• Basic Research •

Protective effect of rosmarinic acid on retinal pigment epithelial injury induced by oxidative stress through activation of REV-ERBα/Nrf2 pathway

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Abstract

● **AIM:** To investigate the protective effects of rosmarinic acid (RA) against hydrogen peroxide (H_2O_2)-induced oxidative stress and cellular senescence in human retinal pigment epithelial (RPE) cells and the involvement of the reverse erythroblastosis virus alpha (REV-ERBα)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway.

● **METHODS:** The adult retinal pigment epithelial cell line-19 (ARPE-19) was pretreated with RA (20, 40, and 80 μmol/L) for 48h and then exposed to H_2O_2 (100–1600 μmol/L) for 24h to establish the oxidative stress model. Cell viability was measured using a cell counting kit-8 (CCK-8) assay, and 200 μmol/L H_2O_2 and 80 μmol/L RA were selected as the optimal conditions. Cells were divided into control, H_2O_2, and RA+H_2O_2 groups; the REV-ERBα agonist SR9009 and antagonist SR8278 were applied to explore the underlying mechanism. Oxidative stress was assessed by flow cytometry for reactive oxygen species (ROS) and by biochemical kits for malondialdehyde (MDA) and superoxide dismutase (SOD). Cellular senescence was evaluated by senescence-associated β-galactosidase (SA-β-gal) staining and Western blotting of P53 and P21. Mitochondrial function was examined by real-time polymerase chain reaction (PCR) for mitochondrial DNA copy number (mtDNA-CN), JC-1 staining for mitochondrial membrane potential (ΔΨm), and immunofluorescence for zonula occludens protein 1 (ZO-1). REV-ERBα and Nrf2 protein levels were detected by Western blot.

● **RESULTS:** RA pretreatment significantly increased cell viability (P<0.01) and decreased ROS and MDA levels, and increased SOD activity (all P<0.001) in H_2O_2-treated cells. RA also reduced the percentage of SA-β-gal-positive cells and the expression of P53 and P21, partially restored mtDNA-CN and ΔΨm, and enhanced ZO-1 expression (P<0.001). SR9009 partially restored REV-ERBα and Nrf2 expression, whereas SR8278 significantly diminished the protective effects of RA (P<0.001).

● **CONCLUSION:** RA protects RPE cells against H_2O_2-induced oxidative stress, senescence, and mitochondrial dysfunction by activating the REV-ERBα/Nrf2 pathway, suggesting that RA may be a promising therapeutic agent for retinal degenerative diseases such as age-related macular degeneration.

● **KEYWORDS:** rosmarinic acid; oxidative stress; senescence; REV-ERBα; Nrf2; age-related macular degeneration

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INTRODUCTION

Age-related macular degeneration (AMD) represents a significant threat to vision, particularly in industrialized nations with increasing life expectancies[1]. This complex disease is influenced by multiple factors, with oxidative stress and chronic inflammation playing critical roles in its development, particularly in the degeneration of the retinal pigment epithelial (RPE)[2-3]. The precise interactions of the pathophysiological events involved in degeneration processes are still not fully clarified. Currently, no conclusive treatment exists to arrest the progression of AMD, and research is ongoing to discover viable therapeutic options for AMD[4]. Cellular senescence is a condition in which cells irreversibly

stop dividing in response to various stressors, such as oxidative stress, and has been identified as a key contributor to aging and age-related diseases[5]. The role of senescent RPE cells in retinal degeneration has been well documented, making them a promising target for therapy in the management of AMD[6].

Rosmarinic acid (RA) is a natural compound derived from the esterification of caffeic acid and 3,4-dihydroxyphenyllactic acid, and it is found in plants belonging to the *Lamiaceae* and *Boraginaceae* families. This compound exhibits various biological and pharmacological effects, such as antioxidant and anti-inflammatory activities[7-8]. A wealth of studies has demonstrated the therapeutic potential of RA across various human diseases, encompassing inflammatory conditions, neurodegenerative diseases, cancer[9], diabetes and ocular neovascularization[10]. Prior research has indicated that the nuclear receptor reverse erythroblastosis virus alpha (REV-ERBα), abundantly expressed in RPE, acts as a novel intrinsic defender against age-related oxidative stress[11]. Additionally, the nuclear factor erythroid 2-related factor 2 (Nrf2) has been identified as a crucial endogenous antioxidant[12]. Considering that senescent RPE cells exhibit compromised functions in AMD, resulting in neurotrophic deficits and abnormal secretion of angiogenic factors, this study aimed to assess whether RA can mitigate the senescence of RPE cells subjected to oxidative stress, thereby providing a protective effect in the context of AMD. Furthermore, we investigated the role of the REV-ERBα/Nrf2 pathway in this protective mechanism.

MATERIALS AND METHODS

Cell Culture and Treatment The adult retinal pigment epithelial cell line-19 (ARPE-19) cell line, which originates from human RPE, was sourced from the cell bank of the Chinese Academy of Sciences in Shanghai, China. Cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12, Gibco, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2. To induce senescence, varying concentrations of hydrogen peroxide (H_2O_2; 100, 200, 400, 800, and 1600 μmol/L) were applied for 24h.

For drug treatment, ARPE-19 cells were pretreated with RA at 20, 40, and 80 μmol/L (Shanghai Yuanye Bio-Technology Co., Ltd., China) for 48h, followed by the optimal concentration of H_2O_2 for 24h. The control group consisted of cells cultured without drug treatment. To evaluate the protective effect of RA against oxidative stress, three groups were established: control, H_2O_2-treated, and RA+H_2O_2-treated. Then, to explore the mechanisms involved, ARPE-19 cells were divided into a control group, H_2O_2 group, and H_2O_2+SR9009 [a REV-ERBα agonist, 20 μmol/L; MedChemExpress (MCE), China] group. Finally, ARPE-19 cells were divided into a control group, H_2O_2 group, RA+H_2O_2 group, and RA+H_2O_2+SR8278 (a REV-ERBα antagonist, 0.5 μmol/L; MCE, China) group. Cells in each group were pretreated with RA for 48h, followed by combined exposure to H_2O_2 with or without SR9009 or SR8278 for 24h.

Cell Viability Assay ARPE-19 cell viability was evaluated using a cell counting kit-8 (CCK-8) assay kit (Qisai Biotechnology Co., Ltd., Wuhan, China). Approximately 5×10^3 cells per well were seeded in 96-well plates containing 100 μL medium and incubated overnight. After treatment with various H_2O_2 concentrations or 48h RA pre-treatment, 10 μL of CCK-8 reagent was added to each well and incubated for an additional 2h in the dark. Absorbance at 450 nm was measured using a Bio-Rad microplate reader, with blank wells as controls. Cell proliferation was determined from the absorbance readings.

Senescence-Associated B-Galactosidase Activity Assay A total of 3×10^5 cells per well were seeded in 6-well culture plates. After treatment, cells were stained with senescence-associated β-galactosidase (SA-β-gal) according to the manufacturer's instructions (Servicebio, Wuhan, China). Blue-stained positive cells were observed under an optical microscope (Nikon, Japan). Five fields per group were analyzed, and the percentage of positive cells was quantified using ImageJ software.

Measurement of ROS by Flow Cytometry Total reactive oxygen species (ROS) in ARPE-19 cells were measured using the non-fluorescent probe 2',7'-dichlorodihydrofluoresce in diacetate (DCFH-DA, Beyotime, Shanghai, China). The cells were cultivated in 6-well plates and allowed to incubate overnight prior to the measurement. After treatment according to experimental groups, cells were collected, washed twice with phosphate-buffered saline (PBS), and incubated in the dark for 20min at 37°C with serum-free medium containing 10 μmol/L DCFH-DA. Following three additional washes, cells were analyzed for mean fluorescence intensity using a CytoFLEX flow cytometer (Beckman Coulter, Brea, USA).

Detection of Malondialdehyde Levels After treating ARPE-19 cells under different experimental conditions, malondialdehyde (MDA) levels were quantified using a biochemical detection kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MDA was measured through its reaction with thiobarbituric acid, producing a colorimetric compound detected at 532 nm.

Detection of SOD Activity The analysis of superoxide dismutase (SOD) activity in ARPE-19 cells was conducted utilizing a biochemical detection kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), adhering closely to the instructions provided by the manufacturer. To quantify the intracellular SOD activity, we measured the absorbance at 450 nm, which allowed for an accurate assessment of the enzyme's functional levels within the cells.

Western Blot After treatment, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Servicebio, Wuhan, China) and centrifuged. Total protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4°C with primary antibodies: P21 (1:2000), P53 (1:5000), REV-ERBα (1:2000), Nrf2 (1:2500; Proteintech, Wuhan, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000; Hangzhou Xianzhi Biotechnology, China). Afterward, membranes were treated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10 000; Beyotime, Shanghai, China) for 2h. Bands were visualized using the enhanced chemiluminescence (ECL) kit (Servicebio, Wuhan, China) and quantified with IPP software (version 6.0; Media Cybernetics, Inc., USA).

Polymerase Chain Reaction Total DNA was extracted from ARPE-19 cells using a kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Mitochondrial DNA (mtDNA) levels were measured *via* real-time polymerase chain reaction (PCR) with 2× Q3 SYBR quantitative PCR (qPCR) Master Mix (TOLOBIO, Shanghai, China) on a ProFlex system (ABI, USA). Primers included mtDNA (forward: 5'-CATACCC ATGGCCAACCTCCTACTCCTCATT-3'; reverse: 5'-TG CTCGCAGTGCGCCGATCAGGGCGTAGT-3') and 18s (forward: 5'-AGGCGCGCAAATTACCCAATCC-3'; reverse: 5'-GCCCTCCAATTGTTCCTCGTTAAG-3'; Tsingke Biotechnology Co., Ltd., Beijing, China). Results were analyzed with the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence Staining ARPE-19 cells were fixed in 4% paraformaldehyde for 15min and washed with PBS containing 0.1% Tween-20. After permeabilization with 0.5% Triton X-100 and blocking with 5% bovine serum albumin (BSA), cells were incubated overnight at 4°C with zonula occludens protein 1 (ZO-1) primary antibody (Boster, 1:100). Following washing, a Cy3-conjugated secondary antibody (Boster, 1:100) was applied for 1h at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5min. Images were captured using an Olympus BX53 microscope and analyzed with ImageJ software (NIH, USA).

Measurement of Mitochondrial Membrane Potential Using JC-1 Staining ARPE-19 cells were seeded at 3×10^5 cells/well in 6-well plates and incubated overnight. Following the treatment described above (48h RA pretreatment plus 24h H_2O_2 exposure), cells were collected, washed with PBS, and stained with JC-1 solution (Qisai Biotechnology, Wuhan, China) for 20min in the dark at 37°C. Red fluorescence indicated intact mitochondrial potential, while green fluorescence indicated depolarization. Flow cytometry assessed mitochondrial stress by measuring red JC-1 aggregates on the FL-2 channel and green monomers on the FL-1 channel.

Statistical Analysis The results were presented as the mean±standard deviation (SD). Data analysis was carried out employing SPSS software version 19.0. To evaluate statistical significance across multiple comparisons, we utilized analysis of variance (ANOVA), followed by the least significant difference (LSD) test for post hoc analysis. A *P*-value of less than 0.05 was considered indicative of statistical significance.

RESULTS

RA Protected ARPE-19 Cells from H_2O_2-Induced Cytotoxicity To establish the ideal concentration of H_2O_2 for inducing oxidative stress, ARPE-19 cells were exposed to various H_2O_2 concentrations for 24h. Based on the results, a concentration of 200 μmol/L was chosen for further experiments since it diminished cell viability to approximately 70% (Figure 1A). To evaluate the protective effect of different concentrations of RA against H_2O_2-induced damage, the cells were pretreated with RA for 48h before being subjected to H_2O_2 exposure for an additional 24h. Results from the CCK-8 assay indicated that increased RA concentrations effectively reduced cell viability loss caused by H_2O_2 (Figure 1B). Therefore, 80 μmol/L RA was chosen for further investigation of its protective effects against H_2O_2-induced cellular senescence.

RA Reduced H_2O_2-Induced Oxidative Stress and Senescence in ARPE-19 Cells In this study, we investigated the protective effects of RA against oxidative stress and cell senescence induced by H_2O_2 in ARPE-19 cells. We found that RA significantly reduced oxidative stress by reducing ROS and MDA levels while enhancing SOD activity. Flow cytometry analysis using DCFH-DA staining revealed that ROS levels were markedly elevated in H_2O_2-treated cells, but this increase was substantially mitigated by RA pretreatment (Figure 2A). Biochemical assays demonstrated that H_2O_2 treatment led to increased MDA levels and decreased SOD activity; however, these adverse changes were reversed following RA pretreatment (Figure 2B, 2C). Collectively, these findings indicate that the antioxidative properties of RA are linked to its protective effect on cell viability in H_2O_2-treated ARPE-19 cells. Furthermore, we evaluated the impact of RA on H_2O_2-induced senescence by measuring the activity of SA-β-gal. The number of SA-β-gal-positive cells increased progressively following H_2O_2 treatment. In contrast, cells pretreated with RA showed a significant reduction in the proportion of SA-β-gal-positive cells compared to those treated with H_2O_2 alone (Figure 2D). Additionally, the expression levels of senescence-associated proteins P53 and P21 were significantly upregulated

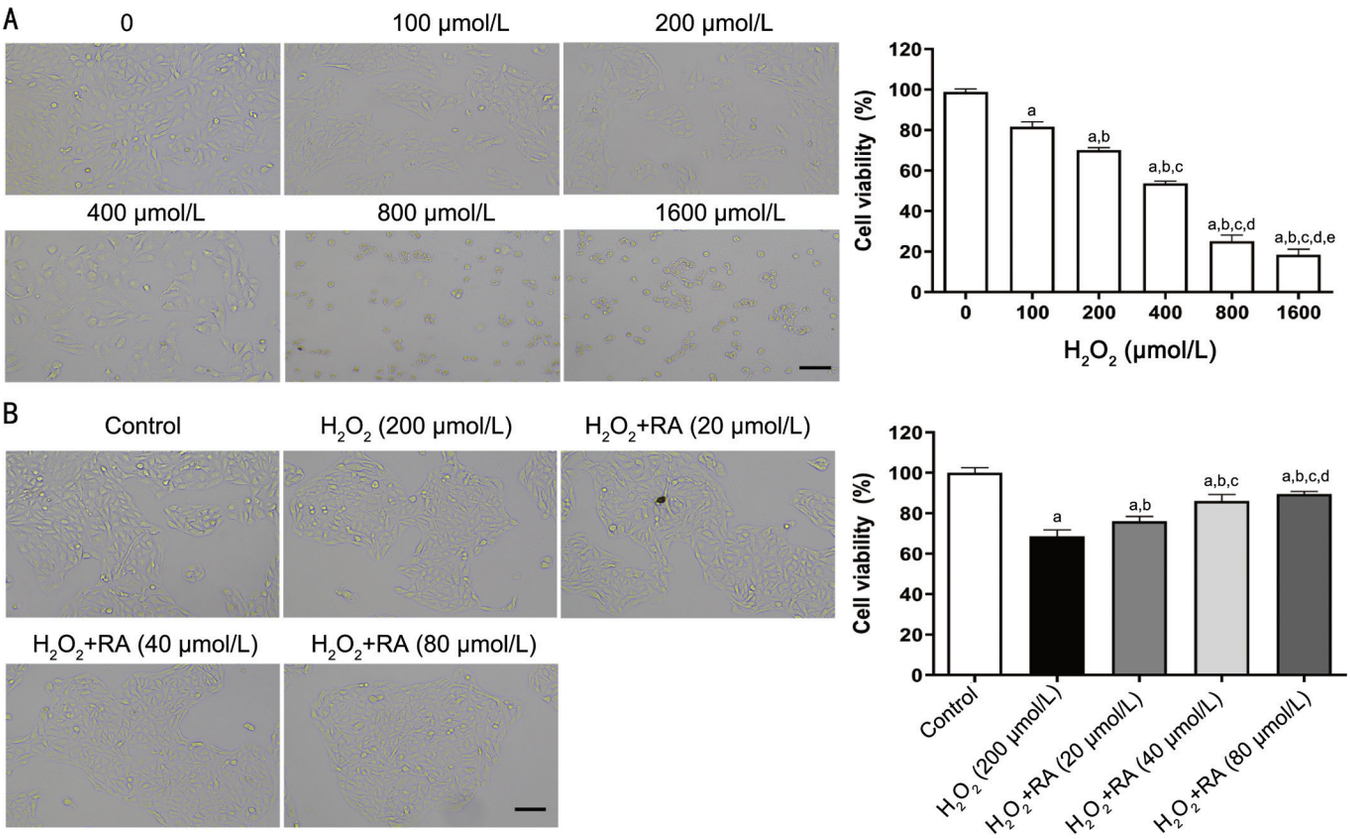


Figure 1 RA prevented reduction of cell viability by H_2O_2 treatment in ARPE-19 cells Cells were treated with different concentrations of H_2O_2 for 24h (A) or pretreated with RA for 48h and then treated with H_2O_2 for an additional 24h (B), followed by the CCK-8 assay. H_2O_2 resulted in reduction in cell viability in a dose-dependent manner and RA had no cytotoxicity up to 80 μmol/L. The reduction in cell viability by H_2O_2 gradually improved with increasing RA pretreatment concentration. Data are graphically presented as mean±SD [n=3, ^aP<0.01, compared with the control group; ^bP<0.01 compared with 100 μmol/L H_2O_2 group, ^cP<0.01, compared with 200 μmol/L H_2O_2 group, ^dP<0.01, compared with 400 μmol/L H_2O_2 group, ^eP<0.01, compared with 800 μmol/L H_2O_2 group in A; ^aP<0.01, compared with the control group, ^bP<0.01 compared with 200 μmol/L H_2O_2 group, ^cP<0.01, compared with the H_2O_2+RA (20 μmol/L) group, ^dP<0.01, compared with the H_2O_2+RA (40 μmol/L) group in B; bar=100 μm]. RA: Rosmarinic acid; ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; CCK-8: Cell counting kit-8; SD: Standard deviation.

in the H_2O_2 treatment group compared to the control group, but were reduced in the RA+H_2O_2 group (Figure 2E). These results suggest that H_2O_2 exposure induces senescence in ARPE-19 cells, and that RA effectively mitigates this effect.

RA Protected against H_2O_2-Induced Mitochondrial Dysfunction and Junctional Disruption in ARPE-19 Cells We investigated the protective effects of RA on H_2O_2-treated ARPE-19 cells, focusing on its ability to mitigate mitochondrial dysfunction and junctional disruption. PCR analysis revealed that mitochondrial DNA copy number (mtDNA-CN) levels were decreased in ARPE-19 cells subjected to H_2O_2 treatment; however, these levels were partially restored following RA treatment (Figure 3A). Furthermore, JC-1 staining showed that the intensity of red fluorescence, indicative of the polymeric form of the JC-1 complex, was prominent in control cells, suggesting high mitochondrial membrane potential (Δψm) in healthy cells (Figure 3B). In contrast, H_2O_2-treated cells exhibited a significant increase in green fluorescence, corresponding to the monomeric form of JC-1, which indicated a state of Δψm depolarization. Notably, cells that underwent RA pretreatment prior to H_2O_2 exposure demonstrated a reduced ratio of JC-1 monomer to polymer, with recovery of red-fluorescent JC-1 aggregates, indicating that RA effectively protects against H_2O_2-induced mitochondrial dysfunction. Additionally, we assessed the expression and localization of ZO-1 in ARPE-19 cells, given the importance of tight junctions in maintaining the integrity of the outer blood-retinal barrier. Figure 3C illustrates that H_2O_2 exposure led to a significant decrease in ZO-1 levels at the cell membrane, whereas RA treatment significantly promoted the accumulation of ZO-1 in these cells. Collectively, these findings indicate that RA plays a crucial role in protecting against mitochondrial dysfunction and enhancing junctional integrity in H_2O_2-treated ARPE-19 cells.

REV-ERBα/Nrf2 Pathway in RA's Protective Effects Against Oxidative Stress in RPE Cells REV-ERBα is highly expressed in RPE cells and has been identified as a novel

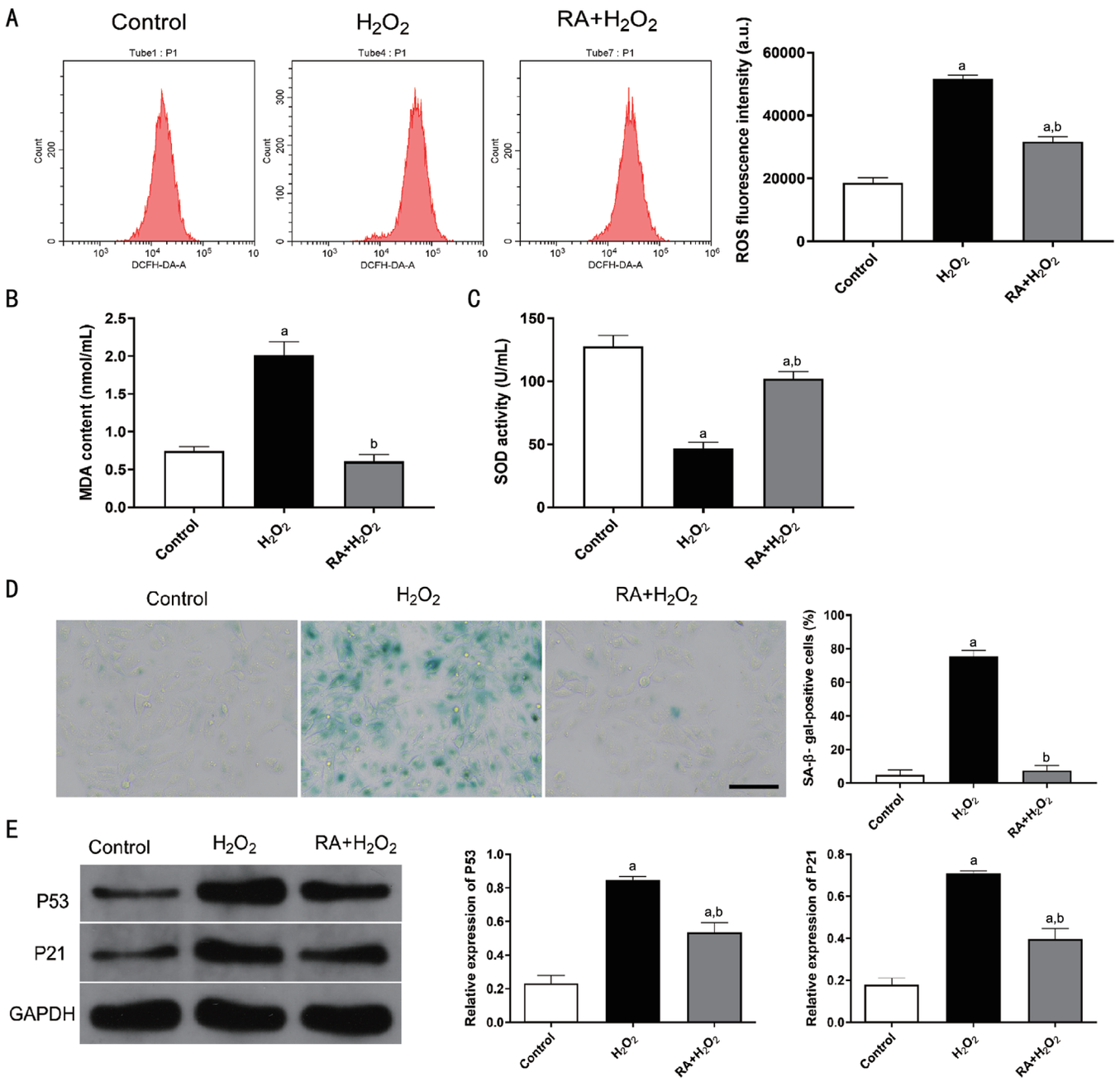


Figure 2 RA reduced H_2O_2-induced oxidative stress and senescence in ARPE-19 cells Cells were treated with H_2O_2 for 24h or pretreated with RA for 48h followed by H_2O_2 treatment for an additional 24h. Oxidative stress was evaluated by flow cytometry following DCFH-DA staining (A), and by measuring MDA (B) and SOD (C) levels using biochemical kits. H_2O_2 treatment significantly increased ROS fluorescence intensity and MDA content, while RA pretreatment effectively reduced these values. Additionally, H_2O_2 treatment decreased SOD activity, which was elevated by RA pretreatment. For senescence assessment, cells were analyzed by optical microscopy following SA-β-gal staining (D) and Western blot (E). H_2O_2 treatment increased the percentage of SA-β-gal-positive cells and upregulated the protein expressions of P53 and P21, while RA pretreatment significantly reduced their levels. Data are presented as mean±SD (n=3, ^aP<0.001 compared with the control group, ^bP<0.001 compared with the H_2O_2 group; bar=100 μm). RA: Rosmarinic acid; ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; MDA: Malondialdehyde; SOD: Superoxide dismutase; ROS: Reactive oxygen species; SA-β-gal: Senescence-associated β-galactosidase; SD: Standard deviation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

endogenous defense against age-related oxidative stress, while Nrf2 is a potent antioxidant molecule. We investigated whether the protective role of RA in oxidative stress-induced damage of RPE cells is mediated through the REV-ERBα/Nrf2 pathway. As shown in Figure 4A, treatment with H_2O_2 suppressed the expression of REV-ERBα and Nrf2 in RPE cells, indicating their involvement in H_2O_2-induced damage to RPE cells. Subsequently, we used the REV-ERBα agonist SR9009 to observe its effects on the expression of REV-ERBα and Nrf2 proteins in ARPE-19 cells following H_2O_2 treatment. The

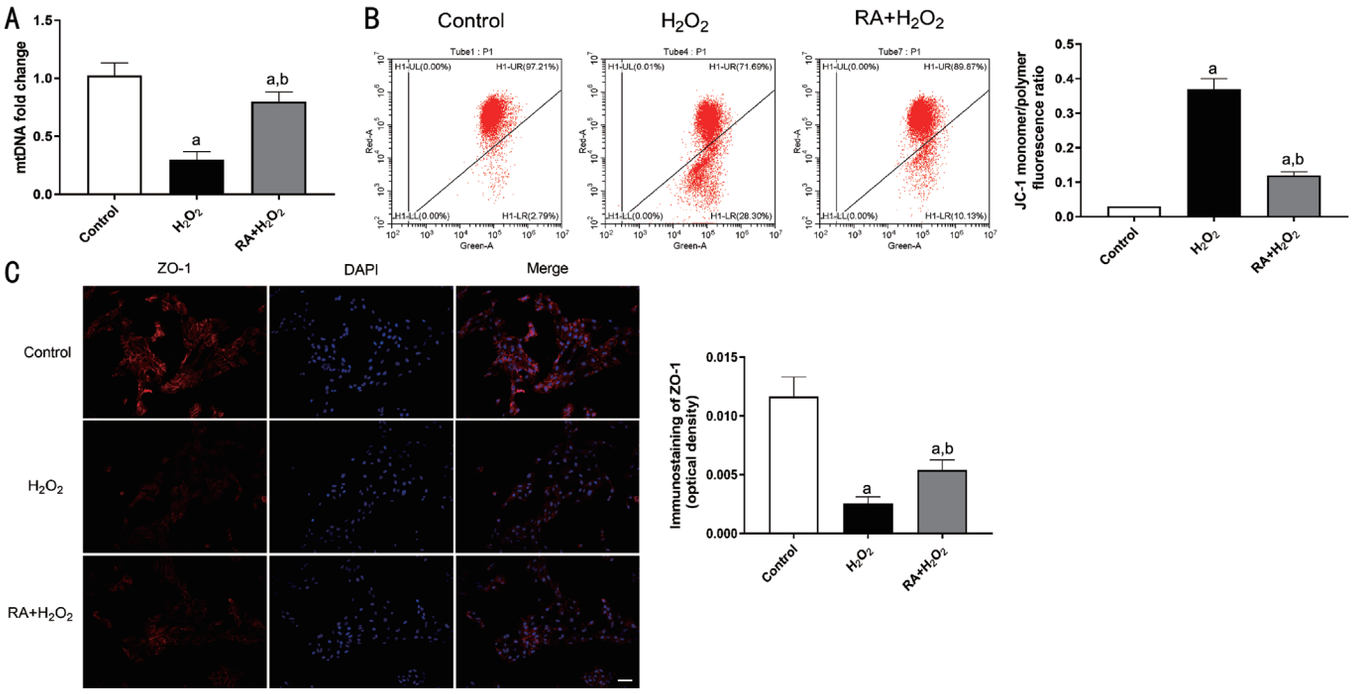


Figure 3 RA suppressed mitochondrial dysfunction and ameliorated junctional disruption in ARPE-19 cells induced by H_2O_2 treatment Cells were treated with H_2O_2 for 24h or pretreated with RA for 48h before H_2O_2 treatment for an additional 24h. Mitochondrial function was assessed by PCR (A) and flow cytometry following JC-1 staining (B). H_2O_2 treatment resulted in decreased mtDNA-CN, which was significantly increased by RA pretreatment. Furthermore, H_2O_2 treatment increased the JC-1 monomer-to-polymer ratio, indicating ΔΨm depolarization, whereas RA pretreatment reduced this ratio, suggesting a protective effect on mitochondrial integrity. Additionally, junctional integrity was evaluated *via* immunofluorescence staining (C), which revealed that H_2O_2 treatment decreased the expression of ZO-1 in the cytomembrane, while RA pretreatment significantly increased its expression. Data are graphically presented as mean±SD (n=3, ^aP<0.001 compared with the control group, ^bP<0.001 compared with the H_2O_2 group; bar=50 μm). RA: Rosmarinic acid; ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; PCR: Polymerase chain reaction; mtDNA-CN: Mitochondrial DNA copy number; ΔΨm: Mitochondrial membrane potential; ZO-1: Zonula occludens protein 1; DAPI: 4′,6-diamidino-2-phenylindole; SD: Standard deviation.

results indicated that the REV-ERBα agonist SR9009 partially restored the levels of both proteins, suggesting that Nrf2 is a downstream target of REV-ERBα. Next, we employed the REV-ERBα antagonist SR8278 to further investigate its effects on the expression of REV-ERBα and Nrf2 proteins in H_2O_2-treated ARPE-19 cells. As shown in Figure 4B, RA pretreatment increased the expression of REV-ERBα and Nrf2, while SR8278 treatment diminished the effect of RA on their protein levels.

RA Ameliorated H_2O_2-Induced Damage of ARPE Cells by Activating REV-ERBα/Nrf2 Pathway Moreover, SR8278 treatment diminished the protective effects of RA on the cells, as evidenced by decreased cell viability, an increased number of SA-β-gal-positive cells, elevated expression of P53 and P21, and enhanced mitochondrial dysfunction. As an antagonist of REV-ERBα, SR8278 modulates cell viability, senescence, and mitochondrial dysfunction in ARPE-19 cells following RA and H_2O_2 treatment. In Figure 5A, cells were pretreated with RA for 48h and then exposed to H_2O_2 with or without SR8278 for an additional 24h, with cell viability assessed using the CCK-8 assay. The data indicated that SR8278 significantly reduced cell viability compared to both the control and RA+H_2O_2 groups. Senescence was evaluated using optical microscopy following SA-β-gal staining (Figure 5B) and Western blot (Figure 5C), which demonstrated that SR8278 treatment increased the percentage of SA-β-gal-positive cells and the expression levels of senescence markers P53 and P21 in RA and H_2O_2 treated cells. Furthermore, mitochondrial dysfunction was assessed by PCR (Figure 5D) and flow cytometry following JC-1 staining (Figure 5E). The results indicated that SR8278 treatment decreased mtDNA-CN and reduced Δψm in cells subjected to RA and H_2O_2 treatment. Taken together, these findings suggest that activation of the REV-ERBα/Nrf2 pathway is one of the protective mechanisms of RA.

DISCUSSION

AMD is a multifactorial disease involving complex interactions between aging, environmental factors, and genetic predisposition, leading to photoreceptor death, RPE degeneration, and choroidal capillary degeneration[13]. The loss of central vision in AMD is primarily attributed to RPE dysfunction and degeneration, and/or choroidal neovascularization, which result in the malfunction and

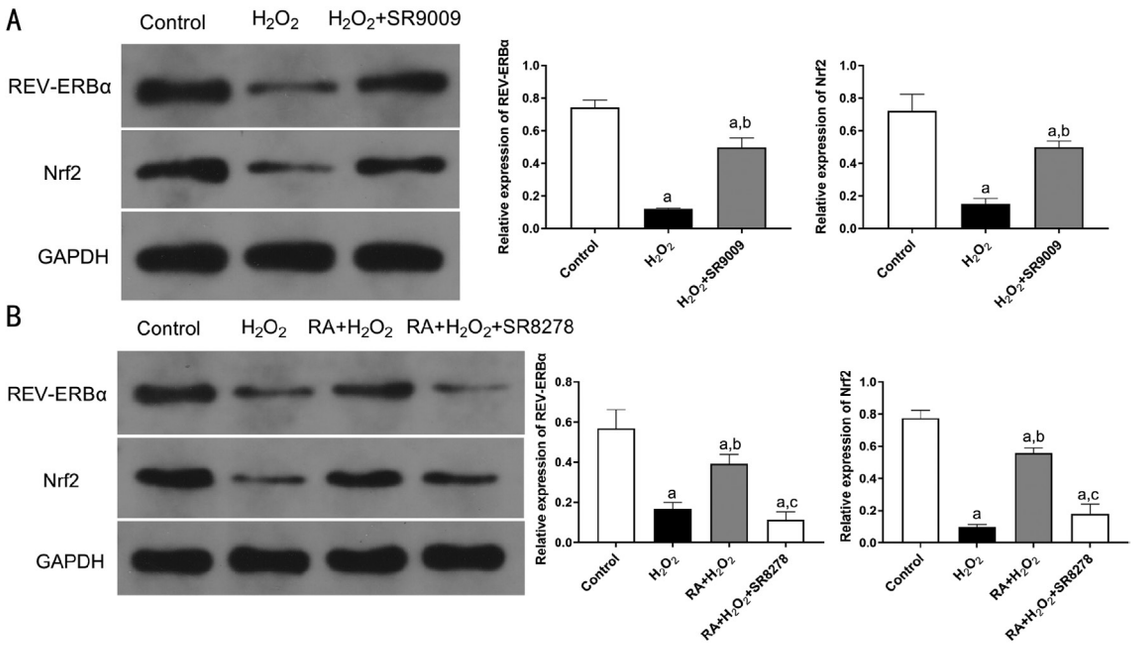


Figure 4 REV-ERBα/Nrf2 pathway in RA's protective effects against oxidative stress in RPE Cells A: Cells were treated with H_2O_2 with or without SR9009, an agonist of REV-ERBα, for 24h, and Western blot was used to assess protein expression. Results showed that SR9009 restored the expression of REV-ERBα and Nrf2 proteins in H_2O_2-treated cells; B: Cells were pretreated with RA for 48h and then exposed to H_2O_2 combined with or without SR8278, an antagonist of REV-ERBα, for an additional 24h. Western blot analysis indicated that SR8278 reduced the expression of both REV-ERBα and Nrf2 proteins following RA treatment. Data are graphically presented as mean±SD (n=3, ^aP<0.001, compared with the control group, ^bP<0.001 compared with the H_2O_2 group, ^cP<0.001 compared with RA+H_2O_2 group). Nrf2: Nuclear factor erythroid 2-related factor 2; REV-ERBα: Reverse erythroblastosis virus alpha; RA: Rosmarinic acid; RPE: Retinal pigment epithelium; H_2O_2: Hydrogen peroxide; SD: Standard deviation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

loss of photoreceptor cells[14]. Oxidative stress refers to an imbalance between pro-oxidants and antioxidants, which can lead to tissue damage under various harmful stimuli[6]. This phenomenon has been linked to numerous diseases, including AMD, where elevated levels of ROS contribute to oxidative injury in various types of retinal cells[15]. Non-radical species such as H_2O_2 function as major second messengers in redox signaling[16]. Recognizing the strong connection between oxidative stress damage to RPE cells and the onset of AMD, researchers frequently utilize human RPE cell lines like ARPE-19 and treat them with H_2O_2 to model the disease[17-18]. In this study, H_2O_2 treatment resulted in reduced cell viability, excessive production of ROS and MDA, and decreased levels of SOD, which are commonly used as markers of oxidative stress.

Cellular senescence is defined by a permanent cessation of proliferation in somatic cells, along with distinct phenotypic alterations[19]. As a cellular defense mechanism, senescence occurs when proliferating cells are subjected to various stressors, thereby preventing unnecessary cellular damage. Research suggests a link between RPE cell senescence and the onset of AMD, characterized by notable morphological changes[20-21]. The reorganization of the cytoskeleton, especially vimentin filaments, results in flattened cells that can grow to more than twice the size of those that are not senescent[22]. Senescent cells typically exhibit increased acidic SA-β-gal activity, partly reflecting an increase in lysosome mass[23]. Moreover, proteins such as p53 and p21 are expressed at elevated levels in these cells[24-25]. In this study, we induced senescence in ARPE-19 cells using H_2O_2, resulting in blurred cell edges, decreased cell density, increased SA-β-gal positive cells, and significantly elevated protein expression of p53 and p21, consistent with findings from other studies[26]. Furthermore, the expression of the retinal barrier protein marker ZO-1 was significantly decreased in senescent ARPE-19 cells[27].

mtDNA-CN, which reflects the quantity of mitochondrial genomes within a cell, serves as a potential biomarker for assessing mitochondrial function and is linked to various age-related diseases, including AMD[28-29]. JC-1 is a cationic fluorescent dye that, when introduced to living cells with sufficient Δψm, localizes specifically within mitochondria. The ratio of red (J-aggregates) to green fluorescence (J-monomers) in JC-1-loaded cells is commonly used to detect Δψm. A reduction in Δψm results in decreased mitochondrial accumulation of JC-1 and diminished formation of J-aggregates[30]. Mitochondrial dysfunction and cellular senescence are key characteristics of aging and are closely related[31], we also found decreased mtDNA-CN and Δψm in H_2O_2-treated ARPE-19 cells.

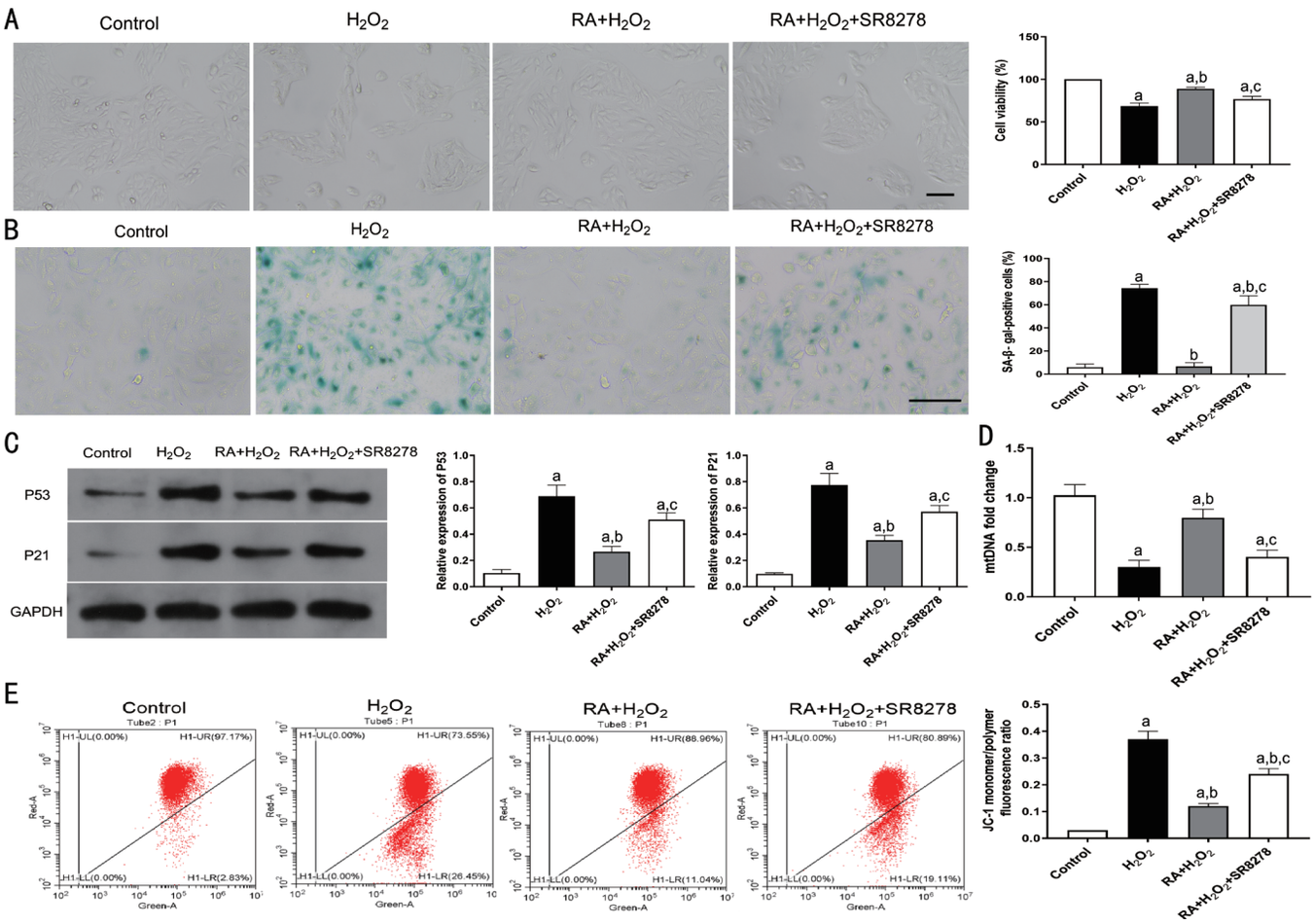


Figure 5 RA ameliorated H_2O_2-induced damage of RPE cells by activating REV-ERBα/Nrf2 pathway Cells were pretreated with RA for 48h and then exposed to H_2O_2 with or without SR8278 for an additional 24h, with cell viability assessed using the CCK-8 assay. Data indicated that SR8278 significantly reduced cell viability compared to the control and RA+H_2O_2 groups (A, 100× magnification). Senescence was evaluated using optical microscopy following SA-β-gal staining (B, 200× magnification) and Western blot (C), which demonstrated that SR8278 treatment increased the percentage of SA-β-gal-positive cells as well as the expression levels of senescence markers P53 and P21 in RA- and H_2O_2-treated cells. Mitochondrial dysfunction was assessed by PCR (D) and flow cytometry following JC-1 staining (E). The results showed that SR8278 treatment decreased mtDNA-CN and reduced ΔΨm in cells subjected to RA and H_2O_2 treatment. Data are graphically presented as mean±SD (n=3, ^aP<0.001 compared with the control group, ^bP<0.001 compared with H_2O_2 group, ^cP<0.001 compared with RA+H_2O_2 group; bar=100 μm). RA: Rosmarinic acid; H_2O_2: Hydrogen peroxide; RPE: Retinal pigment epithelium; Nrf2: Nuclear factor erythroid 2-related factor 2; SA-β-gal: Senescence-associated β-galactosidase; PCR: Polymerase chain reaction; mtDNA-CN: Mitochondrial DNA copy number; ΔΨm: Mitochondrial membrane potential; SD: Standard deviation.

Nuclear receptors have increasingly been acknowledged for their novel roles in maintaining retinal and RPE health and their potential as therapeutic targets[32-33]. Recent nuclear receptor mapping studies have revealed a high expression of REV-ERBα in freshly isolated human RPE cells. REV-ERBα is a redox-sensitive transcription factor that is transcribed and translated in a circadian manner in the liver, skeletal muscle, adipose tissue, and brain, regulating various biological processes including circadian rhythms, metabolism, and inflammatory responses[34]. Despite being identified as a novel molecular target for AMD therapy, REV-ERBα remains largely unexplored with respect to its role in RPE health and disease[11]. The retina and RPE maintain normal oxidative homeostasis due to effective antioxidants and repair systems, with Nrf2 acting as the primary transcriptional regulator of endogenous antioxidants and phase II detoxification enzymes[35]. Consistent with a recent review highlighting the critical role of Nrf2 in AMD[36], our study further distinguishes itself by experimentally identifying REV-ERBα as a functional upstream regulator of this pathway in RPE cells, thereby providing a novel mechanistic link not previously detailed in theoretical summaries. Both clinical and experimental studies on RPE aging and AMD have highlighted Nrf2 as a crucial signaling pathway for cellular protection[37].

In recent years, the strategy of using traditional Chinese medicine to treat AMD has attracted considerable attention[38].

RA is widely distributed in plants, especially in the *Lamiaceae* and *Boraginaceae* families, and is well known for its potent antioxidant activity[39]. While previous studies have demonstrated the antioxidant efficacy of RA in ARPE-19 cells against H_2O_2-induced oxidative stress[40], the specific molecular mechanisms underlying these protective effects remain to be fully elucidated. Here, we confirmed that RA promoted cell viability and inhibited cell senescence in ARPE-19 cells under oxidative stress induced by H_2O_2. However, the potential therapeutic impact of RA on AMD requires further validation through animal experiments and clinical trials. Additionally, the precise molecular mechanisms underlying its effects need to be investigated in more depth.

In conclusion, the findings from this study provide preliminary support for the protective role of RA on RPE cells subjected to oxidative stress. One potential mechanism for this effect may involve the activation of the REV-ERBα/Nrf2 pathway.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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