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Corneal Endothelial Cytotoxicity of Riboflavin/UVA Treatment in vitro

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Cell culture · Crosslinking · Cytotoxicity · Endothelium · Riboflavin · UV treatment

Abstract
Recently, we have developed collagen crosslinking induced by combined riboflavin/UVA treatment, thus increasing the biomechanical rigidity of the cornea to treat progressive keratoconus. The present safety study was performed to evaluate possible cytotoxic effects of combined riboflavin/UVA treatment on the corneal endothelium in vitro. Endothelial cell cultures from porcine corneas were treated with 500 μ*M* riboflavin solution, exposed to various endothelial UVA irradiances (370 nm) ranging from 0.1 to 1.6 mW/cm^2 for 30 min and evaluated 24 h later using trypan blue staining and Yopro fluorescence staining. The effect of either treatment alone (UVA irradiation ranging from 0.2 to 6 mW/cm^2) was also tested. An abrupt cytotoxic threshold irradiance level was found at 0.35 mW/cm^2 after combined treatment with riboflavin plus UVA irradiation and at 4 mW/cm^2 with UVA irradiation alone. Riboflavin alone was not toxic. A cytotoxic effect of the combined riboflavin/UVA treatment on corneal endothelial cells is to be expected with a corneal thickness of less than 400 μm. Therefore, pachymetry should be routinely performed before riboflavin/UVA treatment to exclude patients at risk.

Introduction

Collagen-crosslinking in the cornea using UVA and the photosensitizer riboflavin has been developed by us recently as a novel method leading to a significant increase in the mechanical stiffness of the cornea, as demonstrated in biomechanical stress-strain measurements [1–3], and to an increased resistance to collagenases [4]. Its main clinical application lies in the treatment of progressive keratoconus. In a prospective clinical pilot study including 22 patients with moderate or advanced progressive keratoconus followed up for up to 4 years, the progression of keratoconus could be stopped in all treated eyes; in 70% of the patients, even regression was achieved, with a reduction in the maximal keratometry readings by 2 diopters [5]. In addition, the method can be applied in corneal melting processes [6] and in corneal refractive laser surgery to prevent iatrogenic keratectasia [7].

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In the present study, we investigated the risk involved for the corneal endothelium as part of a comprehensive safety evaluation of the new treatment modality. To this end, we determined in vitro the endothelial cytotoxicity of combined riboflavin/UVA treatment and of either treatment alone.

Materials and Methods

Materials

All primary cultures and serial passaging were carried out in growth media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma, Deisenhofen, Germany) and antibiotics (penicillin-streptomycin). Trypan blue solution (Biochrome, Berlin, Germany) [8] and Yopro (Molecular Probes, Eugene, Oreg., USA) fluorescent stain [9] were used for the viability assay.

Culture Conditions

Porcine eyes were obtained from a local slaughterhouse 3–5 h postmortem. The entire corneoscleral ring was cut out and placed into a concave container used for the storage of contact lenses. A 0.05% trypsin-0.02% EDTA solution was applied onto the endothelium for 5 min to dissociate the cells. The possible contact of trypsin with the cut stromal or scleral surface was carefully avoided to prevent collecting non-endothelial cells. During the last minute, the endothelial cells were mobilized using a glass spatula [8, 10, 11]. Finally, the solution containing suspended endothelial cells was pipetted and transferred to 25-cm^2 cell culture flasks (Nunc, Wiesbaden, Germany) filled with 2 ml DMEM where the digestive activity of trypsin was quenched by 10% fetal calf serum. The primary cultures were placed in a cell culture oven at 37°C and gassed with 6% carbon dioxide. Cell growth was evaluated every other day using an inverted phase contrast microscope (Zeiss Axiovert L5). The media were exchanged every week. Confluence with about 2.5×10^6 cells per flask was reached after 2–3 weeks (fig. 1). For passaging, the confluent stock cultures were dissociated and detached using a 0.05% trypsin-0.02% EDTA solution. The free floating cells were centrifuged at 230 *g*, again transferred to culture flasks and suspended in the cell culture medium. Passaging was performed every 2nd week at a split ratio of 1:3.

For the irradiation, the cells were dissociated, centrifuged, resuspended and finally transferred to 8-well tissue plates at a concentration of 5×10^4 cells/well, where they reached confluence after 8–10 days.

Treatment Groups

We divided the endothelial cells into three different treatment groups: (1) treatment with riboflavin alone; (2) treatment with riboflavin plus UVA, and (3) treatment with UVA alone.

In the cells treated with riboflavin alone, we tested the cytotoxicity of 25, 50, 100 and 500 μ*M* riboflavin without additional UVA irradiation. In the cells treated with riboflavin and UVA, the riboflavin concentration was chosen as close as possible to the condition in the real treatment of human corneas, and it was calculated as follows. In humans, 0.1% riboflavin solution is dropped onto the corneal surface. Using the diffusion equation and the diffusion coefficient [12] of the related dye fluorescein ($D = 6.5 \times 10^{-7}$ cm^2/s), the average riboflavin concentration over 30 min at the human corneal endothelium was 0.024%. Therefore, we used 0.025% riboflavin solution (500 μ*M*) by adding 57 μl 0.2% riboflavin stock solution to 400 μl colorless culture medium without phenol red. Others have previously found cytotoxicity using a 100-μ*M* riboflavin solution plus white light [13], and we found the minimum riboflavin concentration necessary for cell damage after UVA treatment to be 50 μ*M* [unpubl. data]. The riboflavin solution for the UVA irradiation was added to the wells 5 min before the irradiation. The UVA irradiances ranged from 0.3 to 1.6 mW/cm^2 (table 1).

Cells treated with UVA alone were irradiated with various UVA irradiances ranging from 0.2 to 6 mW/cm^2 to exclude the multiplying effect through the photosensitizer riboflavin (table 1).

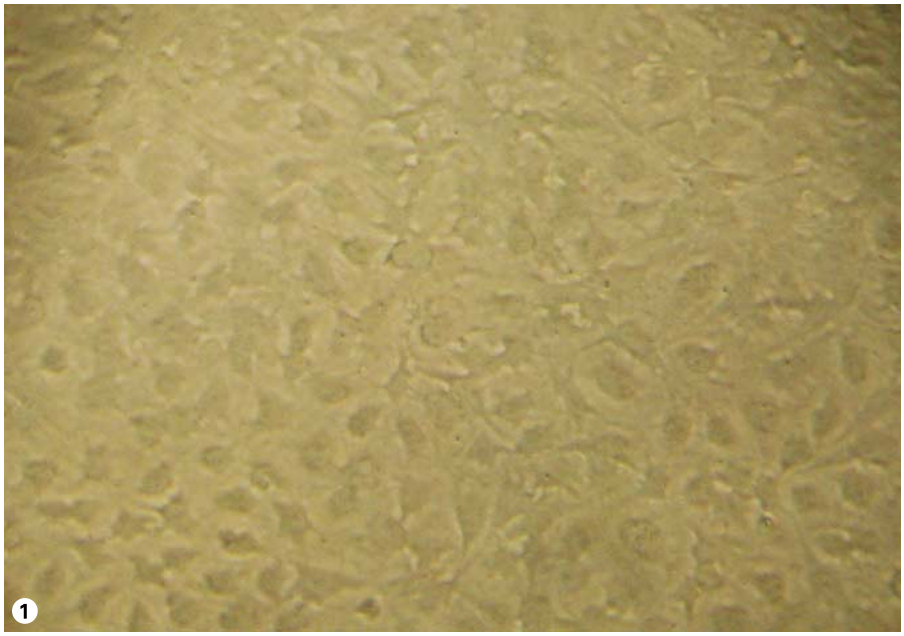
Irradiation Procedure

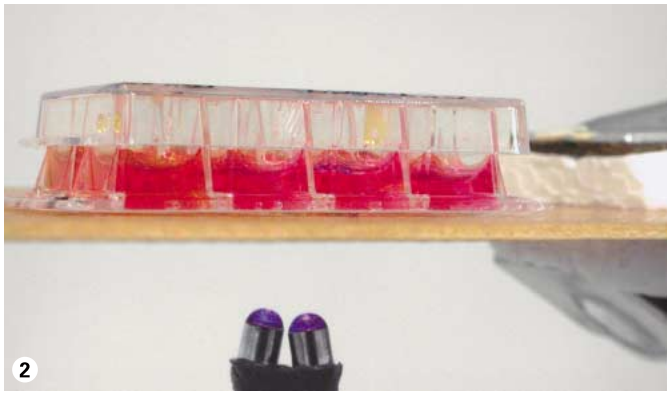
To avoid UVA absorption by the riboflavin solution overlying the monolayer of endothelial cells attached to the floor of the wells, we irradiated the cells from underneath the tissue plate. The UVA absorption by the 100-μm-thin floor of the wells made of borsilicone was measured to be 2% only and is therefore negligible. One UVA double diode (370 nm) was fixed 1 cm under the respective well to be irradiated (fig. 2) using a stand. Before the treatment, to attain UVA irradiances ranging from 0.2 to 6 mW/cm^2, UVA irradiances were controlled with a UVA meter (LaserMate-Q, LASER 2000, Wessling, Germany) at 1 cm distance and if necessary regulated with a potentiometer in series. The actual irradiation lasted 30 min, which is con-

Table 1. Endothelial cytotoxicity of riboflavin combined with UVA and of UVA treatment alone

Irradiance level mW/cm^2	Riboflavin + UVA	UVA
6		+
5		+
4		+[a]
3.5		–
3		–
2		–
1.6	+	
1.2	+	
1.1	+	
1.0	+	–
0.8		–
0.6	+	–
0.55	+	
0.5	+	–
0.45	+	
0.4	+	–
0.35	+[a]	
0.3	–	–
0.2		–

[a] Threshold values.





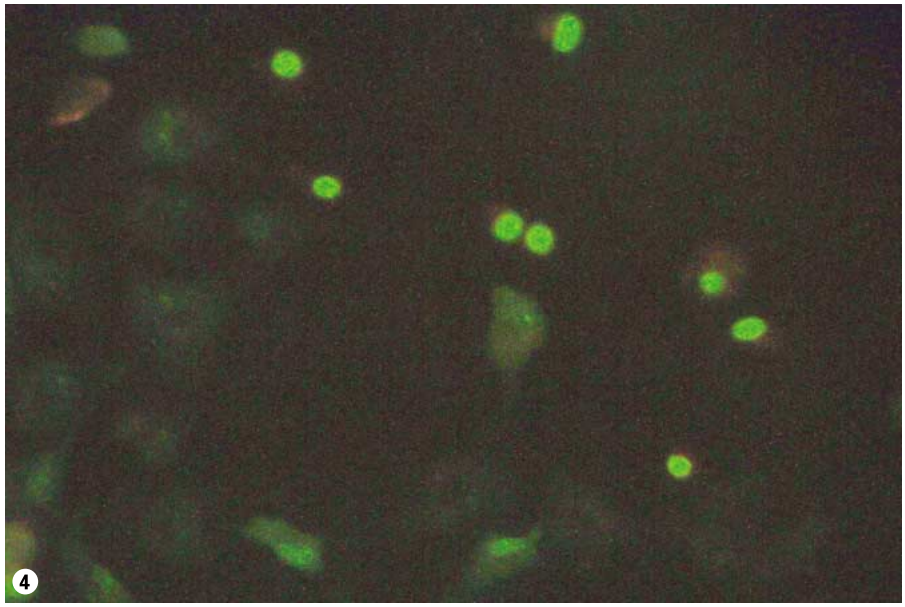


Fig. 1. Representative morphology of the porcine corneal endothelial cells cultured at confluence (phase contrast, ×100).
Fig. 2. Irradiation procedure with double UVA diode under the culture well plate.
Fig. 3. Sector of the circular treatment area (0.35 mW/cm² UVA plus riboflavin) with trypan-blue-stained nuclei of the damaged endothelial cells and loss of cells on the right (trypan blue, ×400).
Fig. 4. Sector of the circular treatment area (0.35 mW/cm² UVA plus riboflavin) with Yopro-stained nuclei of the damaged endothelial cells on the right (Yopro, ×400).

form to the clinical setting. Irradiation doses (J/cm^2) were calculated from the UVA irradiances (mW/cm^2) by multiplying the value with the irradiation time in seconds (= 30 × 60).

Following the irradiation, the riboflavin solution was discarded and replaced by the cell medium.

Supravital Staining

24 h after treatment, supravital staining of the cells was performed to determine the viability of the endothelial cells. To determine possible cell damage 100 µl of 0.25% trypan blue solution dissolved in colorless culture medium was applied per well for 15 min followed by 2-fold rinsing with colorless culture medium. After microscopic evaluation of the trypan blue staining [8], the cells were stained with Yopro [9] adding 1 µl/well followed by one rinse with culture medium. The trypan blue stains were evaluated with the help of an inverse microscope (Leica DMIR) at 40- to 400-fold magnification using differential interference contrast and the green fluorescent Yopro stain using an additional fluorescence filter at 488 nm (N2.1 filter). Photos were taken with a digital camera attached to the microscope (Nikon coolpix 950). The nuclei of the damaged cells were labeled with the stains.

Results

Cellular Characteristics

Before confluence, the scattered cells were spindle shaped and often formed ring-like configurations. After confluence, they appeared round and epithelial like (fig. 1). About 10% of the cells contained some brown-colored, autofluorescent granules typical of lipofuscein deposits. After cytotoxic doses, the damaged cell nuclei stained positively with trypan blue (fig. 3) and the green-fluorescent Yopro (fig. 4). Dead cells became globular in shape before losing adhesion and some of the damaged cells were floating off the floor of the wells. Due to the round shape of the UVA source, the damaged cells were arranged in a circular pattern like a direct imprint of the irradiation cone, with damaged cells in the inner circle of about 40 mm^2 and a sharp demarcation towards the unirradiated surviving cells in the periphery (fig. 3, 4).

Cytotoxicity

The cells treated with riboflavin only were not damaged, and their nuclei were not labeled by the stains. The cells treated with UVA irradiation plus riboflavin showed an abrupt and massive threshold-like damage at ≥0.35 mW/cm^2 (table 1, fig. 3, 4), corresponding to an irradiation dose of 0.63 J/cm^2. At these cytotoxic levels, over 98% of the cells in the irradiation area stained positively for both trypan blue and Yopro in their nuclei, whereas at the next lower irradiation level there were no necrotic cells at all. Therefore, statistical analysis and cell counts were not useful. Similarly, endothelial cells irradiated with UVA alone revealed an abrupt cytotoxic threshold irradiance at ≥4 mW/cm^2, corresponding to a dose of 7.2 J/cm^2. Again, the effect was so massive with virtually all irradiated cells positive for trypan blue and Yopro that cell counts and statistical analysis were not useful.

Discussion

The present study has shown a specific threshold-like cytotoxic effect of combined riboflavin/UVA treatment on corneal endothelial cells starting at 0.35 mW/cm^2 UVA irradiance, corresponding to a dose of 0.63 J/cm^2.

A cytotoxic effect of UV on corneal endothelium is already known from clinical cases with photokeratitis [14] and rabbit experiments [15]. UVA (365 nm) without photosensitizer was reported to induce endothelial cell damage only after relatively high surface UVA doses of 42.5 J/cm^2 [16]. UVB with a shorter wavelength (310 nm) and a correspondingly higher energy content was found to cause endothelial apoptosis already at 0.47 J/cm^2 in rabbit corneas [17].

Remarkably, the cytotoxic irradiance level for combined UVA/riboflavin treatment was by a factor of 10 lower than after UVA treatment alone (0.35 mW/cm^2 or a dose of 0.63 J/cm^2 vs. 4 mW/cm^2 irradiance or a dose of 7.2 J/cm^2), which can be explained by the photosensitizing effect of riboflavin. Similarly, UVA absorption was shown to be increased to 95% in the porcine cornea after riboflavin treatment compared to 32% without riboflavin by us [2]. In cell culture experiments with combined riboflavin/light treatment, a similar massive cytotoxic effect on bovine corneal endothelial cells has been observed after combination therapy with 100 µ*M* riboflavin solution and 2 h white light irradiation. UV-induced cell death is due to oxidative damage caused by the so-called reactive oxygen species, e.g. hydrogen peroxide, superoxide or singlet oxygen generated by the UV light [13]. Riboflavin (vitamin B2) alone produced no cell damage, in agreement with previous experiments [13], which is not surprising because riboflavin is also present in the retina, liver and heart being an essential element in normal nutrition [18].

Our results are of clinical relevance because using the absorption coefficient of 53 cm^{-1} [2] it can be calculated according to the Lambert-Beer equation $I_{depth} = I_{surface} \cdot e^{(-d\mu)}$ that in human corneas the cytotoxic endothelial UVA irradiance of 0.35 mW/cm^2 is reached at 400 µm stromal depth using the standard 3 mW/cm^2 surface irra-

diance [19]. In corneal ulcers and very advanced keratoconus, the corneal thinning may be beyond this limit. In these cases, riboflavin/UVA treatment should be avoided. Fortunately, the cytotoxic threshold of 0.35 mW/cm^2 is reached neither in normal corneas with a corneal thickness of 540 μm on average nor in most keratoconus patients (410–470 μm) [20].

To determine possible cell death, we first conducted trypan blue staining followed by Yopro staining. Both dyes stain positively in the nuclei of damaged cells without interfering with cell viability, whereas undamaged endothelial cells are impervious to these dyes. However, in contrast to larger molecules like trypan blue (molecular weight: 961 D), the smaller DNA dye Yopro (molecular weight: 629 D) is also able to detect earlier stages in the cell death pathway like in apoptosis when larger molecules like trypan blue do not pass the nuclear membrane. Yet, as the threshold for both Yopro and trypan blue was congruent and at the same dose level, the cellular damage in the present in vitro experiment is obviously mainly due to necrosis, because otherwise the Yopro stain should have been positive already at lower irradiance levels than the one found by trypan blue indicating damage of an early phase like in apoptosis [9].

Regarding the preparation of the bovine endothelial cells, we preferred the method of trypsin-induced endothelial cell dissociation [8,10,11]: this method enables to harvest only endothelial cells, whereas in the method using peeling of Descemet's membrane from the stroma [13] often mixed cellular populations with some keratocytes adherent to Descemet's membrane are obtained.

In conclusion, this study has shown that due to the photosensitizing effect of riboflavin the cytotoxic irradiance level is significantly lowered to 0.35 mW/cm^2 for the corneal endothelium after combined riboflavin/UVA treatment, with a tenfold lower cytotoxicity level than after UVA irradiation alone. In human corneas, this cytotoxic threshold can be expected in corneas thinner than 400 μm. Therefore, in the future, routine pachymetry measurements should be performed before considering riboflavin/UVA treatment, thereby excluding patients with extreme corneal thinning who are not suited for the crosslinking treatment because of the risk involved for the endothelium.

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