

# Keratocyte Apoptosis After Corneal Collagen Cross-linking Using Riboflavin/UVA Treatment

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## Abstract

### Purpose:

Combined riboflavin/UVA treatment inducing collagen cross-links in the cornea has been shown to increase the biomechanical rigidity of the cornea and has been used successfully in the treatment of progressive keratoconus. The current study was undertaken to investigate the possible cytotoxic effect of combined riboflavin/UVA treatment on corneal keratocytes in vivo.

### Methods:

Thirty-four New Zealand white rabbits were treated with 0.1% riboflavin solution and surface UVA irradiances ranging from 0.75 to 4 mW/cm<sup>2</sup> (1.35–7.2 J/cm<sup>2</sup>) for 30 minutes. The animals were euthanized either 4 (n = 6) or 24 (n = 28) hours postoperatively. Four additional control eyes underwent epithelial debridement alone. The corneas of the enucleated eyes were evaluated in routine histologic sections. In addition, the TUNEL technique and transmission electron microscopy were used for the detection of keratocyte apoptosis.

### Results:

In the control eyes with corneal epithelial debridement only, apoptotic keratocytes were found in the anterior 50 μm of the corneal stroma 4 hours postoperatively. However, riboflavin/UVA-induced apoptosis was only visible in the rabbit eyes enucleated 24 hours postoperatively. In these eyes, we found apoptosis of keratocytes down to a variable stromal depth depending on the applied UVA irradiance. A cytotoxic UVA irradiance for keratocytes in the range of 0.5–0.7 mW/cm<sup>2</sup> could be deduced.

### Conclusions:

Riboflavin/UVA treatment leads to a dose-dependent keratocyte damage that can be expected in human corneas down to a depth of 300 μm using a surface UVA dose of 5.4 J/cm<sup>2</sup>. Future studies should be done to examine the keratocyte repopulation and exclude possible adverse sequelae of keratocyte loss like stromal scarring or thinning.

**Key Words:** UVA radiation, cross-linking, keratocytes, riboflavin, cornea, apoptosis

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Collagen cross-linking in the cornea using UVA and the photosensitizer riboflavin was developed by us recently for biomechanically strengthening the cornea. In a prospective clinical pilot study including 22 patients with progressive keratoconus and a follow-up of up to 4 years, the progression of keratoconus could be stopped in all treated eyes. Even regression with a reduction of the maximal keratometry readings by 2 diopters was achieved in 70% of patients. Corneal and lens transparency as well as endothelial cell density remained unchanged.<sup>1</sup> In stress-strain measurements of human, rabbit, and porcine cornea,<sup>2–4</sup> we could show a significantly increased biomechanical rigidity. After exposure to collagenases that play a role in both keratoconus and corneal ulceration, a significant delay in enzymatically induced tissue dissolution by 8 days was found in cross-linked corneas indicating an increased resistance to enzymatic digestion.<sup>5</sup> Other applications of the new cross-linking method lie in the treatment of corneal melting processes<sup>6</sup> and in the field of refractive surgery like prevention of postoperative keratectasia after LASIK for high myopes or regression of the refractive effect.<sup>7</sup>

As part of an extensive safety evaluation of the new treatment, we have undertaken the following experimental study to assess the possible damage to the keratocytes after combined riboflavin/UVA treatment.

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## MATERIAL AND METHODS

### Study Design

Thirty-eight eyes of 38 female New Zealand white rabbits weighing 2–2.5 kg were used. Thirty-four eyes of 34 rabbits were treated with central epithelial abrasion, riboflavin, and varying UVA irradiances (see below). Four control eyes in 4 rabbits were treated with corneal abrasion alone. The animals were divided into 2 subsets depending on the postoperative time interval of either 4 or 24 hours, respectively:

1. Six rabbits were irradiated with a surface UVA irradiance ranging from 0.75 to 4 mW/cm<sup>2</sup> (0.75, 1.5, 3, and 4 mW/cm<sup>2</sup>) (Table 1). These animals were all euthanized 4 hours postoperatively. Two additional rabbits underwent corneal epithelial debridement alone and were euthanized 4 hours postoperatively as well (n = 8).
2. Twenty-eight rabbits treated with riboflavin and irradiated with a surface UVA irradiance ranging from 0.75 to 4 mW/cm<sup>2</sup> (0.75, 1.5, 1.88, 2.25, 2.62, 3, and 4 mW/cm<sup>2</sup>) (Table 1) were euthanized 24 hours postoperatively. Two additional rabbits underwent corneal epithelial debridement alone and were euthanized 24 hours postoperatively as well (n = 30).

### Cross-linking Treatment

General anesthesia was performed with a subcutaneous injection of a mixture of diazepam and atropine (1 mg). For premedication, 1.5 mL ketamine hydrochloride 10% (35

mg/kg) and 0.5 mL xylazine hydrochloride (5 mg/kg) were used. In each rabbit, only the right eye was exposed to UV radiant energy. A lid speculum was placed. After removal of the central 5-mm portion of the epithelium using a blunt crescent knife, riboflavin (vitamin B<sub>2</sub>) photosensitizer solution containing 0.1% riboflavin-5-phosphate and 20% dextran T-500 was dropped onto the cornea 5 minutes before the irradiation to achieve good corneal penetration of the solution and every 5 minutes during the irradiation. The eyes were irradiated with UVA (370 nm) for 30 minutes using a double UVA diode (Roithner Lasertechnik, Vienna, Austria) at a distance of 1 cm from the cornea. Three 1.3-V accumulators were used as a power generator. Before the treatment, the desired surface irradiance in the range from 0.75 to 4 mW/cm<sup>2</sup> (Table 1) was controlled with a calibrated UVA meter (LaserMate-Q, LASER 2000, Wessling, Germany) at a 1-cm distance and if necessary regulated with a potentiometer. The animals were euthanized 4 or 24 hours postoperatively with general anesthesia using an overdose of intravenous sodium phenobarbital. All animal procedures were approved by the ethics committee and conformed to the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

### Tissue Preparation

The rabbit eyes enucleated 4 or 24 hours postoperatively were bisected. One half was fixed in 4% neutral-buffered formalin for light microscopy and the other half was

TABLE 1. Depth of Keratocyte Loss in Relation to UVA Irradiance

Group	n	Surface UVA Irradiance (mW/cm <sup>2</sup> )	Depth of Keratocyte Loss (μm)	Calculated Cytotoxic UVA-Irradiance (mW/cm <sup>2</sup> )
1	3	4	350	0.63
2	6	3	300	0.61
3	5	2.62	260	0.66
4	3	2.25	200	0.77
5	3	1.88	170	0.76
6	5	1.5	130	0.75
7	3	0.75	80	0.49

immersed in 2% glutaraldehyde for transmission electron microscopy (TEM). For light microscopy, 4- $\mu\text{m}$  thin paraffin sections were stained with hematoxylin-eosin and periodic acid-Schiff (PAS). The specimens were evaluated using a Zeiss light microscope (Axioskop) at 40- to 1000- fold magnification. The depth of the damaged zone with loss of keratocytes, and few remaining apoptotic keratocytes was measured using a special morphometry reticule

For transmission electron microscopy, small pieces of the central cornea were post-fixed in 4% osmium tetroxide, dehydrated, and embedded in Epon resin. After observation of the semithin sections stained with toluidine blue, 50–70-nm ultrathin sections were prepared, mounted on copper grids, contrasted with uranyl acetate and lead citrate, and assessed using the Morgagni 268D electron microscope (Philips) at 3500–34,000 $\times$  magnification.

### TUNEL Assay

To detect apoptosis with DNA strand breaks in situ, terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL) was performed essentially as described previously.<sup>8</sup> In brief, after the quenching of endogenous peroxidase, sections were incubated with TdT buffer (30 mM Tris, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) at pH 7.2 and incubated with 0.3 u/mL TdT (Sigma, München, Germany) and biotinylated-dUTP (1:200; Boehringer, Mannheim, Germany) in TdT buffer for 60 minutes at 37°C. Labeled nuclei were detected with Vectastain ABC (Vector Labs, Burlingame, CA) and peroxidase activity was visualized by 3-amino-9-ethyl-carbazole (AEC) 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 and gave the expected positive staining of tingibile bodies in the germinal centers.

### Calculation of Cytotoxic UVA Irradiances and Doses

The cytotoxic UVA irradiance was determined by correlating the depth of the keratocyte loss with the corresponding UVA irradiances reached at the specific stromal depth that were calculated according to the equation  $I_{\text{depth}} = I_{\text{surface}} \cdot e^{(-\mu x)}$  (Lambert-Beer law) using the absorption coefficient  $\mu$  of 53  $\text{cm}^{-1}$  that was found by us in earlier UVA transmission measurements following riboflavin treatment (Table 1).<sup>4,9</sup> Irradiation doses ( $\text{J}/\text{cm}^2$ ) were calculated from the UVA irradiances ( $\text{mW}/\text{cm}^2$ ) by multiplying the value with the irradiation time in seconds ( $30 \times 60$ ).

## RESULTS

### Light Microscopy and TUNEL Assay

In the 6 cross-linked corneas, all euthanized 4 hours postoperatively and the 4 control eyes with corneal abrasion alone, euthanized 4 or 24 hours postoperatively, only scattered apoptotic keratocytes were present down to a depth of 50  $\mu\text{m}$  in the anterior stroma as shown by light microscopy in the TUNEL-stained sections and on transmission electron microscopy.

In the 28 cross-linked corneas, all euthanized 24 hours postoperatively, a massive loss of keratocytes with an almost acellular zone and some remaining apoptotic, TUNEL-positive, or completely destroyed necrotic cells were observed in variable depths of the corneal stroma correlating with an increasing surface UVA irradiance (Figs. 1, 2A-C, 3A; Table 1). All these changes were only present in the treated area with a sharp transition zone toward the normal-appearing adjacent tissue without treatment (Fig. 2C). Some regenerating thinned epithelium was present over the edges of the lesion as some epithelial migration had already taken place in the deepithelialized treatment zone within 24 hours (Fig. 2C).

### Transmission Electron Microscopy

Irradiated apoptotic keratocytes revealed apoptotic changes like formation of

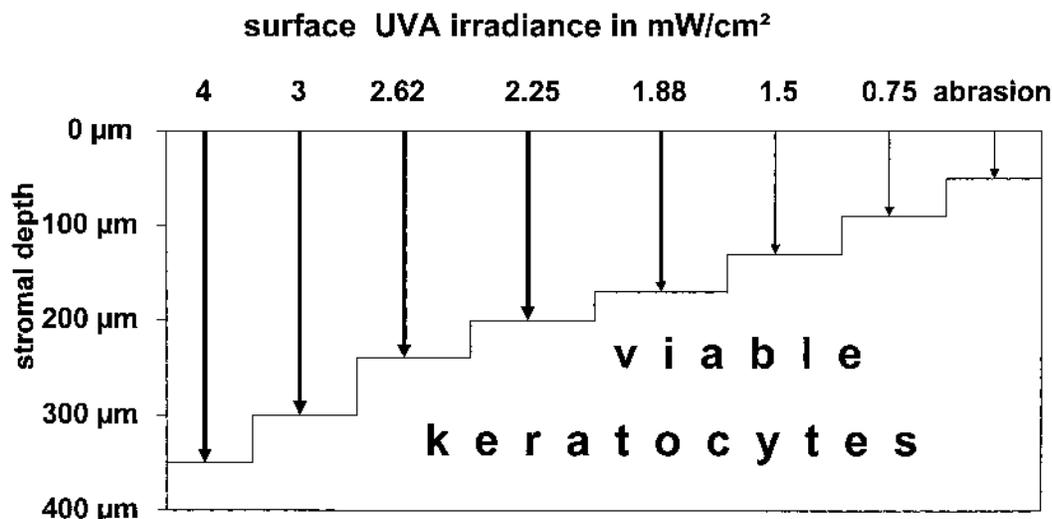


FIGURE 1. Scheme illustrates the depth of keratocyte loss depending on surface UVA irradiance.

apoptotic bodies, chromatin condensation, and cell shrinkage (Figs. 3B,C).

### Cytotoxicity

The cytotoxicity level for keratocytes was calculated for the various stromal depths according to the Lambert-Beer law (see above) and found to be in the range of 0.49–0.77 mW/cm<sup>2</sup> irradiance corresponding to UVA doses of 0.86–1.39 J/cm<sup>2</sup> (Table 1).

### DISCUSSION

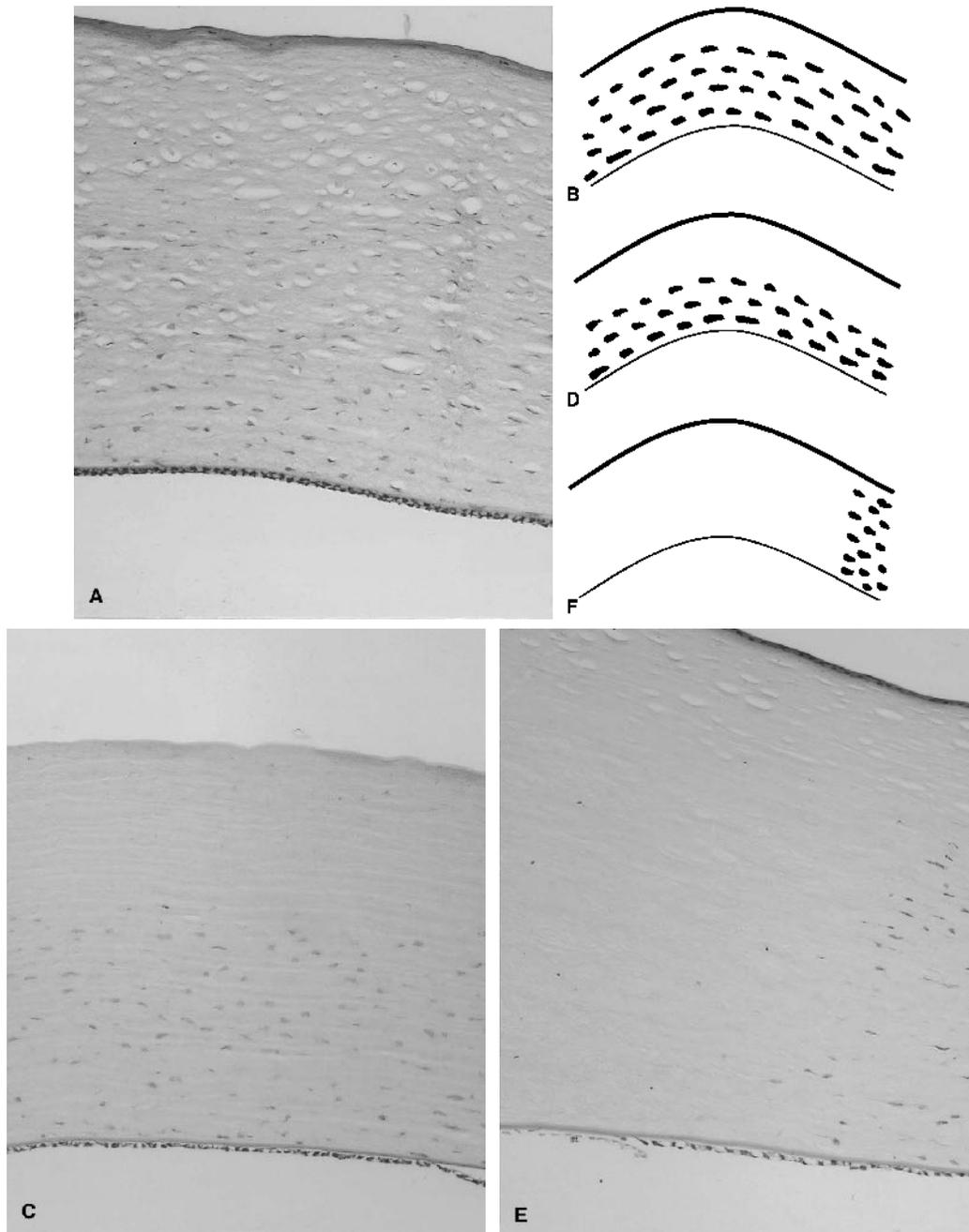
The current study has shown significant keratocyte damage after combined riboflavin/UVA cross-linking treatment correlating closely with the level of UVA irradiance (Figs. 1 and 2; Table 1). Using the Lambert-Beer law and the absorption coefficient of 53 cm<sup>-1</sup> for the UVA irradiation in riboflavin-treated cornea,<sup>4,9</sup> we were able to deduce a cytotoxic irradiance threshold for keratocytes in the range of 0.5–0.7 mW/cm<sup>2</sup> from the depth of the keratocyte loss and the corresponding irradiance levels.

In the eyes of the irradiated animals that had been euthanized 4 hours postoperatively and in the deepithelialized control eyes, keratocyte apoptosis was located only in the anterior 50 μm, whereas in the irradiated ani-

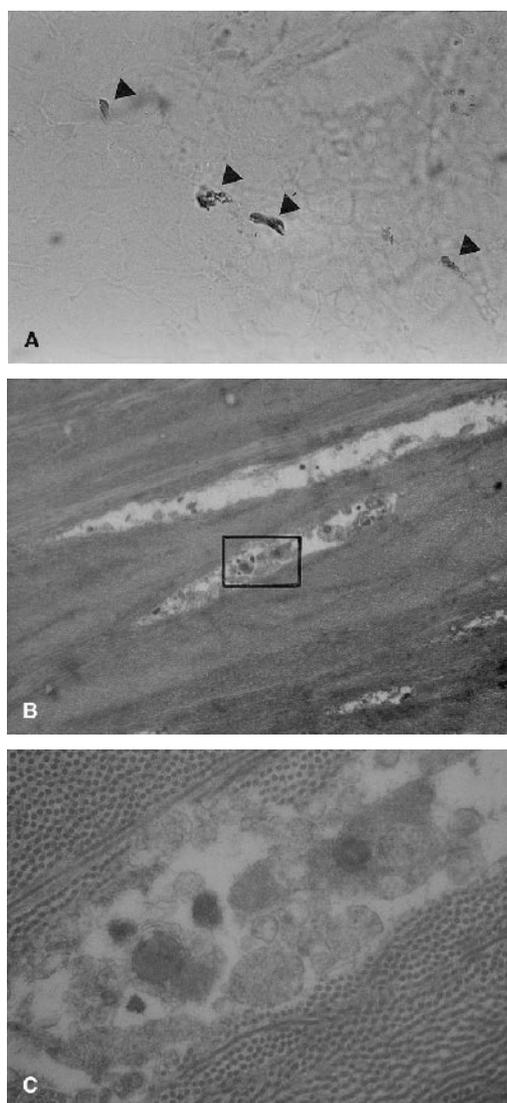
mals euthanized 24 hours postoperatively, extended and deep keratocyte loss and apoptosis was found. Obviously, in the cases euthanized 4 hours postoperatively, keratocyte apoptosis was of the immediate type<sup>10,11</sup> and only due to epithelial scraping, as has been described by others after corneal deepithelialization.<sup>12,13</sup> Accordingly, in the eyes enucleated 24 hours postoperatively, a more delayed type of apoptosis was found that was due to UVA.<sup>10,11</sup> Riboflavin by itself is not cytotoxic, but as a photosensitizer, it increases the absorption of UVA, which induces the cellular damage.<sup>14</sup>

Similar UVA-induced keratocyte damage has been reported after UVA irradiation without a photosensitizing agent. Using pigmented rabbits, Pitts et al<sup>15</sup> found a corneal damage threshold at the surface UVA dose (365 nm) of 42.5 J/cm<sup>2</sup>. Our relatively low cytotoxic UVA surface dose in the range of 1.35–7.2 J/cm<sup>2</sup> can be explained by the multiplying effect on the UVA absorption by riboflavin.<sup>14</sup> UVB-induced keratocyte loss can be found already at lower dose levels because of the shorter wavelength of UVB with a correspondingly higher energy content.

Histologically, keratocyte damage was observed, especially in the anterior one-fourth of the albino rabbit cornea after expo-



**FIGURE 2.** Light micrograph **(A)** and schematic diagram **(B)** of keratocyte loss and apoptosis down to 100  $\mu\text{m}$  24 hours after irradiation with surface UVA irradiance of 0.75  $\text{mW}/\text{cm}^2$  (hematoxylin and eosin,  $\times 200$ ). Light micrograph **(C)** and schematic diagram **(D)** of keratocyte loss and apoptosis down to 170  $\mu\text{m}$  24 hours after irradiation with surface UVA irradiance of 1.88  $\text{mW}/\text{cm}^2$  (hematoxylin and eosin,  $\times 200$ ). Light micrograph **(E)** and schematic diagram **(F)** of transition from the irradiated area with a total keratocyte loss across the entire stroma 24 hours after irradiation with surface UVA irradiance of 4  $\text{mW}/\text{cm}^2$  (hematoxylin and eosin,  $\times 200$ ) to the adjacent normal cornea. Thinned healing epithelium migrating from the right side.



**FIGURE 3. A:** TUNEL-positive apoptotic keratocytes (arrowheads) in the posterior half of the cornea 24 hours after irradiation with surface UVA irradiance of 4 mW/cm<sup>2</sup> (oil immersion,  $\times 1000$ ). **B:** Apoptotic cell bodies, chromatin condensation, and cell shrinkage in the central keratocyte 24 hours after irradiation surface UVA irradiance of 4 mW/cm<sup>2</sup> (TEM, uranyl acetate and lead citrate,  $\times 3500$ ). The frame delineates the portion that is shown magnified in **C**. **C:** Higher magnification of the apoptotic keratocyte illustrates apoptotic cell bodies (TEM, uranyl acetate and lead citrate,  $\times 34,000$ ).

sure to 290–350 nm UVB irradiation over 15 minutes.<sup>16</sup> After UVB irradiation of 0.12 J/cm<sup>2</sup> at 280 nm and 0.47 J/cm<sup>2</sup> at 310 nm, other authors have reported extended UV-induced keratocyte apoptosis through the en-

tire thickness of the cornea 24 hours after the treatment.<sup>17,18</sup>

The clinical importance of massive keratocyte loss is not quite clear because the keratocyte loss can usually be repaired by repopulation from migrating keratocytes of the adjacent cornea.<sup>12,19</sup> In other treatment modalities like corneal abrasion,<sup>20–22</sup> PRK,<sup>12,19</sup> LASIK,<sup>12,19,23</sup> and epikeratophakia<sup>24</sup> or diseases like keratoconus<sup>25</sup> and herpes,<sup>26</sup> keratocyte loss of variable extension has been reported in recent years having a possible influence on scarring, haze,<sup>27</sup> or corneal thinning.<sup>20,25</sup> However, in our clinical study, we have not observed any change in transparency or corneal thickness after the cross-linking treatment.<sup>1</sup> Application of the apoptosis inhibitor zinc chloride is not helpful<sup>28</sup> because then the wanted cross-linking effect would also be weakened.<sup>14</sup>

In conclusion, we have shown that combined riboflavin/UVA cross-linking treatment leads to a dose-dependent keratocyte apoptosis at 0.86–1.39 J/cm<sup>2</sup> and can be expected in human corneas of 500- $\mu$ m thickness down to a depth of 300  $\mu$ m using the usual 3 mW/cm<sup>2</sup> surface irradiance (5.4 J/cm<sup>2</sup> surface dose). A clinical long-term study using confocal in vivo microscopy<sup>29</sup> after riboflavin/UVA treatment is currently underway to evaluate the depth of the keratocyte loss and the repopulation process and to exclude the development of treatment-related stromal scarring, haze, and thinning in humans.

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