

Role of quercetin in protecting ARPE-19 cells against H₂O₂-induced injury via nuclear factor erythroid 2 like 2 pathway activation and endoplasmic reticulum stress inhibition

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Abstract. Age-related macular degeneration (AMD) is a common cause of irreversible blindness in the elderly in the western world. Research has demonstrated that degenerative and progressive conditions of retinal pigmented epithelial (RPE) cells are the key pathogenic mechanisms in AMD. Previous research has indicated the anti-apoptosis, anti-oxidant, anti-inflammatory and anti-cancer properties of quercetin. Therefore, the present study aimed to investigate the protective effects of quercetin on the ARPE-19 human retinal pigment epithelial cell line. ARPE-19 cells were pretreated with various concentrations of quercetin (0-80 μ M) before exposure to 300 μ M H₂O₂. Cell viability was assessed and reactive oxygen species (ROS) were determined. The importance of the NF-E2-related factor 2 (Nrf2) signaling pathway was corroborated by western blotting and immunostaining. The protein expression levels of endoplasmic reticulum-associated stress responsive genes and apoptotic markers were assessed by western blotting. The results demonstrated that in the H₂O₂ group, cell viability was weakened, but preserved in quercetin group in a dose-dependent manner, particularly at 20 μ M. The level of ROS decreased in the quercetin group compared with the control groups. In the quercetin group, the expression levels of Nrf2 and phase II enzymes (NQO1 and HO-1) were increased, whereas the levels of ER stress markers (binding of immunoglobulin protein, CCAAT/enhancer-binding protein homologous protein and phosphorylated eukaryotic translation initiation factor 2 α) were reduced. Cell apoptosis-associated protein expression levels were altered, with the increase of B-cell lymphoma 2 and reduction of Bcl-2 X-associated

protein. In conclusion, quercetin protected ARPE-19 cells from H₂O₂-induced cytotoxicity by activating the Nrf2 pathway, inhibiting ER stress and targeting anti-apoptotic proteins.

Introduction

Age-related macular degeneration (AMD) is a common cause of irreversible blindness in the elderly in the western world. It is estimated that 1.47% of the US population (>40 years) are suffering from AMD, and the likelihood of AMD is expected to increase by 50% (to 2.95 million) by 2020 (1). Although the pathogenic mechanism underlying the progression of AMD is currently unknown, research has demonstrated that degenerative and progressive conditions of retinal pigmented epithelial (RPE) cells are the key pathogenic mechanisms in AMD (2).

RPE cells serve a critical role in protecting the outer retina from photo-oxidative stress, maintaining the viability of photoreceptors and inhibiting retinal edema and neovascularization (3). Oxidative stress is considered to be particularly vital in the development of RPE cell degeneration, dysfunction and apoptosis (4).

NF-E2-related factor 2 (Nrf2) has been demonstrated to regulate the expression of genes encoding detoxification enzymes, antioxidant proteins and other stress-response mediators, including heme oxygenase-1 (HO-1) and NAD(P)H, quinone oxidoreductase 1 (NQO1) (5). *In vivo* and *in vitro* studies have highlighted the central role of Nrf2 in protecting RPE cells from a variety of oxidative challenges. Previous studies have demonstrated that antioxidants could mitigate oxidative stress in RPE cells by upregulating Nrf2-regulated phase II enzymes (6,7). A study demonstrated that RPE cells of aged mice expressed higher levels of the Nrf2 target genes compared with RPE of younger mice, especially under unstressed conditions, thereby indicating an age-associated increase in basal oxidative stress (8). RPE cells of older mice exhibited impaired induction of the Nrf2 signaling pathway following oxidative stress, thereby suggesting that the aged RPE cells are vulnerable to oxidative damage due to impaired Nrf2 signaling (8). The Nrf2 pathway may serve an important role in AMD pathogenesis and function as a promising target for novel pharmacologic or genetic therapeutic strategies.

Endoplasmic reticulum (ER) stress is considered to be an early or initial response of cells to stress or damage.

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Disturbed ER homeostasis could induce the unfolded protein response (UPR) accumulation, thereby triggering cell death responses (9). Growing evidence has suggested that ER stress serves an important role in retinal diseases. Libby and Gould (10) proposed that ER stress could be an important mechanism in the pathogenesis of AMD. A previous study demonstrated that exposure of A2E containing ARPE-19 cells to blue light resulted in significant apoptosis and increased levels of ER stress markers, indicating that photo-oxidative damage to RPE cells was mediated by the ER stress-induced intrinsic apoptotic pathway (11). Therefore, therapeutic agents that inhibit ER stress may protect RPE from dysfunction and apoptosis during the course of development of AMD.

Quercetin is a ubiquitous flavonoid compound, which is widely distributed in different fruits and vegetables, including onions, capers, cranberries, fennel, dark grapes and cocoa. Experimental data has suggested that quercetin serves as a strong free radical scavenger and possesses anti-apoptosis, anti-oxidant, anti-inflammatory and anti-cancer properties (12,13). Although a previous study demonstrated that precubation with quercetin could protect RPE cells from H₂O₂-induced oxidative damage and attenuate cellular senescence increase *in vitro* in a dose-dependent manner (14), the underlying mechanism remains unclear. Furthermore, the association between Nrf2 and quercetin remains to be investigated. The role of quercetin in promoting the expression of Nrf2 and downstream signaling molecules and modulating ER stress and apoptosis proteins remains unclear. To confirm this, the present cultured ARPE-19 cells with quercetin prior to H₂O₂ stimulation and investigated the underlying molecular mechanism.

Materials and methods

Materials. The human retinal pigment epithelial cell line ARPE-19 was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12) trypsin-EDTA was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Quercetin, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO) and H₂O₂ were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). A CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay kit (MTS) was obtained from Promega Corporation (Madison, WI, USA). The NE-PER[™] Nuclear and Cytoplasmic Extraction reagent was obtained from Thermo Fisher Scientific, Inc. The antibodies for Nrf2 (cat. no. ab31163), NQO1 (cat. no. ab80588), HO-1 (cat. no. ab68477) and Laminin B1 (cat. no. ab109293) were obtained from Abcam (Cambridge, MA, USA). Antibodies for binding of immunoglobulin protein (Bip; cat. no. 3177S), CCAAT/enhancer-binding protein homologous protein (CHOP; cat. no. 5554S), phosphorylated (p) eukaryotic translation initiation factor 2 α (eIF2 α ; cat. no. 3398), eIF2 α (cat. no. 5324S), B-cell lymphoma 2 (Bcl-2; cat. no. 15071S), Bcl-2 X-associated protein (Bax; cat. no. 5023S) and β -actin (cat. no. 12620S), and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (cat. no. 4412S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse (cat. no. ab6789)

and anti-rabbit (cat. no. ab6721) secondary antibodies were purchased from Abcam. An enhanced chemiluminescence (ECL) kit and polyvinylidene difluoride (PVDF) membranes were obtained from EMD Millipore (Billerica, MA, USA).

In addition, quercetin was freshly dissolved in 100% DMSO and further diluted with culture medium on the day of experiment. The final concentration of DMSO in each experiment was <0.1%. H₂O₂ was diluted with double-distilled water to the desired concentration at the beginning of each experiment.

Cell culture. ARPE-19 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂/95% air. For monolayer culture, ARPE-19 cells were seeded at high confluence at a density of 4x10⁵ cells/well, and maintained for 3 days to form a monolayer. The culture medium was replaced every 48 h. The cells were passaged once a week, with a split ratio of 1:3.

Cell viability studies. Inhibition of cell proliferation by different concentrations of H₂O₂ or quercetin was measured using an MTS kit according to the manufacturer's protocol. Briefly, the cells were seeded into 96-well culture plates (10⁴ cells/well) in 100 μ l media, and different concentrations of H₂O₂ or quercetin were added. After treatment, 20 μ l MTS solution was added to each well, and the cells were further incubated in 5% CO₂ for 1 h. The plates were read at 450 nm using a microplate reader (Model EL800; Omega Bio-Tek, Inc., Norcross, GA, USA). All the experiments were conducted in triplicate.

Measurement of intracellular reactive oxygen species (ROS) levels. Production was determined by DCFH-DA staining assay. Briefly, following treatment, ARPE-19 cells were incubated with 10 μ M DCFH-DA at 37°C for 30 min in the dark. The cells were washed twice with PBS, resuspended, and subjected to flow cytometry with a CytoFLEX flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) at excitation and emission wavelengths of 488 nm and 525 nm, respectively. The results were expressed as fluorescence intensity of DCF.

Immunofluorescence microscopy. ARPE-19 cells were seeded into 24-well glass slides (Merck KGaA) at a density of 4x10⁵ per well. After treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at room temperature for 20 min. The cells were permeabilized with 0.05% Triton X-100 in PBS for 10 min. After washing with ice-cold PBS, the cells were blocked with 3% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 15 min, followed by incubation with primary antibodies against Nrf2 (1:200) overnight at 4°C. The cells were then washed three times with PBS and incubated for 1 h with an Alexa Fluor 488-conjugated secondary antibody at room temperature. 4'-6-diamidino-2-phenylindole (DAPI; 5 mg/ml; Beyotime Institute of Biotechnology, Nanjing, China) in PBS was used to stain the nuclei. Fluorescence photographs were acquired by a fluorescence microscope.

Protein extraction. Following appropriate treatment, cells were trypsinized and collected by centrifugation at 1,000 x g

for 10 min at room temperature, washed briefly with PBS, resuspended in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100] and supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). The suspension was left on ice for 30 min and then centrifuged at 15,000 \times g for 10 min at 4°C. Cytoplasmic and nuclear extracts were collected using NE-PER™ Nuclear and Cytoplasmic Extraction reagents according to the manufacturer's protocol. All the protein extracts were stored at -80°C until use. Protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology).

Western blotting. Equal amounts of extracted protein samples (40 μ g) were separated on Tris-HCl 10% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 100 V and subsequently transferred onto a PVDF membrane. After blocking with 5% instant non-fat dry milk for 1 h, the membranes were incubated with the following primary antibodies at 4°C overnight: Anti-Nrf2 (1:2,000), anti-NQO1 (1:5,000), anti-HO-1 (1:5,000), anti-Bip (1:1,000), anti-Chop (1:1,000), anti-p-eIF2 α (1:1,000), anti-eIF2 α (1:1,000), anti-Bcl-2 (1:1,000), anti-Bax (1:1,000), anti- β -actin (1:1,000) and anti-Laminin B1 (1:2,000), followed by incubation with the following secondary antibodies for 1 h at room temperature: Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:1,000) and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (1:1,000). Protein bands were detected with an enhanced chemiluminescence (ECL) detection kit (EMD Millipore) using an ECL detection system (GE Healthcare, Chicago, IL, USA). Blots were semi-quantified by densitometry using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.).

Statistics analysis. Each experiment was repeated at least three times. Data are expressed as the mean \pm standard deviation. Statistical analyses were performed using unpaired Student's t-test for two-group data and one-way analysis of variance followed by a post hoc Bonferroni's multiple comparison test for three groups or more. Statistical analysis was performed using GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of H_2O_2 and quercetin on cell viability in ARPE-19 cells. To determine the concentration of H_2O_2 , ARPE-19 cells were treated with 0-1,000 μ M H_2O_2 for 24 h, and the dose-dependent cell toxicity of H_2O_2 was measured by MTS assay. As presented in Fig. 1A, H_2O_2 progressively decreased the viability of ARPE-19 cells. However, for further studies, 300 μ M H_2O_2 was used, where the cell viability was decreased to $63.97 \pm 0.99\%$ ($P < 0.05$). In order to evaluate the potential toxicity quercetin towards ARPE-19 cells, the of cells were incubated with 0-80 μ M quercetin for 24 h, and the cell viability was tested (Fig. 1B). Experimental data demonstrated that quercetin treatment alone had no effect on cell viability; thereby demonstrating that quercetin at the tested concentrations was safe for cultured ARPE-19 cells.

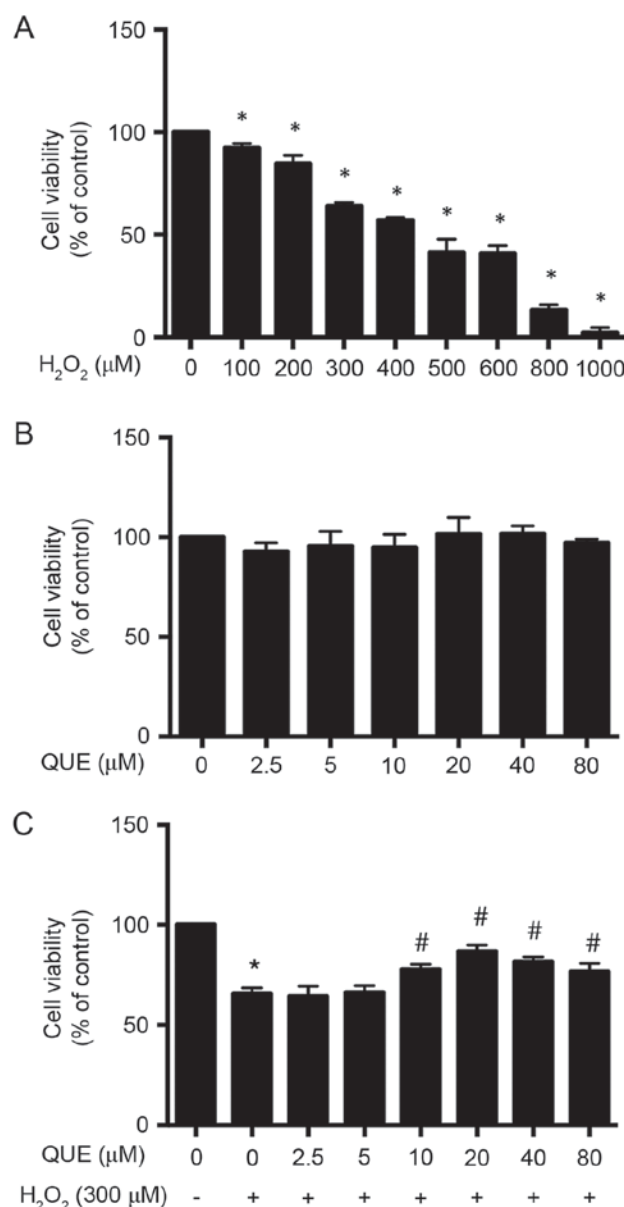


Figure 1. Inhibitory effect of quercetin on H_2O_2 -induced injury in ARPE-19 cells. Cells were treated with (A) H_2O_2 , (B) quercetin and (C) or a combination of the two at various concentrations for 24 h, and cell viability was determined by CellTiter 96® Aqueous One Solution Cell Proliferation assay. Data are expressed as the mean \pm standard deviation. * $P < 0.05$ vs. control, # $P < 0.05$ vs. H_2O_2 -induced cells without pretreatment with quercetin. QUE, quercetin.

Effect of quercetin on inhibiting H_2O_2 -induced toxicity in ARPE-19 cells. To investigate whether quercetin treatment could protect ARPE-19 cells from H_2O_2 -induced cell damage, the cells were first incubated in medium containing different concentrations of quercetin. After 24 h of treatment, the medium was removed and the cells were exposed to 300 μ M H_2O_2 for another 24 h. Cell viability was determined by MTS assay. As presented in Fig. 1C, the cell viability was significantly decreased to $65.53 \pm 1.77\%$ ($P < 0.05$) in cultures treated with H_2O_2 alone as compared with untreated cells. There was no statistically significant difference in cell viability of incubation with quercetin at low concentrations of 2.5 μ M ($64.40 \pm 2.89\%$; $P > 0.05$) and 5 μ M ($66.33 \pm 1.91\%$; $P > 0.05$) compared with the H_2O_2 group; however, pretreatment of

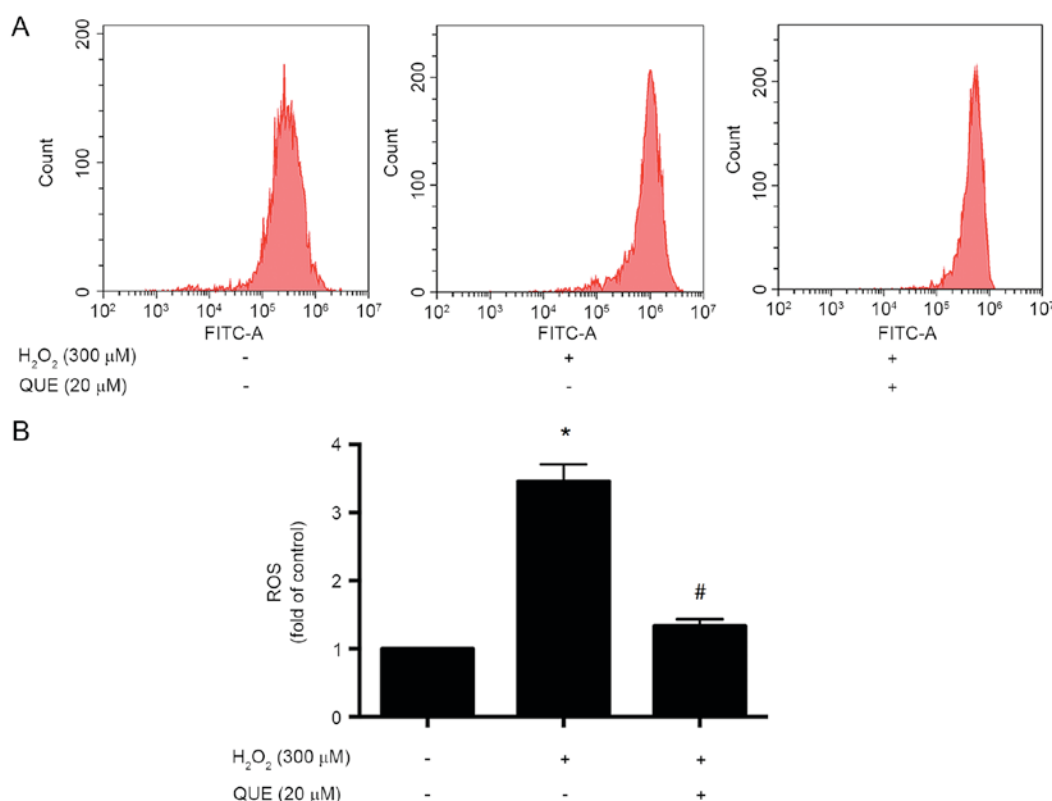


Figure 2. Quercetin reduces H₂O₂-induced intracellular generation of ROS. (A) ARPE-19 cells were pretreated with the 20 μM quercetin for 24 h, followed by 300 μM H₂O₂ administration. ROS production was evaluated using (A) flow cytometry (B) with quantification. Data are presented as the mean ± standard deviation. *P<0.05 vs. control, #P<0.05 vs. H₂O₂-induced cells without pretreatment with quercetin. QUE, quercetin; ROS, reactive oxygen species; FITC, fluorescein isothiocyanate.

quercetin at concentrations of 10, 20, 40 and 80 μM protected up to 77.97±1.36% (P<0.05), 86.87±1.80% (P<0.05), 81.67±1.29% (P<0.05) and 76.67±2.40% (P<0.05) of ARPE-19 cells from H₂O₂-induced cell death, compared with 65.53±1.77% in the control group (300 μM H₂O₂; Fig. 1C). In this study, 20 μM quercetin was used for all the experiments.

Quercetin pretreatment reduces H₂O₂-induced intracellular generation of ROS. To evaluate the effect of quercetin on H₂O₂-induced ROS generation in ARPE-19 cells, the cells were incubated with 20 μM quercetin for 24 h before exposure to 300 μM H₂O₂ for another 24 h. It was observed that treatment of cells with 300 μM H₂O₂ resulted in an increase in the intracellular level of ROS. ROS accumulation was reduced following pretreatment with quercetin at concentration of 20 μM (Fig. 2).

Effect of quercetin on activation of Nrf2 and phase II enzymes. It has been well documented that Nrf2 and dependent genes, NQO1 and HO-1, are important components of the cellular stress response. To investigate the potential effect of quercetin on the expression of Nrf2 and NQO1, the cells were incubated with 20 μM quercetin for different time intervals (0-48 h), and the expression levels of Nrf2 and NQO1 were examined. It was observed that 20 μM quercetin administration induced protein expression of Nrf2 and NQO1 in a time-dependent manner (Fig. 3A).

Furthermore, the location of Nrf2 was examined by immunofluorescence experiments. It was observed that H₂O₂

alone could only slightly enhance the Nrf2 expression in the cytoplasm; however, quercetin treatment significantly stimulated the expression and accumulation of Nrf2 and induced the nuclear translocation of Nrf2 in ARPE-19 cells (Fig. 3B). Western blotting results also revealed that quercetin supplementation enhanced the total expression levels of Nrf2 and significantly increased nuclear levels of Nrf2 in the quercetin pretreatment group, compared with H₂O₂ (Fig. 3C). The expression level of NQO1 was increased significantly as well. HO-1 was also increased, but no significant difference was detected (Fig. 3D).

Effect of quercetin on ER stress markers under H₂O₂ stimulation. To confirm the protective effect of quercetin against H₂O₂-induced ER stress in ARPE-19 cells, expression levels of ER stress markers were assessed by western blotting. The results demonstrated that Bip and CHOP expression levels were downregulated, with eIF2α phosphorylation reduction, following quercetin pretreatment, compared with H₂O₂ alone. This finding demonstrated that quercetin could inhibit ER stress in ARPE-19 cells during H₂O₂-induced injury (Fig. 4A-D).

Effect of quercetin on apoptosis-associated proteins in ARPE-19 cells. To further investigate the effect of quercetin on apoptosis-associated proteins, the expression levels of Bcl-2 and Bax were evaluated. The results demonstrated that in the quercetin-pretreated group, the expression level of Bcl-2 was upregulated, and Bax was downregulated (Fig. 4E-G). The Bcl-2/Bax ratio declined in H₂O₂ group, while it was

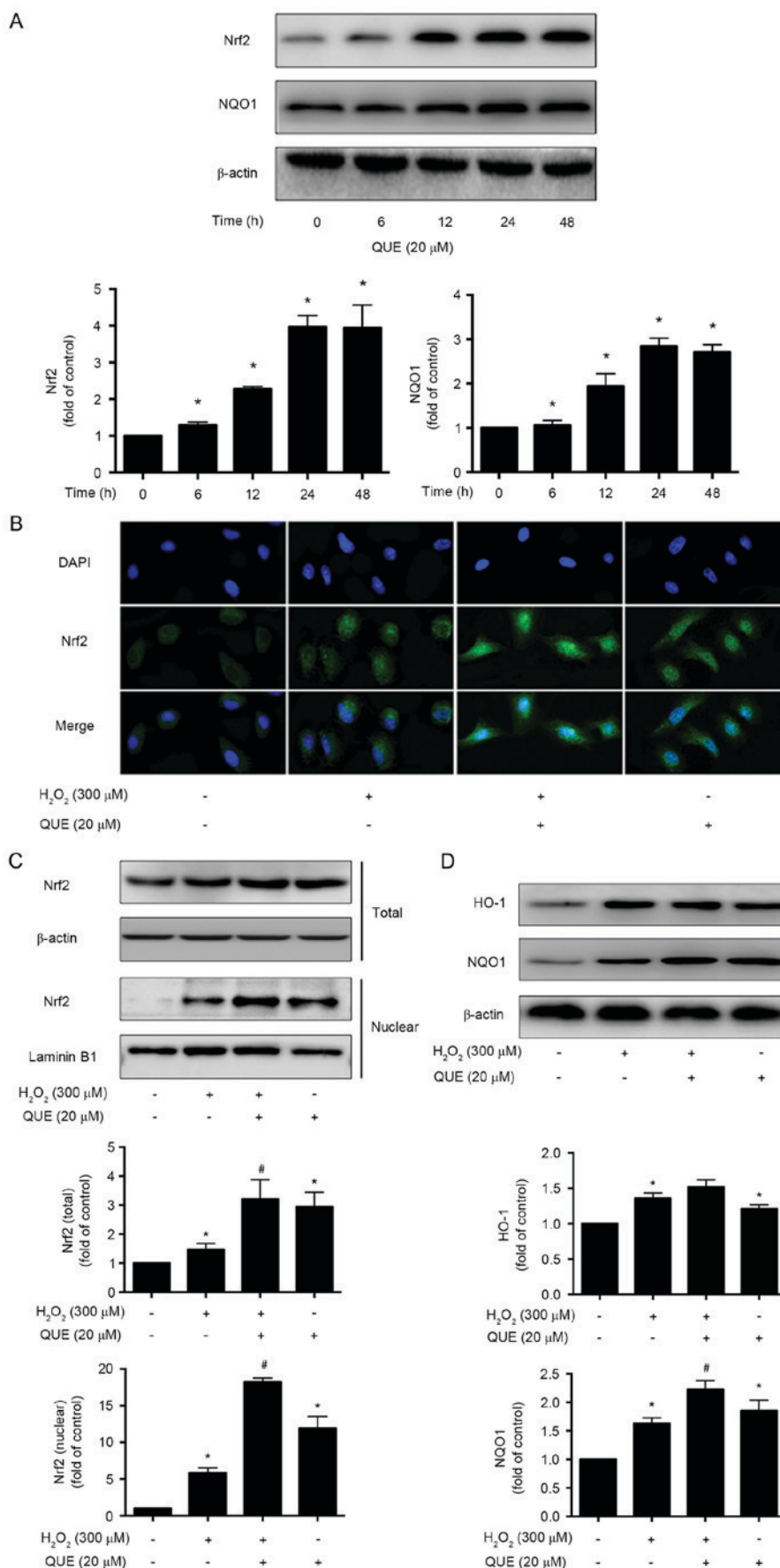


Figure 3. Effects of quercetin on activation of Nrf2 and phase II enzymes in ARPE-19 cells. (A) Western blot images and quantification of Nrf2 and NQO1 protein expression levels in cells incubated with 20 μM quercetin for 0, 6, 12, 24 and 48 h. (B) Representative immunofluorescence images of activation of Nrf2 in ARPE-19 cells. Magnification, x400. Western blot images and quantification of (C) total and nuclear Nrf2, and (D) HO-1 and NQO1 protein expression levels in cells. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. #P<0.05 vs. H₂O₂-induced cells without pretreatment with quercetin. QUE, quercetin; Nrf2, NF-E2-related factor 2; NQO1, NAD(P)H, quinone oxidoreductase 1; HO-1, heme oxygenase-1.

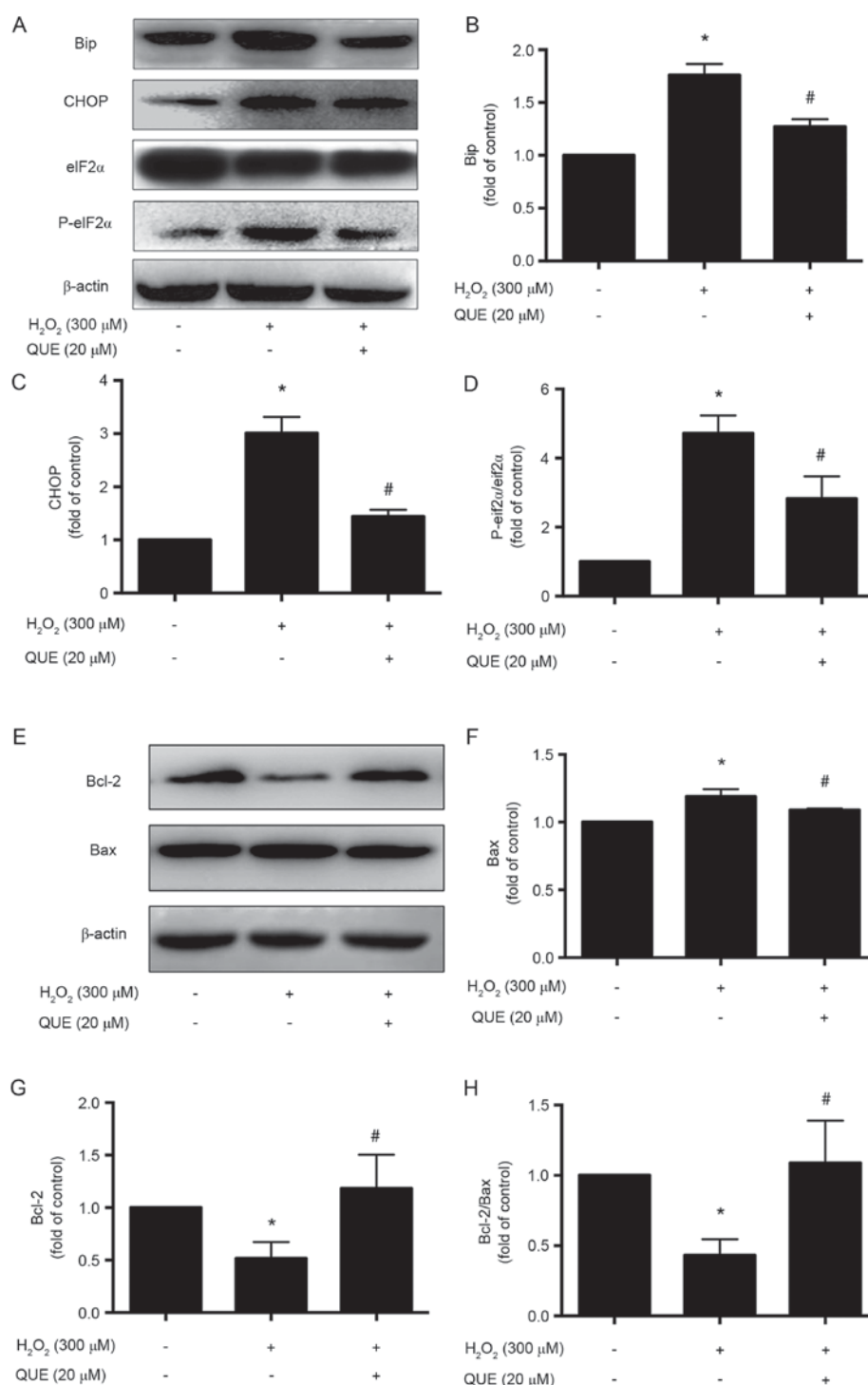


Figure 4. Effects of quercetin on endoplasmic reticulum stress markers and apoptosis-associated proteins in ARPE-19 cells. Cells were incubated with 300 μM H₂O₂ with or without 20 μM quercetin administration. (A) Representative western blot images and quantification of (B) Bip, (C) CHOP and (D) eIF2α and p-eIF2α protein expression levels. (E) Representative western blot images and quantification of (F) Bax, (G) Bcl-2 and (H) the ratio of Bcl-2/Bax protein expression levels. Data are presented as the mean ± standard deviation. *P<0.05 vs. control, #P<0.05 vs. H₂O₂-induced cells without pretreatment with quercetin. QUE, quercetin; Bip, binding of immunoglobulin protein; CHOP, CCAAT/enhancer-binding protein homologous protein; p, phosphorylated; eIF2α, eukaryotic translation initiation factor 2α; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 X associated protein.

significantly increased with the administration of quercetin (20 μM; Fig. 4H).

Discussion

Quercetin is an important dietary polyphenol, which is present in several foods. The present study assessed the cytotoxic

effects of quercetin on ARPE-19 cells and observed that quercetin does not influence the viability of APRE-19 cells. The results demonstrated that a higher concentration of quercetin attenuated H₂O₂-induced cytotoxicity in ARPE-19 cells, particularly at 20 μM concentration.

ROS have been implicated in the etiology of a number of physiological and pathological conditions, and diseases.

Oxidative stress, produced by excess ROS, has been identified to serve a critical role in the injury and degeneration of RPE. In the present study, it was observed that the level of intercellular ROS were increased significantly after H₂O₂ treatment, as demonstrated by the conversion of DCFH-DA into DCF, while pretreatment with quercetin significantly reduced ROS accumulation, indicating that the cytoprotection conferred by quercetin was due to its antioxidant effect.

It is well-known that Nrf2 is a ubiquitous transcription factor, which significantly influences the maintenance of cellular redox status. During homeostasis, Nrf2 is sequestered in the cytoplasm by binding to cytoskeleton-binding Kelch-like ECH-associated protein 1 (Keap1) and degraded through the ubiquitin-26S proteasome pathway. Under oxidative stress, Nrf2 is uncoupled with Keap1 and is rapidly translocated into the nucleus, where it heterodimerizes with Maf proteins and binds to the antioxidant response elements (AREs) in the promoters of its target genes, such as NQO1 and HO-1 (15). A previous study demonstrated that quercetin could activate Nrf2 by upregulating the steady-state level of Nrf2 at the transcriptional level, as well as stabilizing Nrf2 protein post-transcription in HepG2 cells (16). In keeping with these findings, the present study provided direct evidence that quercetin alone had the potential of elevating the expression levels of Nrf2 and NQO1 in a time-dependent manner. It was confirmed that quercetin treatment significantly resulted in translocation of Nrf2 to the nucleus, as detected by immunostaining. In addition, the expression levels of Nrf2 and its target genes were significantly elevated in cells following quercetin pretreatment, compared with H₂O₂ treatment alone. Therefore, quercetin exerts anti-oxidative effects on ARPE-19 cells via the Nrf2-ARE signaling pathway.

There is increasing evidence that induction of ER stress occurs in exposure to oxidative stress, which serves a crucial role in the RPE cell dysfunction and death. Oxidative stress and ER stress are interrelated biological events, and both participate in RPE apoptosis. Previous studies have demonstrated that oxidative stress increases the accumulation of ROS in the ER and subsequently triggers ER stress after H₂O₂ stimulation or A2E and blue light-induced damage (17,18). Huang *et al* (19) revealed that cigarette smoke extract (CSE) exposure induced a dose- and time-dependent increase in ER stress markers, and enhanced ROS, mitochondrial fragmentation and apoptosis of RPE cells, while the ROS scavenger N-acetylcysteine reduced the expression of ER stress protein. These findings suggested a close interaction between oxidative and ER stress in CSE-induced apoptosis (19). In the present study, it was observed that H₂O₂ increased the level of intracellular ROS and triggered ER stress markers expression in ARPE-19 cells. However, quercetin could significantly reduce ROS accumulation, and decrease the expression levels of ER stress markers Bip and CHOP. As ROS have been demonstrated to trigger ER stress, it could be hypothesized that the anti-oxidative activity of quercetin partially contributed to the inhibitory effect on ER stress. In addition, the results demonstrated that CHOP upregulation was in consistent with the increase in phosphorylation of eIF2 α , a downstream target of protein kinase RNA-like endoplasmic reticulum kinase (PERK), suggesting activation of the PERK-eIF2 α -activator of transcription factor 4 (ATF4)-CHOP branch of ER stress

pathways. In response to stressors, PERK phosphorylates eIF2 α to repress translation of proteins. eIF2 α phosphorylation preferentially upregulates the translation of ATF4, which then activates expression of its downstream target genes (20). Thus, pretreatment with quercetin may re-establish ER homeostasis. Hayakawa *et al* (21) demonstrated that quercetin could reduce eIF2 α phosphorylation and ATF4 expression via damaged-inducible gene 34 induction in the brain. However, the ROS-independent effects of quercetin on alleviating ER stress require further investigation.

Bcl-2 and Bax, belonging to the Bcl-2 family, are associated with physiological and pathological apoptosis. Bcl-2 mediates cell survival through sequestration of BH3-only proteins, which are necessary for Bax-mediated mitochondrial permeabilization and apoptosis (22). The anti-apoptotic effects of quercetin have been reported in several cells, including neural, cardiomyoblast and endothelial cells (23,24). The results of the present study have demonstrated that quercetin pretreatment reduced the production of Bax and promoted the generation of Bcl-2, thereby indicating that quercetin might protect ARPE-19 cells by activating pro-survival proteins and inhibiting pro-apoptotic proteins. ER stress can regulate a number of apoptosis-associated proteins that localize on the mitochondrial membrane, particularly the members of the Bcl-2 family. CHOP has been defined as a pivotal mediator of cell death signaling in ER stress, and it also has been suggested that prolonged activation of CHOP promotes apoptosis by downregulating the expression of Bcl-2 (25). Quercetin may upregulate Bcl-2 expression partially by suppressing the expression of CHOP. However, according to previous research, quercetin treatment could result in cell apoptosis in different kinds of cancer cells. Quercetin directly binds to the BH3 domain of Bcl-2 and Bcl-xL proteins, thereby inhibiting their activity and promoting cancer cell apoptosis (26). Therefore, quercetin might have contrasting effects under different conditions.

Xu *et al* (27) demonstrated that quercetin did not effectively reduce the contents of ROS in H₂O₂-treated ARPE-19 cells, whereas the phospholipid complex (PC) of quercetin could significantly decrease their level. Quercetin-PC, but not quercetin, was demonstrated to upregulate the protein expression levels of HO-1 and NQO1. Contradictory to this, the result of the present study demonstrated that 20 μ M quercetin could significantly elevate Nrf2 and NQO1 in a time-dependent manner, ROS accumulation was reduced following pretreatment with quercetin. Consistent with this, Zhu *et al* (28) revealed that quercetin (20 μ M) significantly blocked UVB irradiation (15 mJ/cm²)-induced intracellular ROS generation (28). Therefore, it was hypothesized that quercetin could have antioxidant properties in H₂O₂-treated ARPE-19 cells at relatively low concentrations. Kook *et al* (29) first demonstrated that quercetin was able to protect RPE cells from oxidative damage and cellular senescence *in vitro* in a dose-dependent manner.

In conclusion, the present study confirmed the antioxidant effect of quercetin in ARPE-19 cells and further highlighted the possible mechanisms-activation of the Nrf2 signaling pathway, attenuation of ER stress, and the involvement in regulation of apoptosis. These findings offer some novel therapeutic strategies and methods for various oxidative stress-associated retinal degenerative diseases, such as AMD.

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