

# Rhizoma Paridis total saponins alleviate H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury by upregulating the Nrf2 pathway

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**Abstract.** Rhizoma Paridis total saponins (RPTS) is an active substance isolated from the traditional Chinese medicine Rhizoma Paridis, which possesses multiple biological activities. The aim of the present study was to explore the roles and mechanisms of RPTS in oxidative stress injury of ARPE-19 human retinal pigment epithelial cells. Cell viability, reactive oxygen species (ROS) levels, mitochondrial membrane potential (MMP) and apoptosis were determined by Cell Counting kit-8 assay and flow cytometry, respectively. Enzyme-linked immunosorbent assay was performed to detect the expression of oxidative stress markers. Western blotting and reverse transcription-quantitative polymerase chain reaction were used to determine the expression levels of related genes and proteins. The results revealed that RPTS enhanced cell viability and reduced H<sub>2</sub>O<sub>2</sub>-induced oxidative stress of ARPE-19 human retinal pigment epithelial cells. RPTS increased the MMP of ARPE-19 cells compared with in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. In addition, RPTS suppressed ROS production and apoptosis of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. Additionally, RPTS modulated the expression levels of apoptosis-associated proteins and the nuclear factor 2-related factor 2 (Nrf2) pathway. In conclusion, RPTS alleviated H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury by upregulating the Nrf2 pathway. The potential effects of RPTS on protection against H<sub>2</sub>O<sub>2</sub>-induced apoptosis of ARPE-19 cells suggested that RPTS may be a potential therapeutic target for preventing age-related macular degeneration.

## Introduction

Age-related macular degeneration (AMD) is an age-associated disease of the eyes, which is the most common cause of visual impairment and blindness in the elderly (1). According to a

blind register center survey in Britain, ~50% of blind patients are affected by AMD (2). The main manifestation of AMD is decreased retinal pigment epithelial cell digestive ability of the rod outer segment, which leads to the presence of rod outer segment residual bodies in the protoplasm. These residual bodies deposit in Bruch's membrane and form drusen, which lead to impaired vision or even blindness (3,4). However, to best of our knowledge, the exact pathogenesis of AMD remains unclear. The pathogenesis of AMD has been reported to be related to aging, metabolism, genetic inheritance, smoking, high blood pressure and obesity (5-8). Recent studies have demonstrated that antioxidant supplements may protect retinal pigment epithelial cells from oxidative stress damage (9-11); however, current AMD therapy remains ineffective.

Heme oxygenase-1 (HO-1) is an inducible antioxidant enzyme that exerts cytoprotective effects on various cell types (12-15). The expression levels of HO-1 are mainly regulated by the antioxidant response element (ARE), which activates the nuclear factor 2-related factor 2 (Nrf2). Under normal conditions, Nrf2 is present in the cytoplasm in combination with Kelch-like ECH-related protein 1 (Keap1) (16). Activated Nrf2 translocates into the nucleus and forms heterodimers with transcription factors, which activate the transcription of HO-1 (17). Studies have demonstrated that the Nrf2 pathway participates in the development and progression of oxidative stress injury (18-20). However, the exact mechanisms of the Nrf2 pathway in the modulation of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury in retinal pigment epithelial cells remain unclear.

Rhizoma Paridis total saponins (RPTS) is a major effective constituent isolated from the traditional Chinese medicine, Rhizoma Paridis, which has been demonstrated to possess numerous biological activities (21,22). RPTS exhibits antitumor activity in several cancer types, involving liver (23) and lung cancer (24). A recent study also suggested that RPTS attenuates liver fibrosis by regulating expression of the Ras protein activator-like 1/ERK1/2 signaling pathway in rats (25). However, it is unclear whether RPTS may prevent AMD, and little is currently known about the roles of RPTS in oxidative stress-induced injury on retinal pigment epithelial cells.

In the present study, the association between RPTS and H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury was analyzed. In addition, the roles and mechanisms of RPTS and the Nrf2 pathway in the protection of H<sub>2</sub>O<sub>2</sub>-induced ARPE-19 cells were examined to identify a potential therapeutic strategy for preventing AMD.

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**Key words:** Rhizoma Paridis, saponins, nuclear factor 2-related factor 2, H<sub>2</sub>O<sub>2</sub>, oxidative stress, reactive oxygen species, Fas, caspase-3

## Materials and methods

**Cell culture and reagents.** The human retinal pigment epithelial cell line ARPE-19 was obtained from the Cell Bank of the Chinese Academy of Sciences. ARPE-19 cells were incubated in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in an atmosphere containing 5% CO<sub>2</sub> at 37°C. RPTS was obtained from Nanjing DASF Biotechnology Co., Ltd. (cat. no. dasf0242), and the main components of RPTS were Polyphyllin I, Polyphyllin II, Polyphyllin VII and Polyphyllin H. H<sub>2</sub>O<sub>2</sub> was obtained from Xilong Scientific Co., Ltd.

**Grouping.** Cultured ARPE-19 cells (all at 37°C) were separated into five treatment groups as follows: i) Control, ARPE-19 cells without treatment; ii) H<sub>2</sub>O<sub>2</sub>, ARPE-19 cells treated with 200 μM H<sub>2</sub>O<sub>2</sub> for 12 h; iii) 10 μg/ml RPTS + H<sub>2</sub>O<sub>2</sub>, ARPE-19 cells pretreated with 10 μg/ml RPTS for 6 h and treated with 200 μM H<sub>2</sub>O<sub>2</sub> for 12 h; iv) 20 μg/ml RPTS + H<sub>2</sub>O<sub>2</sub>, ARPE-19 cells pretreated with 20 μg/ml RPTS for 6 h and treated with 200 μM H<sub>2</sub>O<sub>2</sub> for 12 h; and v) 40 μg/ml RPTS + H<sub>2</sub>O<sub>2</sub>, ARPE-19 cells pretreated with 40 μg/ml RPTS for 6 h and treated with 200 μM H<sub>2</sub>O<sub>2</sub> for 12 h.

**Cell viability assay.** Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology) was used to detect the effects of various concentrations of RPTS (10, 20, 40 and 80 μg/ml) at 48 h, and the effects of H<sub>2</sub>O<sub>2</sub> and RPTS + H<sub>2</sub>O<sub>2</sub> at 12, 24 and 48 h, on ARPE-19 cell viability. ARPE-19 cells (~6x10<sup>3</sup> cells/well) in the logarithmic phase were seeded into 96-well plates and maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub> for 12 h. Subsequently, CCK reagent (10 μl) was added to each well and the cells were incubated for 3 h at 37°C. A microplate reader (Bio-Rad Laboratories, Inc.) was used to record the absorbance at 450 nm. Cell viability was determined as the percentage of cell survival compared with the control.

**Enzyme-linked immunosorbent assay (ELISA).** The kits used for the assessment of reactive malondialdehyde (MDA; cat. no. S0131), superoxide dismutase (SOD; cat. no. S0101) and glutathione peroxidase (GPx; cat. no. S0056) in ARPE-19 cells were obtained from Beyotime Institute of Biotechnology. Cultured ARPE-19 cells (2x10<sup>5</sup> cells/well) were seeded into 6-well plates, which were subsequently sealed with adhesive tape and maintained at 37°C for 90 min. Biotinylated antibodies (100 μl) were then added to the wells. The wells were re-sealed and maintained at 37°C for 60 min. Chromogenic substrate from the kit was added to the wells and the plates were maintained for 10-15 min in the dark at 37°C. Stop solution was then added to each well and mixed for 10 min. Optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

**Apoptosis assay.** Flow cytometry (FCM) was performed to assess the apoptotic rates of ARPE-19 cells. After washing with PBS, cultured ARPE-19 cells were trypsinized with 0.25% trypsin (Beyotime Institute of Biotechnology). The supernatant was removed and the cells were suspended in incubation buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) at a

density of 1x10<sup>6</sup> cells/ml. ARPE-19 cells were then incubated with Annexin PE and 7-aminoactinomycin (5 μl; cat. no. 559763; BD Biosciences) at room temperature in the dark for 15 min. Finally, a FACScalibur flow cytometer (BD Biosciences) was used to assess apoptosis, and the data was analyzed using BD CellQuest™ Pro version 1.2 software (BD Biosciences).

**Evaluation of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) in ARPE-19 cells.** PBS was added to cultured ARPE-19 cells until a cell density of 1x10<sup>6</sup> cells/ml was achieved in a 12-well plate. Subsequently, 2',7'-dichlorofluorescein diacetate (cat. no. HY-D0940; MedChem Express LLC) and Fluo-3 acetoxymethyl (cat. no. 70-F1243; Hangzhou MultiSciences Biotech Co., Ltd.) were added to the ARPE-19 cells to evaluate ROS and MMP, respectively. ARPE-19 cells were incubated at room temperature in the dark for 10 min. The supernatant was then removed and 100 μl PBS was added to the cells. FCM was performed to assess ROS levels and MMP in ARPE-19 cells at 488 nm. A total of 10,000 cells were collected from each sample and analyzed using a flow cytometer, and the data were analyzed using Summit V4.3 software (Dako; Agilent Technologies, Inc.).

**ARE-luciferase activity.** ARPE-19 cells (3x10<sup>4</sup> cells/well) were seeded into 24-well plates and ARE dual-luciferase reporter plasmid (50 ng/well; cat. no. 11548ES03; Shanghai Yeasen Biotechnology Co., Ltd.) was transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 24 h post-transfection, the cells were treated with H<sub>2</sub>O<sub>2</sub> + 0, 10, 20 or 40 μg/ml RPTS for 24 h. ARE-luciferase activity was measured at 490 nm following the addition of Luciferase Assay Reagent (Promega Corporation); *Renilla* luciferase was used as an internal control.

**Western blot analysis.** Proteins were extracted from ARPE-19 cells using RIPA buffer (Beyotime Institute of Biotechnology), and a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.) was used to quantify protein concentration. Protein lysates (25 μg/lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to a PVDF membrane (EMD Millipore). The blots were then blocked in TBS + 0.1% Tween-20 containing 5% skimmed milk at 37°C for 1 h and incubated with the following rabbit anti-human antibodies: Anti-Fas (dilution, 1:100; cat. no. ab133619), anti-Fas ligand (FasL; dilution, 1:1,000; cat. no. ab15285), anti-Bax (dilution, 1:1,000; cat. no. ab32503), anti-Bcl-2 (dilution, 1:1,000; cat. no. ab32124), anti-caspase-3 (dilution, 1:500; cat. no. ab13847), anti-Nrf2 (dilution, 1:500; cat. no. ab137550), anti-HO-1 (dilution, 1:2,000; cat. no. ab13243), anti-γ-glutamyl-cysteine synthetase (γ-GCS; dilution, 1:1,000; cat. no. sc-166382; Santa Cruz Biotechnology, Inc.), anti-NAD(P)H quinone dehydrogenase 1 (NQO1; dilution, 1:1,000; cat. no. ab34173) and anti-GAPDH (dilution, 1:2,500; cat. no. ab9485; all Abcam unless specified) at 4°C overnight. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (dilution, 1:5,000; cat. no. ab205718; Abcam) was then added, and the membranes were incubated at room temperature for 1 h. GADPH was used as an internal control. Enhanced chemiluminescence (ECL)

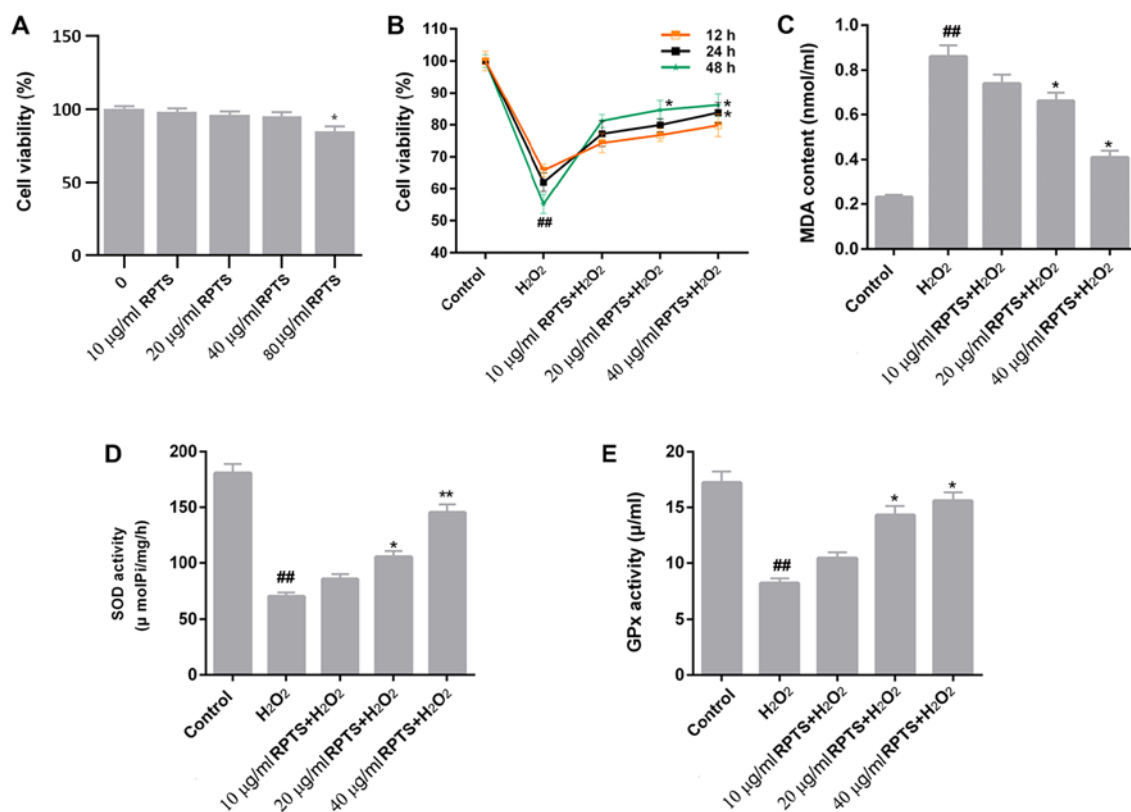


Figure 1. RPTS affects cell viability and oxidative stress of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>. (A) ARPE-19 cells were treated with 10, 20, 40 and 80 µg/ml RPTS. Cell viability was detected using the CCK-8 assay. \*P<0.05 vs. 0 µg/ml RPTS. (B) ARPE-19 cells were treated with H<sub>2</sub>O<sub>2</sub>, or pretreated with 10, 20 and 40 µg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. CCK-8 assay was performed to evaluate the viability of ARPE-19 cells. (C-E) ELISA was performed to assess the levels of (C) MDA, (D) SOD and (E) GPx in ARPE-19 cells. ##P<0.01 vs. Control; \*P<0.05 and \*\*P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; CCK-8, Cell Counting kit-8; ELISA, enzyme-linked immunosorbent assay; GPx, glutathione peroxidase; MDA, malondialdehyde; RPTS, Rhizoma Paradis total saponins; SOD, superoxide dismutase.

reagents (EMD Millipore) in combination with an ECL system (GE Healthcare) were used to analyze the results. Densitometry was performed using Quantity One software version 2.4 (Bio-Rad Laboratories, Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from cultured ARPE-19 cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA using PrimeScript<sup>™</sup> RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol at 42°C for 1 h and 70°C for 10 min. cDNA was amplified using the SYBR Fast qPCR Mix (Invitrogen; Thermo Fisher Scientific, Inc.) on the ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Pretreatment at 94°C for 10 min; followed by 45 cycles at 95°C for 15 sec and 68°C for 45 sec; and a final extension step at 75°C for 10 min. The primers were designed by Invitrogen (Thermo Fisher Scientific, Inc.) as follows: Fas, forward 5'-GACTCA GAACTTGGAAGGCC-3', reverse 5'-ACTTGGTATTCT GGGTCCGG-3' (product, 245 bp); FasI, forward 5'-TGGTTC TGGTTGCCTTGGA-3', reverse 5'-GCATGGACCTTG AGTTGGAC-3' (product, 210 bp); Bax, forward 5'-CATCAT GGGCTGGACATTGG-3', reverse, 5'-CCTCAGCCCATC TTCTCCA-3' (product, 226 bp); Bcl-2, forward 5'-TTCTTT GAGTTCGGTGGGGT-3', reverse 5'-CTTCAGAGACAG CCAGGAGA-3' (product, 207 bp); caspase-3, forward 5'-TGA

GCCATGGTGAAGAAGGA-3', reverse 5'-TCGGCCTCC ACTGGTATTTT-3' (product, 220 bp); and GAPDH, forward 5'-CCATCTTCCAGGAGCGAGAT-3' and reverse 5'-TGC TGATGATCTTGAGGCTG-3' (product, 222 bp). GAPDH was used as the internal control. The mRNA expression levels were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (26).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism version 6.0 software (GraphPad Software, Inc.). Data are presented as the mean ± SD of at least three independent experiments. The experimental data were analyzed by Kruskal-Wallis and Tukey's tests. P<0.05 was considered to indicate a statistically significant difference.

## Results

**RPTS enhances the viability of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>.** CCK-8 assay results reonstrated that cell viability was not affected by 10, 20 and 40 µg/ml RPTS (Fig. 1A). However, 80 µg/ml RPTS reduced cell viability (P<0.05; Fig. 1A). To further examine whether RPTS affected ARPE-19 cell viability, the viability of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and different concentrations of RPTS was measured. Compared with control cells, H<sub>2</sub>O<sub>2</sub> treatment significantly inhibited the viability of ARPE-19 cells (P<0.01); however, in the groups pretreated with RPTS, the viability of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells was enhanced in a dose-dependent manner (Fig. 1B).

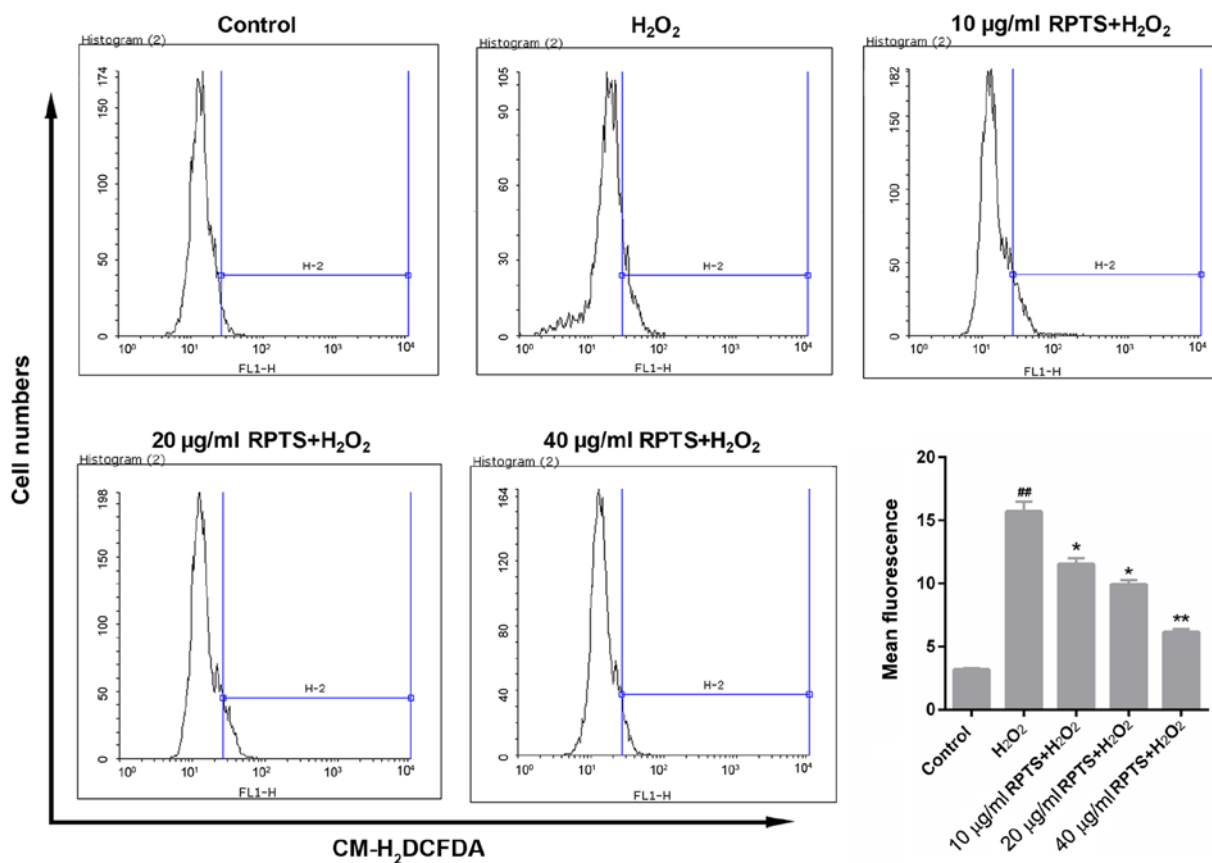


Figure 2. RPTS reduces ROS production in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. Flow cytometry was performed to detect ROS production in untreated ARPE-19 cells, ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, and ARPE-19 cells pretreated with 10, 20 and 40 µg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. <sup>##</sup>P<0.01 vs. Control. <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; ROS, reactive oxygen species; RPTS, Rhizoma Paradis total saponins.

These results suggested that RPTS may increase the viability of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>.

*Oxidative stress marker levels in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells are modulated by RPTS.* As oxidative stress contributes to the development and progression of H<sub>2</sub>O<sub>2</sub>-induced injury, the levels of oxidative stress markers, including MDA, SOD and GPx, were assessed in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and different concentrations of RPTS. The MDA content in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> was significantly higher compared with that in control (P<0.01) cells, whereas RPTS treatment significantly reduced the levels of MDA in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells (P<0.05; Fig. 1C). Conversely, H<sub>2</sub>O<sub>2</sub> treatment significantly reduced the levels of SOD and GPx in ARPE-19 cells compared with the control group (P<0.01). The SOD and GPx levels in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells were enhanced in the groups pretreated with different concentrations of RPTS compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 1D and E). Therefore, RPTS pretreatment reduced MDA content, and increased SOD and GPx levels in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells, which indicated that RPTS may reduce oxidative stress in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells.

*RPTS pretreatment reduces ROS production in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells.* ROS production in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and with different concentrations of RPTS was measured. FCM results demonstrated that H<sub>2</sub>O<sub>2</sub> treatment

significantly elevated ROS levels in ARPE-19 cells compared with in the control group (P<0.01). In the groups pretreated with RPTS, mean fluorescence value indicating ROS levels in the H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells were decreased compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 2). Therefore, RPTS may reduce ROS production in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells.

*RPTS increases the MMP of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells.* FCM analysis of MMP revealed that H<sub>2</sub>O<sub>2</sub> treatment significantly decreased the MMP in ARPE-19 cells (P<0.01), whereas the MMP in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells was significantly enhanced in response to RPTS (P<0.05; Fig. 3). These results suggested that RPTS may modulate the MMP in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, which indicated that RPTS may reduce H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in ARPE-19 cells.

*RPTS suppresses H<sub>2</sub>O<sub>2</sub>-mediated apoptosis of ARPE-19 cells.* The apoptotic rate of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and different concentrations of RPTS was determined by FCM. The results demonstrated that the proportion of apoptotic ARPE-19 cells in the H<sub>2</sub>O<sub>2</sub> group was 16.74%, which was significantly higher compared with 3.75% in the control group (P<0.01; Fig. 4). By contrast, in the groups pretreated with RPTS, the apoptotic rates of ARPE-19 cells were significantly decreased compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 4). This suggested that RPTS may reduce the apoptotic rate of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells in a dose-dependent manner.

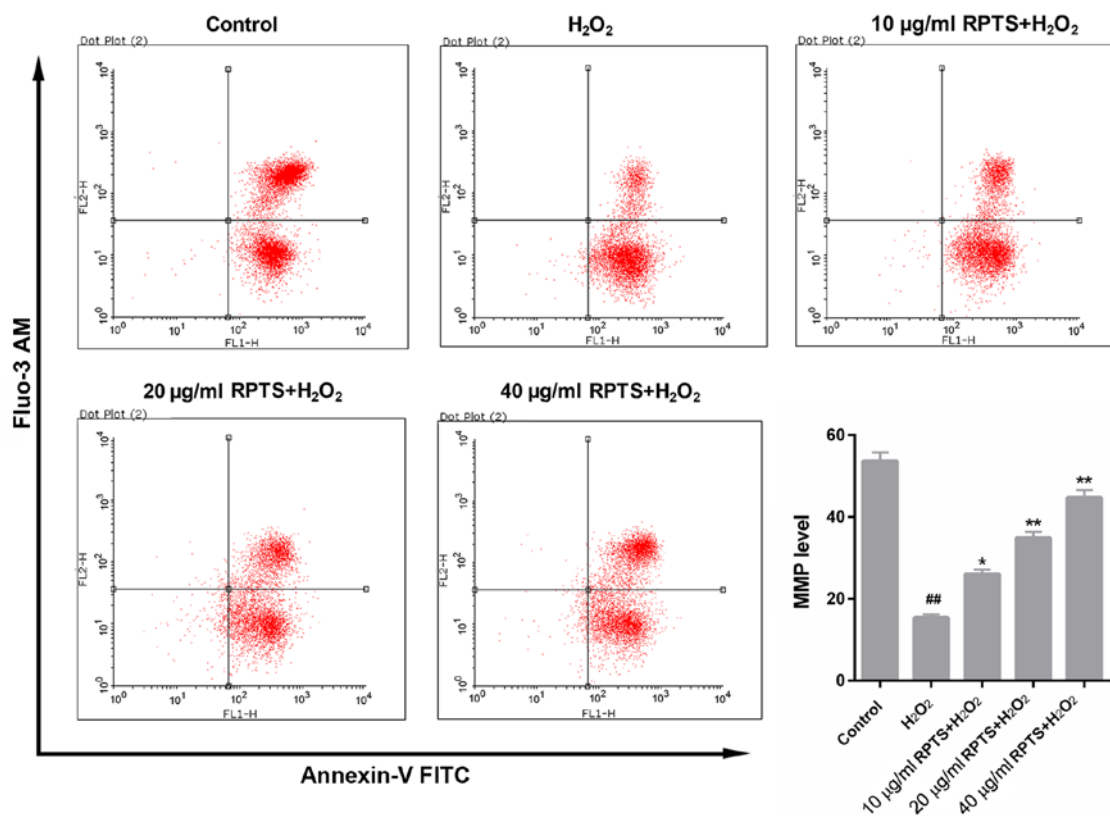


Figure 3. RPTS increases the MMP of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>. Flow cytometry was performed to assess the MMP of untreated ARPE-19 cells, ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, and ARPE-19 cells pretreated with 10, 20 and 40 µg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. <sup>##</sup>P<0.01 vs. Control; <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; Fluo-3 AM, Fluo-3 acetoxyethyl; MMP, mitochondrial membrane potential; RPTS, Rhizoma Paridis total saponins.

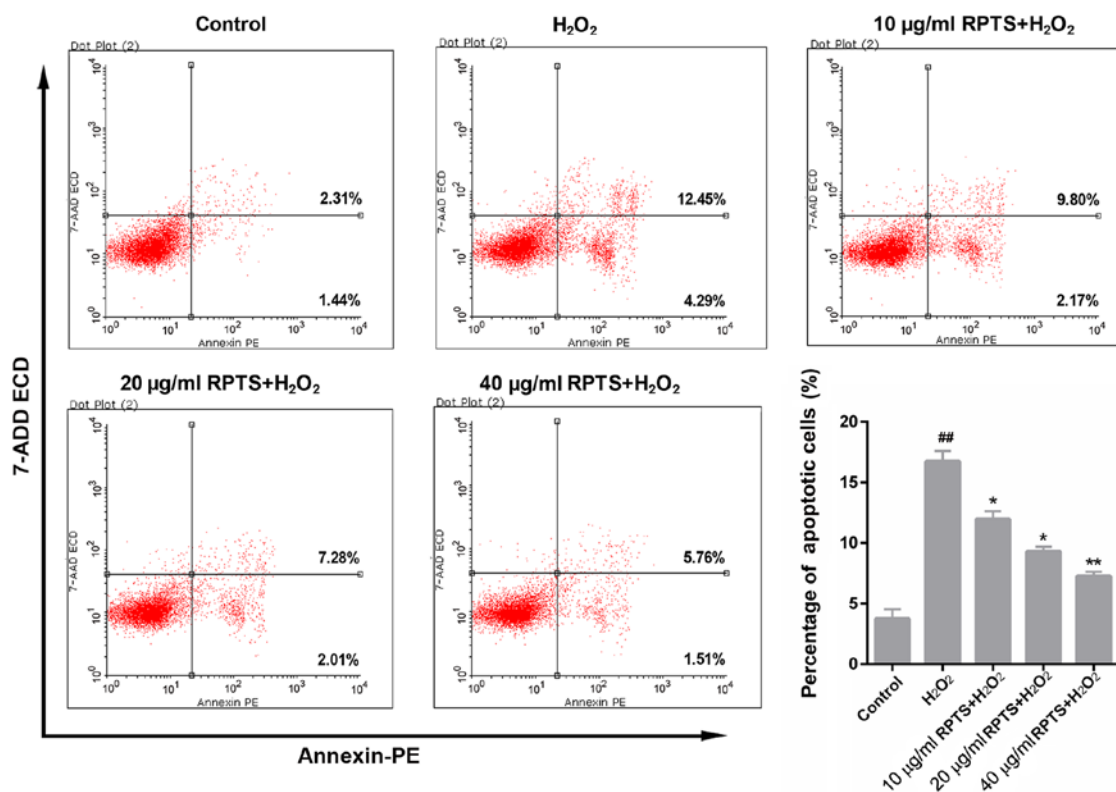


Figure 4. RPTS suppresses H<sub>2</sub>O<sub>2</sub>-mediated apoptosis of ARPE-19 cells. Flow cytometry (Annexin PE/7-ADD) was performed to assess the apoptotic rate of untreated ARPE-19 cells, ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, and ARPE-19 cells pretreated with 10, 20 and 40 µg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. <sup>##</sup>P<0.01 vs. Control; <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; RPTS, Rhizoma Paridis total saponins; PE, Phycoerythrin; 7-ADD, 7-Amino Actinomycin.

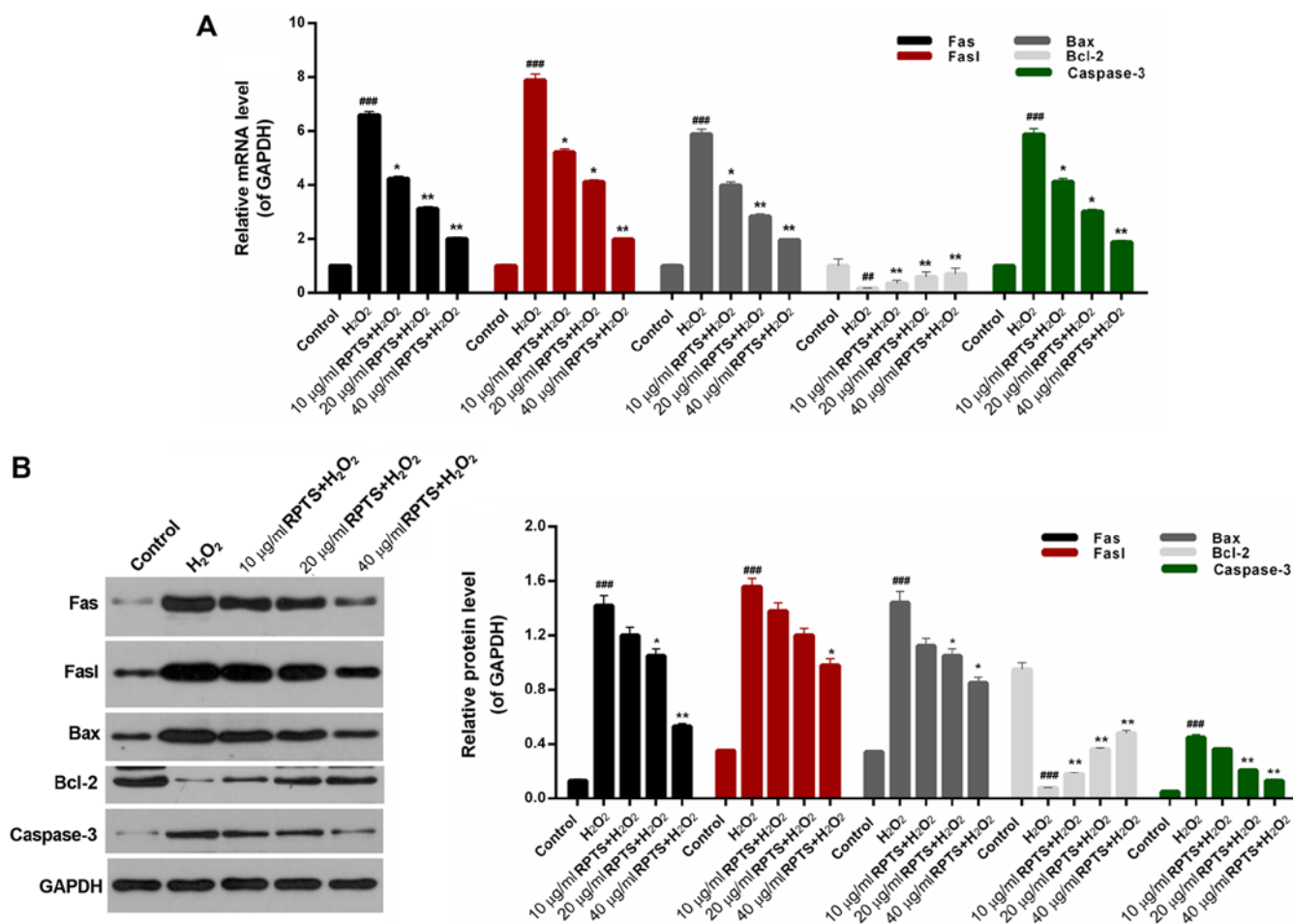


Figure 5. Expression levels of apoptosis-associated proteins are modulated by RPTS. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blotting were performed to detect the expression levels of Fas, FasI, Bax, Bcl-2 and caspase-3 in untreated ARPE-19 cells, ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, and ARPE-19 cells pretreated with 10, 20 and 40 µg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. <sup>##</sup>P<0.01 and <sup>###</sup>P<0.001 vs. Control; \*P<0.05 and <sup>\*\*</sup>P<0.01 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; RPTS, Rhizoma Paradis total saponins.

*Expression levels of apoptosis-associated proteins are modulated by RPTS.* As RPTS suppressed H<sub>2</sub>O<sub>2</sub>-induced apoptosis of ARPE-19 cells, the related mechanisms were further investigated. The expression levels of apoptosis-associated proteins Fas, FasI, Bax, Bcl-2 and caspase-3 were measured in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and different concentrations of RPTS. RT-qPCR data demonstrated that the mRNA expression levels of Fas, FasI, Bax and caspase-3 in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> were significantly upregulated compared with in the control group (P<0.001). However, in the groups pretreated with RPTS, significant decreases in the expression levels of Fas, FasI, Bax and caspase-3 in H<sub>2</sub>O<sub>2</sub>-induced ARPE-19 cells were observed compared with in the H<sub>2</sub>O<sub>2</sub> group. In addition, H<sub>2</sub>O<sub>2</sub> treatment significantly reduced Bcl-2 expression in ARPE-19 cells compared with in the control group (mRNA, P<0.01; protein, P<0.001), whereas RPTS pretreatment upregulated Bcl-2 expression in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 5A). Western blot analysis revealed similar trends with regards to Fas, FasI, Bax, Bcl-2 and caspase-3 expression in ARPE-19 cells in all groups (P<0.05; Fig. 5B). These results indicated that RPTS may suppress the apoptosis of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells by modulating the expression levels of Fas, FasI, Bax, Bcl-2 and caspase-3.

*RPTS modulates the Nrf2 pathway.* An ARE-luciferase assay was performed to confirm that RPTS suppressed H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury. RPTS promoted ARE-luciferase activity in a dose-dependent manner in H<sub>2</sub>O<sub>2</sub>-treated cells compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.01; Fig. 6A). To further investigate the exact mechanisms underlying the effects of RPTS on the protection of ARPE-19 cells against H<sub>2</sub>O<sub>2</sub>, the related signaling pathway was identified. Western blotting results revealed that the expression levels of Nrf2, HO-1, γ-GCS and NQO1 were significantly downregulated following treatment with H<sub>2</sub>O<sub>2</sub> (P<0.001). By contrast, in the groups pretreated with RPTS, HO-1, γ-GCS and NQO1 protein expression levels were significantly increased compared with in the H<sub>2</sub>O<sub>2</sub> group (P<0.05; Fig. 6B-C). Therefore, RPTS may regulate the Nrf2 pathway in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>.

## Discussion

AMD is associated with multiple factors; increasing age may enhance oxidative stress, which leads to irreversible damage to retinal pigment epithelial cells (27). Oxidative stress injury refers to circumstances, such as ischemia and inflammation, in which the organism faces various stimuli when highly active molecules

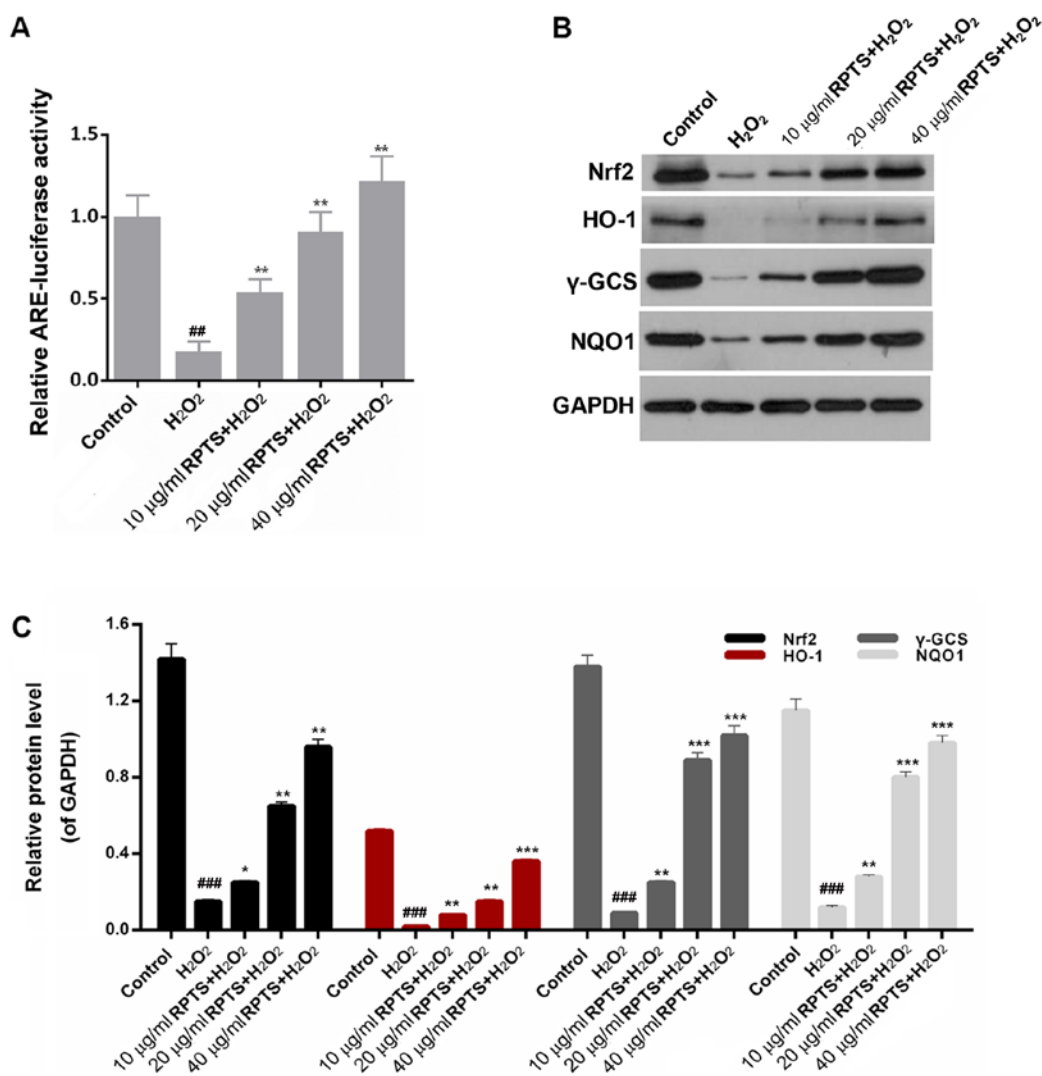


Figure 6. RPTS modulates the Nrf2 pathway. (A) ARE-luciferase activity was examined in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells pretreated with 10, 20 and 40 μg/ml RPTS. Renilla luciferase activity was used as an internal control. (B and C) Western blot analysis was performed to determine the expression levels of Nrf2, HO-1, γ-GCS and NQO1 in untreated ARPE-19 cells, ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, and ARPE-19 cells pretreated with 10, 20 and 40 μg/ml RPTS and then treated with H<sub>2</sub>O<sub>2</sub>. <sup>##</sup>P<0.01 and <sup>###</sup>P<0.001 vs. Control; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. H<sub>2</sub>O<sub>2</sub>. ARPE-19, Adult Retinal Pigment Epithelial cell line-19; ARE, antioxidant response element; HO-1, heme oxygenase 1; Nrf2, nuclear factor 2-related factor 2; γ-GCS, γ-glutamyl-cysteine synthetase; NQO1, NAD(P) H quinone dehydrogenase 1; RPTS, Rhizoma Paradis total saponins.

such as ROS and free radicals are overproduced (28). This results in an imbalance between oxidation and the antioxidant system, which further triggers tissue damage. To investigate the exact mechanisms underlying oxidative stress injury in retinal pigment epithelial cells, an experimental model of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> was established in the current study. RPTS has been reported to exhibit numerous biological activities, including anti-tumor (29), anti-fibrosis and anti-cirrhosis effects (30). However, the roles of RPTS in protection against oxidative stress injury in retinal pigment epithelial cells remain unclear. Therefore, RPTS was selected as the subject of the present study on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury in ARPE-19 cells. Firstly, the viability of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and different concentrations of RPTS was measured; RPTS enhanced the viability of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells, indicating that RPTS may serve a function in protecting ARPE-19 cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress injury. Further assessment of the levels of oxidative stress markers in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and RPTS demonstrated that RPTS reduced the MDA content, and

increased SOD and GPx levels, in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. Further studies are required to identify which component of RPTS is responsible for these results.

Since RPTS reduced the levels of oxidative stress markers, it was hypothesized that RPTS may affect ROS levels and MMP in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>. An appropriate level of ROS is necessary in the aging process, whereas overproduction of ROS can lead to an imbalance in the internal environment, which results in cell death, structural and functional tissue damage, and organ damage (31,32). Therefore, increased endogenous ROS production is a marker of a high level of oxidative stress. Retinal pigment epithelial cells are necessary in the function of vision, and they transport nutrients and ions between photoreceptors and the choriocapillaris (33). Therefore, ROS production in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and RPTS was further analyzed. RPTS distinctly reduced ROS production in ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub>, which suggested that RPTS may serve as a therapeutic agent of oxidative stress injury.

MMP participates in the development and progression of cell apoptosis (34). The MMP of ARPE-19 cells was assessed in the present study; the results demonstrated that RPTS enhanced the MMP of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. These results suggested that RPTS served a function of alleviating the oxidative stress injury induced by H<sub>2</sub>O<sub>2</sub> on ARPE-19 cells.

Previous studies have demonstrated that oxidative stress aggravates the degeneration, dysfunction and apoptosis of age-related retinal pigment epithelial cells (35,36). In the present study, the apoptosis of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> and RPTS was tested, which indicated that RPTS significantly reduced the apoptotic rate of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. In addition, RPTS downregulated the expression levels of Fas, FasL, Bax and caspase-3, and upregulated Bcl-2 expression in H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells. These results suggested that RPTS suppressed the apoptosis of H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells by modulating the expression levels of Fas, FasL, Bax, Bcl-2 and caspase-3.

The Nrf2 pathway serves crucial roles in oxidative stress injury-induced cell apoptosis (37,38). However, no studies have focused on the association between RPTS and the Nrf2 pathway in oxidative stress injury, to the best of our knowledge. Nrf2-driven free radical detoxification pathways are important endogenous homeostatic mechanisms, and previous studies have reported that, under oxidative stress, cellular antioxidant defenses depend primarily on Nrf2 dissociation from Keap1 and its subsequent translocation to the nucleus, where the activation of antioxidant genes occurs (39,40). The results of the present study demonstrated that Nrf2, HO-1,  $\gamma$ -GCS and NQO1 protein levels were significantly decreased by H<sub>2</sub>O<sub>2</sub> stimulation and partially rescued by RPTS pretreatment, which indicated that Nrf2 signaling may be involved in RPTS-induced protection of ARPE-19 cells against oxidative injury. In addition, several studies have reported the effects of H<sub>2</sub>O<sub>2</sub> on Nrf2 expression that were consistent with the present study (41,42).

Under oxidation conditions *in vitro*, cells may be unable to spontaneously produce an antioxidant defense. Therefore, *in vitro* experiments and the use of only one retinal cell line were potential limitations of the present study; further *in vivo* experiments and other retinal cell lines are required to validate the effects of H<sub>2</sub>O<sub>2</sub> and RPTS on the Nrf2 pathway. Other limitations included the lack of different detection methods and markers for oxidative stress and apoptosis.

The results of the present study demonstrated that RPTS reduced oxidative stress and suppressed the apoptosis of ARPE-19 cells treated with H<sub>2</sub>O<sub>2</sub> by increasing Nrf2 and antioxidant enzyme expression. Previous studies have suggested that total saponins of several natural products, such as *Panax notoginseng* saponins (43), *Platycodon grandiflorum* saponins (44) and *Aralia taibaiensis* saponins (45), exert a protective effect on oxidative injury. Therefore, the present study suggested that RPTS may alleviate oxidative stress injury induced by H<sub>2</sub>O<sub>2</sub> by upregulating the Nrf2 pathway. These results may provide novel insight into the pathogenesis of oxidative stress injury and may lead to novel approaches to the treatment of oxidative stress injury.

In conclusion, the present study demonstrated that RPTS alleviated oxidative stress injury induced by H<sub>2</sub>O<sub>2</sub> by upregulating the Nrf2 pathway. These results are crucial for the

understanding the mechanisms underlying RPTS activity in retinal pigment epithelial cells. The potential protective effects of RPTS on H<sub>2</sub>O<sub>2</sub>-treated ARPE-19 cells suggested that RPTS may be an effective therapeutic agent for the treatment of oxidative stress injury, opening up a novel direction in AMD research and treatment.

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### Availability of data and materials

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

### Authors' contributions

BZ, ZW and GW conceived and designed the study. JH, ZL and BY acquired, analyzed and interpreted the data. BZ and ZW drafted and critically revised the manuscript for important intellectual content. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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