Eye (2004) 18, 718–722
© 2004 Nature Publishing Group All rights reserved 0950-222X/04 $30.00
www.nature.com/eye

LABORATORY STUDY

Keratocyte cytotoxicity of riboflavin/UVA-treatment *in vitro*

G Wollensak[1], E Spoerl[1], F Reber[2] and T Seiler[3]

Abstract

Purpose **Collagen crosslinking using ultraviolet- A (UVA) -irradiation combined with the photosensitizer riboflavin is a new technique for treating progressive keratoconus. It has been shown to increase effectively the biomechanical strength of the cornea and to stop or even reverse the progression of keratoconus. As part of a safety evaluation, the present study was undertaken to investigate *in vitro* the possible cytotoxic effect of combined riboflavin/UVA-treatment on corneal keratocytes and to compare it to UVA-irradiation alone.**
Methods **Cell cultures established from porcine keratocytes were treated with 0.025% riboflavin solution and various UVA (370 nm)-irradiances ranging from 0.4 to 1.0 mW/cm^2 and with UVA alone between 2 and 9 mW/cm^2 for 30 min. The cell cultures were evaluated for cell death 24 h after irradiation using trypan-blue and Yopro-fluorescence staining.**
Results **An abrupt cytotoxic irradiance level was found at 0.5 mW/cm^2 for keratocytes after UVA-irradiation combined with the photosensitizer riboflavin, which is 10-fold lower than the cytotoxic irradiance of 5 mW/cm^2 after UVA-irradiation alone.**
Conclusions **A cytotoxic effect of combined riboflavin/UVA-treatment on keratocytes is to be expected at 0.5 mW/cm^2, which is reached in the clinical setting in human corneas down to a depth of 300 μm using the standard surface UVA-irradiance of 3 mW/cm^2.**
Eye (2004) **18,** 718–722. doi:10.1038/sj.eye.6700751
Published online 23 January 2004

Keywords: keratocytes; UVA; riboflavin; necrosis; cell culture; crosslinking

[1]Department of Ophthalmology Technical University of Dresden Dresden, Germany

[2]Department of Anatomy Technical University of Dresden Dresden, Germany

[3]Institut für Refraktive und Ophthalmo-Chirurgie (IROC) Zurich Switzerland

Correspondence: G Wollensak Wildentensteig 4 Berlin D-14195, Germany Tel: +49 351 458 3763 Fax: +49 351 458 4335 E-mail: gwollens@hotmail.com

Received: 3 February 2003 Accepted in revised form: 22 October 2003 Published online: 23 January 2004

Introduction

We have recently developed a new method for the treatment of keratoconus by inducing collagen crosslinking in the cornea using ultraviolet A (UVA) and the photosensitizer riboflavin. Collagen crosslinking leads to an increase in intra- and interfibrillar covalent bonds by photosensitized oxidation and causes a biomechanical stabilization of the cornea. In a prospective clinical pilot study including 22 patients with moderate or advanced progressive keratoconus and with a follow-up time of up to 4 years, the progression of keratoconus could thus be stopped in all treated eyes. Regression with a reduction of the maximal keratometry readings by 2 diopters was achieved in 70% of patients.[1]

In stress–strain measurements of the human,[2] porcine,[2,3] and rabbit[4] cornea, a significant increase of mechanical rigidity was found. An increased resistance to collagenases could be demonstrated.[5] Besides keratoconus and corneal ulcers, the new treatment can be used in corneal melting processes[6] and in the field of refractive surgery to reduce the risk for iatrogenic keratectasia after LASIK (laser assisted in situ keratomileusis).[7]

The impact of various treatment modalities like corneal abrasion, PRK (photorefractive keratectomacy),[8] LASIK,[8,9] and epithelial injury[10–12] on keratocytes has gained considerable interest in recent years having a possible influence on scarring or corneal thinning.[10] Therefore, as part of a comprehensive safety evaluation, we have undertaken the following experimental study to assess the possible damage to corneal keratocytes after combined riboflavin/UVA-treatment and to compare it to the effect of UVA-irradiation alone.

Material 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% foetal calf serum (FCS, from Sigma, Deisenhofen, FRG), phenol red, and antibiotics (penicillin–streptomycin). Trypan-blue solution[13] (Biochrome AG, Berlin, FRG) and Yopro (Molecular probes, Eugene, OR, USA)-fluorescent stain were used for the viability assay.[14]

Explant culture conditions

Explant cultures[13] were established from porcine eyes, which were obtained from a local slaughterhouse within 3–5 h postmortem. The epithelium was removed mechanically using a blunt hockey knife. Pieces 6×6 mm of mid-stromal lamellae were dissected by a pair of scissors, and transferred into a 25 cm^2 cell culture flask (Nunc, Wiesbaden, FRG). The flask was filled with 2 ml DMEM plus 10% foetal calf serum and placed into an incubator gassed with carbon dioxide 6% at 37°C. Cell outgrowth from the stromal explants started after 10–12 days, and confluence with about 2.5×10^6 cells per flask was reached after 21 days. The media were exchanged every week. The confluent stock cultures were passaged every 3 weeks with a split ratio of 1:3. For dissociation and detachment, the cultures were treated with 0.05% trypsin–EDTA solution. The free cells were centrifuged at 230 *g*, transferred into new culture flasks and resuspended. The cell growth was evaluated regularly using an inverted phase-contrast microscope at $\times 100$ magnification (Zeiss Axiovert L5). Before the irradiation, 5×10^4 cells were plated per well in eight-well tissue plates, where they reached confluence after 8–10 days. After another week, the UVA-irradiation was performed.

Treatment groups

The cells were exposed to three different treatments:

1. Riboflavin alone: The cells were exposed to 25, 50, 100, and 500 μM riboflavin solution.
2. Riboflavin plus UVA: The cells were exposed to 0.025% (=500 μM) riboflavin solution plus UVA-irradiances ranging from 0.4 to 1 mW/cm^2 (Table 1).
3. UVA alone: The cells were exposed to UVA-irradiances ranging from 2 to 9 mW/cm^2 (Table 1).

Except for the varying UVA-irradiances, the other parameters like riboflavin concentration or the UVA-wavelength of 370 nm were kept identical to the clinical treatment in the second treatment group.

Irradiation procedure

A riboflavin concentration as close as possible to the conditions of the clinical treatment of human corneas was chosen and calculated as follows. In humans, 0.1% riboflavin solution is applied. Using the so-called diffusion equation and the diffusion coefficient ($D = 6.5 \times 10^{-7}$ cm^2/s) of the related dye fluorescein, we calculated the average riboflavin concentration over 30 min in the stroma as 0.024% riboflavin solution. Therefore, we used 0.025% riboflavin solution (=500 μM) by adding 57 μl of 0.2% riboflavin stock solution to 400 μl colourless culture medium without phenol red. No riboflavin was added to the cultures with UVA-treatment alone. The riboflavin solution for the UVA-irradiation was added to the wells 5 min before the irradiation and was replaced by the cell medium after the irradiation. To avoid UVA-absorption by riboflavin solution overlying the monolayer of keratocytes attached to the floor of the wells, we irradiated the wells from underneath fixating the UVA-double diode (370 nm) 1 cm under the respective wells (Figure 1) with the help of a stand. The UVA-absorption by the 100 μm thin floor of the wells made of borsilicone was measured to be only 2% and was negligible. Before the treatment, the required UVA-irradiances ranging from 0.4 to 9 mW/cm^2 were controlled with a UVA-meter (LaserMate-Q, LASER 2000, Wessling, Germany) at a 1 cm distance and if necessary regulated with a potentiometer in series. The irradiation itself lasted 30 min, which is conform to the clinical setting. Five wells were tested for each irradiance level. The 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).

Table 1 Cytotoxicity for porcine keratocytes after combined riboflavin/UVA-treatment and after UVA alone

Irradiance level (mW/cm^2)	*Riboflavin + UVA*	*UVA*
9		+
8		+
7		+
6		+
5		+
4.5		−
4		−
3		−
2		−
0.8	+	
0.7	+	
0.6	+	
0.55	+	
0.5	+	
0.45	−	
0.4	−	

(+) Necrosis, (−) no cell damage.

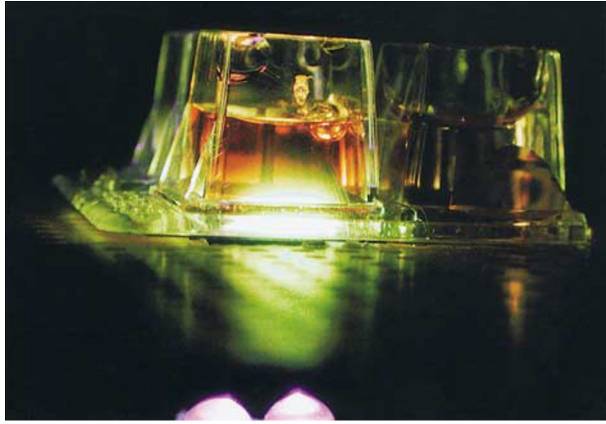

Figure 1 Irradiation procedure with double UVA-diode under the wells of the culture plate.

Figure 3 Peripherial sector of the circular treatment area (riboflavin plus 0.5 mW/cm^2 UVA) with trypan-blue-positive keratocytes and loss of cells (trypan blue, $\times 400$).

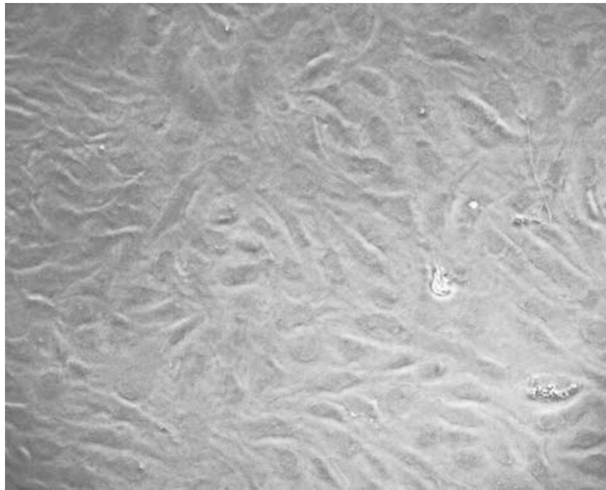

Figure 2 Representative morphology of the cultured porcine keratocytes at confluence (phase-contrast, $\times 100$).

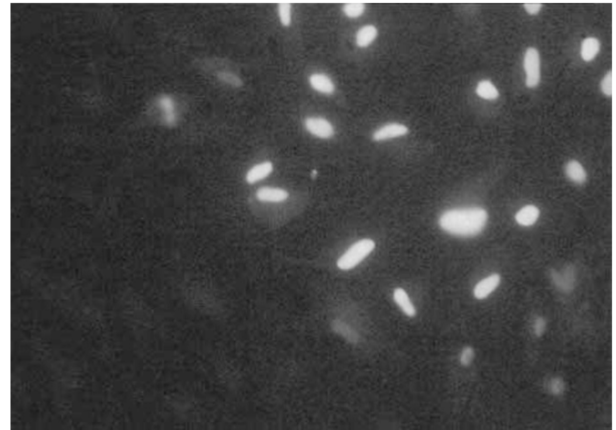

Figure 4 Peripherial sector of the circular treatment area (5 mW/cm^2 UVA) with Yopro-stained keratocyte nuclei (Yopro, $\times 400$).

Supravital staining

To determine possible cell damage after treatment, 100 μl of 0.25% trypan-blue solution dissolved in colourless culture medium was applied per well for 15 min followed by twofold rinsing with culture medium. After microscopic evaluation of the trypan-blue staining,[13] the cells were subsequently stained with Yopro adding 1 μl per well followed by one rinse with culture medium.[14] For trypan blue (Figures 2 and 3), cultures were examined in an inverse microscope (Leica DMIR) at 100–400-fold magnification using differential interference contrast and for Yopro (Figure 4) using fluorescence at 488 nm (N2.1 filter). Photos were taken with a digital camera attached to the microscope (Nikon coolpix 950). Only the nuclei of damaged cells were labelled with the stains.

Results

Cellular characteristics

Before confluence, the scattered cells appeared dendritic, whereas they assumed a spindle-shaped appearance when confluence was reached (Figure 2). About 10% of the cells contained some brown-coloured, autofluorescent granules as is typical for lipofuscein deposits. After cytotoxic doses, the damaged cell nuclei stained positively with trypan blue (Figure 3) and the green-fluorescent Yopro (Figure 4). The cell damage detected by the two stains was always observed in the same cells and at the same irradiance level. In addition, dead cells became more globular in shape and about 5–10% of the damaged dead cells lost adhesion floating off the bottom of the wells. The area of the damaged cells

was shaped like a circle quasi like an imprint of the UVA-beam with surviving cells only in the nonirradiated periphery (Figures 3 and 4).

Cytotoxicity

The cytotoxic irradiance level was found to be 10-fold lower at 0.5 mW/cm^{2}. (=0.9 J/cm^2) for keratocytes after riboflavin combined with UVA-irradiation compared to 5 mW/cm^2 (=9 J/cm^2) for cells with UVA-irradiation alone (Table 1). The cytotoxic effect occurred in a threshold-like manner with a sharp and abrupt transition from damaging to nontoxic irradiance levels. There was no variability in the five different wells per irradiance level and always all cells irradiated with the toxic dose were damaged. There was no cytotoxicity for the cells treated with riboflavin alone without UVA-irradiation.

Discussion

The present study has shown an abrupt threshold-like cytotoxic irradiance level of combined riboflavin/UVA-treatment at 0. 5 mW/cm^2. Using the standard surface irradiance of 3 mW/cm^2 and the absorption coefficient of 53 cm^{-1}, it can be calculated according to the Lambert–Beer equation $I_{\text{depth}} = I_{\text{surface}} e^{(-d\mu)}$ that in human corneas the cytotoxic keratocyte UVA-irradiance of 0.5 mW/cm^2 is reached down to a stromal depth of 300 μm.[4,15] These data fit well to our *in vivo* results in riboflavin/UVA-treated rabbit corneas, where we found exactly the same cytotoxic irradiance of 0.5 mW/cm^2 with a keratocyte loss down to a depth of 300 μm after 3 mW/cm^2 surface irradiance.[16]

UV-induced damage of keratocytes has also been reported by others and depends on the ultraviolet wavelength and dose. While ultraviolet C (UVC) (100–290 nm) is completely absorbed at the corneal surface and 80% of ultraviolet B (UVB) (290–315 nm) in the corneal epithelium, 25–34% of UVA (315–400 nm) is absorbed in the stroma.[17] Accordingly, massive keratocyte damage was observed especially in the anterior portion of the rabbit cornea following exposure to UVA-irradiation at 350 nm.[18,19]

In the present *in vitro* study, the cytotoxic irradiance level after combined riboflavin/UVA-treatment was about 10-fold lower than that after treatment with UVA alone because the cytotoxic effect, which is due to the oxidant effect of UVA-light, is multiplied by the photosensitizer riboflavin due to increased UVA-absorption. Concurrent with this observation, UVA-absorption was shown by us to be increased to 95%[4] in the cornea after riboflavin treatment compared to 25–35% without riboflavin.[17]

Riboflavin (vitamin B_2) alone produced no cell damage as is also known from other experiments[20] and is not surprising because riboflavin is also present in the retina, liver, and heart, being an essential element in normal nutrition.[21]

In terms of the clinical problems of keratocyte loss, only long-term imbalances of the keratocyte reconstitution bear a risk for corneal thinning.[8,10] However, this is not to be expected because the keratocyte loss can be repaired rapidly by repopulation from migrating keratocytes of the adjacent cornea.[8,22] Loss of keratocytes has been observed already in other corneal procedures without having dramatic consequences. So after PRK,[8] LASIK,[8,9] or epithelial injury[10–12] keratocyte apoptosis of variable degree was described. However, treatment-related keratocyte apoptosis might be an issue because of the presumptive role of keratocyte apoptosis in the pathogenesis of keratoconus.[8]

The state of the keratocytes was assessed 24 h following UVA-irradiation because then there is the maximum degree of cell damage as has been shown previously.[16] To determine possible cell death, we first conducted trypan-blue staining followed by Yopro staining.[14] Both stains are positive in the nuclei of damaged cells without interfering with cell viability, whereas undamaged endothelial cells are impervious to these dyes. The DNA-intercalant dye Yopro is also able to detect minor damage like in apoptotic cells due to its relatively low molecular weight (MW 629 *vs* 961).[14] 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.

In conclusion, we have shown that combined riboflavin/UVA-treatment leads to a 10-fold lower threshold for keratocyte cytotoxicity at 0.5 mW/cm^2 compared to 5 mW/cm^2 after UVA-irradiation alone. Using riboflavin/UVA in human corneas, keratocyte damage can be expected down to a depth of 300 μm using 3 mW/cm^2 surface irradiance. Although no corneal thinning or scarring due to keratocyte loss has been observed so far in a 4-year-clinical trial,[1] a long-term study including confocal *in vivo* microscopy in humans after riboflavin/UVA-treatment is currently underway to evaluate the depth of the keratocyte loss, the repopulation process, and to exclude reliably the development of treatment-related stromal scarring, haze, or thinning in humans.

References

1 Wollensak G, Spoerl E, Seller T. Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol* 2003; **135**: 620–627.
2 Wollensak G, Spoerl E, Seller T. Stress–strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg* 2003; **29**: 1780–1785.
3 Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp Eye Res* 1998; **66**: 97–103.
4 Spoerl E, Schreiber J, Hellmund K, Seiler T, Knuschke P. Untersuchungen zur Verfestigung der Hornhaut am Kaninchen. *Ophthalmologe* 2000; **97**: 203–206.
5 Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Curr Eye Res* 2003, accepted for publication.
6 Schnitzler E, Spoerl E, Seiler T. Bestrahlung der Hornhaut mit UV-Licht und Riboflavingabe als neuer Behandlungsversuch bei einschmelzenden Hornhautprozessen, erste Ergebnisse bei vier Patienten. *Klin Mbl Augenheilkd* 2000; **217**: 190–193.
7 Seiler T. Iatrogenic keratectasia: academic anxiety or serious risk? (Editorial). *J Cataract Refract Surg* 1999; **25**: 1307–1308.
8 Wilson SE. Keratocyte apoptosis in refractive surgery. *CLAO J* 1998; **24**: 181–185.
9 Mitooka K, Ramirez M, Maguire LJ, Erie JC, Patel SV, McLaren JW *et al* . Keratocyte density of central human cornea after laser *in situ* keratomileusis. *Am J Ophthalmol* 2002; **133**: 307–314.
10 Kim W-J, Helena MC, Mohan RR, Wilson SE. Changes in corneal morphology associated with chronic epithelial injury. *Invest Ophthalmol Vis Sci* 1999; **40**: 35–42.
11 Campos M, Szerenyi K, Lee M, McDonnell JM, Lopez PF, McDonnell PJ. Keratocyte loss after corneal deepithelialization in primates and rabbits. *Arch Ophthalmol* 1994; **112**: 254–260.
12 Gurelik BG, Bilkgihan K, Sezer C, Akyol G, Hasanreisoglu B. Effect of mechanical *vs* dilute ethanol epithelial removal on keratocyte apoptosis and polymorpho-nuclear leukocyte migration. *Eye* 2002; **16**: 136–139.
13 Borderie VN, Lopez M, Lombet A, Carvajal-Gonzalez S, Cywiner C, Laroche L. Cryopreservation and culture of human corneal keratocytes. *Invest Ophthalmol Vis Sci* 1998; **40**: 1511–1519.
14 Idziorek T, Estaquier J, De Bels F, Ameisen J-C. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J Immunol Meth* 1995; **185**: 249–258.
15 Kolozsvári L, Nógrádi A, Hopp B, Bor Z. UV absorbance of the human cornea in the 240- to 400-nm range. *Invest Ophthalmol Vis Sci* 2002; **43**: 2165–2168.
16 Wollensak G, Spoerl E, Wilsch M, Seiler T. Keratocyte apoptosis after corneal collagen-crosslinking using riboflavin-UVA treatment. *Cornea* 2003, accepted for publication.
17 Tsubai T, Matsuo M. Ultraviolet light-induced changes in the glucose-6-phosphate dehydrogenase activity of porcine corneas. *Cornea* 2002; **21**: 495–500.
18 Ringvold A, Davanger M. Changes in the rabbit corneal stroma caused by UV-radiation. *Acta Ophthalmol* 1985; **63**: 601–606.
19 Pitts DG, Cullen AP, Hacker PD. Ocular effects of ultraviolet radiation from 295-365 nm. *Invest Ophthalmol Vis Sci* 1977; **16**: 932–939.
20 Cho K-S, Lee EH, Choi J-S, Joo C-K. Reactive oxygen species-induced apoptosis and necrosis on bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci* 1999; **40**: 911–919.
21 Dollery C *Therapeutic Drugs*, Vol. 2. Churchill Livingstone: Edinburgh, 1991, pp 24–25.
22 Podskochy A, Fagerholm P. Cellular response and reactive hyaluronan production in UV-exposed rabbit corneas. *Cornea* 1998; **17**: 640–645.