Mitochondrial-Targeted Antioxidant Peptide SS31 Prevents RPE Cell Death under Oxidative Stress

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This work aims at investigating the protective effects of the mitochondria-targeted peptide SS31, on mitochondria function, preventing human retinal pigment epithelial cell-19 (ARPE-19) cell apoptosis. The ARPE-19 cells were subjected to 24 h of intervention with H2O2 of various concentrations (0, 100, 150, 200, 250, 300, and 500 μmol/L). Various concentrations of SS31 (10 nM, 100 nM, and 1 μmol/L) pretreated the cells for 2 h. The MTT assay determined cell viability. ARPE-19 cell apoptosis was observed by 4′,6-diamidino-2-phenylindole (DAPI) staining under fluorescence microscope and detected by Annexin-V/PI staining under flow cytometry. The measurement of reactive oxygen species (ROS) release level used MitoSOX Red (a mitochondrial superoxide indicator) and the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). And with the use of a JC-1 probe, the mitochondrial membrane potential (MMP; ΔΨm) was analyzed. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR were responsible for measuring the levels of apoptosis related genes (Bcl-2, Bax, and Caspase-3).

The cell viability increased significantly with SS31 pretreated (P<0.05). In the SS31 + H2O2 group, the fluorescence of the cell nuclei with DAPI staining was weaker than H2O2 alone group accordance with the decreased ratio of apoptotic cells (P<0.05). The ROS generation decreased in SS31 pretreated group, with the increased ΔΨm. The RT-PCR result showed decreased Bax gene and Caspase-3 gene expression with SS31 pretreatment, while increased antiapoptotic gene Bcl-2 (P<0.05). We provide evidence that SS31 promotes resilience of RPE cells to oxidative stress by stabilizing mitochondrial function.

1. Introduction

Age-related macular degeneration (AMD) bears the major responsibility for progressive and irreversible central vision loss among the aged [1]. The most common characteristics of AMD are the formation of drusen and the alterations of RPE. The etiology of AMD is very complex, and the pathogenesis is having not been fully defined. Current research suggests a close connection between the oxidative damage of RPE cells and the pathogenesis of AMD, and the important cause of AMD is the dysfunction and metabolic abnormalities of RPE cells caused by oxidative damage of mitochondria [2]. Reactive oxygen species (ROS) are the main factor causing oxidative stress of RPE cells in retinal tissue. ARPE-19 is a spontaneously formed retinal pigment epithelial cell line. It is one of the cell models and tools commonly used by scientists that study vision. At present, it is mainly used to study the occurrence of ADM, the mechanism of disease or drug molecules with therapeutic activity, and to decipher cellular signal transduction events.

In addition to the traditional treatments, there are currently some studies on the treatment of AMD with antioxidants [3]. SS31 is a mitochondria-targeted peptide synthesized by Peter W. Schiller and Hazel H. Szeto with 639.8 Da [4]. The polypeptide contains an alternating aromatic cation motif so that it can freely penetrate the cell membrane and be selectively concentrated on the inner membrane of the mitochondria without saturation. By targeting the mitochondria, it can clear ROS and inhibit lipid peroxidation. SS31 can reduce ROS generation both under physiological and pathological conditions. In addition, SS31 plays a variety of biological effects, such as inhibiting...
the transition of mitochondria membrane potential and mitochondria swelling, thereby preventing the release of CytC induced by Ca\textsuperscript{2+}, etc., which can effectively reduce the cell apoptosis [5, 6].

The critical protective role of SS31 in some neurodegenerative diseases, such as ischemic brain injury, ischemiareperfusion injury, and other diseases, has been repeatedly confirmed [7–9]. Researcher reported that SS31 is essential in resisting hippocampal neuron withering by downregulating Bax gene and protein expression and upregulating Bcl-2 gene expression in the Alzheimer’s disease model [10–12]. SS31 also promotes electron transport and ATP synthesis in the mitochondrial inner membrane [9, 13, 14]. Preliminary studies in the treatment of diseases in ophthalmology have shown that SS31 can protect human lens epithelial cells by reducing oxidative damage to mitochondria [15] and 661 W mouse retinal photoreceptor cell lines [16]. In recent years, SS31 and other mitochondrial targeted peptides as a new type of antioxidant protective agent show a potent role on the neurodegenerative diseases [17, 18].

In this study, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was used on ARPE-19 cells to simulate the pathological process of AMD. We detected the protective effects of mitochondrial-targeted peptide SS31 on ARPE-19 cells under oxidative damage through cell viability and morphology observation, apoptosis, and the mitochondrial function detection. We speculated the possible new strategies for preventing and treating AMD from the mitochondrial-targeted antioxidant pathway.

2. Methods

2.1. Cells and Cell Cultivation. ARPE-19 cells, supplied by the American Type Culture Collection (ATCC, Rockville, MD), were cultivated in the medium and conditions as follows: medium: Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY) + 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY); culture conditions: 37°C, 5% CO\textsubscript{2}, and humidified atmosphere [19]. Cells were processed for the following assays when 75-80% confluence was observed.

2.2. Cell Viability. To detect ARPE-19 cell viability under oxidative stress (OS), cells were seeded into the wells of 96-well plates and treated with hydrogen peroxide (0, 100, 150, 200, 250, 300, and 500 µmol/L), with or without 2 h pretreatment of SS31 (10 nM, 100 nM, and 1 µM) for 24 h, followed by cultivation with DMEM (serum-free). Following MTT solution removal 4 h later, dimethyl sulfoxide (0.1 ml/well) was added to the plates, which were then shaken at ambient temperature for 10 min. A microplate spectrophotometer was utilized to read the absorbance at 490 nm of the supernatant.

2.3. Cellular Morphology. After planting ARPE-19 cells into the wells of 6-well plates, they were treated with H\textsubscript{2}O\textsubscript{2} (0, 100, 200, or 400 µmol/L) alone or with 2 h pretreatment with SS31 (10 or 100 nM, or 1 µM) for 24 h. Then, observation of cellular morphology was performed with an inverted phase-contrast microscope (Leica, Frankfurt, Germany).

2.4. Determination of Cell Apoptosis Using 4′,6-Diamidino-2-phenylindole (DAPI Staining). Nuclei morphology and cell apoptosis were determined by DAPI staining. After seeding ARPE-19 cells into the wells of 6-well plates, they were intervened by 200 µmol/L of H\textsubscript{2}O\textsubscript{2} at 37°C for 24 h, with or without 2 h of pretreatment with 1 µmol/L SS31. They were then treated with two phosphate-buffered saline (PBS) rinsing and the subsequent 10 min of light tight cultivation with DAPI (5 µg/ml). Afterward, PBS (Gibco, Grand Island, NY) was used to rinse each sample three times. Cell apoptosis was indicated the nucleus was fragmented or condensed. Observation of nuclei morphology and apoptosis alterations was performed with a fluorescence microscope manufactured by Leica, Frankfurt, Germany.

2.5. Determination of Cell Apoptosis Using Flow Cytometry (FCM). The existence of apoptotic cells was evaluated by early changes of cell-related membrane phospholipid asymmetry in the early stage of apoptosis. Membrane cell phospholipid asymmetry loss is accompanied by exposure to phosphatidylserine. An Annexin-V (AV)/propidium iodide (PI) kit from KeyGEN BioTECH, Jiangsu, China was used for the determination of apoptosis. The ARPE-19 cells were treated with 200 µmol/L for 24 h, alone or with the pretreatment of 1 µmol/L SS31 for 2 h. 1 × 10\textsuperscript{6} cells/ml cells were then gathered for two PBS washes. Five hundred microliters of Binding Buffer was used to resuspend cells, after which 5 µL each of AV and PI was placed into the cell suspension. Then came 15 min of cell incubation in the dark at ambient temperature. Finally, the samples were evaluated using FCM (BD Accuri, Franklin Lakes, New Jersey).

2.6. ROS Detection. In ARPE-19 cells, the ROS was evaluated by observation under fluorescence microscope using MitoSOX Red (KeyGEN BioTECH, Nanjing, Jiangsu, China) and FCM using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) probe. After seeding the cells into 6-well plates, they were intervened by H\textsubscript{2}O\textsubscript{2} (200 µmol/L) for 4 h at 37°C, with or without the pretreatment of 1 µmol of LSS31 for 2 h. First, the ARPE-19 cells were rinsed once with PBS.7. Second, 1 µL of MitoSOX Red was dissolved in 999 µL of Hank’s Balanced Salt Solution (HBSS) to make a concentration MitoSOX Red solution in every well. After shaking the plate lightly, the ARPE-19 cells were subjected to light tight incubation (37°C, 10 min). After light rinsing with warm buffer, they were observed by fluorescence microscope. Meanwhile, intracellular ROS in ARPE-19 cells was evaluated with DCFH-DA probe (KeyGEN BioTECH, Nanjing, Jiangsu, China). Cells were collected and suspended in DMEM containing 10 µmol/L DCFH-DA, cultivated at 37°C for 20 min in dark, and mixed every 3-5 min. The samples were treated with three DMEM rinses for complete removal of DCFH-DA that did not enter ARPE-19 cells and then analyzed immediately using FCM. Setting parameters: Ex = 488 nm, and Em = 525 nm.

2.7. ΔΨ\textsubscript{m} Detection. As a ΔΨ\textsubscript{m} parameter, JC-1, a lipophilic dye that can permeate plasma and mitochondrial membranes, was used to detect ΔΨ\textsubscript{m} changes in cells. The dye,
which glows red when it agglomerates in normal healthy cells with high mitochondria membrane potential, shows green fluorescence in those with low ΔΨm. After seeding cells into the wells of 6-well plates, they were introduced by 200 μmol/L H₂O₂ for 4 h at 37°C, with or without the pretreatment of 1 μmol/LSS31 for 2 h. The JC-1 dye (YEASEN, Shanghai, China) was added to cells adjusted to 1 × 10⁶ cells/ml in 6-well plates in each sample. Then, the cells were treated with light tight incubation (37°C, 20 min), and the subsequent two rinses in particular buffer for immediate FCM analysis. Setting parameters: Ex = 488 nm; Em = 530 nm.

2.8. Reverse Transcription Polymerase Chain Reaction (RT-PCR). After isolating from ARPE-19 cells using a kit (Thermo Fisher Scientific), the total RNA was synthesized into cDNA with another kit (Thermo Fisher Scientific). RNA yield (A260/A280) was spectrophotometrically monitored. RT was performed with the use of a First Strand cDNA Synthesis kit, and 2 μg of RNA was incubated with the following items: oligo (dT) primer (1 μmol/L), 1×10⁶ cDNA Synthesis kit, and 2 μl of RNase-free dH₂O, in a final volume of 20 μl. Reaction conditions of the mixture was as follows: 70°C for 5 min, followed by 37°C for 5 min and then 42°C for 60 min, and finally 70°C for 10 min. RT-PCR: cDNA (2 μl), Taq PCR Master Mix (12.5 μl), sense and antisense primers (1 μl each), and dH₂O₂, in a final volume of 25 μl following the manufacturer’s protocol, for 35 cycles. The thermocycling conditions used for RT-PCR were as follows: 35 cycles at 94°C, 60°C, and 72°C, all for 30 secs. After that, 1% agarose (agarose-to-TAE buffer solution: 1 g: 100 ml) was made and dissolved in a TAE buffer via microwave heating. Then, it was cool about 50°C and added with 5 μl of ethidium bromide. The final RT-PCR product samples underwent electrophoresis. Finally, gel was imaged and analyzed.

2.9. Real-Time PCR. The mRNA was isolated from ARPE-19 cells using the kit. QPCR was performed using a three-step amplifying protocol using PCR Master (Thermo Fisher Scientific). Mixes, as well as Real-time PCR Master Mixes (12.5 μl), template cDNA (1000 ng), and sense and antisense primers (0.5 μl each), were diluted in a 20 μl reaction volume. Reaction conditions: 95°C/10 min; followed by 40 cycles of 95°C/15 secs., 60°C/30 secs., 72°C/32 secs.; and finally 95°C/15 secs., 60°C/60 secs, and 95°C/15 secs. Each sample was repeatedly tested three times and quantification of expression levels used the 2-ΔΔCt method.

2.10. Statistical Processing. Data were statistically analyzed via SPSS v19.0 (SPSS, Chicago, IL). All analyses were performed independently at least three times. A one-way analysis of variance was adopted to test the collected data recorded as mean ± standard error of the mean, with P < 0.05 as the significance level.

3. Results

3.1. SS31 Inhibits Oxidative Damage-Induced ARPE-19 Cell Viability Decline. ARPE-19 cell viability was dose-dependently reduced in H₂O₂ treatment group (P < 0.05). Cell survival rate was reduced by 60% with 200 μmol/L H₂O₂ (65% ± 3.04) versus the control group (95% ± 5.5), as shown in Figure 1(a). While pretreatment with SS31 gave rise to increased cell survival as compared to the H₂O₂ group (67% ± 3.9), followed by (71.9% ± 5.4), (76.4% ± 3.3), and (82.2% ± 3.4) (P < 0.05), and 1 μmol/L SS31 was most obvious, as shown in Figure 1(b). From the statistical analysis data, SS31 showed no obvious side effect on ARPE-19 cell viability, with no notable difference found between control (89.3% ± 4.1) and SS31 (86% ± 4.0) groups (P < 0.05).

3.2. SS31 Morphologically Protects ARPE-19 against H₂O₂-Induced Cell Apoptosis. The number of living cells decreased as H₂O₂ concentration increased. As shown in Figure 2(a), ARPE-19 cells wizened gradually in morphology versus the control. By contrast, the number of living cells increased as the SS31 concentration increased, with cells tending to be normal in morphology gradually, as shown in Figure 2(b).

3.3. SS31 Prevents Cell Apoptosis in ARPE-19 Cells. DAPI staining showed concentrated nuclear chromatin and enhanced fluorescence of apoptotic cells. ARPE-19 cells with 24 h of 200 μmol/L H₂O₂ exposure showed obvious apoptotic properties like concentrated chromatin and enhanced fluorescence. In contrast, the nuclear fluorescence was weak in control and SS31 groups. Pretreatment of SS31 attenuated fluorescence obviously, suggesting the protective role of SS31 towards ARPE-19 cells against oxidative damage (×200), as shown in Figure 3(a). PI and AV-negative (-) cells were considered live cells (bottom left); PI (-) while AV-positive (+) cells were viable apoptotic cells (bottom right); those with PI (+) and AV (+) were primarily nonviable apoptotic cells (top right); and those with PI (+) and AV (-) were nonviable nonapoptotic cells (top left). An evident increase of AV-positive ARPE-19 cells was observed after 24 h of H₂O₂ treatment. By contrast, 1 μmol/L of SS31 (28.0%) was highly protective of ARPE-19 against H₂O₂ (37.7%), as shown in Figure 3(b). Obviously, SS31 showed no effect on cells alone (P < 0.05). A significant difference was present between control and H₂O₂ groups, as well as between SS31 + H₂O₂ and H₂O₂ groups (P < 0.05), as shown in Figure 3(c).

3.4. SS31 Significantly Reduces Mitochondrial ROS. MitoSOX red and DCFH-DA probe were applied to detect the ROS release level in ARPE-19 cells. The control cells showed weak red fluorescence. The red fluorescence intensity was significantly enhanced in H₂O₂ group, indicating that the release level of ROS was significantly increased. After the pretreatment of SS31, cell fluorescence intensity in this group dropped notably compared with H₂O₂ group. SS31 group and control group differed insignificantly in fluorescence intensity, as shown in Figure 4(a). Meanwhile, H₂O₂ group showed significantly stronger cell fluorescence intensity than control group, as indicated by the FCM diagram. After the pretreatment of SS31, the fluorescence intensity was statistically weaker versus the H₂O₂ group, indicating significantly reduced ROS release level (P < 0.05), as shown in Figures 4(b) and 4(c).
3.5. SS31 Elevates $\Delta \Psi_m$ under OS. For the purpose of determining the mitochondrial-mediated pathway in cellular dysfunction and apoptosis under OS, the present study measured $\Delta \Psi_m$ using membrane potential indicator JC-1 by FCM. The image showed statistically reduced $\Delta \Psi_m$ of ARPE-19 cells under H$_2$O$_2$ treatment and increased number of cells with low membrane potential. Compared with the H$_2$O$_2$ group, the proportion of low mitochondrial potential of ARPE-19 cells in the 1 $\mu$M of SS31 + H$_2$O$_2$ group was reduced ($P < 0.05$). The proportion of low mitochondrial membrane potential cells was not statistically different between SS31 and control groups ($P < 0.05$), as shown in Figures 5(a) and 5(b).

3.6. SS31 Regulates the Expression and mRNA Level of Apoptosis-Associated Genes. OS-induced apoptosis was also investigated by detecting apoptosis-associated gene expression. Administration of H$_2$O$_2$ was accompanied by notably enhanced expression and mRNA level of caspase-3 and Bax, both of which are linked to apoptosis, as well as suppressed Bcl-2, an antiapoptotic gene. However, the reverse was true under the intervention of SS31, namely, decreased caspase-3 and Bax levels and elevated antiapoptotic gene Bcl-2 level were observed, as shown in Figures 6(a) and 6(b).

4. Discussion
A large number of studies showed that abnormal changes in structure and dysfunction of RPE caused by oxidative damage is an important factor leading to AMD [19, 20]. In aging retinas, OS and ROS play a vital part in pathology [21]. Herein, ARPE-19 cell viability declined dose-dependently...
Figure 3: Continued.
Figure 3: SS31 inhibits ARPE-19 cell apoptosis. (a) ARPE-19 cell apoptosis detected by DAPI staining. Observation under fluorescence microscope (magnification, ×200). (b, c) Representative FCM images identified an obvious reduction in AV-positive cells in ARPE-19 cells pretreated with 1 μmol/L of SS31, compared with those intervened by 24 h of H₂O₂. Quantitative analysis of AV-positive cells (n = 6, *P < 0.05 vs. the control; **P < 0.05 vs. the H₂O₂). Data are indicated by mean ± SD.

Figure 4: SS31 significantly reduces mitochondrial ROS. (a, b) Representative fluorescence images and FCM images of ARPE-19 cells, revealing an increased fluorescence in H₂O₂ group. Pretreatment with 1 μmol/L SS31 significantly ameliorated the fluorescence (magnification, ×200). (c) Quantitative analysis (n = 6, *P < 0.05 vs. the control; **P < 0.05 vs. the H₂O₂). Data are indicated by mean ± SD.
after exposure to H$_2$O$_2$, indicating that the cells were damaged in various degrees of OS. In this experiment, 200 μmol/L of H$_2$O$_2$ was used to stimulate the cells for 24 h to establish the oxidative damage model of RPE during the pathogenesis of AMD. The viability of cells at this concentration is slightly greater than the half-lethal concentration, which was reasonable. In determining the concentration of SS31, this experiment was based on previous experimental studies [22]. Three concentrations of SS31 (10 nmol/L, 100 nmol/L, and 1 μmol/L) were chosen for protecting the ARPE-19 cells. The cell viabilities in these concentrations were higher than that in the H$_2$O$_2$ group. According to the degree of increase, the optimal concentration of SS31 was 1 μmol/L. At the same time, it was also found that when SS31 was used alone in ARPE-19 cells, it showed no toxicity to the cells.

Mitochondria are considered to be the starting site of endogenous apoptosis pathways and a key factor for cell survival and death. Mitochondria are critical in transduction process and expansion of death signals. Under the
stimulation of apoptotic signals, the mitochondrial membrane potential decreases, ROS release level increases, permeability increases, and various apoptotic factors are released from the mitochondria into the cytoplasm, eventually leading to cell apoptosis [23, 24]. Typical morphological changes of apoptosis, such as chromatin condensation, cell shrinkage, and some organelles, ribosomes, and nuclear fragments, are wrapped by cell membranes to form apoptotic bodies. In this study, under the pretreatment of SS31, the number of apoptotic cells decreased, cell shrinkage was improved, and apoptosis was inhibited versus H2O2 group.

In DAPI staining and FCM detection, the apoptosis rate of ARPE-19 cells after OS increased significantly, while SS31 can significantly reduce the apoptosis rate of cells.

Current research suggests that ROS is an important mediator of apoptosis, and it can act on various links in the mitochondrial apoptosis pathway. The signaling molecules in mitochondrial apoptosis also affect the generation of ROS. In this experiment, SS31 was shown to reduce the release level of ROS in ARPE-19 cells under OS. ROS release level was similar in SS31 and normal groups. It was preliminarily believed that SS31 can reduce the damage to retinal tissue under OS to a certain extent by reducing the release level of ROS.

In vitro experiments have confirmed that in the early stage of apoptosis, before the appearance of nuclear pathological changes, decrease of mitochondrial membrane potential is the manifestation of early cell apoptosis [25, 26]. In this experiment, after pretreatment of SS31, the green fluorescence was significantly weakened and the red fluorescence was enhanced, indicating a notably higher mitochondrial membrane potential than the H2O2 group, and SS31 had a certain protective effect against mitochondrial function and apoptosis in ARPE-19 cells. The mitochondrial membrane potential differed insignificantly between SS31 and control groups.

Recent evidence has shown the vital role of the mitochondrial pathway in the mechanism of cell survival. The caspase family and the Bcl-2 protein family currently received much attention among many apoptosis regulating genes. Of them, caspase-3 is the most crucial apoptotic executive protease in cell apoptosis, and the most important pair of apoptosis-regulating genes, which are opposite in function to each other, are the Bcl-2 gene and Bax gene [27]. Our research identified markedly elevated Bax and Caspase-3 expression and mRNA levels in ARPE-19 cells after cells were treated with H2O2, while decreased antiapoptotic gene Bcl-2, indicating that OS caused cell apoptosis. After the pretreatment of SS31, the results reversed, showing that SS31 plays a significant part in modulating apoptosis-related gene levels.

Previous studies have confirmed that SS31 can be detected in eye tissue after subcutaneous injection. Observation under confocal microscope and other methods confirmed that SS31 mainly enters the mitochondrial inner membrane after entering the cell [28]. In this experiment, SS31 shows no significant effect on mitochondrial function and apoptosis, and there was no cytotoxicity.

5. Conclusion

In our experiment, the protective actions of SS31 on ARPE-19 cells under OS were reported, which showed a potential value in treatment of AMD through enhanced RPE cell function. Since the molecular biological mechanism of SS31 was not studied in this experiment, genechip technology can be used to further explore SS31 in subsequent experiments.

Data Availability

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

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Yuan He and Zejun Chen contributed equally to this work. YH conceived and designed the study. She also reviewed and edited the manuscript. Zejun Chen performed all experiments and wrote the paper. Yun Xu, Yuan Ren, Ruixue Zhang, Zhuoya Quan, and Beilei He performed the experiments. All authors read and approved the manuscript.

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