killed at 24 hours.

Treatment Procedure

General anesthesia was induced with a subcutaneous injection of a mixture of diazepam (1 ampule Faustan®) and atropine (½ ampule, 1 mg). For premedication, 1.5 mL ketamine hydrochloride 10% (Rompun®) (35 mg/kg) and 0.5 mL xylazine hydrochloride (5 mg/kg) were used. The rabbits were placed on the left side with the left eye closed; the right eye was held open with a lid holder (Figure 1). The central 5.0 mm portion of the epithelium was removed with a blunt hockey knife; 5 minutes before the irradiation, a riboflavin photosensitizer solution containing 0.1% riboflavin-5-phosphate and 20% dextranT-500 was placed on the cornea to achieve good corneal penetration of the solution; this was repeated every 5 minutes during the irradiation. The eyes were exposed to a surface UVA (370 nm) irradiance ranging from 0.75 to 4.0 mW/cm^2 (Table 1) for 30 minutes using a double UVA diode (Roithner Lasertechnik) 1.0 cm from the cornea (Figure 1). The respective endothelial UVA irradiances between 0.09 and 0.50 mW/cm^2 were calculated according to the equation $I_{depth} = I_{surface} \times e^{(-d\mu)}$, where depth ($d$) was 400 μm and the UVA-absorption coefficient (μ) was 53 cm^{-1}, determined in earlier UVA-transmission measurements following riboflavin treatment.[2] Before treatment, the desired irradiance was controlled with a calibrated UVA meter (LaserMate-Q, Laser 2000) at 1.0 cm and, if necessary, regulated with a potentiometer.

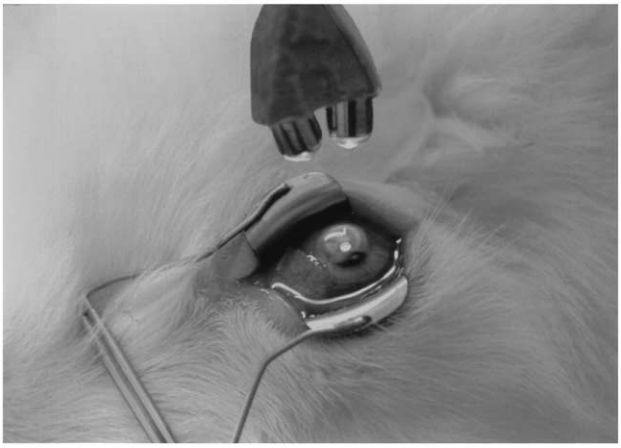

Figure 1. (Wollensak) Irradiation procedure showing a double UVA diode 1.0 cm from the riboflavin-treated rabbit cornea.

In the animals killed after 4 hours, anesthesia was maintained with supplemental injections. The other animals were killed under general anesthesia with an overdose of sodium phenobarbital 24 hours postoperatively. All animal procedures were approved by the ethics committee and conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

After enucleation, the rabbit eyes were bisected and fixed in neutral buffered 4% formalin for light microscopy

Table 1. Treatment groups, surface epithelial and endothelial UVA irradiance, and endothelial cytotoxicity in rabbits (only animals with 24 hours post-mortem).

Group	Number of Animals	Surface Epithelial UVA Irradiance (mW/cm^2)	Endothelial UVA Irradiance (mW/cm^2)	Endothelial Cytotoxicity
1	3	4.0	0.50 ± 0.07	+++
2	6	3.0	0.36 ± 0.04	+++
3	5	2.62	0.32 ± 0.04	0
4	3	2.25	0.27 ± 0.03	0
5	3	1.88	0.23 ± 0.03	0
6	5	1.5	0.18 ± 0.02	0
7	3	0.75	0.09 ± 0.01	0

and 2% glutaraldehyde for transmission electron microscopy (TEM).

For light microscopy, 4 μm paraffin sections were stained with hematoxylin and eosin. The specimens were analyzed using a Zeiss light microscope (Axiomat) at ×20 to ×1000 magnification.

For electron microscopy, small pieces of central cornea were postfixed in 4% osmium tetroxide, dehydrated, and embedded in Epon resin. Semithin sections were stained with toluidine blue. Ultrathin sections of 50 to 70 nm were contrasted with uranyl acetate and lead citrate and assessed using the Morgagni 268D electron microscope (Philips) at ×2800 magnification.

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT) deoxy-UTP-nick-end labeling for detecting apoptosis was performed essentially as described by Ihling et al.[8] Briefly, after the endogenous peroxidase was quenched, sections were incubated with TdT buffer (30 mN TRIS, 140 mM sodium cacodylate, 1 mM cobalt chloride) at pH 7.2 and incubated with 0.3 eu/mL TdT (Sigma) and biotinylated-dUTP (1:200; Boehringer) in TdT buffer for 60 minutes at 37°C. Labeled nuclei were detected with Vectastain ABC (Vector Labs), and peroxidase activity was visualized by 3-amino-9-ethylcarbazole to yield a reddish-brown reaction product. The sections were lightly counterstained with hematoxylin. As a positive control, tissue sections of follicular hyperplasia of the appendix were used. Negative control slides were also included.

Pachymetry

The central corneal thickness of the rabbit corneas was determined preoperatively using an ultrasound pachymeter (Pachette, Technomed).

Results

Histology

In the 6 treated animals killed at 4 hours and the 2 cases with corneal epithelial debridement alone, neither TUNEL-positive endothelial cells nor loss of endothelium was observed.

In the animals killed at 24 hours, significant endothelial cell necrosis with complete loss of endothelial cells (Figure 2) and a few remaining apoptotic endothelial cells were observed in the treatment area in the eyes that had an endothelial UVA dose of 0.9 J/cm^2 (0.5 mW/cm^2) and of 0.65 J/cm^2 (0.36 mW/cm^2), which was twice as high as the therapeutic endothelial dose in humans of 0.32 J/cm^2 (0.18 mW/cm^2) (Table 1).

Figure 2. (Wollensak) Complete endothelial cell loss in the treatment zone with sharp demarcation toward untreated area (arrow) (endothelial UVA dose of 0.9 J/cm^2).

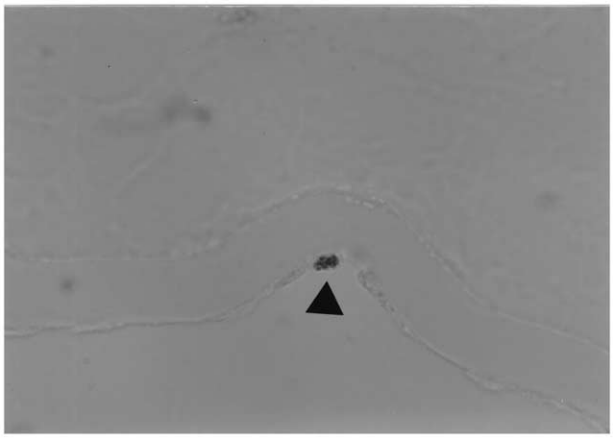

Figure 3. (Wollensak) TUNEL-positive apoptotic endothelial cell (arrow) (endothelial UVA dose of 0.65 J/cm^2, original magnification ×100, oil immersion).

In the animals exposed to an endothelial UVA dose of 0.16 to 0.58 J/cm^2 (0.09 to 0.32 mW/cm^2), the endothelial cells were intact.

TUNNEL Assay and TEM

Using the TUNEL-straining, the few remaining endothelial cells in the 2 groups (0.9 J/cm^2 and 0.65 J/cm^2) were TUNEL-positive, showing brown nuclear staining (Figure 3).

On TEM, apoptotic endothelial cells showed typical features of apoptosis-like chromatin condensation, formation of apoptotic bodies, and cell shrinkage (Figure 4).

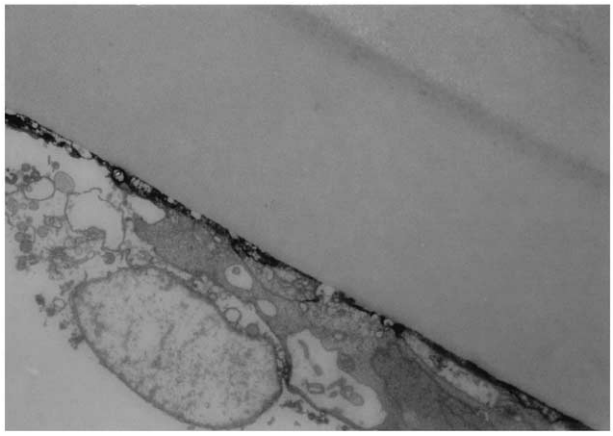

Figure 4. (Wollensak) Apoptotic endothelial cell with apoptotic bodies and chromatin condensation (TEM, original magnification ×2800) (endothelial UVA dose of 0.65 J/cm^2).

Pachymetry

The mean central corneal thickness was 400 μm ± 20 (SD).

Discussion

This study showed a specific threshold-like cytotoxic effect of combined riboflavin–UVA treatment on corneal endothelium starting at an endothelial UVA dose of 0.65 J/cm^2 (0.36 mW/cm^2). Using the absorption coefficient of 53 cm^{-1}, which we found in earlier UVA-transmission measurements following riboflavin treatment,[2] it can be calculated that in human corneas thinner than 400 μm, the cytotoxic endothelial UVA irradiance of 0.36 mW/cm^2 is reached using the standard surface irradiance of 3.0 mW/cm^2.

Fortunately, the cytotoxic threshold is not reached in normal corneas with an average corneal thickness of 540 μm or in most keratoconus patients (410 to 470 μm).[9] However, in corneal ulcers and advanced keratoconus, the corneal thinning may be beyond this limit. In such cases, riboflavin–UVA treatment should be avoided and alternative methods such as amnion transplantation for ulcers or keratoplasty for keratoconus considered. Alternatively, in thinner corneas with at least 350 μm corneal thickness, a reduced surface UVA dose of 3.6 J/cm^2 (2 mW/cm^2), which is the lowest dose to produce a significant mechanical stiffening effect[1] and increase resistance to enzymatic digestion,[4] might be tried cautiously; the endothelial UVA dose would then be only 0.54 J/cm^2 (0.3 mW/cm^2). In addition, in keratoconus patients with extreme thinning in a small area only, the treatment might be used because the endothelial cell loss in a small circumscribed area might be compensated for by the migration of adjacent undamaged endothelial cells.

The observed cytotoxic effect on the endothelium appeared to be due to the combined effect of UVA and the photosensitizer riboflavin, which increases UVA absorption in the cornea to 95%[2] compared to 32% without riboflavin.[10] From in vivo irradiation experiments in animal eyes, it is known that UVA irradiation (320 to 400 nm) alone can induce endothelial cell damage only after a relatively high surface UVA dose of 42.5 J/cm^2 (5.4 J/cm^2 in our experiment).[11,12] In addition, in cell culture experiments with combined riboflavin/light treatment, a significant cytotoxic effect on bovine corneal endothelial cells was observed after application of a minimum riboflavin solution of 50 μM and 2 hours of white-light irradiation; riboflavin or white light alone produced no damage in the cells.[7] Endothelial damage was also reported in cases with solar keratitis[13] with a surface ultraviolet-B (UVB) dose between 0.12 and 0.56 J/cm^2.[14] In rabbit corneas exposed to 310 nm UVB with 0.47 J/cm^2, TUNEL-positive endothelial cells were found 24 hours later.[15] The extensive damage after exposure to UVB is explained by the shorter UVB wavelength, with a correspondingly higher energy content.

In the animals killed at 4 hours, no apoptotic endothelial cells were observed, whereas maximum cytotoxic damage was found at 24 hours, as described after UVA irradiation; this so-called delayed type of apoptosis[15–18] is in contrast to the immediate type of apoptosis observed in keratocytes after epithelial scraping.[19,20]

In conclusion, this study demonstrated that combined riboflavin–UVA treatment should be safe for the endothelium as long as the dose is less than the endothelial cytotoxic dose of 0.65 J/cm^2. In human corneas, the endothelial cytotoxic UVA dose is only reached in corneas thinner than 400 μm. Therefore, pachymetry measurements should be performed routinely before riboflavin/UVA treatment to identify unsuitable cases.

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