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ORIGINAL ARTICLE

SS-31 protect retinal pigment epithelial cells from H_2O_2-induced cell injury by reducing apoptosis

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Abstract

Evidence has shown that effects from oxidative stress induced damage of retinal or human retinal pigment epithelial (RPE) cells. Antioxidant supplementation is a plausible strategy to avoid oxidative stress and maintain the function of retina. d-Arg-2,6-dimethyltyrosine-Lys-Phe-NH2 (SS-31) has been used in the treatment of many diseases. In this study, we found that SS-31 attenuated hydrogen peroxide (H_2O_2)-induced loss of cell viability, reduced oxidative damage and cell apoptosis in RPE cells. HO-1, Trx-1 and Nrf-2 expression levels significantly increased on pre-treatment with SS-31 compared with the H_2O_2 group. SS-31 inhibited apoptosis through the down-regulation of Bax and the upregulation of Bcl-2. Our results suggest that SS-31 had a protective effect against H_2O_2 treatment in ARPE-19 cells by enhancing the anti-oxidative enzymes expression and decreasing apoptosis, which could be considered a promising therapeutic intervention for retinal degeneration.

KEYWORDS
Apoptosis, oxidative stress, retinal pigment epithelial cells, SS-31

1 | INTRODUCTION

Age-related macular degeneration (AMD) is a complex retinal degeneration disease associated with retinal pigment epithelium (RPE) cell dysfunction or degeneration.[1] Late-stage AMD has a substantial influence on vision, which combined with choroidal neovascularization (CNV) under the retina and results in acute or severe vision loss.[2,3]

Retinal pigment epithelium cells located between retina photoreceptors and the choriocapillaris/Bruch's membrane complex supply the retina with essential cellular maintenance for photoreceptor nutrient transport.[4-6] Evidence has shown that effects from oxidative stress induced damage in the RPE/retina.[7] Damage by oxidative stress causes DNA cleavage and lipid peroxidation, resulting in irreversible damage to cells.[8,9]

ARPE-19 cells are highly vulnerable to oxidative stress. Oxidative damage induced ARPE-19 cell death and chronic inflammation and is considered as a pathological cause in the progression of AMD.[10-12] In this study, we use hydrogen peroxide (H_2O_2) as oxidative stress inducer to learn the possible antioxidant stress mechanism in ARPE-19 cells.

Antioxidant supplementation is a plausible strategy to avoid oxidative stress and maintain the function of the retina.[13] To date, however, there has been no effective approach for suppressing oxidative stress-induced RPE cell injury without undesirable side effects.

d-Arg-2,6-dimethyltyrosine-Lys-Phe-NH2 (SS-31) is a kind of cell-permeable mitochondria-targeting antioxidant peptide that could reduce oxidative stress, inhibit reactive oxygen species (ROS) regeneration and mitochondrial depolarization and protect multiple cell types against various external insults.[14,15] In this study, we studied the role of SS-31 in protecting RPE cells from the oxidative insults and explored the mechanisms underlying the antioxidant effect of SS-31.

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2 | RESULTS

2.1 | SS-31 inhibited the cytotoxicity of ARPE-19 cells induced by H_2O_2

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test results showed that because SS-31 is a cell-permeable mitochondria-targeting antioxidant peptide, it is safe for use in ARPE-19 cells. H_2O_2 impaired cell viability, and exposure to 300 μM H_2O_2 induced an approximate 50% cell viability loss (Figure 1A). Therefore, 300 μM H_2O_2 was selected for subsequent experiments. We then selected 0.01 μM, 0.1 μM, 1 μM and 10 μM of SS-31 to test the safety of drug in ARPE-19 cells, and the results showed that it is safe for use in cells (Figure 1B). SS-31 pre-treatment of ARPE-19 cells showed dose-dependent protective effects against H_2O_2 damage. When cells were treated with 0.01 μM SS-31, the cell viability was 55.50 ± 3.10, and the difference compared with cells treated with 300 μM H_2O_2 (50.50 ± 0.87) was not statistically significant. When cells were treated with 0.1 μM SS-31, the cell viability was 60.97 ± 3.97. The difference between this value and that of cells treated with 300 μM H_2O_2 without SS-31 pre-treatment was statistically significant (Figure 1C). Therefore, 0.1-10 μM SS-31 was used in the following experiments. We also detected the morphology of ARPE-19 cells, cells pre-treated with SS-31 were similar to that of the normal controls without H_2O_2, cells morphology were spindle-shaped and uniform, after treatment with H_2O_2, cells density decreased and their shape became smaller. As the concentration of SS-31 increased, changes in cells density and morphology were less pronounced (Figure 1D).

2.2 | SS-31 protected H_2O_2-induced apoptosis in ARPE-19 cells

In Figure 2, H_2O_2-treated group showed an increase in apoptotic cells, whereas SS-31 pre-treatment decreased the cell apoptosis rate in a dose-dependent manner (Figure 2A,B). Hoechst-propidium iodide (PI) staining also confirmed the inhibitory effect of SS-31 on ARPE-19 cell apoptosis.

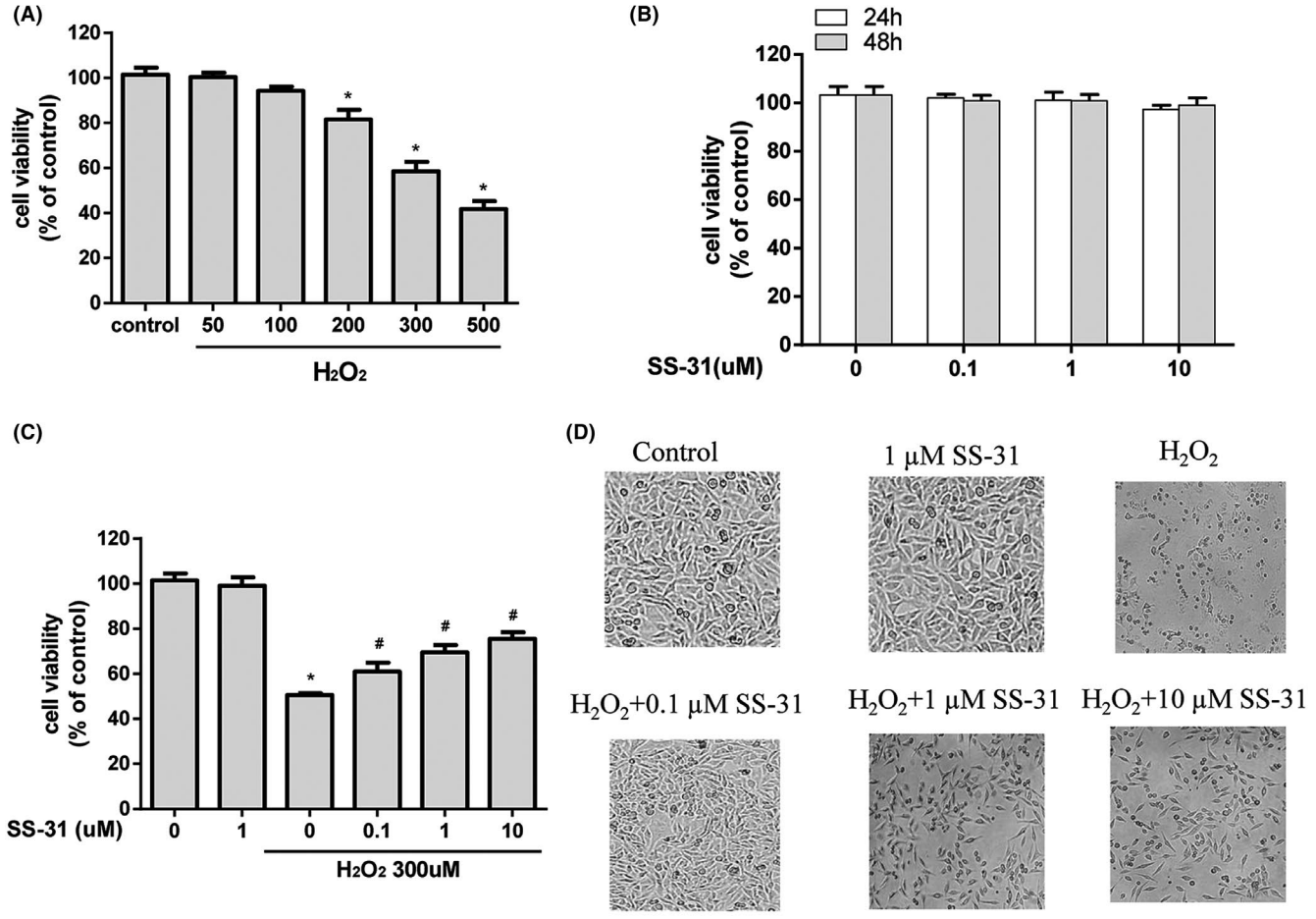

FIGURE 1 SS-31 protects ARPE-19 cells from H_2O_2 damage. A, MTT assay for the survival rate of ARPE-19 cells in vitro after H_2O_2 exposure for 24 hours. B, MTT assay for the survival rate of ARPE-19 cells after SS-31 (0, 0.1, 1, 10 μM) pre-treatment for 24 hours and 48 hours. C, Cells were pre-treated with SS-31 (0-10 μM) for 24 hours and then exposed to H_2O_2 (300 μM) for 24 hours. *P < .05 vs control; #P < .05 vs H_2O_2 group. D, Morphological observation of ARPE-19 cells, 24 hours; 48 hours

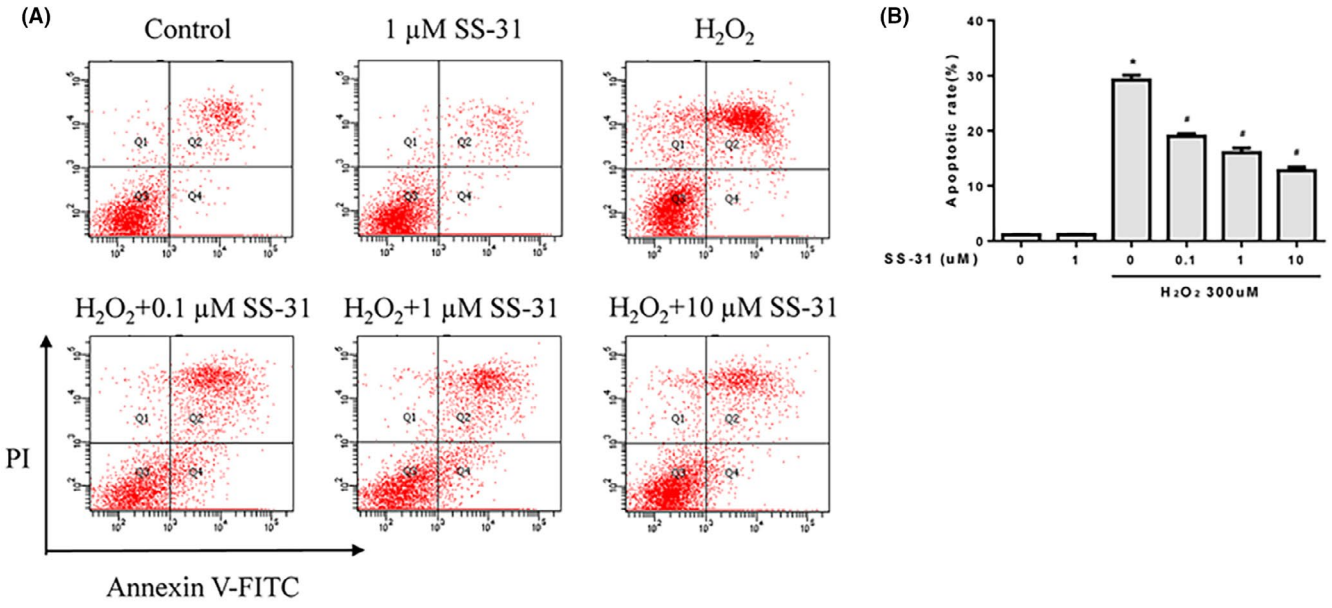

FIGURE 2 SS31 inhibited H_2O_2-induced apoptosis in ARPE-19 cells. ARPE-19 cells were incubated with SS-31 (0-10 μM) for 24 hours and then exposed to H_2O_2 (300 μM) for 24 hours. A, Flow cytometric analysis was used to detect apoptosis rate. B, Quantitative analyses of the apoptosis rate in ARPE-19 cells (n = 3). $^{*}P < .05$ vs control; $^{\#}P < .05$ vs H_2O_2 group

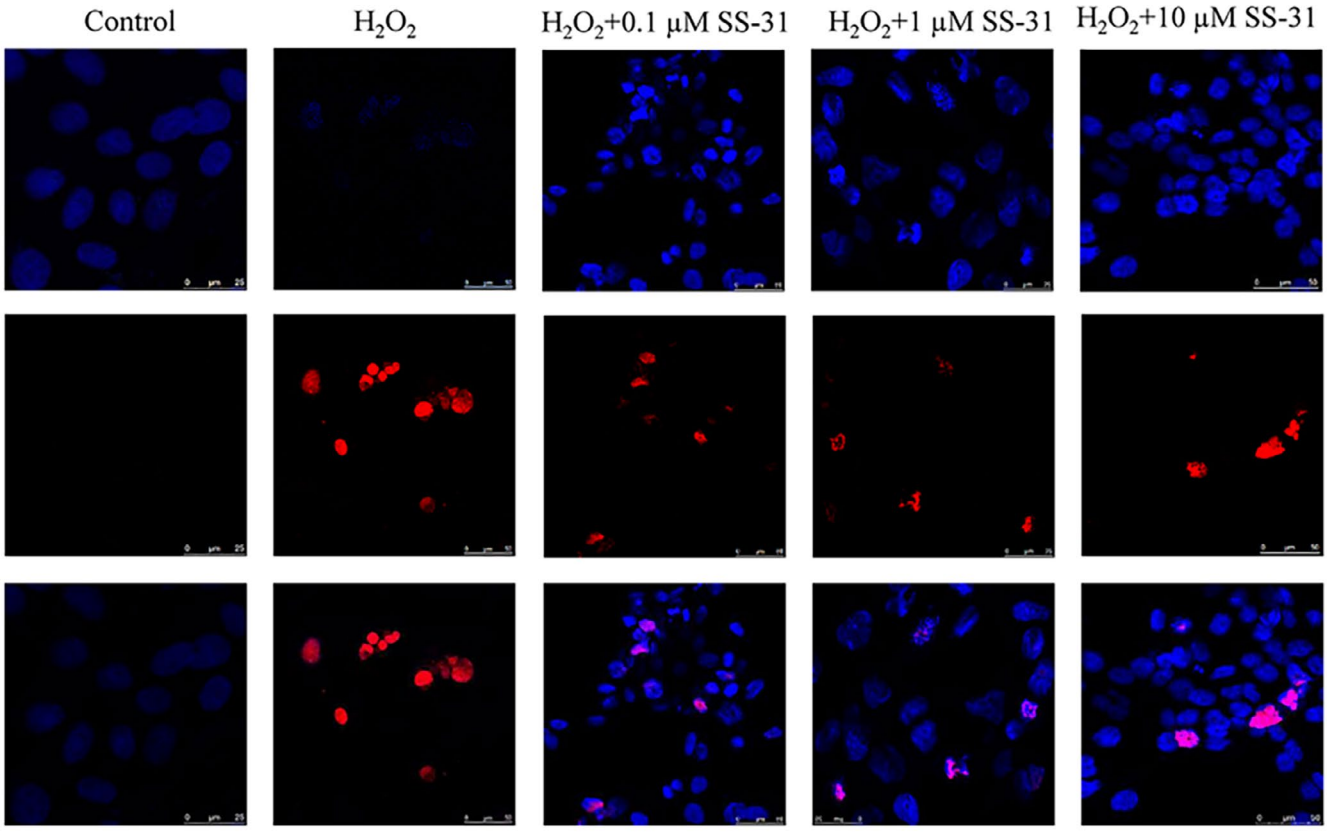

FIGURE 3 Representative Hoechst-PI images of ARPE-19 cells. ARPE-19 cells were pre-treated either with or without SS-31 (0-10 μM) for 24 hours and then exposed to H_2O_2 (300 μM) for 24 hours. The blue colour indicates nuclei stained with Hoechst, and the red colour indicates PI-stained cells, representing apoptotic and necrotic cells

ARPE-19 cells stained with Hoechst had a blue colour and were present during early apoptosis. Cells stained with PI had a red colour and were present during late apoptosis or necrosis. Our results showed that the percentage of cells positive for Hoechst and PI was decreased after cells were treated with SS-31 (Figure 3).

2.3 | SS-31 reduced ROS levels

The results showed that DCF fluorescence intensity (light green colour) in ARPE-19 cells increased significantly after treatment with 300 μM H_2O_2. However, cells pre-treated with SS-31 (0.1 μM, 1 μM and 10 μM) showed markedly reduced DCF f

luorescence intensity (Figure 4).

2.4 | Wound healing

We evaluated the effects of SS-31 on wound healing in cultured ARPE-19 cells to determine the proliferative and migratory activities of cells (Figure 5A). Cells in the control group and cells exposed to 1 and 10 μM SS-31 after exposure to 300 μM H_2O_2 showed an increase in cell migration up to 60% in scratch-wound healing assay. In contrast, cells exposed to 0.1 μM SS-31 after exposure to 300 μM H_2O_2 for 24 hours showed less complete healing patterns, and no increase was found after 24 hours of exposure (Figure 5B).

2.5 | SS-31 protected ARPE-19 cells by reducing apoptosis

Western blotting was used to detect the expression of Bax and Bcl-2. The results showed that the level of Bax in H_2O_2 group was upregulated and Bcl-2 was downregulated, whereas, SS-31 treatment significantly reduced Bax expression and increased Bcl-2 expression (Figure 6).

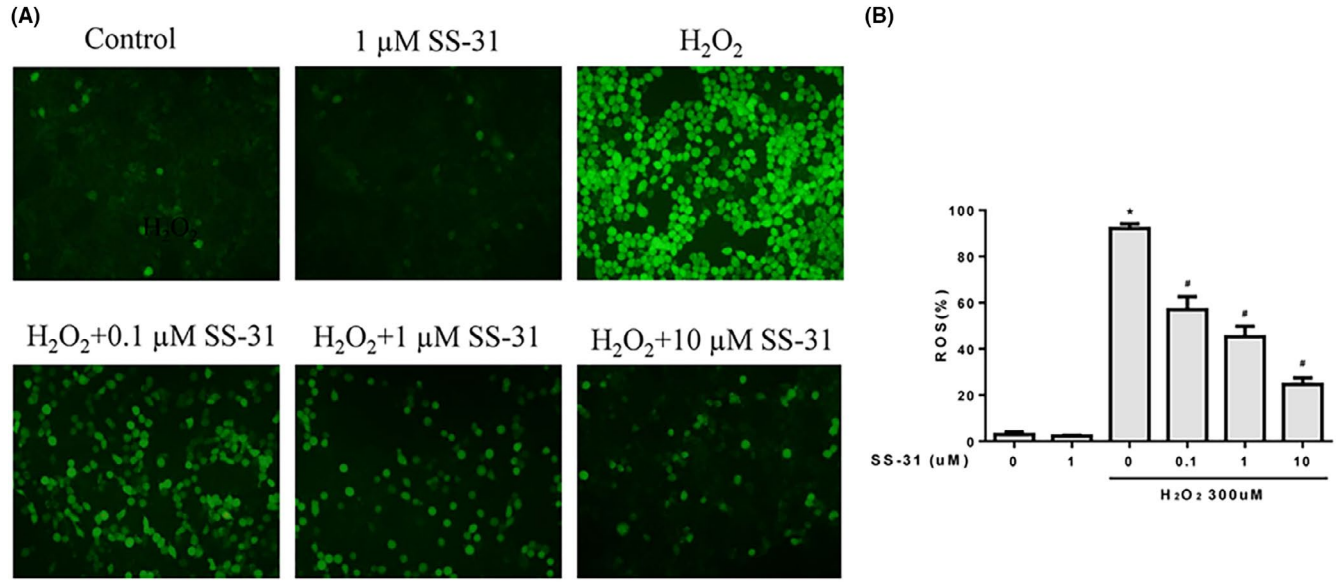

FIGURE 4 Effects of SS-31 on H_2O_2-induced ROS generation in ARPE-19 cells. Cells were pre-treated with SS-31 and then stimulated either with or without H_2O_2 (300 μM) for 24 hours. ROS generation was detected using fluorescence microscope. Magnification, 100×

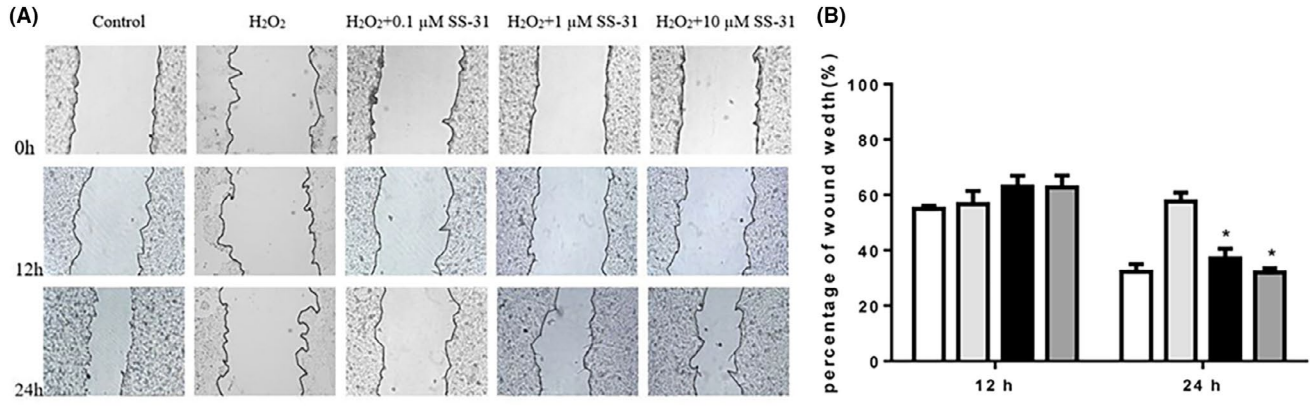

FIGURE 5 ARPE-19 cells were incubated with SS-31 (0-10 μM) for 12 hours and 24 hours and then exposed to H_2O_2 (300 μM) for 24 hours. A, The proliferation and migration ability was determined using a wound-healing assay. B, Quantitative analyses of the proliferative and migratory activities of cells. *P < .05 vs H_2O_2 group control; H_2O_2; H_2O_2 + 0.1 μM SS-31; H_2O_2 + 1 μM SS-31; H_2O_2 + 10 μM SS-31. □ Control; ▨ H_2O_2; ■ H_2O_2 + 0.1 μM SS-31; ▩ H_2O_2 + 1 μM SS-31; ▨ H_2O_2 + 10 μM SS-31

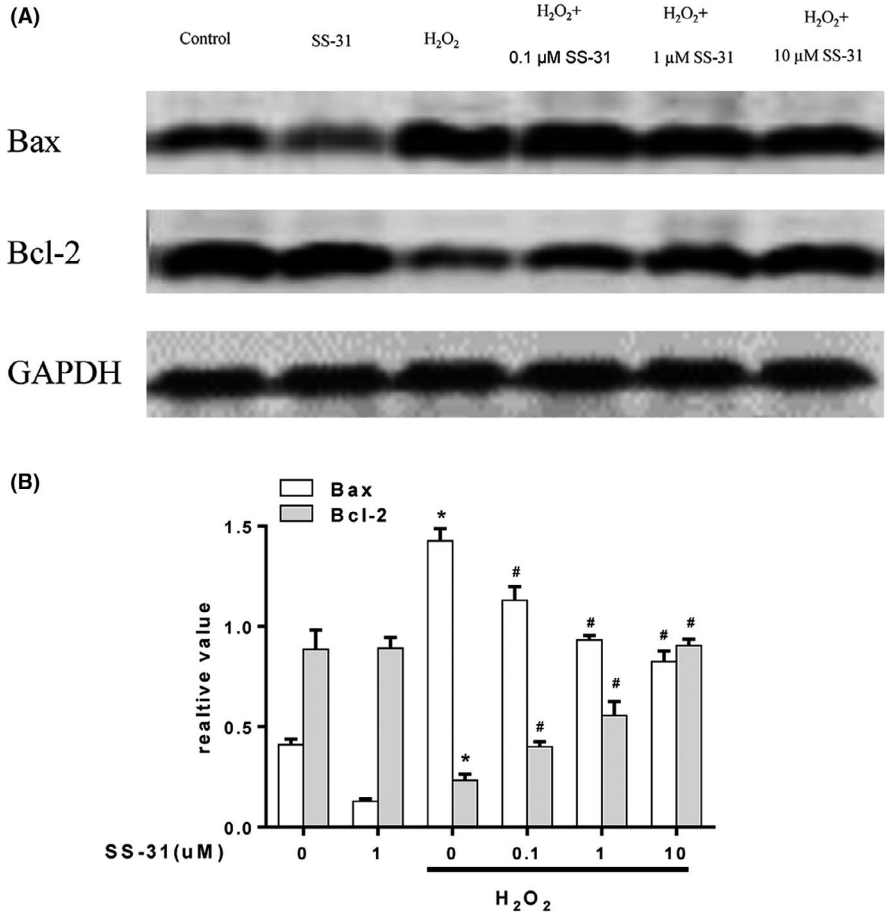

FIGURE 6 Western blot analysis of apoptotic proteins of ARPE-19 cells. A, Bax and Bcl-2 protein levels were examined by western blot. B, Statistical analysis of western blot data (n = 3). *P < .05 vs control; #P < .05 vs H_2O_2 group Bax; Bcl-2

3 | DISCUSSION

The mechanisms of AMD remain unclear, and environmental and genetic factors (e.g., continual exposure to light, smoking, inflammation, apoptosis and oxidative damage) all play important roles in AMD and significantly contribute to AMD pathogenesis.[16] Among these factors, oxidative damage was recognized as the key factor in AMD.[17]

Retinal tissue consists of a unique fatty acid component and has the highest oxidative consumption. In addition, because of its frequent exposure to light and its high-fat content, it is particularly vulnerable to oxidative stress.[18] The primary site of AMD pathology is found in ARPE-19 cells, and the normal construction and function of ARPE-19 cells play crucial roles in retinal functions. Oxidative stress-induced ARPE-19 cell dysfunction and the loss of normal physiological function in aging cells could result in central visual loss.[19] The development of an effective therapeutic to avoid oxidative stress and maintain the function of ARPE-19 cells is a particularly important task in slowing the progression of AMD.

H_2O_2, one of the most important species in ROS, is regarded as a non-radical member of the active oxygen family and can directly cause oxidative injury to cells. Exposure to H_2O_2 is used to evaluate oxidative damage susceptibility and antioxidant activity of RPE cells. Many studies have found that ROS generated by H_2O_2 leads to epithelial cell damage and protein degradation, and the damage resembles that found in AMD. Previous studies showed that ARPE-19 cells treated with H_2O_2 stimulate ROS formation and cell death. Because the H_2O_2-induced retina model involves oxidative stress, it can be used to test whether antioxidants delay the development of dry AMD.[20]

The effective approach for slowing the progression of AMD involves antioxidant supplements, and drugs capable of inhibiting the level of ROS in the retina are considered to be effective and the main treatment for oxidative damage of the retina.[21] SS-31 has several advantages, including the ability to inhibit reperfusion injury and mitochondrial swelling, protection against mitochondrial depolarization and substantial scavenging of ROS. [22-24]

The antioxidative effect of SS-31 in the eye has been reported in several studies. Chen and colleagues have shown that SS-31 can protect human trabecular meshwork cell lines induced by oxidative stress by inhibiting caspase-3 activation.[25] Li and colleagues tested the effect of SS-31 in human retinal endothelial cells and found that SS-31 could reduce the damage induced by high glucose by decreasing ROS production, decreasing caspase-3 expression and increasing Trx-2 expression.[26]

Oxidation after H_2O_2 exposure is an early event preceding apoptosis, it can be activated by excessive ROS levels, viral infections, UV radiation or DNA damage. [27,28] During early stage AMD, ARPE-19

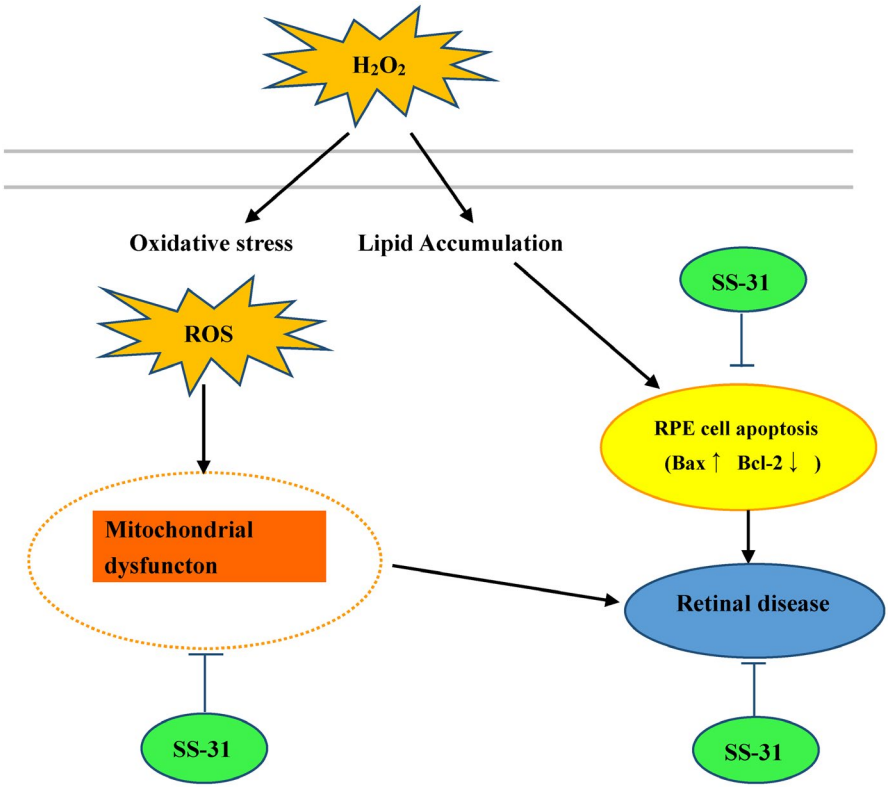

FIGURE 7 Summary of the effects of SS-31 on H_2O_2-induced oxidative damage in RPE cells. SS-31 increased mitochondrial membrane potential, inhibited H_2O_2-induced RPE cell damage and decreased the apoptosis rate in ARPE-19 cells

cells gradually lost normal function and died of apoptosis. As shown in our results, we conclude that SS-31 inhibits apoptosis through the downregulation of Bax and the upregulation of Bcl-2 in H_2O_2-treated ARPE-19 cells (Figure 7). This effect may occur through activation of the PI3 K signalling pathway.

We concluded that H_2O_2 could induce ARPE-19 cell apoptosis, pre-treatment with SS-31 induced a substantial protection against H_2O_2-induced oxidative damage in ARPE-19 cells, and this protection likely occurs through a reduction in apoptosis. We established that SS-31 had a protective effect against H_2O_2 treatment in ARPE-19 cells by decreasing apoptosis and enhancing antioxidative enzyme expression in vitro. This study provided evidence that SS-31 may be a potential drug for the treatment of retinal degenerative disorders, such as AMD.

4 | MATERIALS AND METHODS

4.1 | Materials and reagents

ARPE-19 cells were obtained from Shanghai Institute of Chinese Academy Cell Biology, SS-31 and fetal bovine serum were obtained from Invitrogen, MTT was purchased from Solarbio, annexin V–fluorescein isothiocyanate (FITC)/PI were obtained from BD Biosciences, Hoechst-PI was obtained from Beyotime Institute of Biotechnology, 2',7'-dichlorofluorescein diacetate (H2DCFDA) was purchased from Invitrogen, anti-Bax and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology.

4.2 | Cell culture and treatment

ARPE-19 cells were routinely maintained in DMEM (10% fetal bovine serum and 1% penicillin-streptomycin solution) in a standard incubator. Cells were divided into a control group, a 300 μM H_2O_2 (oxidative stress) group and a 300 μM H_2O_2 co-treated with SS-31 (0.1 μM, 1 μM or 10 μM) group.

4.3 | Cell viability assay

Cells (1×10^5 cells/well) were incubated in 96-well microplates for 24 hours, then treated with H_2O_2 (0 μM, 50 μM, 100 μM, 200 μM, 300 μM and 500 μM) for another 24 hours. MTT assay was used to evaluate cell viability. Cell viability was also evaluated with different concentrations of SS-31 (0.1 μM, 1 μM or 10 μM) for 24 hours. To study the protective effect of SS-31 on the toxicity induced by H_2O_2, ARPE-19 cells were pre-treated with different concentrations of SS-31 for 24 hours and then exposed to H_2O_2 (300 μM) for 24 hours. Cells were incubated for 4 hours with 10 μL of MTT (5 mg/mL), and the absorption was evaluated by a microplate reader (SPECTROstar Omega, BMG LabTech GmbH) at 490 nm wavelength.

4.4 | Cell apoptosis detected by FITC/PI staining

Cells were incubated with SS-31 (0 μM, 0.1 μM, 1 μM, 10 μM) for 24 hours and then treated with H_2O_2 (300 μM) for 24 hours. Cells

were collected and suspended in 400 μL binding buffer (containing 5 μL FITC and 5 μL PI) in the dark for 20 minutes. Cell apoptosis percentage was recorded and analysed by flow cytometry.

4.5 | Hoechst-PI staining

Apoptosis and necrosis were detected with a Hoechst-PI apoptosis detection kit. Cells were treated as described before and then incubated with Hoechst 33258 (10 μg/mL) and PI (2.5 μg/mL) in the dark for 20 minutes. Images were acquired using an Operetta High-Content Imaging System (Olympus, Tokyo, Japan).

4.6 | Intracellular ROS measurement

Cells were incubated with 15 μM fluorescent probe H_2DCFDA for 30 minutes in the dark at 37°C, resuspended in PBS and analysed through flow cytometry and fluorescence microscope (Olympus, Tokyo, Japan).

4.7 | Wound healing assay

ARPE-19 cells were cultured at a density of 1×10^5 cells/well in 6-well plates and plated under serum starvation for 12 hours. The wound gap was simulated by scratching a 10 μL pipette tip down the centre of each well. Images of the same location for each scratch were acquired at 0, 12 and 24 hours using a common microscope, and the width of the wound was obtained using ImageJ software.

4.8 | Western blotting assay

Cells were lysed, and protein (30 mg) was loaded onto a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membrane blocked with 5% milk for 1 hour at room temperature. Mouse anti-Bax polyclonal antibody (1:100, sc-7480) and mouse anti-Bcl-2 polyclonal antibody (1:100, sc-71022) were used as primary antibodies, and goat anti-mouse antibodies (1:10,000, Zhongshan Golden Bridge, Guang Zhou, China) were used as secondary antibodies for 2 hours at room temperature. Blots were developed using the enhanced chemiluminescence (ECL) detection system, and band intensities were detected and exposed to X-ray film (Marsh Bio Products, Rochester, NY). Protein bands were quantified by GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

4.9 | Statistical analysis

All the data are presented as the mean ± SEM from three independent tests. One-way ANOVA or two-tailed Student *t* tests was used for statistical analysis using GraphPad Prism 5 software (GraphPad Software, USA).

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interests.

DATA AVAILABILITY STATEMENT

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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