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Research Article

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Impact of riboflavin-UVA-photodynamic inactivation (PDI) (collagen crosslinking technique) on viability, cell cycle phase, apoptosis and proliferation of human corneal endothelial cells

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ABSTRACT

The purpose of our study was to determine the impact of riboflavin-UVA photodynamic inactivation (PDI) (Collagen crosslinking technique) on viability, cell cycle phase, apoptosis and proliferation of human corneal endothelial cells (HCECs), in vitro. A HCEC line was cultured in DMEM/Ham's F12 medium supplemented with 5% fetal calf serum. HCECs cultures underwent 370 nm-UVA-light illumination for 4.1 minutes during exposure to 0.05% or 0.1% riboflavin and 20% dextran containing PBS. Twenty-four hours after riboflavin-UVA-PDI, viability was determined by the Alamar blue assay, cell cycle phase and apoptosis of the cells using the APO-DIRECT^{TM} Kit, and two and twenty-four hours after PDI, HCECs proliferation by the BrdU Cell Proliferation Assay Kit. Twenty-four hours after the use of 0.1% riboflavin concentration without illumination and after 0.05% and 0.1% riboflavin-UVA-PDI, HCECs viability decreased significantly ($P<0.01$ for all) compared to controls. Twenty-four hours following riboflavin-UVA-PDI, the percentage of HCECs at the G_1 cell cycle phase decreased significantly using 0.05% or 0.1% riboflavin concentration ($P=0.02$ and $P=0.03$), the percentage of HCECs at the G_2/M phase increased significantly using 0.05% riboflavin concentration ($P=0.03$), compared to controls. Two and twenty-four hours after riboflavin-UVA-PDI using 0.05% or 0.1% riboflavin concentration, HCEC proliferation decreased significantly ($P=0.02$ for all). There was no significant difference in percentage of apoptotic HCECs at any of the treated groups compared to controls 24 hours after riboflavin-UVA-PDI ($P=0.10$).Crosslinking arrests HCECs at the G_2/M phase, decreases viability and proliferation, however does not trigger apoptosis of human corneal endothelial cells in vitro.

Key words: riboflavin, UVA, human corneal endothelial cells, viability, apoptosis,

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INTRODUCTION

During corneal collagen crosslinking (CXL) the photosensitizer riboflavin and ultraviolet A (UVA) light illumination of 370 nm wavelength is used. CXL has been introduced as a new technique to effectively increase the biomechanical rigidity of the cornea in order to delay or stop the progression of keratoconus [1, 2]. Recent results of several European [2-4] and the American studies [5] support the efficacy and safety of the CXL procedure in the clinical treatment of keratoconus.

In addition, the use of CXL has been investigated as a potential new therapeutic option for the treatment of infectious bacterial [6-8], mycotic [9] or acanthamoeba keratitis [10]. With this application, it may be called riboflavin-UVA-photodynamic inactivation (PDI). Riboflavin-UVA-PDI uses riboflavin as photosensitizer and ultraviolet-A (UVA) light (370 nm) for excitation. During the so called photodynamic inactivation the produced

reactive oxygen species (ROS) are responsible for eradication of the microorganisms, but can also lead as side effect to damages of the host tissue [11, 12].

Human corneal endothelial cells (HCECs) are located as a single cell layer at the posterior surface of the cornea and play a crucial role in maintaining corneal transparency by regulating barrier and pump functions [13-15]. Many studies described the sensibility of HCECs to stress [16-19]. Therefore, using CXL in the treatment of keratoconus and the same CXL technique as riboflavin-UVA-PDI in infectious keratitis, cytotoxic effects on HCECs should be avoided or reduced as much as possible.

The purpose of this project was to determine the impact of riboflavin-UVA-PDI on viability, cell cycle phase, apoptosis and proliferation of human corneal endothelial cells, *in vitro*.

EXPERIMENTAL SECTION

Materials

Dulbecco's Modified Eagle Medium: (Nutrient Mixture F-12 (DMEM/F12)); fetal calf serum (5%); penicillin/streptomycin (P/S) (1% of 10,000 U penicillin/ml and 10 mg/ml streptomycin); 0.05% trypsin/ 0.02% ethylenediaminetetra-acetic acid (EDTA) were purchased from PPA Laboratories (Pasching, Austria), Alamar blue from Invitrogen (Karlsruhe, Germany) and propidium iodide from Molecular Probes, Inc. (Eugene, Oregon, USA). The APO-DIRECT^{TM} kit and all tissue culture plastics were from PPA Laboratories (Pasching, Austria) and fibronectin was from Sigma-Aldrich Chemie (Deisenhofen, Germany). Cell Proliferation ELISA-BrdU (colorimetric) was obtained from Roche Diagnostics (Mannheim, Germany). Riboflavin-5-phosphate and Dextran were purchased from Sigma-Aldrich (Heidelberg, Germany).

Culture of Human Corneal Endothelial Cells

An immortalized human corneal endothelial cell line (HCEC-12, Technical University Dresden, Dresden, Germany) (previously established by SV40 transfection) prepared from a healthy cornea of a 91-year-old Caucasian woman was used for the experiments. Cells were cultured in DMEM/Ham's F12 medium supplemented with 5% FCS and 1% P/S. The culture plates were coated using 20 µg/ml fibronectin. Medium was changed every 2 to 3 days until HCECs reached confluence, and then the cells were subcultured following dispersal with 0.05% trypsin-EDTA for 3 to 5 minutes and passages 4-20 of HCECs were used for experiments.

Riboflavin-UVA photodynamic inactivation

HCECs were seeded in tissue culture plates and allowed to grow for 48 hours before riboflavin-UVA-PDI. During riboflavin-UVA-PDI, the cells were washed once with PBS and then cultured in 0.05% and 0.1% riboflavin concentration and 20% dextran containing PBS, followed by exposure to 370 nm-UVA-light illumination (8.0 mW/cm^2 or a dose of 2 J/cm^2) for 4.1 minutes. Our UVA-light illumination box was developed by the Department of Physics of the University of Kaiserslautern ("Zentrales Innovationsprogram Mittelstand"; grant number: KF2152004MD0). Following UVA-light illumination, the cells were washed twice with PBS, then fed with culture medium, and let grow at 37 °C for 2 or 24 hours before measurements.

Determination of viability (phototoxicity)

Cell viability was evaluated using the Alamar blue assay as follows: HCECs were seeded in 24-well cell culture plates at a concentration of 1.0×10^5 cells/cm^2. At 24 hours after riboflavin-UVA-PDI, Alamar blue solution was diluted with culture medium for a final concentration of 10% and 500 µl of this solution was added to each well. After 3 to 4 hours of incubation, 200 µl of conditioned culture medium from each well was transferred into two wells of 96-well plates. As a negative control, Alamar blue solution was added to a well without cells. Thereafter, all plates were exposed to an excitation wavelength of 560 nm, and the emission at 616 nm was recorded using a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Wellesley, MA, USA).

Flow Cytometric Analysis

To determine the cell cycle phase and the relative number of apoptotic cells (APO-DIRECT^{TM} kit assay), the HCECs were seeded in 6-well cell culture plates with a concentration of 7.5×10^3 cells/cm^2 and underwent riboflavin-UVA-PDI as described above. Treated HCEC were harvested 24 hours following riboflavin-UVA- PDI. First, the culture medium was discarded and the cells were trypsinized before centrifugation. Then, the cells were re-suspended in 1 ml of 1% paraformaldehyd and placed on ice for 30-60 minutes. Thereafter, cells were washed twice with PBS and stored for 30 minutes at -20 °C following adding 1 ml ice cold 70% ethanol. After removing the ethanol carefully by aspiration, fixed cells were resuspended twice in 1.0 ml Wash-Buffer. The control cells and the probes were resuspended in 50 µl DNA-Labeling-Solution and the cells were washed twice before resuspending the cell pellet in 500 µl PI/RNase Staining Buffer (0.3 ml for lower cell amount). Cells were incubated in the dark for at

least 30 minutes at room temperature prior to analysis using a FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany).

Cell Proliferation

The proliferation of the HCECs was determined using the cell proliferation Elisa BrdU kit 2 and 24 hours after riboflavin-UVA-PDI, by the measurement of BrdU incorporation in the newly synthesized cellular DNA. HCECs were plated in a 96-multiwell plate at a density of 5×10^3 cells/well. Riboflavin-UVA-PDI was performed as described above. Then, the test was performed according to the manufacturer's protocol. BrdU was added to the HCECs at the tissue plates and incubated at 37 °C for 4 hours (BrdU incorporation). After removing the culture medium, the cells were fixed with FixDenat, provided with the test kit, followed by the incubation with anti-BrdU-POD, which binds the incorporated DNA. After adding the substrate solution, the immune complexes were detected using an Elisa reader, Model 550 (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistical Analysis

For statistical analysis the SPSS software version 13.0 was used. Quantitative data were expressed as means ± standard deviation (SD). Statistical analysis was performed using the Wilcoxon Mann Whitney Test. $P<0.05$ was considered statistically significant.

RESULTS

HCEC viability

Using inversion microscopy, HCEC morphology remained unchanged in all analysed groups. HCECs viability 24 hours following riboflavin-UVA-PDI is displayed in **Figure 1** (n=7). Twenty-four hours after the use of 0.1% riboflavin concentration without illumination or following 0.05% and 0.1% riboflavin-UVA-PDI, HCECs viability decreased significantly ($P<0.01$ for all) compared to controls.

Cell cycle phase of HCECs

The percentage of HCECs at different cell cycle phases 24 hours after riboflavin-UVA-PDI is displayed in **Figure 2** (n=7). The use of riboflavin or UVA-light illumination only did not have significant impact on the cell cycle phase of HCECs. Twenty-four hours following riboflavin-UVA-PDI, the percentage of HCECs at the G_1 phase decreased significantly using 0.05% or 0.1% riboflavin concentration (P=0.02 and P=0.03), the percentage of HCECs at the G_2/M phase increased significantly using 0.05% riboflavin concentration (P=0.03) and the percentage of cells at the S phase did not change (P=0.66), compared to controls.

HCEC apoptosis

The percentage of apoptotic HCECs 24 hours after riboflavin-UVA-PDI is shown in **Figure 3** (n=7). Using riboflavin or UVA-light illumination separately, the percentage of apoptotic HCECs did not change significantly compared to controls. The percentage of apoptotic cells was also not significantly different from controls following 0.05% or 0.1% riboflavin-UVA-PDI (P=0.10).

HCEC proliferation

Proliferation of HCECs 2 and 24 hours after riboflavin-UVA-PDI is shown in **Figure 4** (n=4). Using UVA-light-illumination or the photosensitizer riboflavin separately, the proliferation of HCECs remained unchanged compared to controls for both time points. Two hours after riboflavin-UVA-PDI, the proliferation of HCECs was significantly inhibited using 0.05% or 0.1% riboflavin concentration (P=0.02 for all) and it was also decreased at 24 hours following riboflavin-UVA-PDI using 0.05% or 0.1% riboflavin concentration compared to controls (P=0.02 for all).

DISCUSSION

Our study demonstrated that riboflavin-UVA photodynamic inactivation using the collagen crosslinking technique arrests human corneal endothelial cells at the G_2/M phase, decreases viability and proliferation, however does not trigger apoptosis of the HCECs, *in vitro*. To the best of our knowledge, this is the first study analyzing the impact of corneal crosslinking on viability, cell cycle phase, apoptosis and proliferation of *human* corneal endothelial cell cultures.

Interestingly, decreased viability of HCECs could be demonstrated without illumination with the single application of 0.1% riboflavin concentration. In addition, viability also decreased at 0.05% and 0.1% riboflavin-UVA-PDI. In comparison, our previous study using a photosensitizer with higher photosensitizing efficacy (Chlorin e6; Ce6), showed decreased viability of HCECs at 150 nM Ce6 concentration following 670 nm wavelength illumination [19].

The higher photosensitizing efficacy of Ce6 results in production of higher concentration of reactive oxygen species and this may be one reason for the differences in viability of the HCECs for both photosensitizers.

We determined that proliferation of HCECs was inhibited 2 and 24 hours after the use of 0.05% or 0.1% riboflavin concentration and UVA-light illumination. As our HCEC line was an immortalized human corneal endothelial cell line [20] with a different manner of proliferative capacity compared to primary HCECs, the impact of our study analyzing HCECs proliferation is limited. However, using the same HCEC cell line and PDI with the photosensitizer Ce6 and illumination at 670 nm, we detected inhibition of proliferation at 100 nM Ce6 concentration 24 hours after PDI. This is a contrast to the much lower riboflavin concentration in the present study.

The cell cycle consists of four distinct phases (G_1, S, G_2 and M phases), which help to control the accuracy of DNA replication and cell division. The induction of G_2/M phase arrest is known to inhibit cell growth and induce cell cytotoxicity [21, 22]. In the present study, we detected an arrest of HCECs at the G_2/M phase following 0.05% riboflavin-UVA-PDI and an increasing trend in the percentage of G_2/M phase cells at 0.1% riboflavin-UVA-PDI. Similar to the above cited publications [21, 22], the induction of the G_2/M phase also resulted in inhibited proliferation and decreased viability of HCEC following riboflavin-UVA-PDI in the present study.

In contrast to Ce6-PDI, where apoptosis could be detected at 250 nM Ce6 concentration, we did not find significant increase of the percentage of apoptotic HCECs after riboflavin-UVA-PDI. This finding shows that the lower photosensitizing efficacy leads to less damages of the cell nucleus and does not trigger programmed cell death of HCECs. So with other words, riboflavin-UVA-PDI is presumably much less cytotoxic for *human* corneal endothelial cells *in vitro*.

In contrast, the cytotoxic effect on *porcine* corneal endothelial cells is already known by the use of 4 mW/cm^2 UVA irradiance or the use of 0.025% riboflavin solution (500 µM) and UVA (0.35 mW/cm^2) treatment [17]. An in vivo study of *rabbit* corneas [23] also demonstrated a cytotoxic effect on corneal endothelium using 0.1% riboflavin-5-phosphate and 20% dextran T-50 in combination with 3.0 mW/cm^2 surface irradiance of the cornea. In our present *in vitro* study, we used 8.0 mW/cm^2 UVA irradiance (370 nm wavelength) for 4.1 minutes with an application of 0.05% and 0.1% riboflavin and 20% dextran concentration.

While the above mentioned study by Wollensak et al. [17] using porcine endothelial cells applied 0.63 J/cm^2 and our *in vitro* study 2.0 J/cm^2 irradiation dose, results of both studies differ: Wollensak et al. [17] could detect riboflavin-UVA-PDI induced apoptosis of porcine endothelial cells, which was not verified in our study with human endothelial cells. This may be partially explained through properties of the different tissues or origin of the cells.

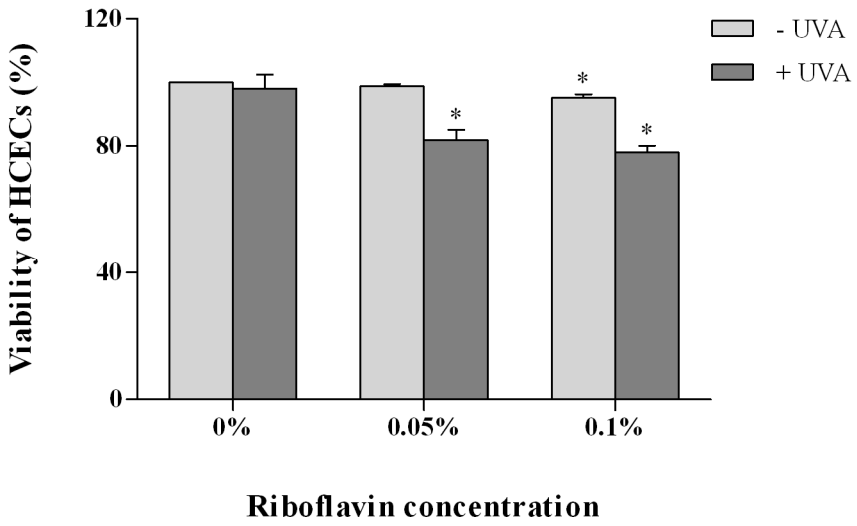

Figure 1. Viability of HCECs 24 hours following riboflavin-UVA-PDI (Alamar blue assay). Twenty-four hours after the use of 0.01% riboflavin concentration without illumination or following 0.05% and 0.01% riboflavin-UVA-PDI, HCECs viability decreased significantly (P<0.01 for all) compared to controls. This experiment has been repeated 7 times.

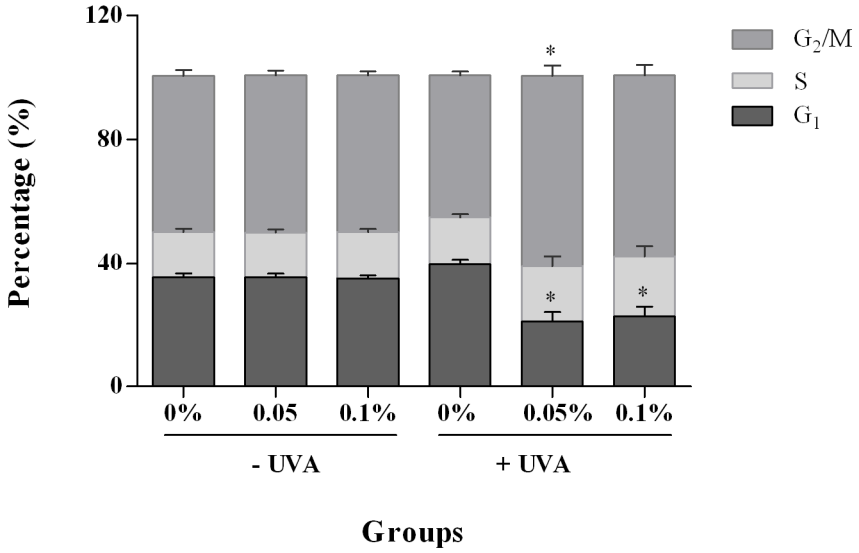

Figure 2. Percentage of HCECs at different cell cycle phases was analyzed 24 hours following riboflavin-UVA-PDI by quantifying propidium iodide incorporation in the cells using flow cytometry (APO-DIRECT^{TM} kit assay). Using riboflavin or UVA-illumination only, we did not detect significant changes in the cell cycle phase compared to control HCECs. Twenty-four hours following riboflavin-UVA-PDI, the percentage of HCECs at the G_1 phase decreased significantly using 0.05% or 0.1% riboflavin concentration (P=0.02 and P=0.03), the percentage of HCECs at the G_2/M phase increased significantly using 0.05% riboflavin concentration (P=0.03) and the percentage of cells at the S phase did not change (P=0.66), compared to controls. This experiment has been repeated 7 times.

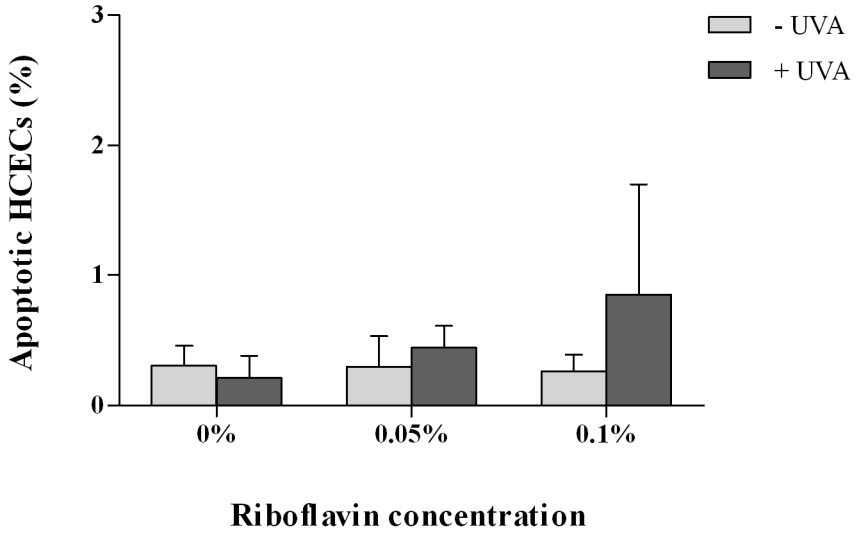

Figure 3. Percentage of apoptotic HCECs 24 hours after riboflavin-UVA-PDI (APO-DIRECT^{TM} kit assay). There was no significant difference in the percentage of apoptotic HCECs at any of the treated groups compared to controls (P=0.10). This experiment has been repeated 7 times.

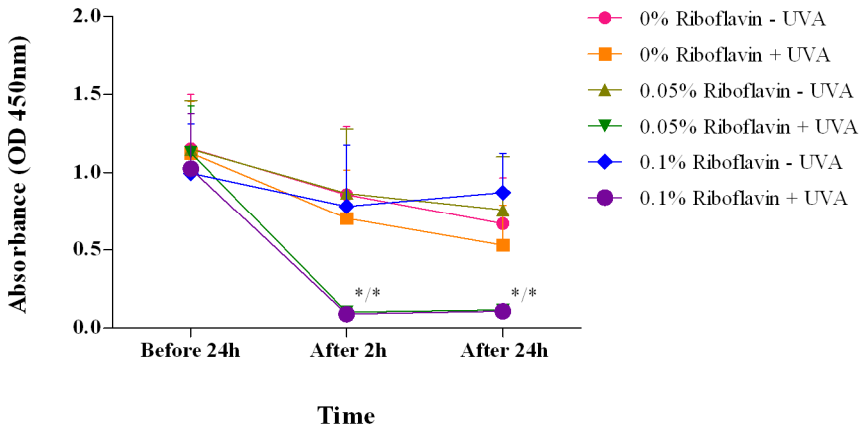

Figure 4. Proliferation of HCECs 2 and 24 hours following riboflavin-UVA-PDI (BrdU Cell Proliferation Assay Kit). Using UVA-light-illumination or the photosensitizer riboflavin alone the proliferation of HCECs remained unchanged compared to controls for both time points. Two hours after riboflavin-UVA- PDI, the proliferation of HCECs was significantly inhibited using 0.05% or 0.1% riboflavin (P=0.02 for all), and it was also decreased at 24 hours following riboflavin-UVA-PDI using 0.05% or 0.1% riboflavin concentration (P=0.02 for all), compared to controls. This experiment has been repeated 4 times.

In conclusion, riboflavin-UVA photodynamic inactivation using the collagen crosslinking technique arrests *human* corneal endothelial cells at the G_2/M phase, decreases viability and proliferation, however, does not trigger significant apoptosis of HCECs, *in vitro*. In order to avoid endothelial cell damage, riboflavin must not penetrate too deep into the human cornea, which is in accordance with the knowledge to clinical practice.

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