Resveratrol protects against oxidative damage of retinal pigment epithelium cells by modulating SOD/MDA activity and activating Bcl-2 expression

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Abstract. – OBJECTIVE: Age-related macular degeneration (AMD) is mainly characterized by dysfunction of retinal pigment epithelium (RPE) cells. This study aimed to investigate the protective effects of resveratrol on oxidative damaged RPE cells.

MATERIALS AND METHODS: Human D407 cells were divided into normal control (NC), H_2O_2 treated (H_2O_2, treating with H_2O_2 at a final concentration of 200 mol/l) and resveratrol treatment groups (treating with resveratrol at a concentration of 12.5, 25, 50 and 100 mg/l). Malondialdehyde (MDA) and superoxide dismutase (SOD) activities were examined using enzyme-linked immunosorbent assay (ELISA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and cell count kit-8 (CCK-8) were used to examine cell viability. Cell cycle phase distribution and apoptosis of D407 cells were evaluated using flow cytometry assay. B-cell lymphoma-2 (Bcl-2) and cleaved caspase 3 expression were detected using quantitative real-time PCR (qRT-PCR) and Western blot assay, respectively.

RESULTS: Resveratrol significantly decreased inhibitive ratios of D407 cell growth compared to that of H_2O_2 group ($p<0.05$). Resveratrol significantly increased SOD activity compared to that of H_2O_2 group ($p<0.05$). Resveratrol significantly reduced MDA activity compared to that of H_2O_2 group ($p<0.05$). Resveratrol affected cell cycle phase distribution of D407 cells compared to that of H2O2 group ($p<0.05$). Resveratrol significantly decreased the early stage and late stage apoptosis rates compared to that of H_2O_2 group ($p<0.05$). Resveratrol significantly enhanced Bcl-2 levels and decreased cleaved caspase 3 levels compared to that of H_2O_2 group ($p<0.05$).

CONCLUSIONS: Resveratrol protected against the oxidative damage of RPE cells by modulating SOD/MDA activity and activating Bcl-2 expression.

Key Words

Resveratrol, Age-related macular degeneration, Oxidative damage, Retinal pigment epithelium, Oxidant.

Introduction

In clinical, the age-related macular degeneration (AMD) has become the most important reason for the severe vision loss of elderly individuals[1,2]. AMD is mainly characterized by the dysfunction and oxidative injury of the retinal pigment epithelium (RPE) cells[3,4]. RPE cells play critical functions in visual sense development and could maintain blood-retinal barrier, transfer nutrients from choroid to photoreceptors, and recognize the light-cycle[5]. Therefore, the dysfunction or functional impairment of RPE of visual functions is always considered to be caused by the RPE cell migration[6], which is a critical biological process affected by the apoptosis or cell death. The ultraviolet or the short-wavelength radiation always causes reactive oxygen species (ROS) production and induces the oxidative injury of the RPE cells[7,8]. Meanwhile, the oxidative injury of RPE cells is also induced by the free-radical reactions and caused by the endogenous-chromophores via the lens or the cornea[9]. The previous study[10] also reported that the antioxidants could significantly inhibit or slow the AMD progression. Therefore, discovering the effective drugs that could protect against the oxidative injury of RPE is a potential strategy for AMD. Resveratrol (3,5,4'-trihydroxystilbene), mainly extracted from the peanuts, red wine, grapes, plays important roles in anti-inflammation, anti-oxidant, anti-carcinogenic activity, anti-diabetic nephropathy, anti-aging and anti-chemopreventive activity[11,12]. Previous works[13-15] also reported that the resveratrol benefits to the treatment of cancer, heart disease, diabetes. However, the effects of resveratrol on the oxidative damaged RPE cells associated AMD have not been fully clarified till now. In this

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study, we exhibited that resveratrol significantly enhances cell viabilities, increases SOD activities, reduces MDA activities and inhibits early and late apoptosis by triggering Bcl-2 expression and suppressing cleaved caspase 3 expressions in RPE cells undergoing H_2O_2 treatment.

Materials and Methods

Cell Culture

The human retinal pigment epithelial cell line, D407, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The D407 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco BRL. Co. Ltd., Grand Island, NY, USA) supplementing with streptomycin (100 μg/ml, Beyotime Biotech. Shanghai, China), penicillin (U/ml, Beyotime Biotech. Shanghai, China) and 10% fetal bovine serum (FBS, Gibco BRL. Co. Ltd., Grand Island, NJ, USA) in carbon dioxide cell incubator (ShelLAB, Cornelius, OR, USA) with 5% CO_2 at 37°C. This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital (Chengdu, China).

Cell Treatment and Trial Grouping

The D407 cells were normal control (NC, treating without any regents) group, H_2O_2 treated group (H_2O_2, treating with H_2O_2 at final concentration of 200 mol/l) and resveratrol treatment groups (treating with resveratrol at final concentration of 12.5, 25, 50 and 100 mg/l). The resveratrol was purchased from Sigma-Aldrich. (St. Louis, MO, USA) and dissolved in the ethanol (Sinopharm. Chemical Reagent Co. Ltd., Shanghai, China) for treating the D407 cells.

Lipid Peroxidation and Antioxidant Enzyme Activity Assessment

In this study, the lipid peroxidation, malondialdehyde (MDA)[16] and antioxidant enzyme, superoxide dismutase (SOD)[17] were examined by using enzyme linked immunosorbent assay (ELISA) method. The SOD activity was evaluated with SOD detection ELISA kit (Cat. No. TO1060, Leagene Biotech. Co. Ltd., Beijing, China) according to instruction of manufacturer. MDA activity was evaluated by using MDA assay kit (Cat. No. A003-1, Jiancheng Bioengineering Int., Nanjing, China) due to the manufacturer's instruction. Finally, the absorbance of the samples was captured at wavelength of 450 nm by using the spectrophotometer (Mode: ZF-90, Shanghai Wujiu Automation Equipment Co. Ltd., Shanghai, China).

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

At 24 h post the D407 culture, the MTT assay was conducted to evaluate the optium concentration of resveratrol. In brief, the MTT (Sigma-Aldrich, St. Louis, MO, USA) dissolved into the phosphorylate-buffered saline (PBS, ZSGB Bio. Co. Ltd., Beijing, China) and added to medium to treat D407 cells (at final concentration of 5 mg/ml) at 37°C for 4 h. Then, a total of 150 μl dimethylsulfoxide (DMSO, Amresco Inc., Solon, OH, USA) was added to the cells to dissolve the formazan crystals. The optical density (OD) values were evaluated at wavelength of 450 nm by utilizing the microplate reader (Bio-Tek Inc., Winooski, VT, USA). Results were represented as the inhibitive ratio by comparing with the Normal control D407 cells (%).

Cell Count Kit-8 (CCK-8) Assay

D407 cells were digested, washed and seeded with density of 4×10^5 cells per milliliter for one well of 96-well plates (Corning Costar, Corning, MA, USA). At 2 h, 8 h and 24 h post the resveratrol treatment, CCK-8 solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium and incubated for 4 h. The cell viability of D407 cells was tested by using the spectrophotometer (Mode: ZF-90, Shanghai Wujiu Automation Equipment Co. Ltd., Shanghai, China) at wavelength of 450 nm.

Cell Cycle Phase Analysis Using Flow Cytometry Assay

D407 cells were adjusted to the density of 5×10^5 cells/ml and treated using 70% ethanol (Sinopharm. Chemical Reagent Co., Ltd., Shanghai, China) for 12 h at 4°C. Next, D407 cells were centrifuged at 1000 r/min for 5 min and treated using cell cycle and apoptosis analysis kit (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min in dark at 4°C. Finally, the propidium iodide (PI, BD Biosciences, San Jose, CA, USA) stained cells were analyzed by utilizing the FACScan flow cytometer (Becton Dickinson, Brea, CA, USA). Results were represented as the percentage of different cell cycle phase of D407 cells.

Apoptosis Evaluation Using Flow Cytometry Assay

D407 cell apoptosis was measured by using flow cytometry assay at 2 h, 8 h and 24 h post the resveratrol treatment. In brief, the D407 cells were re-suspended in PBS buffer containing the Annexin V-FITC and PI according to the instruction of cell cycle and apoptosis analysis kit (BD Biosciences, Franklin Lakes, NJ, USA). The Annexin V-FITC and PI stained cells were incubated at room temperature for 5 min in dark. The ratio of FITC-labeled D407 cell membrane-phosphatidylserine residues (%, represented as apoptosis) was analyzed by utilizing the FACScan flow cytometer (Becton Dickinson, Brea, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNAs of D407 cells were extracted by using the commercial regents (Sino Pharm. Chem. Reagent Co. Ltd., Shanghai, China) due to manufacturer's instructions. Complementary DNA (cDNA) was synthesized by employing Reverse Transcription Kit (Western Biotech., Chongqing, China) according to the instruction of manufacturer. The SybrGreen PCR master Mix (Western Biotech. Chongqing, China) was used to amplify and examine the B-cell lymphoma-2 (Bcl-2) and cleaved caspase 3 genes. The primers used for gene amplification were listed in Table I. Thermal cycling (CT) parameters were assigned as the followings: denaturation step at 94°C for 4 min, followed by 35 cycles of amplification of 94°C for 20 s, 60°C for 30 s and 72°C for 30 s. The relative expressions of Bcl-2 and caspase 3 were calculated by using gel scanning system (Mode: GDS8000, UVP Corporation, Sacramento, CA, USA) and employing the formula of $2^{-\Delta\Delta ct}$.

Western Blot Assay

D407 cells were lysed with radioimmunoprecipitation assay (RIPA, Applygen Gene Tech. Co. Ltd., Beijing, China) and the products were centrifuged at 8000 r/min for 15 min. The supernatant of D407 cells was separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Amresco Inc., Solon, OH, USA). The isolated protein was transferred onto polyvinylidene difluoride membrane (PVDF, DuPont, Wilmington, DE, USA) by using the Trans-Blot SD cell instrument (Bio-Rad Laboratories, Hercules, CA, USA). Then, the PVDF membranes were treated with 5% bovine serum albumin (BSA, Amersham Pharmacia Biotech., Piscataway, NJ, USA) at room temperature for 60 min. PVDF membranes were incubated with the rabbit anti-human Bcl-2 monoclonal antibody (1:3000; Cat. No. ab32124, Abcam Biotech., Cambridge, MA, USA), rabbit anti-human cleaved caspase 3 polyclonal antibody (1:2000, Cat. No. ab2302, Abcam Biotech., Cambridge, MA, USA) and rabbit anti-human glyceraldehyde phosphate dehydrogenase (GAPDH) polyclonal antibody (1:2000; Cat. No. ab9485, Abcam Biotech., Cambridge, MA, USA) at 4°C overnight. After that, the polyvinylidene difluoride (PVDF) membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:2000, Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 60 min. Enhanced chemiluminescence (ECL, Thermo Scientific Pierce, Rockford, IL, USA) was utilized to incubate PVDF membrane in dark for 2 min. Relative grey density was scanned with GDS8000 gel scanning system (UVP corporation, Sacramento, CA, USA) and analyzed with Labworks™ Analaysis Software (Labworks™, Upland, CA, USA).

Statistical Analysis

The data were described as mean ± standard deviation (SD) and analyzed by using the SPSS software 20.0 (SPSS Inc., Chicago, Ull, USA). Student's *t*-test was used for statistical analysis between two groups. Tukey's post-hoc test was used to validate the analysis of variance (ANOVA) for comparing the data among groups. All of data were obtained from at least six independent experiments or test. A statistical significance was defined when $p<0.05$.

Table I. The primers and sequences for qRT-PCR assay.

Genes		Sequences	Length (bp)
Bcl-2	Forwards	AGGGACGGGGTGAACTGG	175
	Reverse	CTACCCAGCCTCCGTTATCC	
Caspase 3	Forwards	AGAACTGGACTGTGGCATTGAG	166
	Reverse	GGCACAAAGCGACTGGATG	
β-actin	Forwards	TGACGTGGACATCCGCAAAG	205

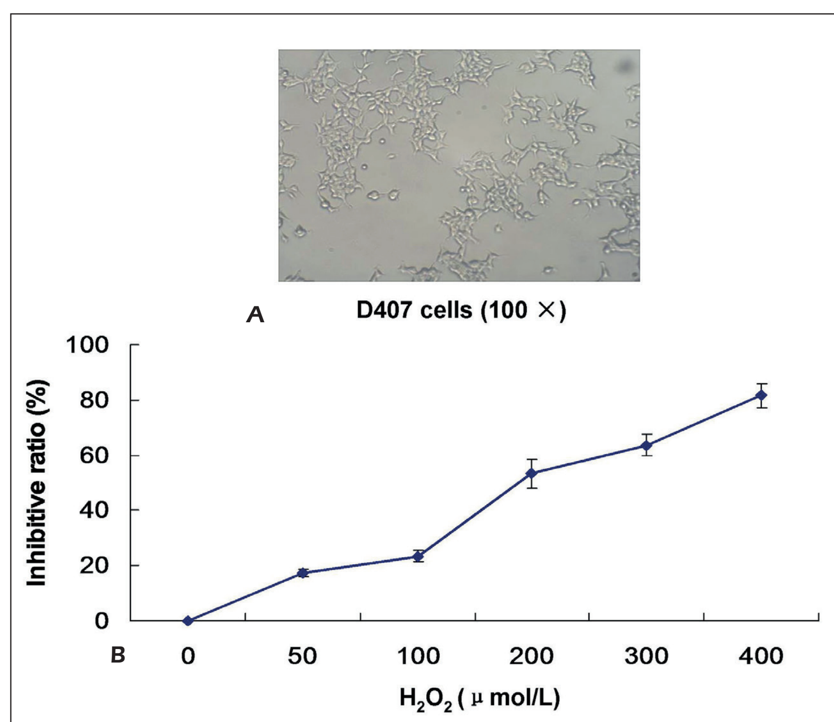

Figure 1. D407 cell culture and optimum concentration of H_2O_2 selection. **A**, Image for the cultured D407 cells. **B**, Statistical curve for the inhibitive ratios according to the MTT assay.

Results

Resveratrol Treatment Promoted D407 Cells Growth

D407 cells were cultured (Figure 1A) and the optimum concentration of H_2O_2 was evaluated by using MTT assay. The results showed that the H_2O_2 concentration of 200 μmol/l was the optimum concentration for inducing the inhibition of D407 cell growth (Figure 1B). Therefore, in the following experiments, the H_2O_2 (200 μmol/l) was used to treat the D407 cells and the inhibitive effects of resveratrol on cell growth were examined. The results indicated that the different concentrations of resveratrol (12.5, 25, 50 and 100 mg/l) significantly decreased the inhibitive ratios of D407 cell growth compared to that of H_2O_2 group ($p<0.05$), at 2 h (Figure 2A), 8 h (Figure 2B) and 24 h (Figure 2C) after the treatment, respectively.

Resveratrol Treatment Enhanced SOD Activity

In this experiment, the activity of represented antioxidant enzyme, SOD, was examined by using ELISA method. In the early stage, after 2 h treatment, the resveratrol (at concentration of 25, 50 and 100 mg/l) significantly increased the SOD activity compared to that of H_2O_2 group (Figure 3A, $p<0.05$). Meanwhile, 8 h (Figure 3B) and 24 h (Figure 3C) post the treatment, the resveratrol (at concentration of 50 and 100 mg/l) significantly enhanced the SOD activity compared to that of H_2O_2 group ($p<0.05$).

Resveratrol Treatment Reduced MDA Activity

The activity of represented lipid peroxidation, MDA, was evaluated with ELISA method. The results indicated that the resveratrol treatment (at concentration of 25, 50 and 100 mg/l) significantly reduced the MDA activities of D407 cells at 2 h (Figure 4A), 8 h (Figure 4B) and 24 h (Figure 4C), compared to that of H_2O_2 group ($p<0.05$).

Resveratrol Treatment Affected Cell Cycle Phase Distribution of D407 Cells

When comparing with the H_2O_2 group, the D407 cells in resveratrol groups illustrated the significant G1-phase reducing, at 2 h, 8 h and 24 h post the treatment (Figure 5, $p<0.05$). D407 cells from the H_2O_2 group illustrated the G1-phase arrest, and the S phase percentages were significantly decreased compared to the NC group (Figure 5, $p<0.05$). However, the S phase percentages D407 cells in resveratrol treatment groups were significantly enhanced compared to that of H_2O_2 group (Figure 5, $p<0.05$).

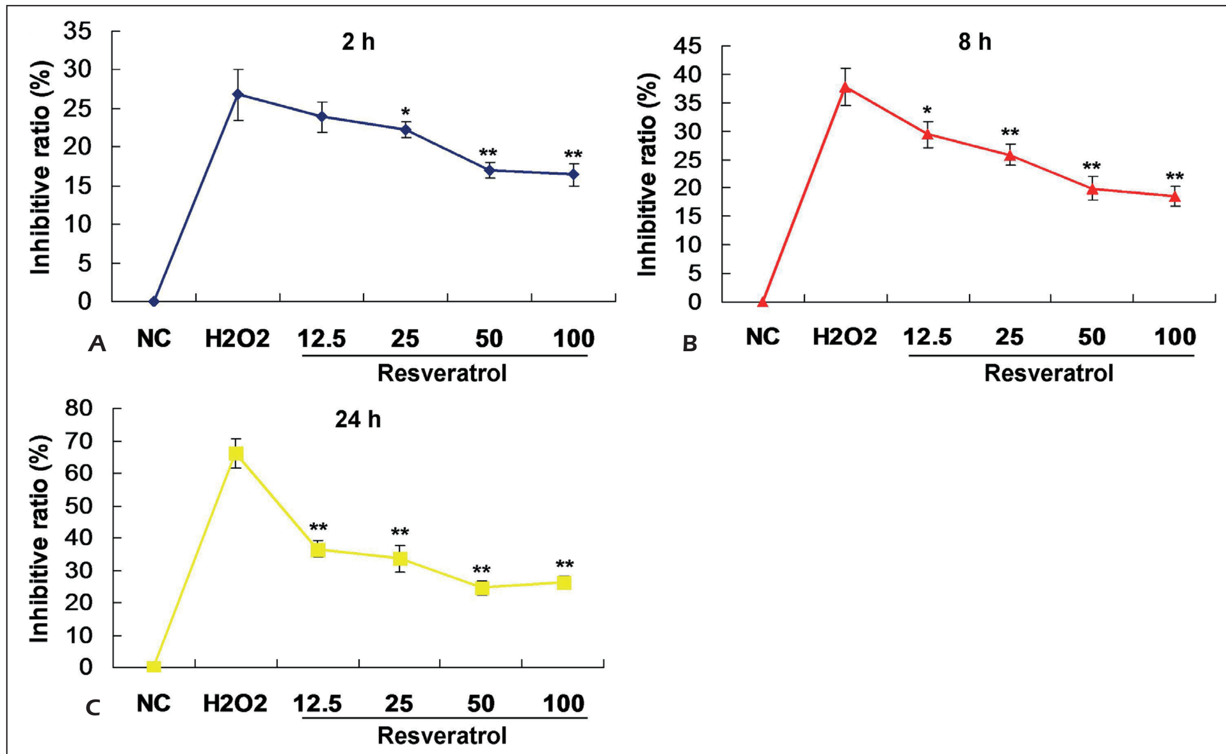

Figure 2. Inhibitive ratios for the resveratrol treated D407 cells at 2 h, 8 h and 24 h post treatment. **A**, Inhibitive ratios for D407 cells at 2 h post resveratrol treatment. **B**, Inhibitive ratios for D407 cells at 8 h post resveratrol treatment. **C**, Inhibitive ratios for D407 cells at 24 h post resveratrol treatment. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

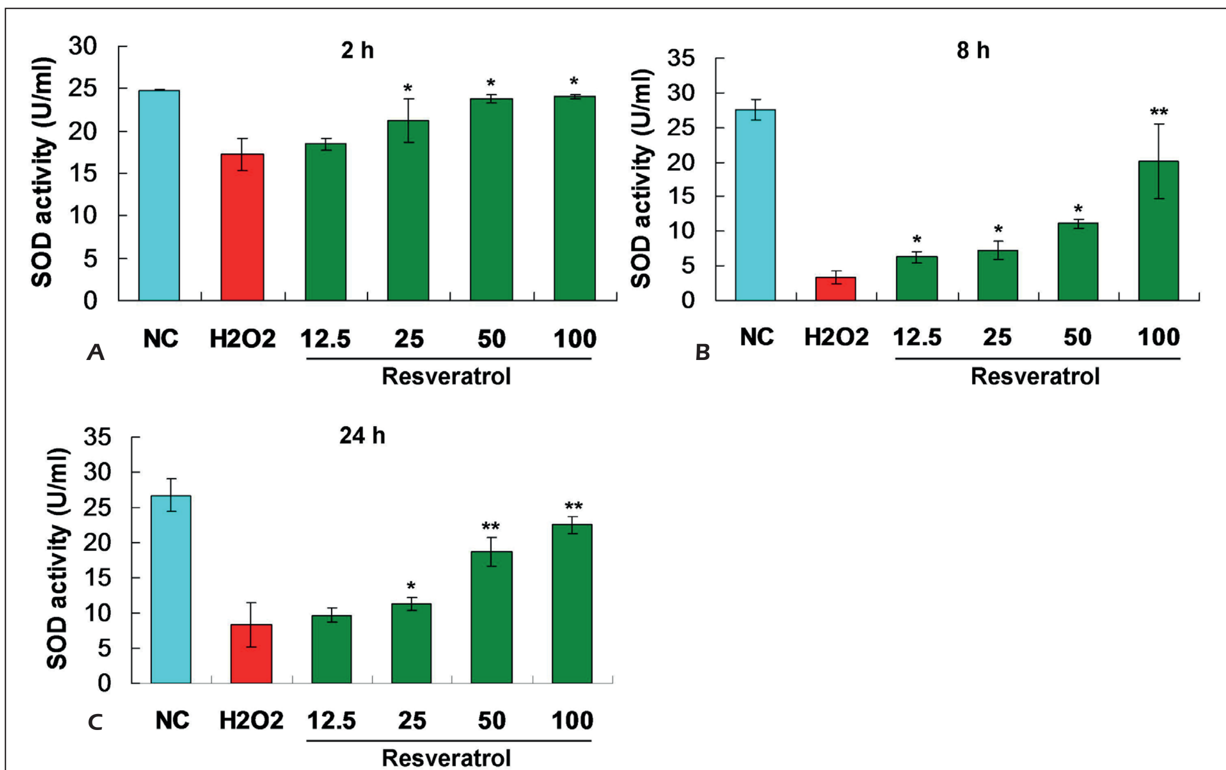

Figure 3. Evaluation for the SOD activity in resveratrol treated D407 cells by using ELISA. **A**, SOD activity of D407 cells at 2 h post resveratrol treatment. **B**, SOD activity of D407 cells at 8 h post resveratrol treatment. **C**, SOD activity of D407 cells at 24 h post resveratrol treatment. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

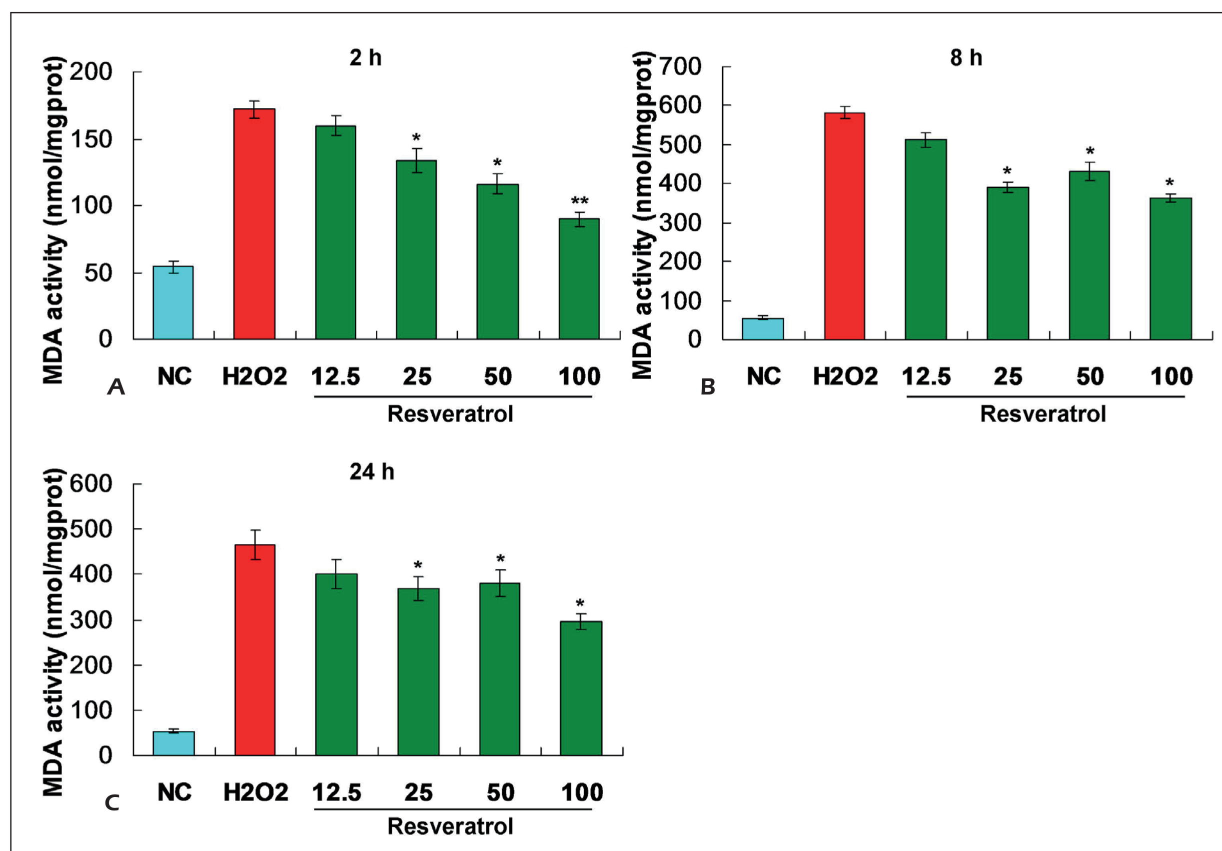

Figure 4. Evaluation for the MDA activity in resveratrol treated D407 cells by using ELISA. **A**, MDA activity of D407 cells at 2 h post resveratrol treatment. **B**, MDA activity of D407 cells at 8 h post resveratrol treatment. **C**, MDA activity of D407 cells at 24 h post resveratrol treatment. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

Resveratrol Treatment Inhibited Early and Late Apoptosis

The results indicated that the resveratrol treatment significantly decreased the early stage apoptosis rate compared to that of H_2O_2 group ($p<0.05$) at 2 h (Figure 6A) and 8 h (Figure 6B) post the treatment. Meanwhile, at 2 h (Figure 6A), 8 h (Figure 6B) and 24 h (Figure 6C) after the resveratrol treatment, the late stage apoptosis rates were also significantly decreased compared to that of H_2O_2 group $p<0.05$).

Resveratrol Improved Cell Growth by Activating Bcl-2/Caspase 3 Pathway

The apoptosis biomarker, Bcl-2, and apoptosis up-stream molecule, caspase 3[18], were analyzed by using Western blot assay and qRT-PCR, respectively. The Western blot assay results (Figure 7A) indicated that the at 24 h post the resveratrol treatment, Bcl-2 levels (Figure 7B) were significantly increased and cleaved caspase 3 levels (Figure 7C) were significantly decreased compared to that of the H_2O_2 group ($p<0.05$). Moreover, the qRT-PCR assay also showed that at 2 h, 8 h and 24 h after resveratrol treatments, Bcl-2 levels (Figure 8A) were significantly enhanced and cleaved caspase 3 levels (Figure 8B) were significantly reduced compared to that of the H_2O_2 group ($p<0.05$).

Discussion

The oxidative injury or stress is considered to be the critical risk factor to the aging-correlated disorders, such as neurodegenerative diseases, cardiovascular diseases, diabetes mellitus and the AMD[19-21]. The produced oxidants could penetrate the cell membrane and induce the production of lipid peroxidation and reactive oxygen species (ROS), induce apoptosis-associated signaling pathways, and finally cause the DNA dam-

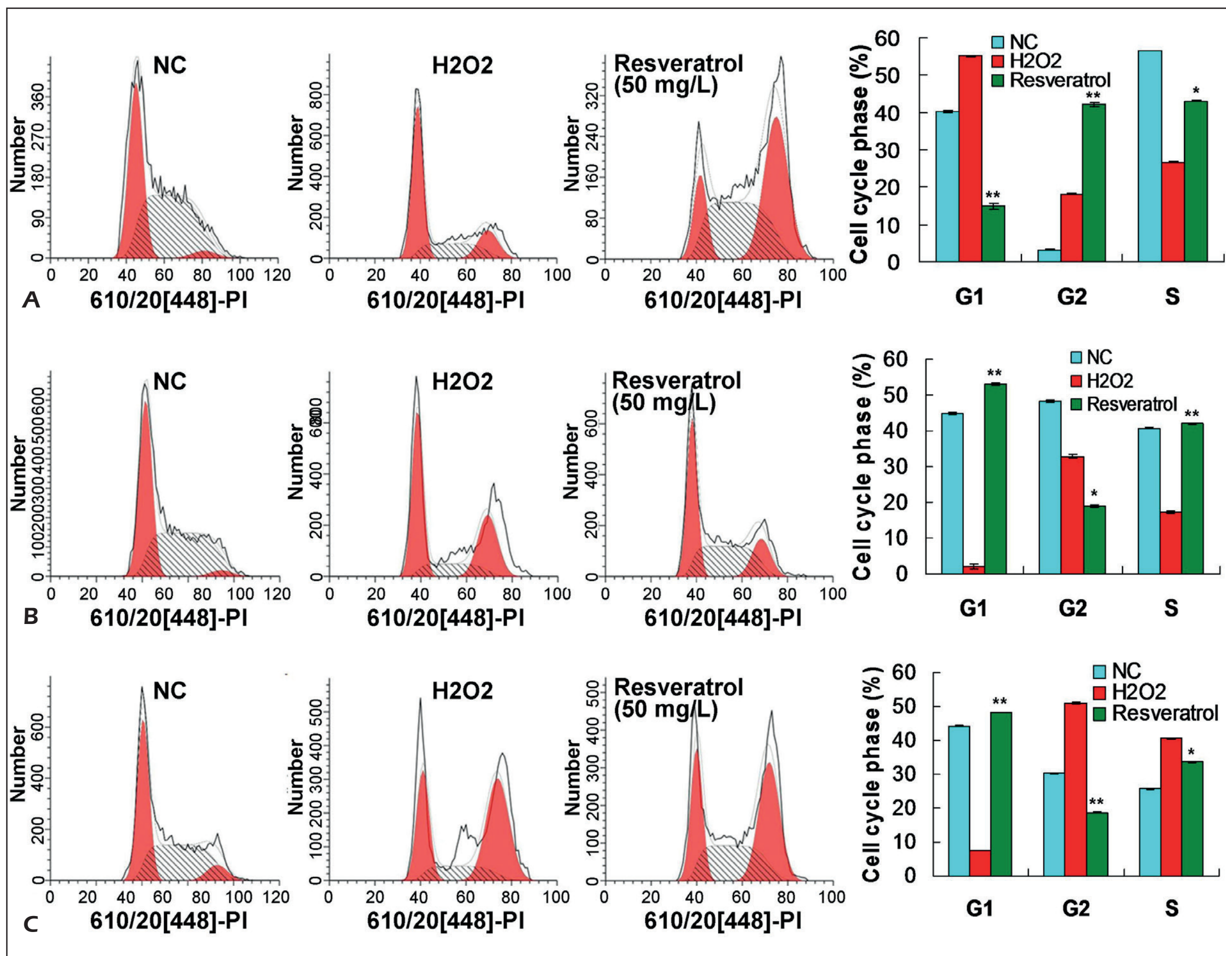

Figure 5. Cell cycle phage distribution evaluation by using flow cytometry assay. **A**, Flow cytometry images and statistical analysis for cell cycle phage distribution at 2 h post resveratrol treatment. **B**, Flow cytometry images and statistical analysis for cell cycle phage distribution at 8 h post resveratrol treatment. **C**, Flow cytometry images and statistical analysis for cell cycle phage distribution at 24 h post resveratrol treatment. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

age[22,23]. The resveratrol is a natural compound that plays effective roles in anti-inflammation, anti-aging, anti-fibrotic process, anti-obesity[24,25]. In the ophthalmological research, resveratrol has been proven to be effective for inhibiting the hypoxic-choroidal vascular endothelial cell growth by suppressing the vascular endothelial growth factor (VEGF) secretion[26,27]. However, in the normal human VEGF, resveratrol could improve RPE cell growth by reducing the oxidative damage[28]. In this study, we proved that resveratrol obviously enhanced the D407 cell growth by regulating SOD and MDA activities, and activating the Bcl-2 associated apoptosis signaling pathway. In the present study, we demonstrated the oxidative damages of D407 cells by incubating with H_2O_2 at optimum concentration of 200 μmol/l[29]. Then, the resveratrol was administrated to the oxidative damaged D407 cells to observe its effects on the cell growth. The results showed that resveratrol treatment significantly decreased the inhibitive ratios and promoted the D407 cells growth, which are consistent with the previous study[30] that reporting the regulative effects of resveratrol on cell growth. Due to the oxidative stress always induces the changes of lipid peroxidation[16] and antioxidant enzyme, therefore, we evaluated their associated molecules, SOD and MDA, in D407 cells post resveratrol treatments. Actually, resveratrol has been reported to be effective on ameliorating oxidative stress in many diseases[31,32]; however, its role was not clarified in AMD. Our findings showed that resveratrol significantly enhanced the SOD levels and sig-

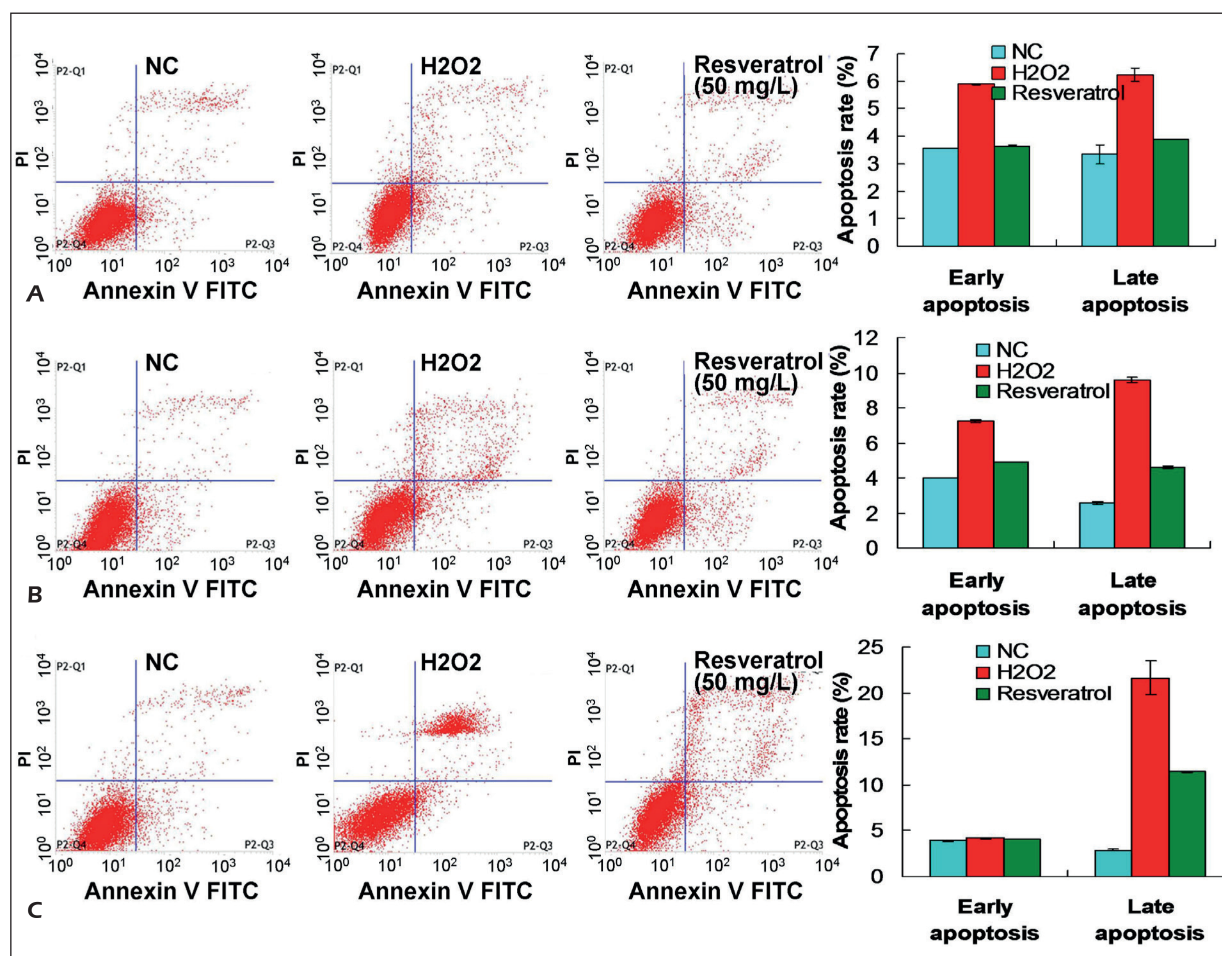

Figure 6. Determination for the early and late stage apoptosis by using flow cytometry assay. **A**, Flow cytometry images and statistical analysis for early and late stage apoptosis at 2 h post resveratrol treatment. **B**, Flow cytometry images and statistical analysis for early and late stage apoptosis at 8 h post resveratrol treatment. **C**, Flow cytometry images and statistical analysis for early and late stage apoptosis at 24 h post resveratrol treatment. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

nificantly reduced the MDA levels compared to H_2O_2 treated cells. This result suggests that the resveratrol could effectively inhibit the oxidative damage by enhancing SOD levels and reducing MDA levels in D407 cells. The previous study[33] reported that the oxidative damage or oxidative stress always induces the cell apoptosis; therefore, we analyzed the apoptosis of D407 cells. Our results indicated that resveratrol treatment significantly inhibited the early and late apoptosis of D407 cells compared to that of H_2O_2 treated cells. Meanwhile, resveratrol treatment affected cell cycle phase distribution of D407 cells. Totally, these results suggest that resveratrol modulated the cell cycle phase distribution and apoptosis of D407 cells, which are consistent with the previous report[34]. In order to clarify the mechanism for resveratrol-mediated protective effects on cell growth, the biomarker of apoptosis, Bcl-2[35], was examined. The findings illustrated that resveratrol significantly increased the Bcl-2 levels of H_2O_2 treated D407 cells, suggesting that the resveratrol improves cells growth by activating the mitochondria-associated apoptosis pathway[36]. Moreover, to specify the detailed process of mitochondria-associated apoptosis, the apoptosis upstream molecule cleaved caspase 3 was examined. Our results exhibited that resveratrol significantly decreased the levels of cleaved caspase 3 compared to that of H_2O_2 treated D407 cells. These findings hint that resveratrol enhanced cell growth by ameliorating cleaved caspase 3 expression and triggering Bcl-2 expression.

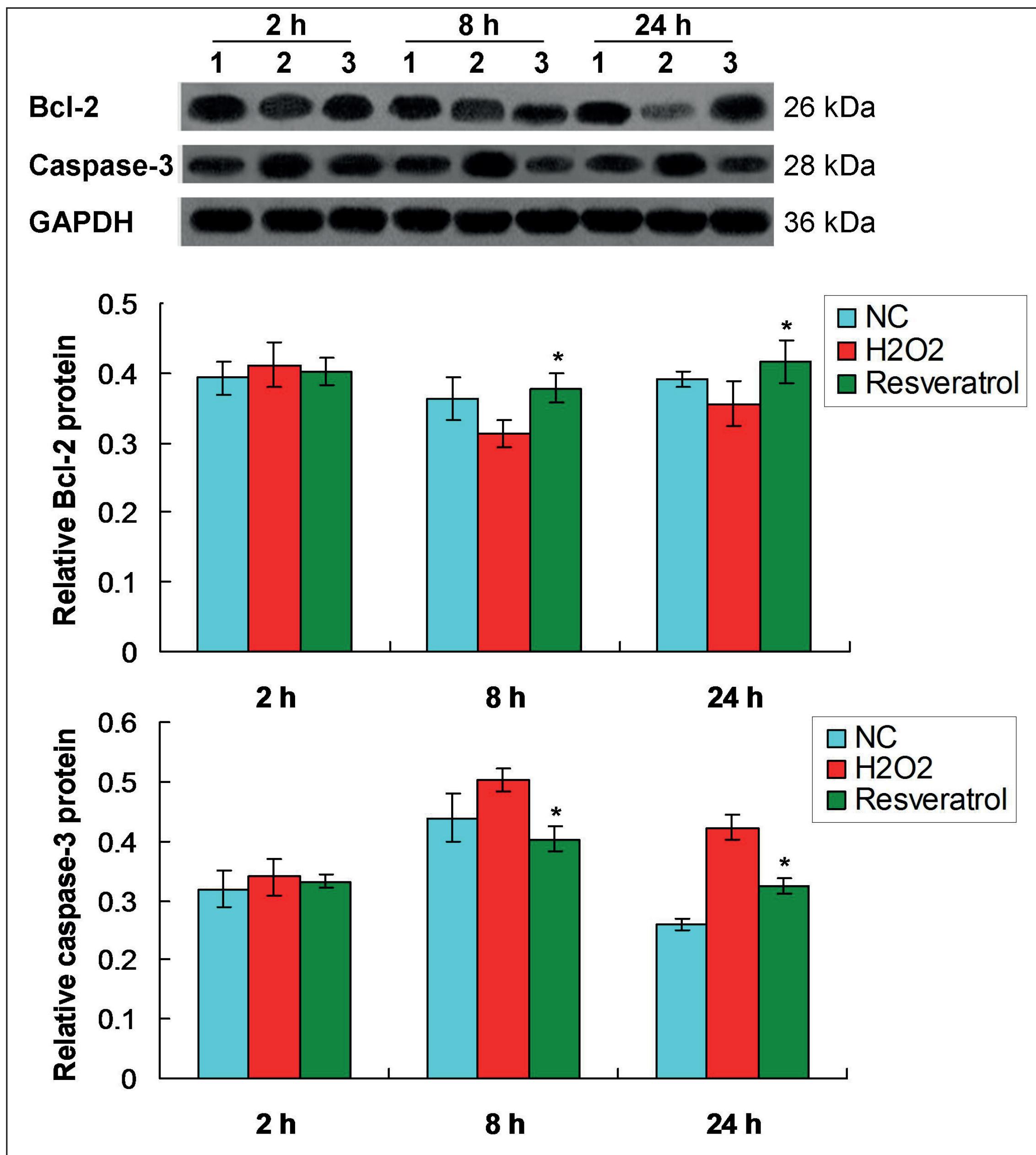

Figure 7. Western blot analysis for detecting the Bcl-2 and cleaved caspase 3 expression in D407 cells undergoing resveratrol treatment. **A**, Western blot images. **B**, Statistical analysis for the Bcl-2 expression. **C**, Statistical analysis for the cleaved caspase 3 expression. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

Conclusions

We constructed oxidative damage cell model by administrating by using H_2O_2 regent. Resveratrol can efficiently enhance D407 cell viability, decrease cell apoptosis and modulate cell cycle arrest. Meanwhile, resveratrol significantly increased SOD activity, decreased MDA activity, and activated Bcl-2 expression and inhibited cleaved caspase 3 expression. In summary, we demonstrated that resveratrol protected against the oxidative damage of RPE cells by modulating SOD/MDA activity and activating Bcl-2 expression.

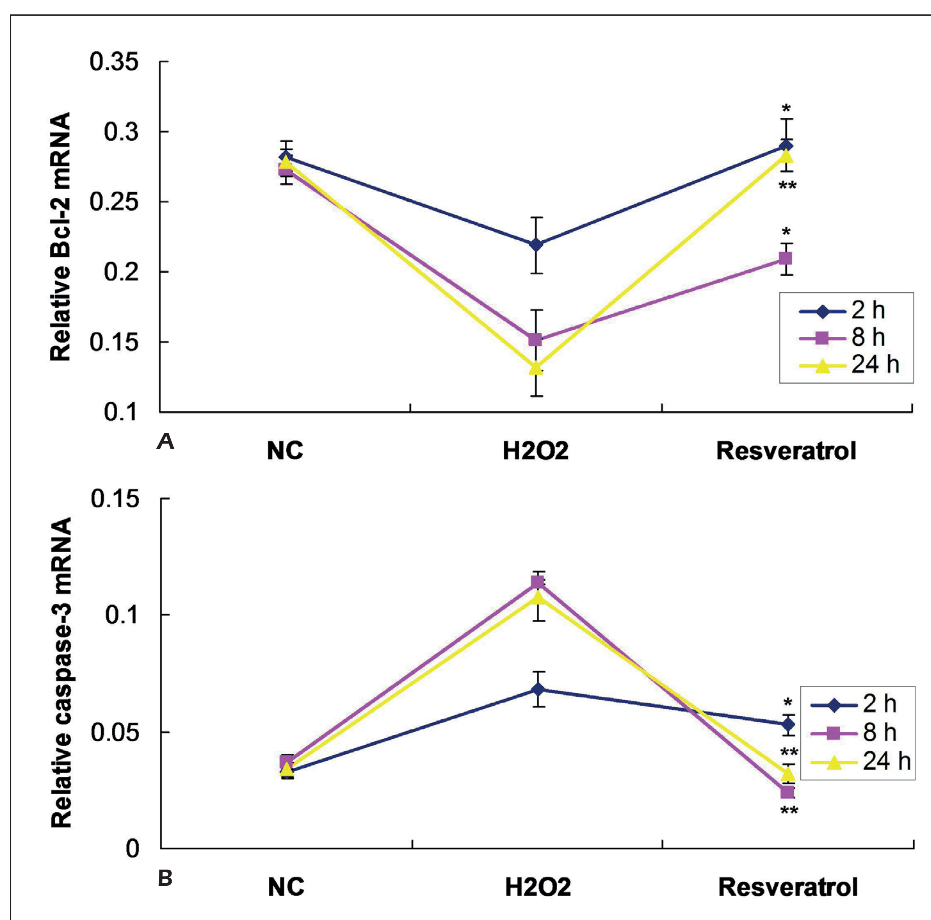

Figure 8. qRT-PCR analysis for evaluating the Bcl-2 and cleaved caspase 3 mRNA levels in D407 cells undergoing resveratrol treatment. ***A***, qRT-PCR analysis images. ***B***, Statistical analysis for the Bcl-2 mRNA expression. ***C***, Statistical analysis for the cleaved caspase 3 mRNA expression. $^{**}p<0.01$, $^{*}p<0.05$ *vs.* H_2O_2 group.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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