

Salidroside prevents hydroperoxide-induced oxidative stress and apoptosis in retinal pigment epithelium cells

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Abstract. Salidroside (SAL) is the major pharmacologically active constituent of *Rhodiola rosea*, which possesses a wide range of pharmacological functions, including anti-aging, anti-inflammatory, antioxidant, anticancer and neuroprotective activities. However, the effects and mechanisms of SAL on oxidative stress in retinal pigment epithelial (RPE) cells exposed to hydrogen peroxide (H₂O₂) remain unclear. The present study investigated the protective effects of SAL and the underlying mechanisms against H₂O₂-induced oxidative stress in human RPE cells. ARPE-19 cells were treated with various doses of SAL for 24 h and then exposed to 200 μ M H₂O₂ for 24 h. Cell viability was analyzed by a MTT assay, and the intracellular levels of reactive oxygen species were measured using CellROX orange reagent. Cell apoptosis was analyzed by annexin V/propidium iodide double staining, followed by flow cytometry. The levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein, phospho (p)-protein kinase B (Akt), Akt, p-glycogen synthase kinase (GSK)-3 β and GSK-3 β were evaluated using western blotting. The results demonstrated that SAL markedly attenuated H₂O₂-induced loss of cell viability. SAL also ameliorated H₂O₂-induced oxidative stress and cell apoptosis in RPE cells. In addition, pretreatment with SAL significantly increased the phosphorylation levels of Akt and GSK-3 β in H₂O₂-treated ARPE-19 cells. In conclusion, the present study demonstrated that SAL protected RPE cells against H₂O₂-induced cell injury through the activation of the Akt/GSK-3 β signaling pathway. This suggests that SAL may be a potential therapeutic strategy for the treatment of age-related macular degeneration.

Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss after the age of 60 years (1). Although the exact pathogenic mechanism of AMD is still unknown, numerous reports provide evidence that oxidative stress plays an important role in the pathophysiology of AMD. Retinal pigment epithelial (RPE) is a monolayer of differentiated cells located between the neural retina and Bruch's membrane, performing essential functions for the maintenance of the normal visual process (2). During AMD, excessive oxidative stress occurs, resulting in the accumulation of reactive oxygen species (ROS), causing damage to RPE cells (3-5). Excessive ROS causes an oxidative cascade, mediated in part by ROS-induced activation of NF- κ B, STAT, and AP-1 transcription factors leading to oxidative injury to macromolecules in RPE cells, which ultimately contributes to the pathogenesis of AMD (6). Thus, inhibiting H₂O₂-induced RPE cell injury may be a therapeutic approach for the treatment of AMD.

Salidroside (SAL) is the major phenylpropanoid glycoside and pharmacological active constituent of *Rhodiola rosea*. Previous studies have been shown that SAL possesses a wide range of pharmacological functions, including anti-aging, anti-inflammatory, antioxidant, anti-cancer and neuroprotective activities (7-11). For example, Zhang *et al* (12) reported that pretreatment with SAL dose-dependently upregulated the production of antioxidant enzymes and inhibited the elevation of intracellular ROS level in Abeta-induced human neuroblastoma cells. However, the effects and mechanisms of SAL on oxidative stress in RPE cells exposed to hydrogen peroxide (H₂O₂) remain unclear. Therefore, the purpose of this study was to investigate its protective effects and the underlying mechanisms against H₂O₂-induced oxidative stress in human RPE cells. The results indicated that SAL protects RPE cells against H₂O₂-induced cell injury through the activation of Akt/GSK-3 β signaling pathway.

Materials and methods

Cell culture. The human RPE cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 (Life Technologies, Carlsbad, CA, USA) supplemented

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with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Lonza, Basel, Switzerland). The cells were cultured in a humidified incubator at 37°C and 5% CO₂.

Cell viability assay. Cell viability was evaluated using the MTT assay. In brief, ARPE-19 cells at a density of 1×10⁴ cells/well were incubated with or without SAL (12.5–100 µg/ml) for 24 h and then treated with 200 µM H₂O₂ for 6 h. Next, 50 µl of MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well, and the plate was incubated for 4 h at 37°C. Then the supernatant was removed and 100 µl DMSO (Sigma-Aldrich; Merck KGaA) was added to dissolve formazan. The absorbance was read at 490 nm using an enzyme linked immunosorbent assay plate reader (Olympus, Tokyo, Japan). The experiment was performed in triplicate.

Cell cytotoxicity assay. The cytotoxicity of treated ARPE-19 cells was evaluated via determining the activity of lactate dehydrogenase (LDH) enzyme released into medium with the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The experiment was performed in triplicate.

Cell apoptosis assay. After treatment, ARPE-19 cells were harvested by trypsinization. Then, the cells were centrifuged, washed twice with PBS and resuspended in 1X Binding Buffer. After adding 5 µl of Annexin V-FITC solution and 5 µl of PI solution according to the instructions of Annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, Nantong, China), cells were incubated in the dark for 30 min at room temperature. The experiment was performed in triplicate.

Detection of ROS. After treatment, ARPE-19 cells were loaded with 5 mM CellROX orange reagent for 30 min at 37°C. Then, the fluorescence intensity of CellRox Green in each well was measured using SpectraMax 5 (Molecular Devices, Downington, PA, USA) following manufacturer's instructions. The experiment was performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from ARPE-19 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized with the Prime Script RT reagent kit (Takara Bio Inc., Otsu, Japan). PCR amplification was carried out by ABI PRISM 7900 thermocycler using SYBR Premix Taq (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: for Bcl-2 forward, 5'-CAAAGGTGGATCAGATTC AAG-3' and reverse, 5'-GGTGAGCATTATCACCCAGAA-3'; for Bax forward, 5'-TGGCAGCAGTGACAGCAGCG-3' and reverse, 5'-TACGGAGGTGGAGTGGGTGT-3'; and for GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CAC CCTGTTGCTGTAGCCAAA-3'. The comparative threshold cycle method (ΔΔC_q) was used to determine the levels of gene expression (13). The experiment was performed in triplicate.

Western blot analysis. ARPE-19 cells were homogenized and lysed with RIPA lysis buffer (Beyotime Institute of

Biotechnology). Then, protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins (30 µg/lane) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to Immobilon P EMD Millipore (Billerica, MA, USA). After blocking with 5% non-fat milk in PBS with Tween-20 buffer at room temperature for 1 h, the blots were incubated for 60 min at room temperature with primary antibody against the following: Bcl-2, Bax, Akt, phosphorylated Akt, GSK-3β, phosphorylated GSK-3β or GAPDH (diluted 1:1,000 in TBST; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology) for 1 h at room temperature. Detection was performed using the ECL western blotting detection system (Thermo Fisher Scientific, Inc.). Each band density was quantified using Quantity One software (Bio-Rad, Richmond, CA, USA) and normalized to GAPDH. The experiment was performed in triplicate.

Statistical analysis. Statistical analysis was made using Prism (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard deviation. Significance was determined by one-way analysis of variance followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Pretreatment with SAL markedly attenuated H₂O₂-induced loss of cell viability. To study the effect of SAL on RPE cell viability, ARPE-19 cells were treated with increasing concentrations of SAL (12.5, 25, 50 or 100 µg/ml). As shown in Fig. 1A, 100 µg/ml of SAL significantly decreased cell viability. Cell viability of ARPE-19 cells was not significantly impaired by concentrations of SAL <100 µg/ml. Then, we detected the effect of various concentrations of H₂O₂ on RPE cell viability. The results showed that 200 µM H₂O₂ could significantly reduce cell viability. Since the effects of 200 and 300 µM H₂O₂ were not significantly different, 200 µM H₂O₂ was chosen for subsequent experiments (Fig. 1B). In addition, the effect of SAL on cell viability in H₂O₂-treated ARPE-19 cells was evaluated. The results of MTT assay demonstrated that the viability of RPE cells treated with 200 µM H₂O₂ significantly decreased compared with the untreated group. However, pretreatment with SAL obviously increased the viability of RPE cells in a dose-dependent manner (Fig. 1C). We further analyzed whether SAL pretreatment could influence H₂O₂-induced cellular cytotoxicity. As shown in Fig. 1D, the exposure to 200 µM H₂O₂ greatly increased LDH release. However, the LDH release gradually down to 274.3, 223.7, and 164.1% with increasing concentrations of SAL, respectively.

Pretreatment with SAL ameliorated H₂O₂-induced oxidative stress in RPE cells. It is known that oxidative stress plays a pivotal role in AMD pathogenesis. Thus, we examined the effect of SAL in on oxidative stress in H₂O₂-treated ARPE-19 cells. As shown in Fig. 2, as compared with the control group, the level of intracellular ROS was significantly increased in

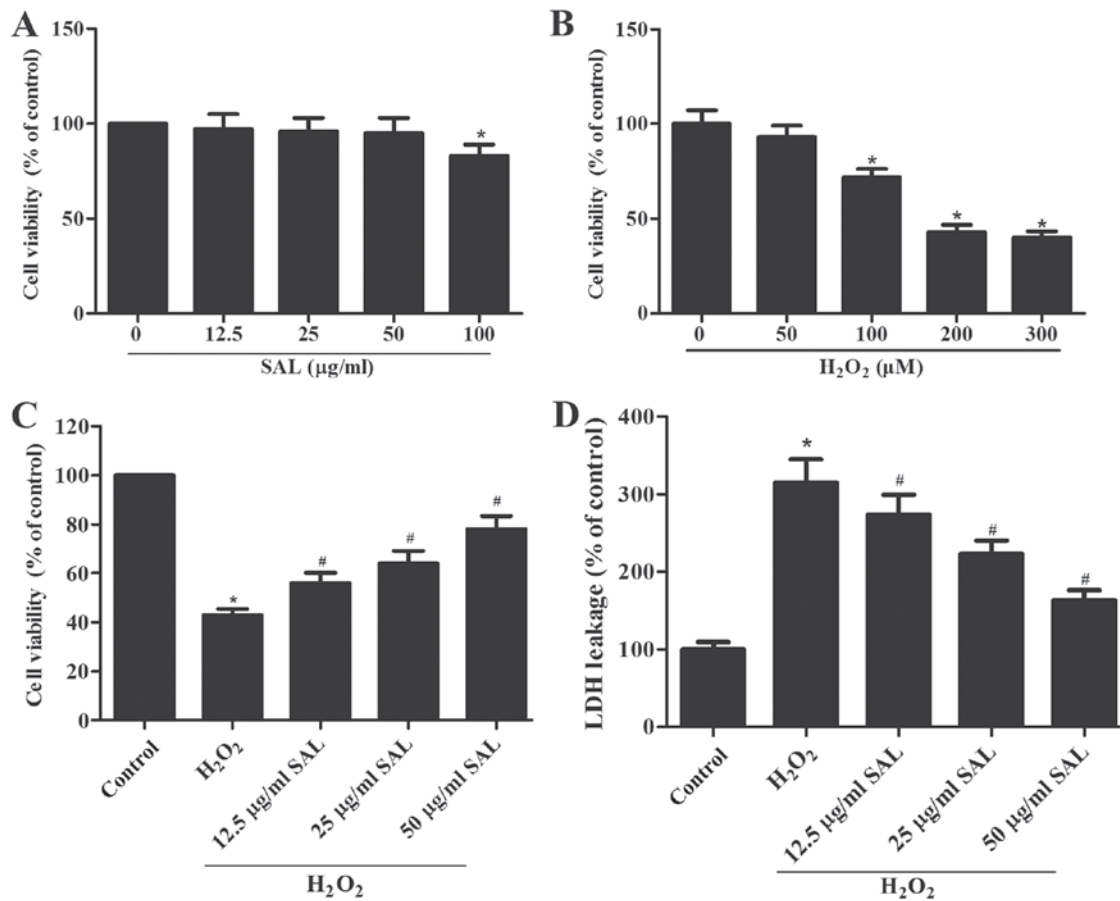


Figure 1. Pretreatment with SAL markedly attenuates H_2O_2 -induced loss of cell viability. (A) ARPE-19 cells were treated with various doses of SAL (0, 12.5, 25, 50 and 100 $\mu\text{g/ml}$) for 24 h and the cell viability was analyzed by an MTT assay. (B) ARPE-19 cells were incubated with various concentrations of H_2O_2 (0, 50, 100, 200 and 300 μM) for 24 h and cell viability was detected by an MTT assay. (C) ARPE-19 cells were incubated with various doses of SAL (0, 12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h and then exposed to 200 μM H_2O_2 for 24 h. Cell viability was analyzed by an MTT assay. (D) Cell cytotoxicity was analyzed by an LDH assay. Data are expressed as the mean \pm standard deviation from a minimum of three independent experiments. * $P < 0.05$ vs. the control group. # $P < 0.05$ vs. the H_2O_2 group. SAL, salidroside; H_2O_2 , hydrogen peroxide.

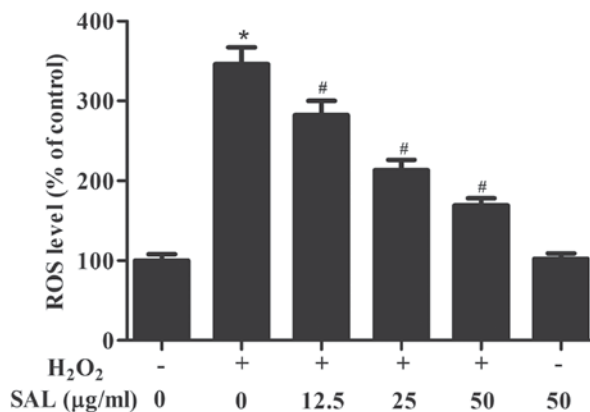


Figure 2. Pretreatment with SAL ameliorates H_2O_2 -induced oxidative stress in retinal pigment epithelial cells. ARPE-19 cells were incubated with various doses of SAL (0, 12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h and then exposed to 200 μM H_2O_2 for 24 h or only treated with 50 $\mu\text{g/ml}$ SAL for 24 h. The intracellular levels of ROS were measured using CellROX orange reagent. Data are expressed as the mean \pm standard deviation from a minimum of three independent experiments. * $P < 0.05$ vs. the control group. # $P < 0.05$ vs. the H_2O_2 group. SAL, salidroside; H_2O_2 , hydrogen peroxide; ROS, reactive oxygen species

H_2O_2 -treated ARPE-19 cells. However, pretreatment with SAL markedly reduced H_2O_2 -induced ROS level in ARPE-19 cells.

In addition, SAL alone treatment did not affect ROS level in ARPE-19 cells.

Pretreatment with SAL inhibited H_2O_2 -induced cell apoptosis in RPE cells. H_2O_2 has been shown to induce apoptosis in RPE cells. Thus, we examined the effect of SAL on ARPE-19 cell apoptosis induced by H_2O_2 . As shown in Fig. 3, the exposure to 200 μM H_2O_2 for 24 h would lead to a significant higher rate of apoptosis, compared with the control cells. Pretreatment with SAL dramatically reversed H_2O_2 -induced apoptosis. In addition, we observed that pretreatment with SAL significantly upregulated the expression of Bcl-2 and downregulated the expression of Bax, as compared with the H_2O_2 group (Fig. 3).

Pretreatment with SAL activated Akt/GSK-3 β signaling pathway in RPE cells. It has been reported that the activation of Akt/GSK-3 β signaling pathway plays an important role in the progression of AMD (14), we examined the effects of SAL on Akt/GSK-3 β signaling pathway in H_2O_2 -treated ARPE-19 cells. The results of western blot analysis indicated that the phosphorylation levels of Akt and GSK-3 β were not significantly activated in H_2O_2 -stimulated ARPE-19 cells. Pretreatment with SAL significantly increased the phosphorylation levels of Akt and GSK-3 β in H_2O_2 -treated ARPE-19

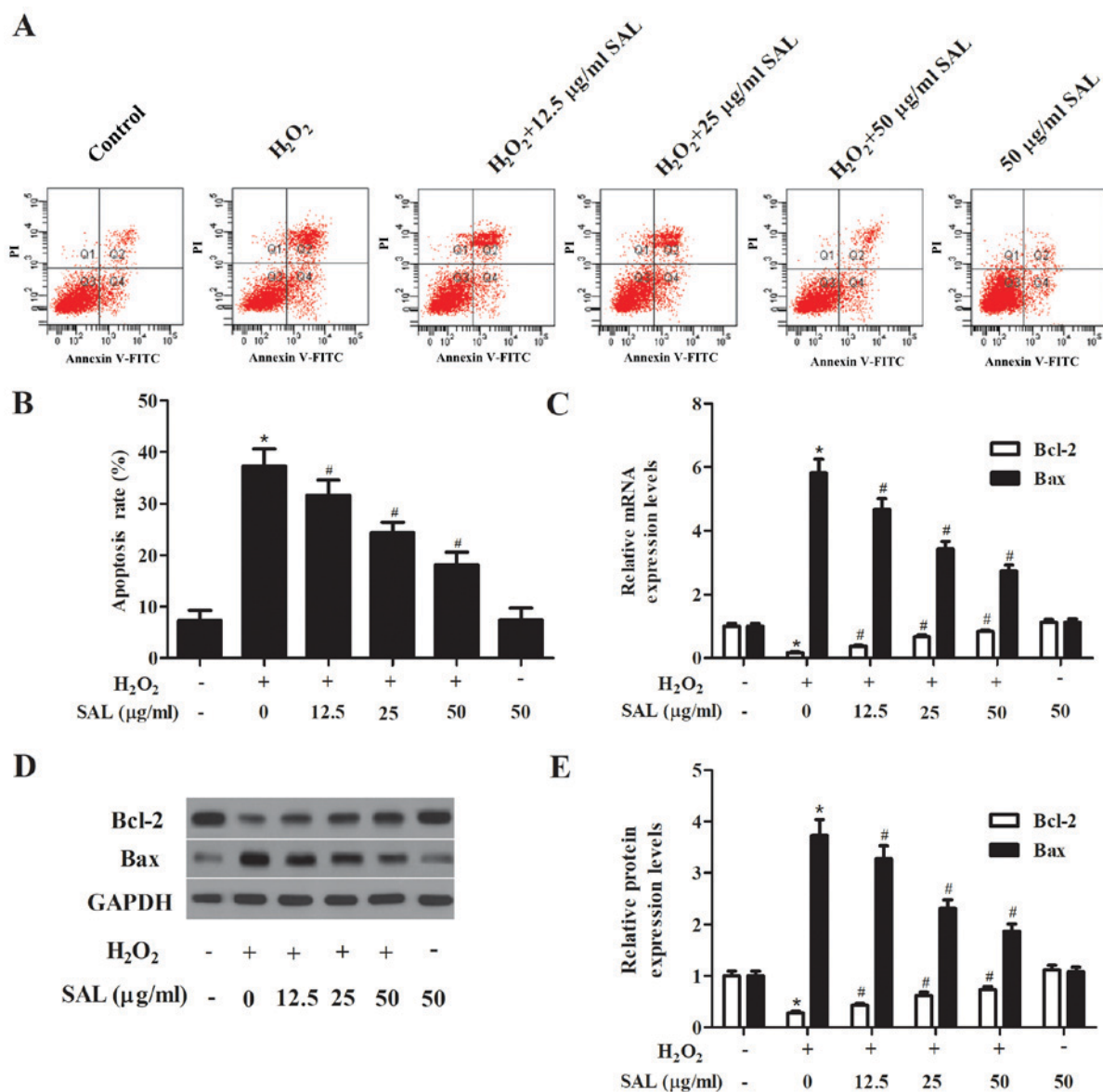


Figure 3. Pretreatment with SAL inhibits H₂O₂-induced cell apoptosis in retinal pigment epithelial cells. ARPE-19 cells were incubated with various doses of SAL (0, 12.5, 25 and 50 µg/ml) for 24 h and then exposed to 200 µM H₂O₂ for 24 h or only treated with 50 µg/ml SAL for 24 h. (A) Cell apoptosis was analyzed by Annexin V/PI double staining, followed by flow cytometry. (B) Quantification analysis of cell apoptosis. (C) The mRNA levels of Bcl-2 and Bax were evaluated using reverse transcription-quantitative polymerase chain reaction. (D) Protein expression of Bcl-2 and Bax were evaluated by western blot analysis. (E) Quantification analysis of Bcl-2 and Bax was performed using Quantity One software. Data are expressed as the mean ± standard deviation from a minimum of three independent experiments. *P<0.05 vs. the control group. #P<0.05 vs. the H₂O₂ group. SAL, salidroside; PI, propidium iodide; Bcl-2, B cell lymphoma-2; H₂O₂, hydrogen peroxide.

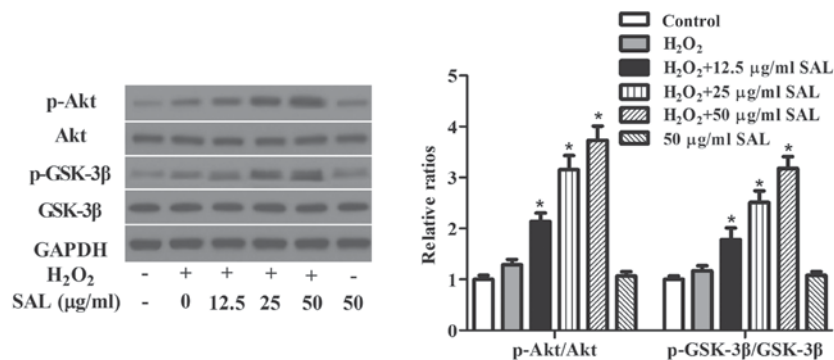


Figure 4. Pretreatment with SAL activates the Akt/GSK-3β signaling pathway in retinal pigment epithelial cells. ARPE-19 cells were incubated with various doses of SAL (0, 12.5, 25 and 50 µg/ml) for 24 h and then exposed to 200 µM H₂O₂ for 24 h or only treated with 50 µg/ml SAL for 24 h. The protein expression of p-Akt, Akt, p-GSK-3β and GSK-3β were evaluated by western blot analysis. Data are expressed as the mean ± standard deviation from a minimum of three independent experiments. *P<0.05 vs. the control group. p-, phosphorylated; Akt, protein kinase B; GSK, glycogen synthase kinase; SAL, salidroside; H₂O₂, hydrogen peroxide.

cells. Additionally, SAL alone treatment did not affect the activation of Akt/GSK-3 β pathway (Fig. 4).

Discussion

To our knowledge, we have shown for the first time that SAL markedly attenuated H₂O₂-induced loss of cell viability. SAL also ameliorated H₂O₂-induced oxidative stress and cell apoptosis in RPE cells. Furthermore, pretreatment with SAL significantly increased the phosphorylation levels of Akt and GSK-3 β in H₂O₂-treated ARPE-19 cells.

Previous studies reported that H₂O₂ can decrease RPE cell viability, which induce AMD progression (15-17). In line with these previous studies, our present study confirmed that H₂O₂ significantly decreased cell viability, as evidenced by the MTT assay. Meanwhile, we observed that pretreatment with SAL obviously increased the viability and reduced LDH release in H₂O₂-induced ARPE-19 cells in a dose dependent manner. These observations suggest that SAL protected human RPE cells from H₂O₂-induced oxidative stress through increasing the viability and reducing LDH release.

Numerous studies have demonstrated that oxidative stress is a major stimulus in the pathogenesis of AMD (18-20). SAL is a strong antioxidative supplement in Chinese traditional medicine. It was confirmed that SAL effectively attenuated the production of ROS in human umbilical vein endothelial cells (HUVECs) under conditions of oxidative injury induced by H₂O₂ (21). Another study reported that SAL effectively inhibited oxidative stress in cardiac H9c2 cells induced by H₂O₂ insult (22). In accordance with previous studies, in the present study, we observed that pretreatment with SAL markedly reduced H₂O₂-induced ROS level in ARPE-19 cells. These data suggest that SAL effectively protected human RPE cells from H₂O₂-induced oxidative stress via antioxidant effect.

Previous studies showed that oxidative stress induces mitochondrial dysfunction and promotes apoptosis correlating with increased Bax expression and decreased Bcl-2 expression in human RPE cells (23,24). Bcl-2 is a key anti-apoptotic member of the Bcl-2 family that regulates the intrinsic apoptosis pathway. In addition, it was reported that SAL increased the ratio of Bcl2/Bax in H₂O₂-induced retinal endothelial cells (25). Similarly, in the present study, we observed that pretreatment with SAL upregulated the expression of Bcl-2 and downregulated the expression of Bax in H₂O₂-treated ARPE-19 cells. These data suggest that SAL effectively protected human RPE cells from H₂O₂-induced oxidative stress via anti-apoptotic effect.

The Akt/GSK-3 β signaling pathway plays a crucial role in a variety of cellular processes such as cell proliferation and apoptosis (26-28). Akt activation enhances RPE cell survival and thus may protect RPE cells from oxidant-induced cell death in the pathogenesis of AMD (29). Previous studies demonstrated that adding H₂O₂ to RPE cells caused Akt activation (29,30). In the present study, we observed that Akt phosphorylation was moderately enhanced in the stimulation of H₂O₂. In addition, pretreatment with SAL significantly increased the phosphorylation levels of Akt and GSK-3 β in H₂O₂-treated ARPE-19 cells. These results suggest that SAL protected RPE cells against H₂O₂-induced cell injury through the activation of Akt/GSK-3 β signaling pathway.

In conclusion, this study demonstrated that SAL could stimulate the recovery of RPE cells under oxidative stress through the activation of Akt/GSK-3 β signaling. These data suggest that SAL may be a potential therapeutic strategy for the prevention and therapy of AMD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DT conceived and designed the experiments. YY and DL performed the experiments. DL analyzed the data. All authors have 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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