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Effects of TF-TP on Human Retinal Pigment Epithelial Cell Damage Induced by Blue Light

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Authors' Contribution
LL conceived the idea, supervised the study and wrote the manuscript. KM performed the experimental work. GA helped in collection of samples and literature review. XZ performed all the statistical tests.

Key words
Tissue factor targeted peptide (TF-TP), Blue light, Retinal pigment epithelial (RPE), Potential mechanism, CCK-8 method

ABSTRACT

The objective of this study was to investigate the effects of tissue factor targeted peptide (TF-TP) on human retinal pigment epithelial (RPE) cell damage induced by blue light and the potential mechanism. Human RPE cells were divided into blank control group, blue light model group and TF-TP group. The survival rate of human RPE cells and the optimal dose of TF-TP were detected by CCK-8 method. The morphology in the cells were observed under the inverted microscope electron microscope. The apoptosis of the cells was assayed by Hoecht staining. The expressions of TF, Bax and Bcl-2 in the cells were determined by Western blot. After treatment with TF-TP 150 μmol/L, the cell monolayer adhered to the wall, which was fusiform or polygonal. The suspended cells and cell debris were significantly reduced compared with the blue light model group. Hoecht staining showed that the apoptosis rate of the blue light model group and TF-TP 150 μmol/L group was significantly higher than that of the blank control group ($P<0.01$). The apoptosis rate of TF-TP 150 μmol/L group was significantly lower than that of blue light model group, and the difference was statistically significant ($P<0.01$). The results of Western Blot assay showed that the TF and Bax proteins in the blue model group were significantly higher than those in the blank control group, and the expression of Bcl-2 protein was significantly decreased ($P<0.01$). Compared with the blue light model group, the expression of TF and Bax protein in TF-TP 150 μmol/L group was significantly decreased, and the expression of Bcl-2 protein was significantly increased ($P<0.01$). It was concluded that pretreation of TF-TP could lessen cell apoptosis and increase cell survival rate and therefore plays a protective role to blue light-induced human RPE cells possibly by inhibiting Bax and Bcl-2 pathways mediated by TF.

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INTRODUCTION

Retinal pigment epithelium (RPE) cells are epithelial cells with secretory function at the outermost layer of the retina. They are composed of monolayer pigment epithelial cells and can be divided into three parts: The top, the body and the base (Place and Kanneganti, 2019). RPE has a unique function of self-phagocytosis and re-synthesis. It is more likely to produce oxidative stress damage when exposed to light stimulation and high oxygen environment for a long time. It is the basis for the development of various retinal diseases. Age-related macular degeneration (AMD) is one of the important eye diseases associated with age-related blindness. The WHO has reported that AMD blinds account for about 8.7% of the global blind. At present, many scholars believe that the formation of AMD disease is closely related to the destruction of RPE cell structure and function (Simó *et al*., 2010; Rohrer, 2018). Blue light is easy to cause photochemical damage of the macula of the retina, and its induction of apoptosis and related signal pathways of RPE cells has become a hot spot for clinical scholars. At present, the etiology and pathogenesis of AMD are still unclear. Relevant data show that tissue factor (TF) can regulate the expression of vascular endothelial growth factor (VEGF) in AMD patients, inhibit the formation of choroidal neovascularization, and slow down the

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progression of AMD (Daniel *et al*., 2018). In addition, TF could increase the expression of RPE cells and oxidative stress-induced photoreceptor apoptosis in AMD patients, accompanied by the imbalance of pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2 (Liang *et al*., 2016). In this paper, a human RPE cell injury model was established by blue light irradiation. The injured TFE cells were treated with tissue factor targeted peptide (TF-TP) to investigate the effect of TF-TP on blue-damaged RPE cells and its potential mechanism.

MATERIALS AND METHODS

Experimental reagents and instruments

Relevant surgeons or fresh isolated eyeballs donated for research, provided by the Eye Bank of the General Hospital of Guangzhou Military Region. The study was informed by the contributors or donors and their families. TF-TP was donated by Professor Rao Benqiang of the General Aviation Hospital. 20% fetal bovine serum and low glucose DMEM medium were provided by Gibco, USA. Mouse anti-rabbit TF, β-actin monoclonal antibody, rabbit anti-human Bax, Bcl-2 antibody were provided by Cell Signaling (CST), USA.

The spectrophotometer was provided by Taiwan Taishi Light Instrument Company. The gel electrophoresis analyzer was supplied by Media Cybernetics, USA. The multi-function microplate reader and cell incubator are supplied by Thermo Fisher Scientific. The inverted microscope (IX70121) was purchased from Olympus Corporation of Japan. The transmission electron microscope (JEM1400) was supplied by Japan Electronics Co., Ltd. The chemical luminescence instrument (Minichemi 320) was provided by Beijing Saizhi Entrepreneur Technology Co., Ltd.

Cell isolation and culture

Fresh eyeballs were immersed in PBS containing 1% penicillin-streptomycin diabody for 1 h under sterile conditions. After washing, the sclera was circumcised 3 mm behind the limbus to remove the vitreous and retinal tissue. The PBS buffer was washed again 3 times, 1% trypsin was added to the optical cup, and digested at room temperature for 1 h. The digestion was terminated using DMEM/F12 containing 10% fetal bovine serum and 1% penicillin streptomycin in double solution. The mixture was centrifuged at 1000 r/min for 10 min. In a 25 cm^2 flask having a CO_2 volume fraction of 5% and a saturated humidity of 37°C, after normal culture for 3 days in an incubator, normal liquid exchange culture was carried out. The blue light irradiation intensity reached (4.0 ± 0.5) mw/cm^2.

Detection method

CCK-8 method was adopted to detect RPE cell survival rate and TF-TP optimal dose. The logarithmic growth cycle cells were selected and digested with the digestive juice, added to the medium, and seeded at a density of 5×10^4/ml on a 96-well plate. After the cells were fused into a single layer, TF-TP10, 100, 150, 200, and 300 μmol/L of the culture solution were separately added, and 100 μl of each well was cultured. A blank control well (no drug added, conventional culture) and blank wells (no drug and cells) were established and placed in a common incubator for 24 h. The culture solution and the CCK-8 solution were mixed at a ratio of 10:1, and the dose of the mixture per well was 110 μl. The culture plate was incubated for 2 h in a dark incubator, and the absorbance value was measured by a microplate reader. Cell viability = (experimental well - blank well) absorbance value / (blank control well - blank well) absorbance value.

The remaining steps were the same as above. After adding different concentrations of TF-TP medium, blank control wells (no added drugs, conventional culture method) and blank wells (no drugs and cells) were established, and they were placed in a common incubator for 24 h. The cells were irradiated into a blue incubator for 12 h to detect the optimal dose of TF-TP.

Hoecht staining was used to detect apoptosis. The cells in the logarithmic growth cycle were digested by digestive juice, and then inoculated on a 6-well plate for blue light irradiation. The culture solution was added to a 4% paraformaldehyde fixative for 10 min, and washed twice with PBS. Hoecht staining solution was added to avoid light for 15 min and then dried at room temperature. The apoptotic cells were condensed by light blue nucleus, and each group was randomly selected for 6 fields to calculate the apoptotic rate.

Western Blot was used to detect the expression of TF, Bax and Bcl-2 proteins. Each group of human RPE cells was washed with PBS, scraped with cell lysate, and protein lysate was added to decompose the cells on ice. 10% SDS-PAGE protein electrophoresis and transmembrane quantification, milk blocking, mixing, adding primary antibody at 4°C overnight, secondary antibody incubation at 37°C for 1 h, detection of TF, Bax, Bcl-2 protein expression.

Statistical method

Statistical analysis was performed using SPSS21.0 statistical software. The data of the normal distribution was represented by (x±s). Levene test was introduced. One-way analysis of variance was used to analyze the cell viability, apoptosis rate and expression of various proteins in the blank control group, blue light model group and TF-

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TP μmol/L group. Multiple comparisons between groups were performed using LSD-t test. P<0.05 was considered to be statistically significant.

RESULTS

Survival rate of human RPE cells

The CCK-8 assay showed that the cell viability of the blank control group was (100.00±0.00) %. Compared with the cell survival rate of the blue model group, the cell survival rate of the TF-TP group was significantly higher, and the difference was statistically significant (P<0.05) (Table I). Among them, TF-TP 150μmol/L group had the highest survival rate and could be used as the optimal concentration for RPE cells.

Morphological changes of RPE cells

The blank control cells grew monolayer and showed a polygonal shape with clear cell contours. A lot of Cell volume and nuclear shrinkage, increased suspension cells and cell debris were exhibited under the inverted microscope electron microscope in the model group. After treatment with TF-TP 150μmol/L, the cell monolayer adhered to the wall, which was fusiform or polygonal. Suspension cells and cell debris were significantly reduced compared to the blue light model group (Fig. 1).

Apoptosis of RPE cells

Hoecht staining showed that the apoptosis rate of the blue light model group and the TF-TP 150μmol/L group was significantly higher than that of the blank control group (P<0.01), and the apoptosis rate of the TF-TP 150μmol/L group and the blue light model group. The difference was statistically significant (P<0.01, Table I).

Expression of TF, Bax and Bcl-2 proteins

The results of Western Blot showed that the TF and Bax proteins in the blue model group were significantly higher than those in the blank control group, and the expression of Bcl-2 protein was significantly decreased (P<0.01). Compared with the blue light model group, the expression of TF and Bax protein in TF-TP 150 μmol/L group was significantly decreased, and the expression of Bcl-2 protein was significantly increased (P<0.01). The results are shown in Figure 2 and Table II.

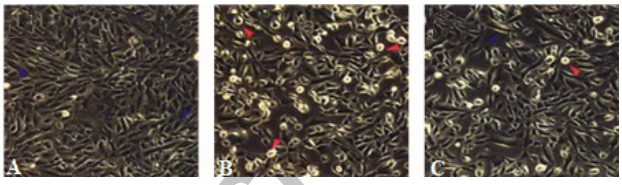

Fig. 1. Morphological changes of RPE cells (×200) in blank control group (A); blue model group (B), and TF-TP 150μmol/L group (C).

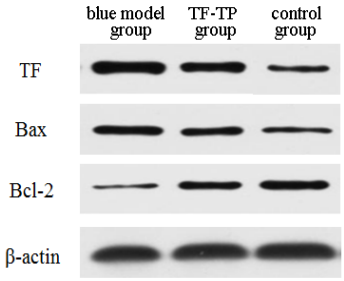

Fig. 2. Expression of TF, Bax and Bcl-2 proteins in each group detected by Western Blot test.

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Table I. Comparison of survival and apoptosis rates of human RPE cells.

	Control (n=10)	Blue model (n=10)	TF-TP (μmol/L)					F	P
			10 (n=10)	100 (n=10)	150 (n=10)	200 (n=10)	300 (n=10)		
Survival rate (%)	100.00±0.00	42.68±6.44*	56.13±7.78**	58.86±4.62**	62.34±3.46**	57.77±5.48**	55.84±2.79**	199.23	<0.002
Apoptosis rate (%)	0.98±0.17	9.87±0.71***	-	-	5.62±0.77***#	-	-	205.17	<0.001

*, Comparison with the blank control group P<0.05; **, P<0.05 compared with the blue model group; ***, indicates P<0.05 compared with the blank control group; #, indicates comparison with the blue light model group P<0.05.

Table II. Expression of TF, Bax and Bcl-2 proteins in different treatment groups (x±s).

	Control (n=10)	Blue model (n=10)	TF-TP (μmol/L)	F	P
			150 (n=10)		
TF	0.40±0.06	0.96±0.06*	0.55±0.05#	105.25	<0.001
Bax	0.73±0.05	1.34±0.05*	0.88±0.03#	142.35	<0.001
Bcl-2	1.10±0.05	0.48±0.04*	0.60±0.04#	64.56	<0.001

*, indicates P<0.05 compared with the blank control group; #, indicates comparison with the blue light model group P<0.05.

DISCUSSION

It is well known that retinal photo damage is closely related to the occurrence and development of various vitreoretinopathy. Under normal conditions, the structural integrity of RPE cells is important to maintain normal retinal function. Hypoxia, physical factors, oxidative stress, drugs and many other factors can cause damage to RPE cells. Once they die, they cannot be regenerated. The defective areas can only be filled by the expansion and migration of adjacent RPE cells (Gao *et al*., 2019; Fazeli and Wehman, 2017). Among them, RPE cell injury is the starting point of pathological changes of dry AMD. AMD is a macular disease with increasing incidence with age, which has become the main cause of low vision and blindness in the elderly. Related data show that RPE cells not only have the functions of transporting nutrients, storing optical pigments, scavenging free radicals, phagocytosis and degrading the extracellular segment of photoreceptor cells, but also forming a blood-retinal barrier with Bruch's membrane, which of great significance to the self-balancing and visual maintenance of the retina (Kaarniranta *et al*., 2017). Epidemiological studies have shown that the occurrence of AMD is related to the exposure of blue light or ultraviolet light. Retinal light damage is related to the intensity, time and distance of light radiation. Due to its high energy and penetrability, blue light could cause photochemical damage to the retina and apoptosis through various mechanisms (Velez-Montoya *et al*., 2014). In this study, we found that blue light irradiated RPE cells at a certain radiation intensity for 12 h, resulting in a significant decrease in cell volume and cell nucleus, and a significant increase in suspended cells and cell debris by establish a blue light-induced RPE cell injury model.

TF is a 47KD transmembrane glycoprotein that is a receptor for factor VII on the cell surface. TF can promote coagulation by activating the exogenous coagulation cascade, and can initiate the exogenous coagulation pathway by binding to FVIIa in plasma to promote the formation of hypercoagulable state in patients, which participating in inflammation, apoptosis, and pathological angiogenesis (Zhang *et al*., 2016; Pittala *et al*., 2018). Related data show that abnormal expression of TF is associated with oxidative stress and inflammatory response, and its level is highly expressed in apoptotic RPE cells and receptors, and high levels of TF can further aggravate the inflammatory response and promote the production of reactive oxygen species. TF-TP has a high affinity for TF, and TF-TP is a novel drug obtained by screening method, which could successfully inhibit the high expression of TF (Søgaard *et al*., 2018). The CCK-8 assay showed that the cell survival rate of TF-TP group was significantly higher than that of the blue model group ($P<0.05$). Among them, TF-TP 150 μmol/L group had the highest survival rate and could be used as the optimal concentration for RPE cells. Hoecht staining showed that the apoptosis rate of the blue light model group and the TF-TP 150 μmol/L group was significantly higher than that of the blank control group ($P<0.01$). The apoptosis rate of TF-TP 150 μmol/L group was significantly lower than that of blue light model group ($P<0.01$), suggesting that TF-TP can significantly increase the survival rate of blue-induced human RPE cell injury and reduce the apoptosis rate.

Apoptosis is an autonomously ordered cell death controlled by genes to maintain intracellular homeostasis. Apoptosis is an active process and closely related to the activation, expression and regulation of a range of proteins (Sitarek *et al*., 2016). Bcl-2 is widely distributed in mitochondrial outer membrane, nuclear membrane and endoplasmic reticulum membrane, and can play a role in inhibiting apoptosis by encoding protein Bcl-2. Among them, Bax is an important pro-apoptotic factor in the Bcl-2 family. It has been confirmed that photoreceptor cells could cause a decrease in Bcl-2 expression and an increase in Bax expression (Yang *et al*., 2019; Dwivedi *et al*., 2019). The results of Western Blot assay showed that the TF and Bax proteins in the blue model group were significantly higher than those in the blank control group, and the expression of Bcl-2 protein was significantly decreased ($P<0.01$). Compared with the blue light model group, the expression of TF and Bax protein in TF-TP 150 μmol/L group was significantly decreased, and the expression of Bcl-2 protein was significantly increased ($P<0.01$). It suggested that TF-TP could significantly inhibit the apoptosis of human RPE cells induced by blue light, which may be related to the Bcl-2/Bax apoptosis pathway.

CONCLUSION

Pretreation of TF-TP could lessen cell apoptosis and increase cell survival rate and therefore plays a protective role to blue light-induced human RPE cells possibly by inhibiting Bax and Bcl-2 pathways mediated by TF.

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Ethical statement

The study was carried out in compliance with guidelines issued by ethical review board and institutional biosafety committee of Zhejiang Jinhua Eye Hospital and Anhui Fuyang Futian Eye Hospital

IRB approval

Ethics Committee approval was obtained from the Institutional Ethics Committee of "The Second Affiliated Hospital of Xi'an Medical University, The Second Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine" to the commencement of the study.

Data availability

The data of the study would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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