



Establishment of a blue light damage model of human retinal pigment epithelial cells *in vitro*

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ABSTRACT. To establish a blue-light damage model of human retinal pigment epithelium (RPE). Fourth-generation human RPE cells were randomly divided into two groups. In group A, cells were exposed to blue light (2000 ± 500 lux) for 0 (control), 3, 6, 9, and 12 h, and cell culture was stopped after 12 h. In group B, cells were exposed to blue light at the same intensity and time periods, but cell culture was stopped after 24 h. TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed to determine the most suitable illuminating time with apoptotic index. Flow cytometry was used to determine apoptotic ratio of RPEs. In group A, the apoptotic index of cells that received 6, 9 and 12 h of blue light was higher than that of control. The apoptotic index of cells receiving 9 and 12 h was higher than that of 6 h ($P = 0.000$). In group B, the apoptotic index and RPE cell apoptosis ratio of

cells exposed to 6, 9 and 12 h of blue light were higher than that of 3 h ($P = 0.000$); and cells receiving 9 and 12 h had higher values than that of 6 h. This study demonstrated that the best conditions to establish a blue light damage model of human retinal pigment epithelial cells *in vitro* are 2000 ± 500 lux light intensity for 6 h, with 24 h of cell culture post-exposure.

Key words: Light-induced damage; Retinal pigmented epithelium; Apoptosis; Damage model

INTRODUCTION

Chronic light damage causes lesions in retinal pigment epithelium (RPE) and Bruch's membrane, and exacerbates age-related macular degeneration, which is an important pathological basis in the occurrence and development of choroidal neovascularization (CNV) (Kernt et al., 2010). During atrophy, degeneration, and damage of the retina, cells die via apoptosis (Wenzel et al., 2005). Lipofuscin, the product of the outer segment of photoreceptor cells, produce auto-fluorescence, which when exposed to blue light, fluoresces. This is the basis for establishing blue light damage models of RPE cells *in vitro*. This study investigated the method of establishing light damage model of RPE cells *in vitro*, which would be important for investigating the mechanisms of blue-light-induced apoptosis in RPE cells.

MATERIAL AND METHODS

Materials

Cell sources ethics statement

RPE cells were collected from two healthy men (Cai et al., 2005), aged 25 and 35, who passed away due to accidents. We obtained the eyes within 12 h of death; the procedures were within the ethical standards established by the unit's human trials Committee.

Reagents and instruments

Reagents and instruments used in the study were as follows: Dulbecco's modified Eagle's medium nutrient mixture F-12, trypsin, paraformaldehyde (Sigma, Germany); fetal bovine serum (Hyclone, USA); TUNEL apoptosis assay kits (Roche, USA); IX7r0-142 inverted optical microscope (Olympus, Japan); micropipetter (Eppendorf, Germany); medical blue light tube (Yingze, Tianjin); TES1330A luminance meter (Taiguang, Taipei); high speed refrigerated table top centrifuge (5804R, Eppendorf, Germany); Beckman Gallios flow cytometer (Beckman, USA).

Methods

Primary cell culture

Primary RPE cells were cultured as described previously (Cai et al., 2005). Briefly, eyeballs were excised via pars plana, which is located 5-6 mm from the back of the corneal

limb. The anterior segment was discarded, followed by vitrectomy and removal of the retinal neuroepithelium. The RPE cells were digested with 0.25% trypsin and 0.02% EDTA, and were seeded at a density of 50,000 cells/mL with complete medium including 20% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.3 mg/mL glutamine. Cells were incubated in culture dishes (50 cm²) at 37°C and 5% CO₂. RPE cells were subcultured when they grew confluent, and cells were used for experimentation after 4 passages. The RPE cells in each control group came from the same individual and cell passage.

Immunohistochemistry detection of the RPE cells

RPE cells were grown on glass cover slips (2 x 2 cm) prior to fixation with acetone. Cells were permeated with 0.1% Triton X-100 (diluted with 0.1% sodium citrate), and were stained with primary mouse anti-human keratin antibodies and bio-labeled goat anti-mouse secondary antibodies conjugated to 3,3'-diaminobenzidine. Coverslips were washed with tap water, followed by deionized water. Lastly, slides were coated and imaged.

Light treatment of RPE cells

Fourth-generation human RPE cells were randomly divided into two groups. Group A: RPE cells were exposed to blue light with the intensity of 2000 ± 500 lux; cells were cultured for 12 h after exposure. Group B: cells were exposed to the light with an intensity of 2000 ± 500 lux; cells were cultured for 24 h after light exposure. Groups A and B were subdivided into the following groups: A0 and B0 (no light exposure), A3 and B3 (light exposure for 3 h), A6 and B6 (6 h), and A12 and B12 (12 h).

A medical blue light tube (450 ± 20 nm, 20 W) was placed on a special framework, and the TES1330A illuminometer was used to measure the light intensity at the cell surface. Distance between the tube and the cell surface was adjusted according to light intensity. To ensure a stable growth environment for RPE cells, the blue light device was placed inside the CO₂ incubator. Temperature was kept between 36.5 and 37.5°C.

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining

Glass coverslips with RPE cells were prepared as mentioned above. Cells were permeated with 0.1% Triton X-100, for 5 min at 25°C followed by the addition of 50-µL TUNEL reaction-solution to each cover slip. Cells were incubated for 60 min at 37°C in a wet box. The coverslips were then washed in PBS for 3 min 3 times, and 50 µL of alkaline phosphatase (ALP) convert agent, Converter-AP, was added to each cover slip. Cells were incubated for 30 min at 37°C, followed by the addition of 1:50 NBT/BCIP (diluted with substrate buffer) for 10 min at room temperature. The coverslips were washed with tap water, counterstained, dried, and coated. Finally, 1 mg/mL deoxyribonuclease was added to TUNEL-positive coverslips and allowed to react for 10 min.

Apoptotic index (AI)

Ten fields were randomly chosen at 100X magnification, and the average percentage of apoptotic RPE cells were calculated as the apoptotic index.

Flow cytometry

RPE cells were stained with Annexin V-FITC/PI according to manufacturer instructions. PI (250 μg) was diluted in 1 mL Annexin V binding buffer (diluted 2X with distilled water). RPE cells were digested and collected, then centrifuged at 1000 rpm at 4°C. Cells were washed twice with cold PBS (pH 7.4); the supernatant was discarded, and cells were re-suspended in cold Annexin V binding buffer at a concentration of 1×10^5 - 10^6 cells/mL. FITC Annexin V (5 μL) and PI (5 μL) were added to 490 μL cell suspension, which was gently vortexed and allowed to incubate for 15 min on ice in the dark. Cells were analyzed by flow cytometry. The pass-band filter for excitation was set at 448 nm; emission of FITC and PI were detected at 488 nm and 515 nm, respectively.

Statistical analysis

Statistical analyses were performed using the SPSS software (version 16.0). Data are reported as means \pm standard deviation. Apoptotic indices (AIs) of RPE cells with different exposure times were analyzed with one-way analysis of variance. When the variances were equal, 2:2 comparison by least-significant difference (LSD) was conducted. Comparisons by the Dunnett test was performed when sample variances were unequal. Statistical significance was achieved when $P < 0.05$.

RESULTS

TUNEL assay

The results of TUNEL assay were showed in Figures 1 and 2.

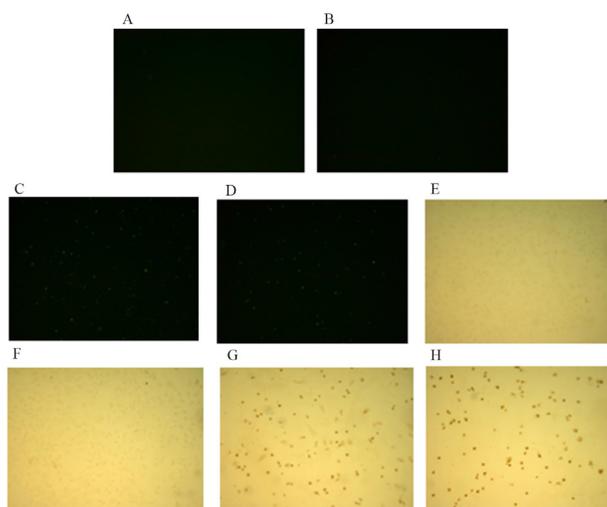


Figure 1. Cell apoptosis and necrosis analyzed by TdT-mediated dUTP nick-end labeling (TUNEL), diaminobenzidine (DAB) staining in RPE cells of A12 and B12 groups. Cultured cells were exposed to 2000 ± 500 lux blue light for 3 (A, E), 6 (B, F), 9 (C, G), and 12 (D, H) h. Cells were cultured for 12 h after light exposure.

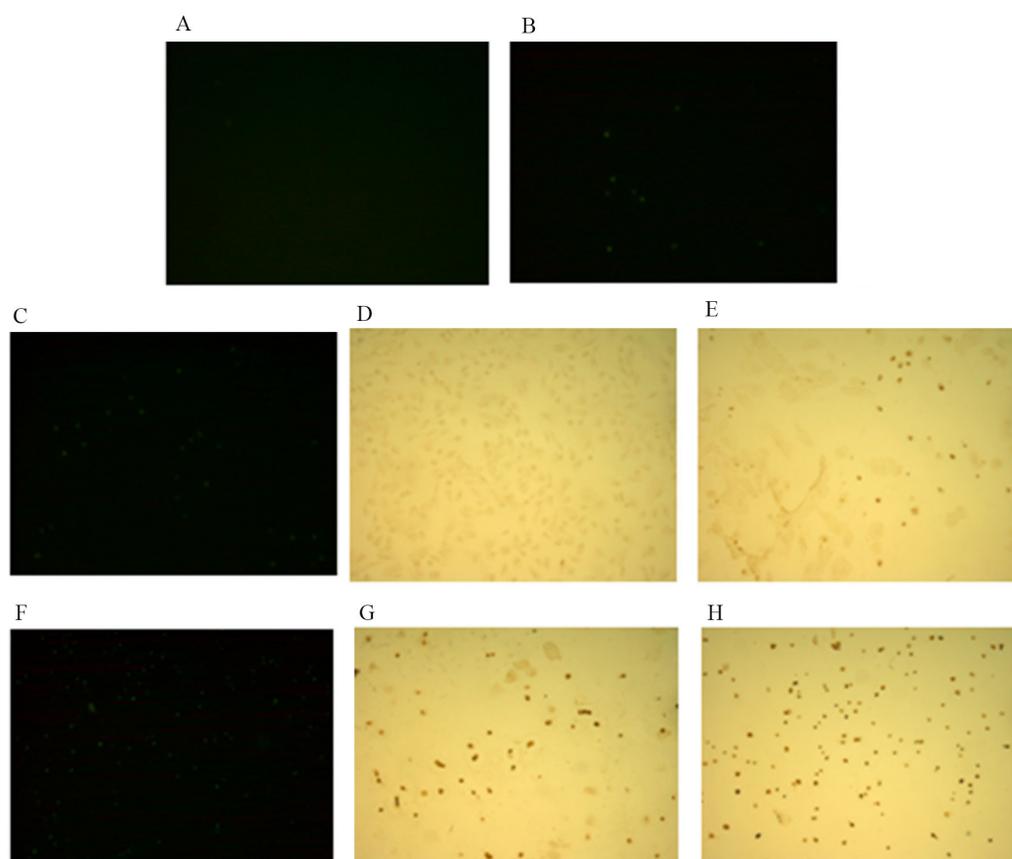


Figure 2. Cell apoptosis and necrosis analyzed by TUNEL and DAB staining in 24 h cultured RPE cells. Cultured cells were exposed to 2000 ± 500 lux blue light for 3 (**A, E**), 6 (**B, F**), 9 (**C, G**), and 12 (**D, H**) h. Cells were cultured for 24 h after light exposure.

AI

AI of subgroups cultured for the same duration

In cells of group A, which was cultured for 12 h after light exposure, the AI between A6, A9, and A12 differed significantly from that of A0 ($P = 0.000$). However, no difference was found between A3 and A0 ($P = 0.162$). In cells of group B, which were cultured for 24 h after light exposure, AI was significantly increased in B3, B6, B9, and B12 when compared to that of the control group ($P = 0.000, 0.000, 0.000, 0.000$). As indicated by Table 1, we also found that AI was significantly increased in B6, B9, and group B12 when compared to that of B3 ($P = 0.000, 0.000, 0.000$).

Table 1. AI of subgroups cultured for the same time after different exposure time.

Exposure time (h)	Fields (N)	AI index	
		12-h culture	24-h culture
0	10	1.15 ± 0.77	0.83 ± 0.57
3	10	2.88 ± 1.73	4.12 ± 1.56*
6	10	15.20 ± 1.66*	50.93 ± 1.60*
9	10	52.81 ± 2.51*	71.08 ± 1.06*
12	10	76.40 ± 2.99*	90.45 ± 0.80*

*P < 0.01 compared with the control group. Data are reported as means ± SD.

AI of subgroup exposed for the same time

Intra-group comparisons between subgroups of group A showed that AIs were similar between cells in group A3, A9 and A12. However, difference in AI was detected between group A and B after 6 h of light exposure (Figure 3).

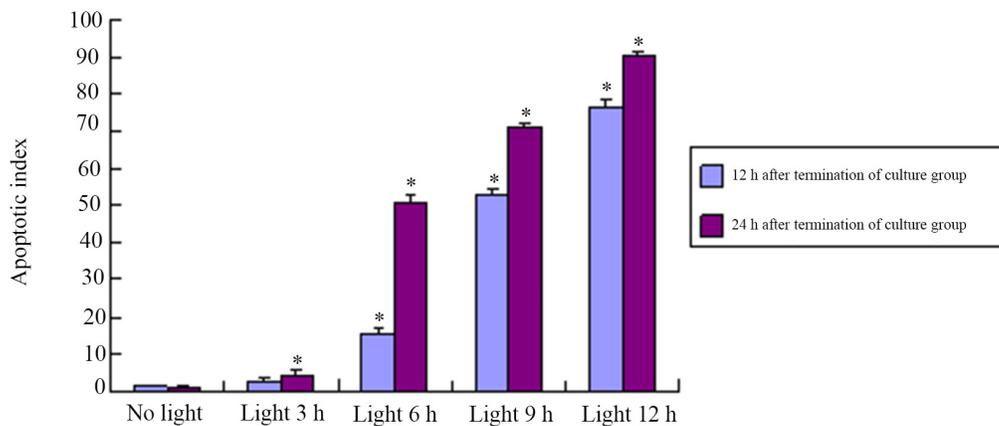


Figure 3. Subgroup AI analysis of same exposure time and different culture time. *P < 0.01 compared with the control group.

AI of subgroup cultured for 24 h after exposure

AIs of the 6-, 9-, and 12-h exposure subgroups were all found to be higher as compared to the 3-h exposure and the control subgroups (P = 0.000, 0.000, 0.000, 0.000, 0.000, 0.000). AIs of the 9- and 12-h exposure subgroup were found to be higher than the 6-h exposure subgroup (P = 0.000, 0.000). In addition, AI of the 12-h exposure subgroup was higher as compared with the 9-h exposure subgroup (P = 0.034) (Table 2, Figure 4).

Table 2. AI of subgroups cultured for 24 h with different light exposure time.

Exposure time (h)	Sample (N)	AI
0	10	13.03 ± 2.95
3	10	16.70 ± 1.60
6	10	40.70 ± 0.88*
9	10	71.80 ± 2.23*
12	10	76.60 ± 3.41*

*P < 0.01 compared with no exposure group. Data are reported as means ± SD.

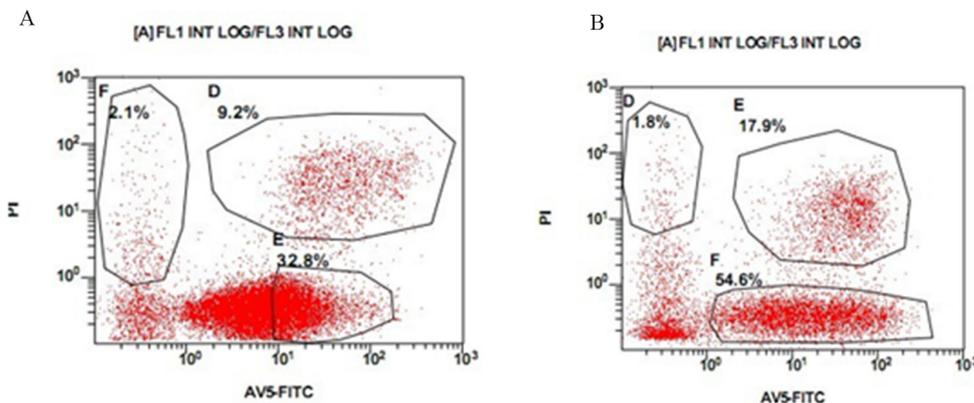


Figure 4. Scatter plot of retinal pigment epithelial cells in subgroups A6, B6, A9, and B9, as detected by flow cytometry. E indicates early apoptotic cells, D indicates late apoptotic RPE cells, and F indicates necrotic cells. The x-axis indicates Annexin V fluorescent intensity; and y-axis, PI fluorescent intensity.

DISCUSSION

Though the eyes of human beings absorb UV wavelengths below 400 nm through the cornea and the lens to protect the retina from high intensity ultraviolet (UV) light injury, the retina is still at risk for injury by visible light (Boulton et al., 2001). Studies showed that short wavelength radiation may damage the retina and can further aggravate age-related macular degeneration (Braunstein and Sparrow, 2005). In addition, free radicals and lipid peroxides have been shown to cause injury to RPE cells (Osborne et al., 2008). These injuries may be mediated by rhodopsin (Jang et al., 2005) among many other factors. Therefore, it is important to establish a light damage model of human RPE cells *in vitro* in order to study the pathogenesis of retinal degenerative diseases and age-related macular degeneration.

This study aimed to establish a stable blue-light damage model of human RPE cells *in vitro*. Following exposure to blue light, RPE cells cultured *in vitro* undergo morphological changes of apoptosis such as karyophy sepsis, hyperchromatic nuclear fragmentation, and becoming crescent or cap-shaped. Our experimental parameter included light intensity, exposure time, as well as culture time after light exposure. Cai et al. (2006) reported no significant difference between treatment groups exposed to light with an intensity of 500 ± 100 lux and the control group, while an intensity of 2000 ± 500 lux is suitable for establishing the light exposure model. Roehlecke et al. (2009) showed that blue light with an irradiance of 1 mW/cm^2 would result in ROS production and changes in matrix metalloproteinase in RPE cells, but not apoptosis. Fu et al. (2010) established a regression equation of light intensity and light irradiance, where light intensity (Lx) = light irradiance (mW/cm^2) $\times 530.699 + 28.839$. Therefore, we set our light intensity at 2000 ± 500 Lx in this study, while varying the exposure and culture time.

In a previous study by Cai et al. (2006), RPE cells cultured *in vitro* were exposed to blue light with an intensity of 2000 ± 500 Lx for 6, 12, and 24 h. Mitochondrial membrane potential and concentration of cytochrome C in the RPE cells were then examined. It was determined that after 6 h of light exposure, there was a reduction in mitochondrial membrane potential and an increase in cytochrome C concentration. In RPE cells cultured for 12, 24, and

36 h after 6 h of light exposure, increase in cytochrome C concentration has also been found. In order to study the appropriate exposure time and culture time after light exposure, cells in this study were exposed to blue light for 3, 6, 9, and 12 h; cells were then cultured for 12 or 24 h.

Fragmentation of chromosomal DNA during apoptosis is a gradual and phased process. When DNA undergoes double-stranded or single-stranded fragmentation, fluorescein sodium and DNA derivatives can be used to label the 3'-terminal. Using TUNEL to stain apoptotic cells or apoptotic bodies *in situ* could reveal the typical biochemistry and morphological characteristics of cell apoptosis. Apoptosis of RPE cells were detected by TUNEL assays in this study. In group A, in which cells were cultured for 12 h after light exposure, RPE cells exposed to 9 h of blue light underwent morphological changes of apoptosis characterized by karyophy sepsis, hyperchromatic nuclear fragmentation, and development of crescent shapes. Increased exposure time was correlated to increased cell apoptosis and induction of necrosis. In group B, in which cells were cultured for 24 h after light exposure, RPE cells that experienced 6 h of light exposure also showed morphological changes of apoptosis. Similarly, greater light exposure time was followed by increased cell apoptosis and necrosis. We found that the AI of the subgroup cultured for 12 h after a 9-h light exposure was similar to that of the subgroup that was cultured for 24 h after a 6-h light exposure.

Phosphatidylserine (PS) is normally only found on the inner face of the plasma membrane in healthy cells. However, during early apoptosis, membrane asymmetry is lost, and PS translocates to the cell surface. This unique change is a marker of apoptotic cells. Once on the cell surface, PS can be easily detected with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. Cell apoptosis can then be analyzed by flow cytometry. As PI binds to necrotic/dead cells, Annexin V-FITC and PI double staining can differentiate between apoptotic and necrotic cells. For the group that was cultured for 24 h, there were more apoptotic cells and less necrotic cell in the 6-h light exposure subgroup as compared with the 9-h exposure subgroup.

In this study, we successfully established a blue light damage model of human RPE cells *in vitro*, and suggest that the optimal condition is 2000 ± 500 lux light intensity, 6-h light exposure, and 24 h of post-exposure culture. Using this parameter, we can establish an easily duplicated and stable blue light damage model of RPE cells (Gong et al., 2013; Wu et al., 2014). This will provide the experimental basis for future studies on the mechanisms of blue-light-induced apoptosis.

Conflicts of interest

The authors declare no conflict of interest.

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