• Basic Research •

Down regulation of UCP2 expression in retinal pigment epithelium cells under oxidative stress: an *in vitro* study

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Abstract

• AIM: To evaluate the expression of uncoupling protein 2 (UCP2) in a retinal pigment epithelium cell line (ARPE-19), under oxidative stress (OS).

• METHODS: ARPE-19 cells were divided into groups treated with various concentrations of hydrogen peroxide (H_2O_2; 0, 150, 300, 500, 700, and 900 μmol/L) for 24h, to induce oxidative damage and cell viability was assessed by MTT assay. UCP2 mRNA expression in cells treated with H_2O_2 was investigated by reverse transcription-polymerase chain reaction (RT-PCR). UCP2 protein expression was assessed by Western blotting and ROS levels analyzed by flow cytometry (FCM). Further, UCP2-siRNA treated cultures were exposed to H_2O_2 (0, 75, 150, and 300 μmol/L) for 2h and cell viability determined by MTT assay.

• RESULTS: Cells treated with higher concentrations of H_2O_2 appeared shrunken; their adhesion to adjacent cells was disrupted, and the number of dead cells increased. The results of cell viability assays demonstrated that the numbers of cells were decreased in a dose-dependent manner following treatment with H_2O_2. Compared with untreated controls, cell viability was significantly reduced after treatment with >300 μmol/L H_2O_2 (P<0.05). Cell metabolic activity was decreased with increased concentrations of H_2O_2 as detected by MTT assay. Levels of OS were further decreased in cells treated with UCP2-siRNA compared with those treated with H_2O_2 alone (P<0.05). The results of RT-PCR and Western blotting demonstrated that UCP2 expression was reduced in H_2O_2-treated groups compared with controls (P<0.05). FCM analysis showed that cell reactive oxygen species (ROS) levels were increased in H_2O_2-treated groups and further upregulated by UCP2-siRNA treatment (P<0.05).

• CONCLUSION: Expression levels of UCP2 are decreased in ARPE-19 cells treated with H_2O_2. ROS levels are further increased in cells treated with UCP2-siRNA relative to those treated with H_2O_2 alone. UCP2 may have a protective role in ARPE-19 cells during oxidative injury.

• KEYWORDS: uncoupling protein 2; retinal pigment epithelium cells; oxidative stress

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness among people over 55y in western countries[1]. The number of patients with AMD continues to increase, and it is estimated approximately 198 million people currently suffered from the disease[2]. AMD is generally considered to be caused by various biochemical, immunogenic, and environmental factors[3]. A number of retinal pathologies, including AMD, are associated with mitochondrial dysfunction[4], which induces increased levels of reactive oxygen species (ROS), mitochondrial DNA damage, and defective metabolic activity[5]. The most recent studies point to the key role of oxidative stress (OS) in the pathogenesis of AMD[6-7].

Uncoupling protein 2 (UCP2), a member of the anion carrier family that localizes to the inner mitochondrial membrane, can decrease ROS production[8], and is widely expressed in rodents and adult human tissues. UCP2 has protective effects on numerous cells, through its function in controlling the production of superoxide and other downstream ROS. We have shown that mitochondrial function declines in aged retinal pigment epithelium (RPE) cells and that OS aggravates this condition, supporting a potential role for mitochondrial dysfunction in RPE cell damage and AMD onset[9-10]. Therefore, we hypothesized that UCP2 may have a protective role in RPE cells during H_2O_2 injury.

The aims of our experiment were to investigate UCP2 expression in RPE cells exposed to OS *in vitro* and to explore how UCP2 is involved in the protection of these cells.

MATERIALS AND METHODS

Cell and Chemical Reagents The RPE cell line, ARPE-19, was purchased from ATCC (Manassas, Virginia, USA). Hydrogen peroxide (H_2O_2) was from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco BRL (Carlsbad, CA, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell Culture Culture medium consisted of DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Cultures were maintained at 37℃ in a humidified atmosphere of 95% air and 5% CO_2, and passages 3-10 were used for experiments.

Hydrogen Peroxide Treatment ARPE-19 cells were plated in 96-well plates at 1.2×10^4 cells/well for 12h. H_2O_2 was added at 0, 150, 300, 500, 700, or 900 μmol/L for 24h.

MTT Assay Cell viability was quantitatively assessed using MTT assays. In each assay, PBS-treated and untreated cells were used as blank controls and negative controls, respectively. Cells were washed three times with PBS, then incubated with 20 μL MTT (5 mg/mL) reaction mixture at 37℃ for 4h. Reactions were terminated by adding 150 μL DMSO after aspiration of the reaction liquid. Subsequently, optical density (OD) was measured at 490 nm, using an Epoch microplate spectrophotometer system (BioTek, Winooski, USA). Five samples were measured from each group and average OD values calculated.

Analysis of UCP2 mRNA Expression Total mRNA was isolated from cells using TRIzol extraction according to the manufacturer's protocol. First-strand cDNA was synthesized using an iScript cDNA synthesis kit (BioRad), and reverse transcription-PCR (RT-PCR) was performed using the SuperScript First Strand Synthesis System for RT-PCR (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal RNA loading control. cDNA aliquots (2 μL) were subsequently amplified in a total reaction volume of 25 μL, containing 2×TaqPCRMaster Mix (12.5 μL), 1.0 μL each of forward and reverse primers, and ddH_2O (8.5 μL). The following primers were used to amplify human UCP2: forward, 5'-CTACAAGACCATTGCACGAGAGG-3' and reverse, 5'-AGCTGCTCATAGGTGACAAACAT-3'; expected product size, 396 bp. Control primers for GAPDH were: forward, 5'-CAAGGTCATCCATGACAACTTTG-3' and reverse, 5'-GTCCACCACCCTGTTGCTGTAG-3'; expected product size, 496 bp. Each reaction was denatured at 95℃ for 5min, amplified using 35 cycles of 95℃ for 30s, 60℃ for 30s, and 72℃ for 30s, and underwent a final extension at 72℃ for 10min. PCR products were separated by electrophoresis on 1% agarose gels, visualized under UV light, and compared against a Ready-Load 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). To quantitate levels of UCP2, bands identified by electrophoresis were analyzed using the Bio-Rad chemiluminescence imaging system, and Image Lab software, Version5.1 (Bio-Rad).

Western Blot Analysis of UCP2 Expression ARPE-19 cells were lysed using Cytobuster lysis buffer (Novagen, Madison, WI, USA) and protein concentrations estimated using a Dc Protein Assay Kit (BioRad). Protein samples (30 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies (1:1000) for 3h at room temperature after blocking with 5% (wt/vol) nonfat dried milk, followed by washing and incubation with horseradish peroxidase secondary antibodies for 1h at room temperature. Bound antibodies were visualized using the Bio-Rad ECL detection system and protein levels quantified with Image J software.

ROS Measurement ARPE-19 cells were detached by trypsinization, and 2×10^6 cells/mL incubated with H2-DCF-DA (0.4 μmol/L) at 37℃ for 30min. Cells were analyzed by flow cytometry (FCM; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) using 488-nm excitation and 530-nm emission wavelengths. Data were analyzed using FCS Express software.

Treatment of Cells with UCP2-siRNA UCP2-siRNA was purchased from GeneCopoeia and transfected according to the manufacturer's recommendations. ARPE-19 cells were grown to approximately 80% confluence, then incubated with 1.0 μg scrambled siRNA or UCP2-siRNA in the presence of EndoFectin Lenti transfection reagent (3 μL/mL). Transfection was conducted without serum in the growth medium. After transfection (3h), a one-half volume of DMEM containing 30% FBS was added to the cells, and cultures grown for 48h. UCP2-siRNA efficacy was estimated by confocal microscopy. ARPE-19 cells mock transfected with siRNA were used as negative controls. After transfection (24h) UCP2-siRNA treated cultures were exposed to H_2O_2 (75, 150, 300 μmol/L) for 2h and cell viability measured by MTT assay.

Statistical Analysis All experiments were conducted in triplicate and each assay was repeated twice. Data are expressed as mean±standard deviation (SD). Differences between groups were analyzed by one-way ANOVA and post-hoc multiple comparisons performed using the Tukey HSD test when ANOVA testing indicated significance (P<0.05) using SPSS version 22.0 for Windows.

RESULTS

Morphological Changes in ARPE-19 Cells Treated With Different H_2O_2 Concentrations ARPE-19 cells were seeded in 6-well plates at 1.0×10^5 cells/well and cultured for 24h. Then, H_2O_2 (0, 150, 300, 500, 700, and 900 μmol/L) was added and cells grown for a further 24h before observation by

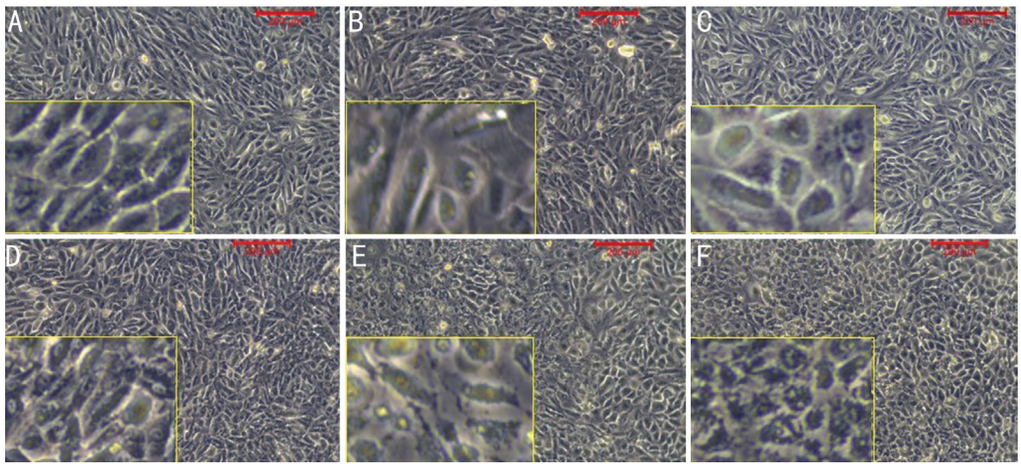

Figure 1 Morphological changes in ARPE-19 cells following treatment with various concentrations of H_2O_2 H_2O_2 [0 (A), 150 (B), 300 (C), 500 (D), 700 (E), and 900 μmol/L (F)] was added and cells cultured for 24h. The number of ARPE-19 cells decreased with increased H_2O_2 concentration and their morphology became irregular and shrunken, with increased numbers of dead cells. Scale bar: 200 μm. Magnification, ×100.

microscopy to evaluate morphological changes. Cells treated with higher concentrations of H_2O_2 appeared shrunken cells, with disruption of adhesion between adjacent cells, and the numbers of dead cells was increased (Figure 1).

Effect of H_2O_2 on ARPE-19 Cell Metabolic Activity MTT assay demonstrated that ARPE-19 cell metabolic activity clearly decreased after H_2O_2 treatment. As concentrations of H_2O_2 increased, the number of viable cells decreased. Following post-hoc correction for multiple comparisons, there was a significant difference in the viability of cells treated with 300 μmol/L H_2O_2 compared with untreated controls (n=5; P=0.000); however, no significant differences were found on comparison of 300 *vs* 500 μmol/L, 500 *vs* 700 μmol/L, or 700 *vs* 900 μmol/L H_2O_2 treatment groups (all P>0.05; Figure 2). Nevertheless, increased concentration of H_2O_2 was associated with reduced cell metabolic activity, as detected by decreased absorbance (OD) values, indicating that H_2O_2 could induce apoptosis of ARPE-19 cells *via* OS (Figure 2).

UCP2 mRNA Expression in ARPE-19 Cells Exposed to Various H_2O_2 Concentrations RT-PCR results revealed that the expression of UCP2 was decreased in a dose-dependent manner on treatment with H_2O_2. Following, post-hoc multiple comparisons, mRNA levels of UCP2 were significantly reduced compared with the untreated control group (n=5, P<0.05; Figure 3A). No significant differences were detected between cells treated with different concentrations of H_2O_2 (P>0.05; Figure 3B).

UCP2 Protein Expression in ARPE-19 Cells Treated with H_2O_2 Western blotting showing that the expression of UCP2 was significantly decreased after treatment with 75, 150 and 300 μmol/L H_2O_2 compared with untreated controls (P<0.05); however, there were no significant differences in UCP2 expression levels between the different treatment groups (P>0.05; Figure 4).

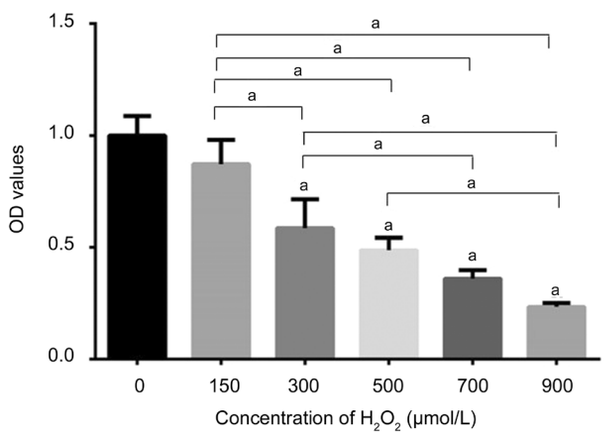

Figure 2 Analysis of cell metabolic activity Cell metabolic activity gradually decreased with increasing H_2O_2 concentration. Compared with the untreated control group, cell viability was significantly reduced by treatment with 300 μmol/L H_2O_2 (n=5, ^aP<0.05).

Effect of UCP2-siRNA on ROS Levels in H_2O_2-treated ARPE-19 Cells Measurement of ROS by FCM, according to H2-DCF-DA fluorescence, in ARPE-19 cells treated with H_2O_2 (0, 75, 150, 300 μmol/L) demonstrated that ROS levels were significantly increased in groups treated with H_2O_2 compared with untreated controls (P<0.05; Figure 5). The ROS levels of cells treated with UCP2-siRNA exhibited an additional increase compared with those treated with 300 μmol/L H_2O_2 alone (P<0.05; Figure 6).

Effect of UCP2-siRNA on Viability in H_2O_2-treated ARPE-19 Cells ARPE-19 cells treated with UCP2-siRNA exhibited reduced UCP2 expression compared with controls (Figure 7). H_2O_2 (75, 150, 300 μmol/L) was added to UCP2-siRNA treated cultures 24h after transfection for 2h. The control group was not treated with either UCP2-siRNA or H_2O_2. MTT assay showed that UCP2-siRNA-treated cell metabolic activity clearly decreased after treatment with H_2O_2. Cells treated with UCP2-siRNA exhibited a further decrease in viability,

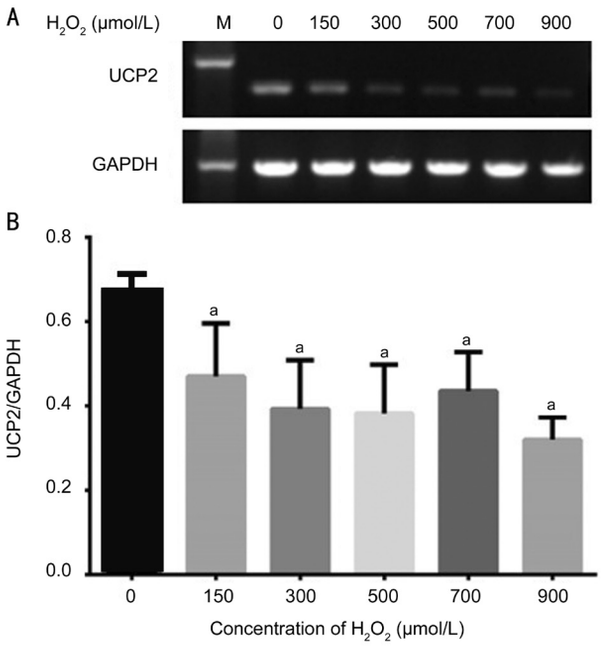

Figure 3 UCP2 mRNA levels in ARPE-19 cells under OS A: RT-PCR showing that down regulation of UCP2 expression in ARPE-19 cells under H_2O_2-induced OS; B: The expression of UCP2 was decreased in a dose-dependent manner in response to H_2O_2 compared with the untreated control group ($^aP<0.05$) and no significant differences were detected between cell groups treated with different concentrations of H_2O_2 ($P>0.05$).

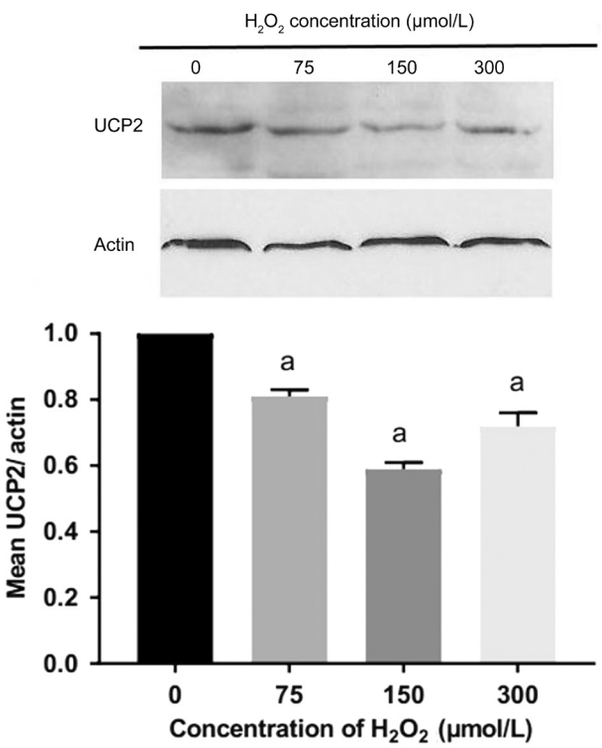

Figure 4 Western blot showing that the expression of UCP2 was significantly decreased after treatment with 75, 150, 300 μmol/L H_2O_2 relative to the untreated control group ($^aP<0.05$).

compared with those treated with H_2O_2 alone. Cell viability was significantly reduced relative to controls (n=5, $P<0.05$; Figure 8).

DISCUSSION

The molecular mechanisms underlying AMD remain poorly understood. Multiple influences contribute to AMD, including biochemical, immunogenic, and environmental factors, as well as OS. OS is a major contributor to the pathologies associated with AMD. The degeneration of RPE cells in the sub retinal pigment epithelial space and choroid is an initial pathological characteristic of AMD and the leading cause of severe vision loss in older people. Moreover, OS is implicated as a major inducer of RPE cell death.

Mitochondria are the main source of ROS production and excess ROS can induce mitochondrial damage and lead to disease[11-12]. High ROS levels result in cell membrane damage *via* lipid peroxidation, which enhances oxidative injury, causing energy depletion and increasing ROS production. Uncoupling proteins (UCPs) localize to the mitochondrial inner membrane and belong to a superfamily of mitochondrial anion transporters that uncouple ATP synthesis from oxidative phosphorylation, by controlling the leakage of protons across the inner mitochondrial membrane[13-14]. UCPs are important natural antioxidants involved in the maintenance of ROS homeostasis, alongside scavenging enzymes (*e.g.* superoxide dismutase and catalase) and low molecular weight antioxidants (*e.g.* ascorbic acid and glutathione)[15]. To date, five UCP subunits (UCP1-5) have been discovered in mammals, among which, UCP2 is widely distributed in various tissues and has been extensively studied[16]. UCP2 is an important mediator of oxidative damage. It is established that increased UCP2 expression can attenuate oxidant tissue damage[17-18].

Available animal models fall short of accurately representing the characteristics of AMD, as they lack human genetic polymorphisms and long-term exposure to OS and environmental factors[19]; however, some cell models have been described. Culture of RPE cells with H_2O_2 is an established model for the study of OS. Our previous research demonstrated that UCP2-deficient RPE cells had increased ROS levels and that increasing UCP2 expression using pigment epithelium-derived factor dampened ROS production in aging and deficient cells exposed to OS[20]. In the present study, we found that UCP2 mRNA levels were reduced in ARPE-19 cells under OS, relative to controls. UCP2 decreases ROS production by regulating ATP generation in mitochondria. In this study, UCP2 expression was decreased in ARPE-19 cells exposed to OS. Further, we analyzed the metabolic activity of cells treated with UCP2-siRNA and H_2O_2, demonstrating that it was further decreased in UCP2-siRNA-treated cells exposed to OS, compared with those treated with H_2O_2 alone, according to increased ROS levels detected in UCP2-siRNA treated cells. These results clarify that UCP2 functions to partially protect ARPE-19 cells under OS. Our previous study also showed that UCP2 could

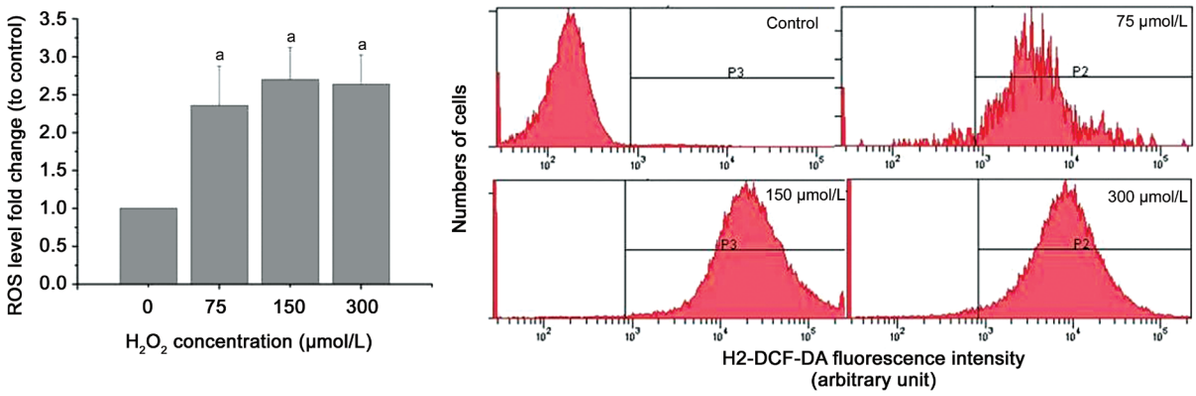

Figure 5 Flow cytometry measurement of ROS levels by detection of H2-DCF-DA fluorescence Data are shown as fold-change in fluorescence levels compared with controls. ROS levels in ARPE-19 cells were significantly increased in cells treated with H_2O_2 compared with untreated controls ($^aP<0.05$).

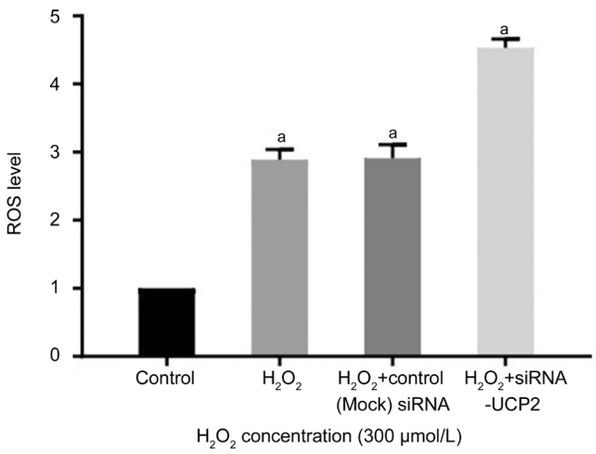

Figure 6 Flow cytometry measurement of ROS levels by H2-DCF-DA fluorescence The ROS levels in cells treated with UCP2-siRNA were increased compared with those exposed to 300 μmol/L H_2O_2 alone ($^aP<0.05$).

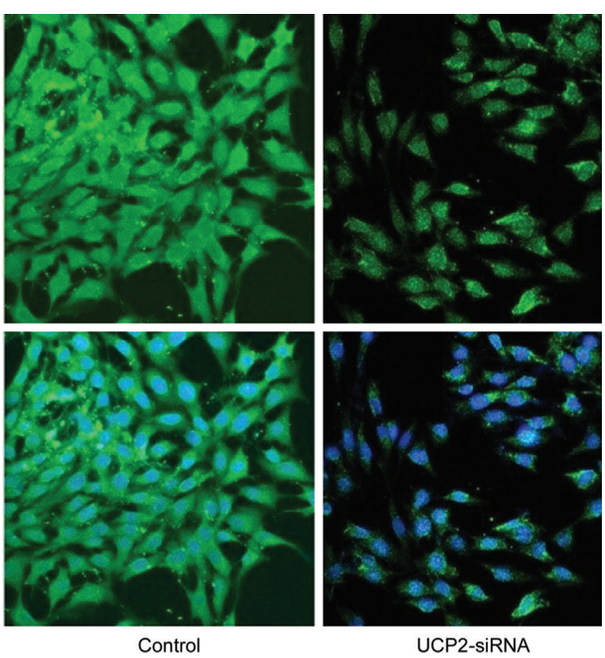

Figure 7 UCP2-siRNA transfected ARPE-19 cells Cells transfected with UCP2-siRNA showed reduced UCP2 expression compared with controls.

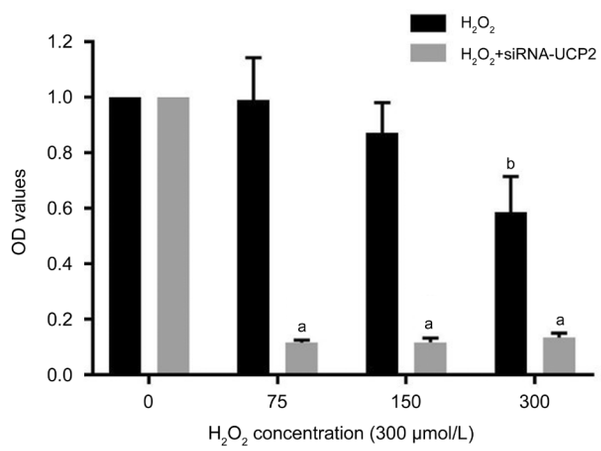

Figure 8 H_2O_2 (75, 150, 300 μmol/L) was added to UCP2-siRNA treated cultures 24h after transfection and incubated for 2h MTT assay showing that metabolic activity in UCP2-siRNA treated cells was clearly decreased following H_2O_2 exposure. Cells treated with UCP2-siRNA exhibited a further decrease compared with those treated with H_2O_2 alone. Cell viability was significantly reduced, relative to controls (n=5, $P<0.05$). $^aP<0.05$ *vs* H_2O_2 treatment, $^bP<0.05$ *vs* control.

reduce ROS generation; therefore, we speculated that reduced expression of UCP2 was associated with elevated ROS, resulting in decreased cellular anti-oxidation function. This finding is consistent with those of several previous studies, where over expression of UCP2 was reported to attenuate oxidant tissue damage[17-18]; however, we did not detect significant differences among cell groups treated with different concentrations of H_2O_2, implying that its function in regulating ATP generation or maintaining energy balance may not be the primary role of UCP2 in human RPE cells, where it may function to reduce ROS generation, promote apoptosis, or perform other roles. One interpretation of our results could be that excessive H_2O_2 oxidative damage causes cells death, thereby decreasing total cell numbers. Overall, our results demonstrate that decreased of UCP2 expression may lead to cell death.

The present study demonstrates that UCP2 gene expression is decreased in human ARPE-19 cells in response to H_2O_2-induced OS *in vitro*. ROS levels in these cells were further increased in UCP2-siRNA treated cells. Hence, the UCP2 gene may have a protective role in RPE cells under OS.

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Conflicts of Interest: Liu Y, None; **Ren Y,** None; **Wang X,** None; **Liu X,** None; **Xu Y,** None; **He Y,** None.

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