



Silibinin alleviates DNA damage, mitochondrial dysfunction, and apoptosis caused by oxidative stress in human retinal pigment epithelial cells

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Abstract

Background Silibinin, a flavonolignan, is known to have a variety of pharmacological activities, including antioxidant activity, but its antioxidant mechanism in the eye is unclear.

Objective This study aimed to evaluate whether silibinin could protect human retinal pigment epithelial ARPE-19 cells from oxidative injury.

Results Silibinin attenuated cell viability reduction and DNA damage in ARPE-19 cells treated with hydrogen peroxide (H_2O_2), while inhibiting intracellular reactive oxygen species (ROS) production and preserving diminished glutathione (GSH). Silibinin also antagonized H_2O_2 -induced inhibition of the expression and activity of antioxidant enzymes, such as GSH peroxidase and manganese superoxide dismutase, which was associated with inhibition of mitochondrial ROS production. Moreover, silibinin rescued ARPE-19 cells from H_2O_2 -induced apoptosis by restoring the reduced Bcl-2/Bax ratio and reducing caspase-3 activation. In addition, silibinin suppressed the release of mitochondrial cytochrome *c* into the cytoplasm, which was achieved by interfering with mitochondrial membrane disruption.

Conclusion These findings imply that silibinin has potent ROS scavenging activity with the potential to protect against oxidative stress-mediated ocular diseases.

Keywords Silibinin · Human RPE ARPE-19 cells · ROS · DNA damage · Apoptosis

Introduction

Oxidative stress due to impairment of antioxidant defense systems and/or excessive accumulation of reactive oxygen species (ROS) acts as a cause of damage to intracellular biomolecules and organelles. Cells exposed to continuous oxidative stress ultimately result in cell death accompanied by mitochondrial dysfunction and DNA damage (Chaudhary et al. 2023; Zia et al. 2022). Eyes, which undergo active oxidative metabolism to form vision, are constantly exposed to solar radiation and are vulnerable to oxidative stress. Accumulating evidence has shown that oxidative stress has been

implicated in the pathogenesis of various eye disorders, including age-related macular degeneration (AMD) and dry eye (Dammak et al. 2023; Hsueh et al. 2022). In addition, it has been demonstrated that ROS act as important executors in the initiation of mitochondria-dependent apoptosis. Therefore, improving mitochondrial function while increasing antioxidant potential may be a promising strategy for protection against oxidative stress-mediated eye diseases (Zia et al. 2022; Demine et al. 2019). To this end, interest is growing in using natural products to discover ideal drugs that can overcome oxidative stress without side effects.

Silybum marianum (L.) Gaertn., also known as milk thistle, belongs to the Asteraceae family and has been used as a remedy for various ailments, especially for treating hepatic diseases such as cirrhosis and hepatitis and protecting the liver from toxic substances (Nawaz et al. 2023; Wang et al. 2020). These pharmacological effects are closely related to a complex of polyphenolic flavonoids called silymarin, first discovered in the achenes of this plant (Nawaz et al. 2023; Wadhwa et al. 2022). The potential of silymarin as an

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antioxidant comes from its ability to scavenge small molecule free radicals (Nawaz et al. 2023; Wang et al. 2020). Silibinin, a natural flavonolignan also called silybin, is one of the major and most active flavonoids identified in the silymarin complex (Nawaz et al. 2023; Islam et al. 2021). Accumulated research has proven that silibinin possesses a wide range of pharmacological activities, including antidiabetic, cardioprotective, antihepatotoxic, anti-inflammatory, neuroprotective, antiviral and anticarcinogenic effects (Singh et al. 2023; Křen and Valentová 2022; Takke and Shende 2019). In particular, the improvement of chronic diseases by silibinin is closely related to its antioxidant activity by eliminating ROS through the regulation of antioxidant signaling pathways. For example, alleviation of oxidative stress-mediated mitochondrial impairment and apoptosis by silibinin could be achieved by blocking ROS production through restoring activity of intracellular antioxidant enzymes (Li et al. 2023; Chen et al. 2020). Silibinin could also successfully reverse nephrotoxicity and hepatotoxicity by scavenging free radicals and increasing intracellular antioxidant defenses in cisplatin-induced acute kidney injury model and liver tissue of itraconazole-treated rats, respectively, thereby protecting renal and hepatic cells from apoptosis (Yang et al. 2022; Sozen et al. 2015). Mao et al. (2018) have reported that the anti-apoptotic effect of silibinin in advanced glycation end product (AGE)-treated osteoblasts was due to inhibition of mitochondrial ROS (mtROS) generation by blocking AGE receptor-dependent mitochondrial dysfunction. In addition, silibinin exhibited anti-aging effects by suppressing oxidative and inflammatory properties in hydrogen peroxide (H_2O_2)-treated embryonic fibroblast cells (Baeeri et al. 2018). Guo et al. (2020) also demonstrated that the protective capacity of silibinin against H_2O_2 -induced apoptosis in trophoblast cells was related to the enhanced mitochondrial membrane potential (MMP). Similar to their results, the neuroprotective effect of silibinin against H_2O_2 in neuroblastoma cells was found to be associated with reduced mitochondrial damage and inhibition of ROS generation by preserving catalase and superoxide dismutase (SOD) levels (Tie et al. 2022).

Recently, Pooja et al. (2022) showed that silibinin was able to inhibit epithelial-mesenchymal transition and excessive proliferation of lens epithelial cells, which is the key process of posterior lens opacification. In addition, it has been shown that silibinin had a protective effect against acute retinal ganglion cell damage caused by blue light and prevented corneal damage caused by nitrogen mustard (Shen et al. 2019; Goswami et al. 2018). Additionally, silibinin had a preventive effect against endotoxin-induced uveitis in human retinal pigment epithelial (RPE) cells and counteracted retinal edema and angiogenesis in a hypoxia-induced AMD model using RPE cells (Chen et al. 2017; Lin et al. 2013). These studies indicate that silibinin has

potent preventive and therapeutic effects against various eye diseases, but the underlying mechanism of silibinin for its antioxidant activity is not fully elucidated. Therefore, this study focused on the antioxidant properties of silibinin under conditions mimicking the oxidative environment induced by H_2O_2 exposure using human RPE ARPE-19 cells.

Materials and methods

Cell culture

ARPE-19 cells were cultured according to a previously described method (Park et al. 2023). Silibinin and H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to prepare stock solutions, which were then diluted to appropriate concentrations in culture medium for treating cells. To trigger oxidative stress, ARPE-19 cells were cultured in medium containing H_2O_2 . Silibinin, *N*-acetyl-L-cysteine (NAC) and Mito-TEMPO (ZnPP, Thermo Fisher Scientific, Waltham, MA, USA) were added at 1 h before H_2O_2 exposure. The final concentration of DMSO diluted in the culture medium did not exceed 0.05%, which did not show significant toxicity to ARPE-19 cells.

Assessment of cytotoxicity

To evaluate the cytotoxicity of ARPE-19 cells stimulated with H_2O_2 in the presence or absence of silibinin, cell viability was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the method of Cao et al. (2023). After treatment, morphological changes of cells were observed using an optical microscope (Carl Zeiss, Oberkochen, Germany). In addition, the degree of lactate dehydrogenase (LDH) release into the culture medium for cells cultured under the same conditions was quantitatively compared using the LDH Assay kit (Abcam, Inc., Cambridge, UK).

Comet assay

To investigate the degree of DNA damage, a Comet Assay kit (Trevigen, Inc., Gaithersburg, MD, USA) was used. In brief, cells were mixed in a low melting-point agarose solution, spread evenly on comet slides according to the manufacturer's instructions. Electrophoresis was then performed and cells were immediately stained with the fluorescent dye provided in the kit, followed by visualization of fluorescent images with a fluorescence microscope (Carl Zeiss).

Immunostaining for analysis of phosphorylation of histone H2AX (γ H2AX) expression

To assess the expression level of γ H2AX, cells were exposed to H₂O₂ in the presence or absence of silibinin, fixed, and then permeabilized (Park et al. 2023). Cells were then probed with an anti- γ H2AX antibody (Thermo Fisher Scientific) and reacted with Alexa Fluor 594-conjugated secondary antibody (Sigma-Aldrich). After staining the nuclei using 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), images were observed under a fluorescence microscope. γ H2AX-stained nuclei are red-fluorescent, and DAPI-stained nuclei are blue-fluorescent.

8-Hydroxyguanosine (8-OHdG) assay

To analyze the degree of oxidative DNA damage, the level of intracellular 8-OHdG was evaluated using the 8-OHdG ELISA Kit (Abcam, Inc.) according to the manufacturer's instructions. In brief, DNA isolated from H₂O₂-treated cells with or without silibinin was digested using DNA Digestion Mix and the level of 8-OHdG was measured with a competitive enzyme immunoassay. The optical density (OP) of each treatment group at 450 nm was recorded. Results are expressed as ng of 8-OHdG/mL.

Quantitative analysis of apoptosis

The frequency of apoptotic cells was determined by flow cytometry using the Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Staining/Detection kit (Abcam, Inc.). In brief, collected cells were double-stained with annexin V and PI in the dark according to the manufacturer's protocol. After staining, annexin V-positive cells were regarded as apoptosis-induced cells using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Protein isolation and immunoblotting

To examine expression levels of proteins of interest using immunoblotting, total cellular proteins were isolated using RIPA lysis buffer (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's experimental protocol. Mitochondrial and cytoplasmic fractions were extracted using a Mitochondrial Fractionation Kit (Thermo Fisher Scientific). After protein quantification, immunoblotting was performed according to the method described previously (Mukherjee et al. 2022). Cytochrome *c* oxidase IV (COX IV) and β -actin were used as housekeeping proteins for mitochondrial and cytoplasmic fractions, respectively. Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Cell Signaling Technology, and Abcam, Inc. (Table 1).

Caspase-3 activity

Caspase-3 activity in cells exposed to H₂O₂ with or without silibinin was measured using the Caspase-3 Assay Kit (Thermo Fisher Scientific) based on the spectrophotometric detection of *p*-nitroaniline (*p*-NA) liberated from the substrate of caspase-3 (*N*-acetyl-Asp-Glu-Val-Asp-*p*-NA). According to the manufacturer's instructions, cell lysates were obtained using reagents provided in the kit. The OP of free *p*-NA was then quantified at 405 nm. Based on OP values, caspase-3 activity in each treatment group was determined relative to that of the untreated control group.

MMP assay

To determine mitochondrial activity, MMP was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) dye (Abcam, Inc.). The fluorescence intensity of JC-1 monomeric and aggregate forms was examined by flow cytometry according to the manufacturer's protocol. Percentages of JC-1 monomers were expressed to represent cells that lost MMP.

Table 1 List of antibodies used in this study

Antibody	Species raised	Dilution	Product code	Source
Bcl-2	Mouse monoclonal	1:1000	sc-509	Santa Cruz Biotechnology Inc
Bax	Mouse monoclonal	1:1000	sc-7480	Santa Cruz Biotechnology Inc
Caspase-3	Rabbit polyclonal	1:1000	#9662	Cell Signaling Technology Inc
PARP	Mouse monoclonal	1:1000	sc-8007	Santa Cruz Biotechnology Inc
Cytochrome <i>c</i>	Mouse monoclonal	1:500	sc-13560	Santa Cruz Biotechnology Inc
PGx1	Rabbit polyclonal	1:1000	#63,536	Cell Signaling Technology Inc
MnSOD	Rabbit polyclonal	1:1000	ab13533	Abcam, Inc
COX IV	Rabbit polyclonal	1:500	#4844	Cell Signaling Technology Inc
β -actin	Mouse monoclonal	1:1000	sc-47778	Santa Cruz Biotechnology Inc

Intracellular ROS production analysis

To analyze the scavenging activity of silibinin on intracellular ROS levels induced by H_2O_2 , cells treated with H_2O_2 for 1 h in the presence or absence of silibinin were stained with 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich). According to the manufacturer's protocols, the levels of ROS were quantitatively evaluated by DCF-fluorescence representing the percentage of control cells through flow cytometry (Sukjamnong et al. 2022). Fluorescence images were also taken with a fluorescence microscope to visually detect the difference in emitted DCF fluorescence intensity.

Evaluation of GSH/GSSG disulfide (GSSG) ratio

The difference in the ratio of reduced GSH to oxidized GSSG in cells exposed to H_2O_2 for 24 h with or without silibinin was quantified using the GSH/GSSG Assay kit (Abcam, Inc.). In brief, after reacting cells of each treatment group under conditions recommended by the manufacturer, levels of GSH and GSSG were determined based on standard curves of GSH and GSSG. GSH/GSSG ratio was presented as a relative value to the control group.

Measurement of the activities of GSH peroxidase (GPx) and manganese (MnSOD)

Colorimetric kits purchased from Abcam, Inc. were used to measure the activities of GPx and MnSOD. In brief, fractions of cells exposed to H_2O_2 for 24 h with or without silibinin were prepared according to the manufacturer's recommendations and the activity of each enzyme was presented as relative values to the control.

MitoTracker Red assay

To investigate the effect of silibinin on inhibition of mitochondrial activity by H_2O_2 treatment, MitoTracker™ Red (Molecular Probes, Eugene, OR, USA) staining was performed. According to the manufacturer's recommendations, cells were stained with MitoTracker Red after pretreatment with silibinin for 1 h and then exposed to H_2O_2 for 3 h. After staining, the nuclei were stained with DAPI and the fluorescence images were acquired using a fluorescence microscope.

Measurement of mitochondrial superoxide levels

To examine the effect of silibinin on mitochondrial peroxide production by H_2O_2 using MitoSOX™ Red (Thermo Fisher Scientific), cells cultured for 1 h in medium with or without silibinin were treated with H_2O_2 for 1 h. Briefly, after staining

cells with MitoSOX according to the method suggested by the manufacturer, the emitted fluorescence of MitoSOX was detected by flow cytometry. In addition, cells were stained with MitoSOX and then additionally stained with DAPI, and the fluorescence images were compared using a fluorescence microscope.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Statistical comparisons were performed using GraphPad Prism version 8.0 (GraphPad Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Inhibition of H_2O_2 -induced cytotoxicity by silibinin in ARPE-19 cells

MTT assay was performed to investigate the inhibitory effect of silibinin on H_2O_2 -induced cytotoxicity in ARPE-19 cells. MTT assay results showed that silibinin decreased cell viability in a concentration-dependent manner when the treatment concentration was above 30 μ M, but there was no significant change compared to the control group when the treatment concentration was 20 μ M or less (Fig. 1A). Therefore, the optimal pretreatment concentration to examine the inhibitory effect of silibinin on H_2O_2 -induced cytotoxicity was selected as 20 μ M or less. The treatment concentration of H_2O_2 to mimic oxidative damage was set at 0.5 mM, which showed a survival rate of about 60% compared to the control group, referring to previous studies (Park et al. 2023). As presented in Fig. 1B, silibinin pretreatment at concentrations of 10 μ M and 20 μ M increased the decrease in cell viability caused by H_2O_2 treatment to approximately 70% and 82%, respectively. To further investigate the cytotoxic protective effect of silibinin, LDH leakage levels were measured and results showed that silibinin pretreatment significantly attenuated the H_2O_2 -induced increase in LDH leakage levels (Fig. 1C). In addition, cells cultured in normal medium were uniform in size and tightly arranged, but as the size after H_2O_2 treatment became more diverse, the shape became irregular and adhesion was lost. However, in the presence of silibinin, the morphology of cells treated with H_2O_2 was clearly improved as showed in Fig. 1D. These results clearly indicate that silibinin had a cytoprotective effect against oxidative stress in ARPE-19 cells.

Protection of H_2O_2 -induced DNA damage by silibinin in ARPE-19 cells

To investigate whether the blocking efficacy of silibinin against H_2O_2 -induced cytotoxicity in ARPE-19 cells was

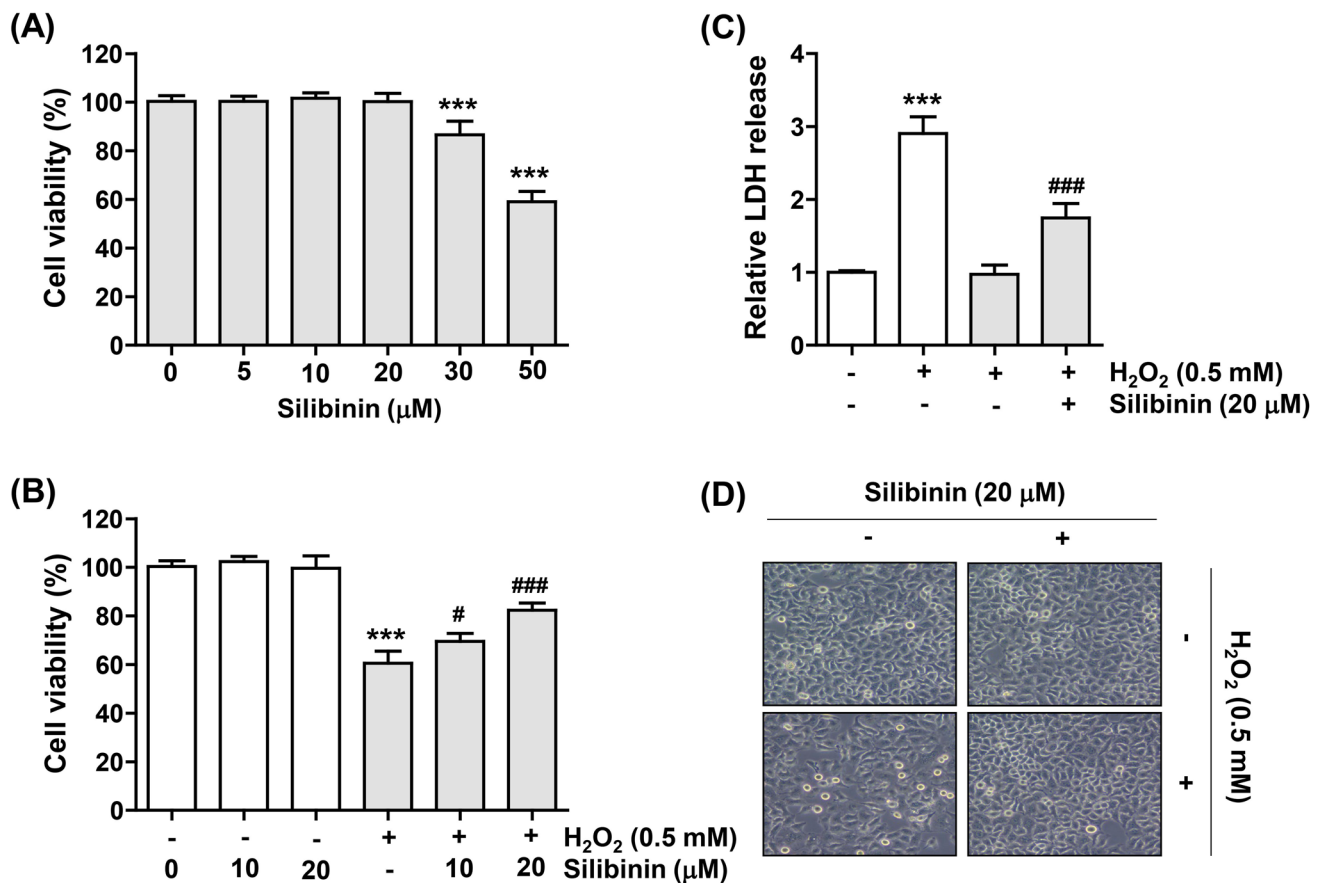


Fig. 1 Suppression of H₂O₂-induced cytotoxicity by silibinin in ARPE-19 cells. **A** and **B** Results of MTT assay after treating cells with various concentrations of silibinin for 24 h **A** or pre-treating cells with silibinin (10 μM and 20 μM) for 1 h and then treating them with 0.5 mM H₂O₂ for 24 h **B**. **C** LDH release from cells

cultured under the indicated conditions was measured in the supernatant. ****p* < 0.001 vs. control group; #*p* < 0.01 and ###*p* < 0.001 vs. H₂O₂ treatment group. **D** Morphological images of cells cultured in H₂O₂-treated medium with or without silibinin

related to attenuation of DNA damage, it was first evaluated the inhibitory effect of silibinin on H₂O₂-induced comet tail formation. Compared to the control group, H₂O₂ treatment induced DNA damage, as evidenced by an increase in tail length, indicating double-strand breaks in DNA, but this was clearly reduced in cells pretreated with silibinin (Fig. 2A). To provide additional evidence for the inhibition of H₂O₂-mediated DNA damage by silibinin, the expression of γH2AX, another indicator of double-strand breakage of DNA, and the level of 8-OHdG, a biomarker of oxidative guanine base damage, were further evaluated. As shown in Fig. 2B and C, silibinin pretreatment remarkably blocked the increase in γH2AX fluorescence intensity in the nucleus and 8-OHdG content caused by H₂O₂ treatment, demonstrating that silibinin was able to block DNA damage caused by oxidative stimulation.

Inhibition of H₂O₂-induced apoptosis by silibinin in ARPE-19 cells

To determine whether silibinin inhibited H₂O₂-induced apoptosis of ARPE-19 cells, flow cytometry analysis was performed after staining with Annexin V/PI. As shown in Fig. 3A and B, apoptosis was greatly increased in H₂O₂-treated cells compared to the control group. However, it was largely abolished by silibinin pretreatment. Immunoblotting was then applied to assess the effect of silibinin on the expression changes of key apoptosis regulatory factors in H₂O₂-treated cells. As depicted in Fig. 3C, H₂O₂ treatment markedly reduced the expression level of Bcl-2, which promotes cell survival, compared to untreated cells, but slightly increased the protein level of Bax, which promotes apoptosis. However, the effect of H₂O₂ treatment on Bcl-2

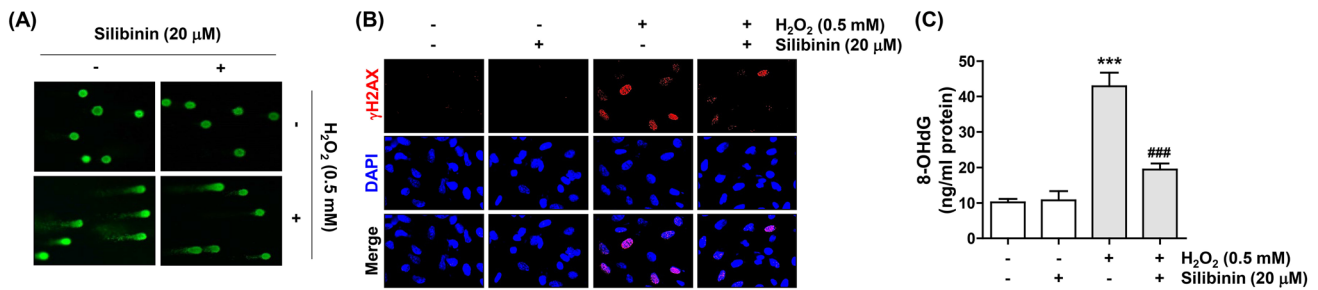


Fig. 2 Protection of H₂O₂-induced DNA damage by silibinin in ARPE-19 cells. Before treating cells with 0.5 mM H₂O₂ for 24 h, they were incubated in the presence or absence of 20 μM silibinin for 1 h. After treatment, the inhibitory effect of silibinin on H₂O₂-induced DNA damage was determined. **A** Representative images of comet assay were presented. **B** Representative immunofluorescence images

stained with γH2AX (red) were taken under a fluorescence microscope. Nuclei were stained with DAPI (blue). **C** The amounts of 8-OHdG in culture supernatants were measured using an 8-OHdG assay kit. ****p* < 0.001 vs. control group; ###*p* < 0.01 vs. H₂O₂ treatment group

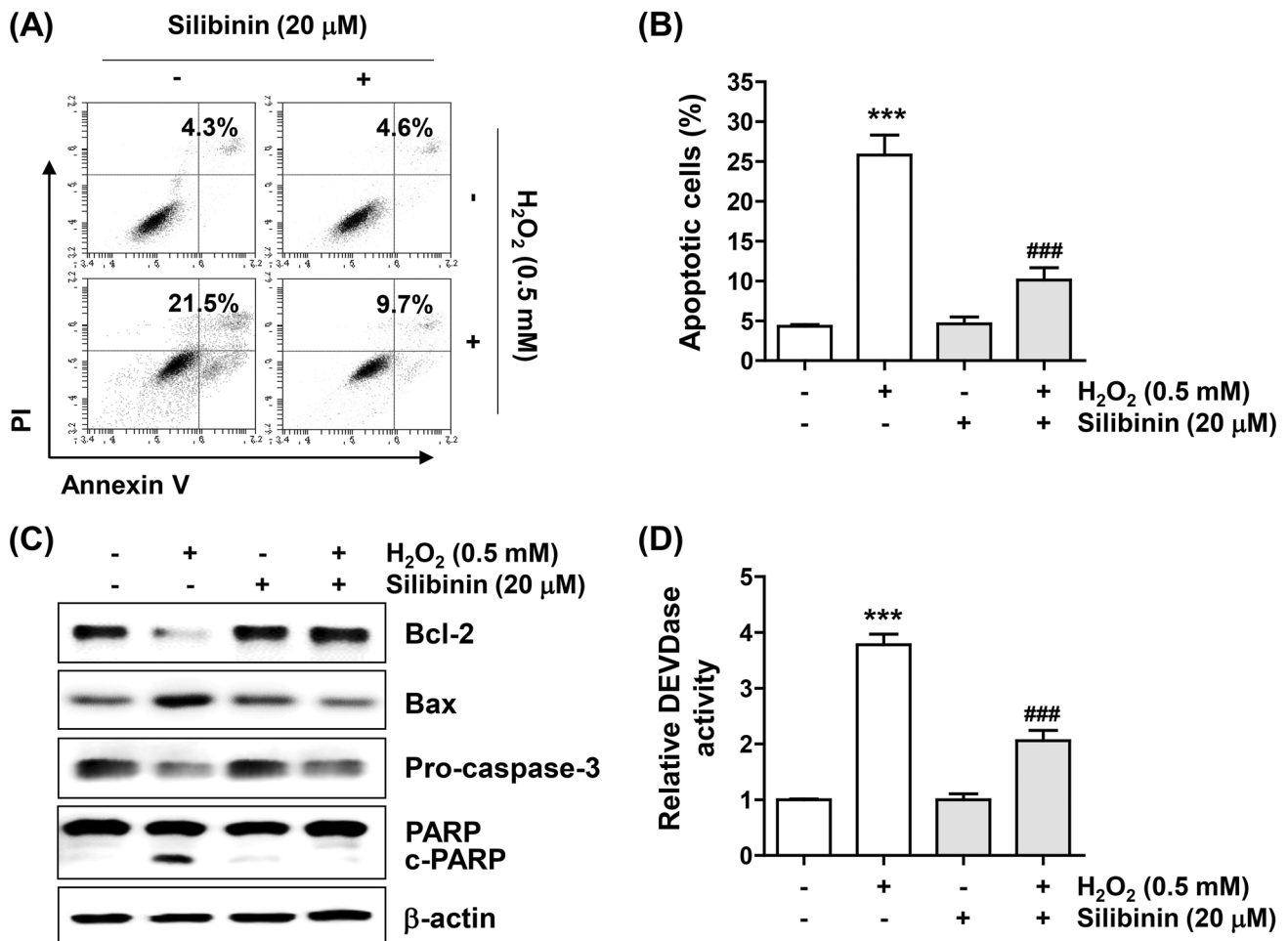


Fig. 3 Inhibition of apoptosis by silibinin in H₂O₂-treated ARPE-19 cells. Cells were treated with silibinin for 1 h before treatment with H₂O₂ for 24 h. **A** and **B** Annexin V/PI staining was followed by flow cytometry. Representative histogram (**A**) and quantitative analysis results (**B**) were shown. **C** Changes in expression of the indicated

proteins were obtained through immunoblotting using antibodies corresponding to each protein. **D** Differences in caspase-3 activity in each treatment group were presented. ****p* < 0.001 vs. control group; ###*p* < 0.01 vs. H₂O₂ treatment group

family members was mitigated in the presence of silibinin. And as shown in Fig. 3C and D, as the expression level of pro-caspase-3 protein was greatly reduced by H₂O₂ administration, its activity increased significantly and cleavage of poly(ADP-ribose) polymerase (PARP), a substrate protein of caspase-3, was induced. However, these changes caused by H₂O₂ treatment were also effectively attenuated when silibinin was applied simultaneously.

Reduction of H₂O₂-induced mitochondrial damage by silibinin in ARPE-19 cells

To analyze whether the inhibitory effect of silibinin against H₂O₂-induced apoptosis was associated with its inhibitory ability against mitochondrial dysfunction, MMP, an indicator of mitochondrial function, were monitored using the fluorescent dye JC-1, which is sensitive to mitochondrial polarity. Flow cytometry results showed that the proportion of JC-1 aggregates was down-regulated in H₂O₂-treated cells, whereas the frequency of JC-1 monomers, indicating the frequency of cells lacking MMP, was significantly up-regulated as showed in Fig. 4A and B. In good agreement with these results, the fluorescence intensity of MitoTracker Red probe, which binds to mitochondria with an active membrane potential, was noticeably reduced in H₂O₂-treated cells (Fig. 4C). In addition, cytochrome *c* expression was promoted in the cytosolic fraction by H₂O₂ insult with the parallel decrease into mitochondrial fraction (Fig. 4D and E), demonstrating that cytochrome *c* present in mitochondria was released into the cytoplasm by H₂O₂ treatment due to disruption of mitochondrial membrane stability. However,

these changes caused by H₂O₂ were all clearly abolished in cells pretreated with silibinin, indicating that functional damage to mitochondria induced by H₂O₂ was alleviated by silibinin.

Suppression of ROS generation and restoration of GSH/GSSG ratio by silibinin in H₂O₂-treated ARPE-19 cells

To examine the antioxidant activity of silibinin, DCF-DA, a cell-permeant indicator for ROS, was used to evaluate the intracellular total ROS levels produced by H₂O₂. As shown in Fig. 5A and B, the frequency of oxidized DCF-positive cells, an indicator of intracellular ROS production, was significantly higher than that of untreated cells. However, it was dramatically abrogated in silibinin-pretreated cells, and treatment with silibinin alone had no significant effect on ROS production. As a result of reconfirming the ROS scavenging ability of silibinin through fluorescence microscopy, a significant increase in DCF fluorescence intensity (green) was observed in cells treated with H₂O₂ as seen in the micrograph shown in Fig. 5C, and the increase was greatly reduced in the presence of silibinin. Because GSH is oxidized to GSSG under oxidative stress conditions, GSH depletion is widely used as an indicator of oxidative stress, so the effect of silibinin on the change of GSH/GSSG ratio by H₂O₂ treatment was further evaluated. As shown in Fig. 5D, the reduced GSH/GSSG ratio upon the administration of H₂O₂ was significantly restored by silibinin pretreatment. These results suggest that depletion of intracellular GSH might be responsible for enhanced ROS generation

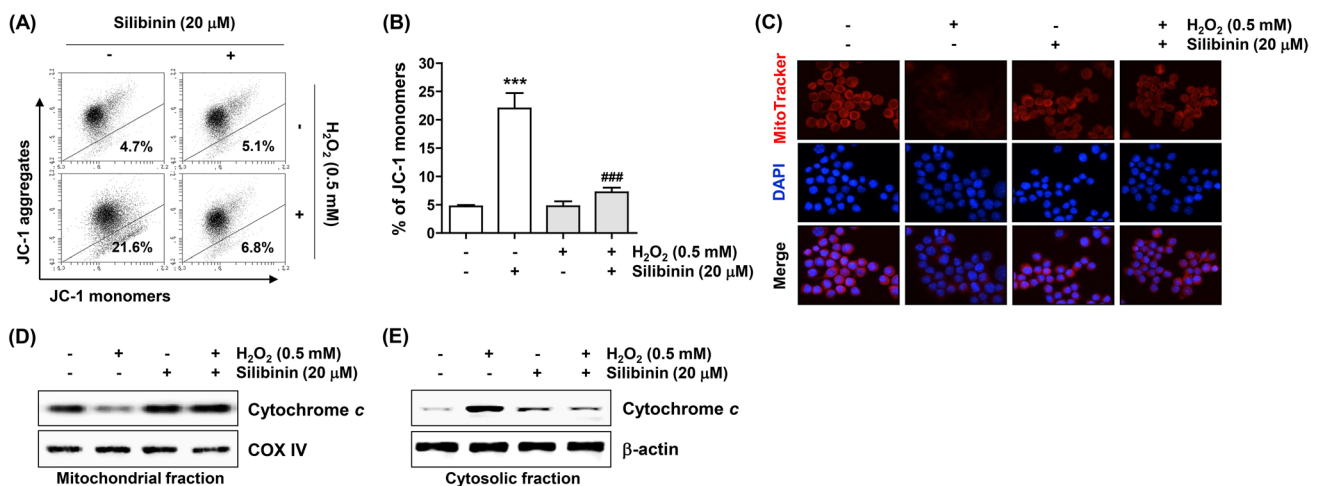


Fig. 4 Attenuation of H₂O₂-induced mitochondrial impairment by silibinin in ARPE-19 cells. Cells cultured for 1 h in medium with or without silibinin were treated with H₂O₂ for 24 h. **(A and B)** Representative histograms of flow cytometry using JC-1 staining in each treatment group **(A)** and quantitative results of JC-1 monomer ratios **(B)** were shown. *** $p < 0.001$ vs. control group; #### $p < 0.01$ vs.

H₂O₂ treatment group. **C** Mitochondria and nuclei were stained with MitoTracker Red (red) and DAPI (blue), respectively, and representative fluorescence images were presented. **D and E** Mitochondrial and cytoplasmic fractions were separated and the expression pattern of cytochrome *c* was investigated by immunoblotting

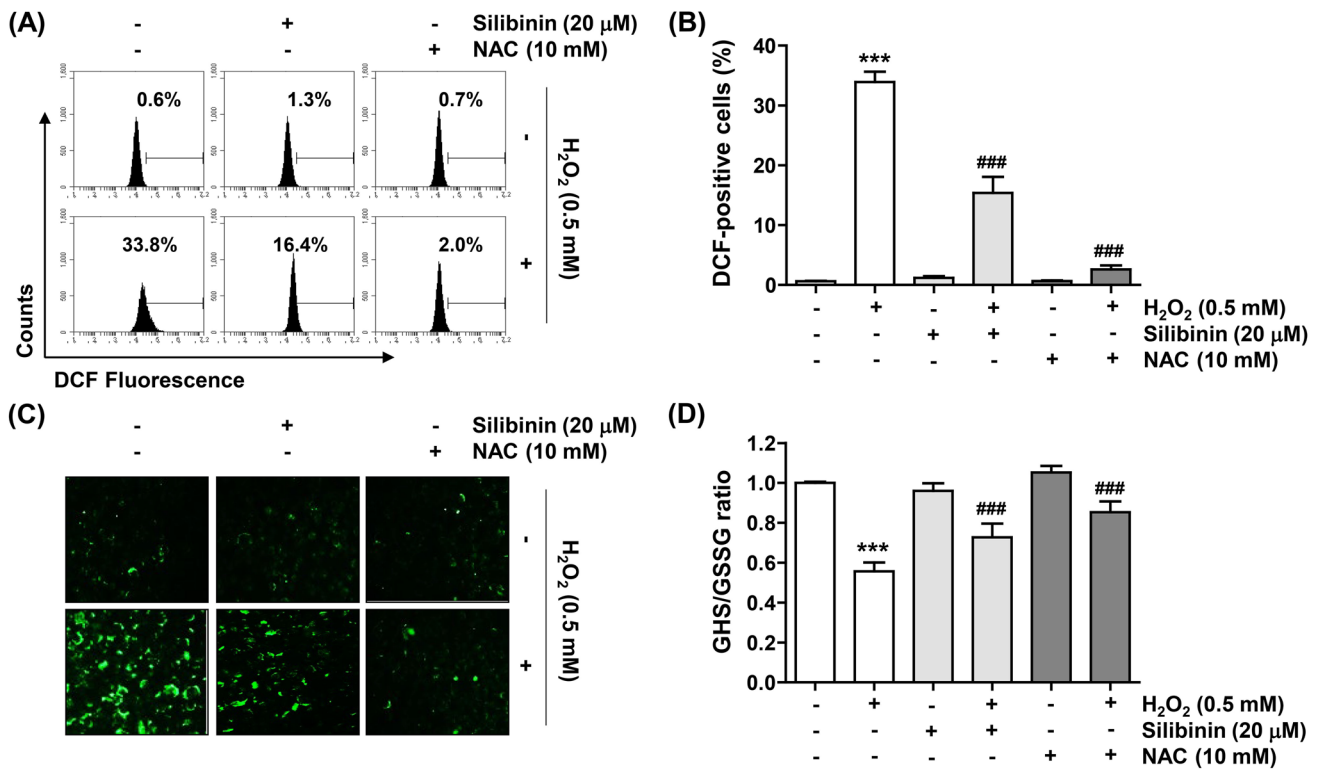


Fig. 5 Improvement of H_2O_2 -induced intracellular ROS generation and GSH/GSSG ratio reduction in ARPE-19 cells by silibinin. Cells were treated with H_2O_2 for 1 h (A–C) or 24 h (D) following a 1 h preincubation with silibinin or NAC. (A and B) Flow cytometry was performed after DCF-DA staining. Representative flow cytometry results of each treatment group (A) and frequencies of DCF-positive cells

(B) were shown. C Representative images obtained under a fluorescence microscope after DCF-DA staining. D The change in the ratio of GSH and GSSG content in each treatment group was measured using a colorimetric kit. *** $p < 0.001$ vs. control group; ### $p < 0.01$ vs. H_2O_2 treatment group (D)

with H_2O_2 treatment, as shown in the results of pretreatment with NAC, a ROS scavenger, and silibinin could lower the intracellular oxidative stress caused by H_2O_2 .

Inhibition of H_2O_2 -induced reduction of GPx and MnSOD activities and increase in mtROS production by silibinin in ARPE-19 cells

To further investigate the antioxidant activity of silibinin, we evaluated the role of GPx, which catalyzes the reduction of H_2O_2 to oxygen and water, and MnSOD, a key enzyme that detoxifies oxygen superoxide, a major byproduct of mitochondrial respiration. As expected, H_2O_2 treatment greatly decreased the expression and activity of GPx and MnSOD (Fig. 6A–C), indicating that activation of MnSOD and GPx were negatively correlated with ROS levels. However, silibinin pretreatment counteracted their expression and enzymatic activity reduced by H_2O_2 , indicating that silibinin could protect ARPE-19 cells against oxidative environments by attenuating excessive generation of intracellular and mtROS. Next, we used MitoSOX, a specific fluorescent probe for detecting mitochondrial superoxide production, to evaluate whether the

protective effect of silibinin on the regulation of mitochondrial homeostasis was related to the protection of mtROS production. As can be seen from the flow cytometry results shown in Fig. 6D and E, H_2O_2 markedly promoted mitochondrial superoxide generation. However, such effect was greatly mitigated by silibinin pretreatment. In parallel with these results, the red fluorescence of oxidized MitoSOX was found to be enhanced in cells treated with H_2O_2 but decreased in the presence of silibinin (Fig. 6F), suggesting that the source of ROS may be mitochondria. In particular, Mito-TEMPO, a potent mitochondria-targeted antioxidant, more obviously prevented H_2O_2 induced mitochondrial superoxide production (Fig. 6D–F). Overall, the current data demonstrated that H_2O_2 -induced cytotoxicity in ARPE-19 cells was mediated at least through the mitochondrial damage pathway by the generation of mtROS.

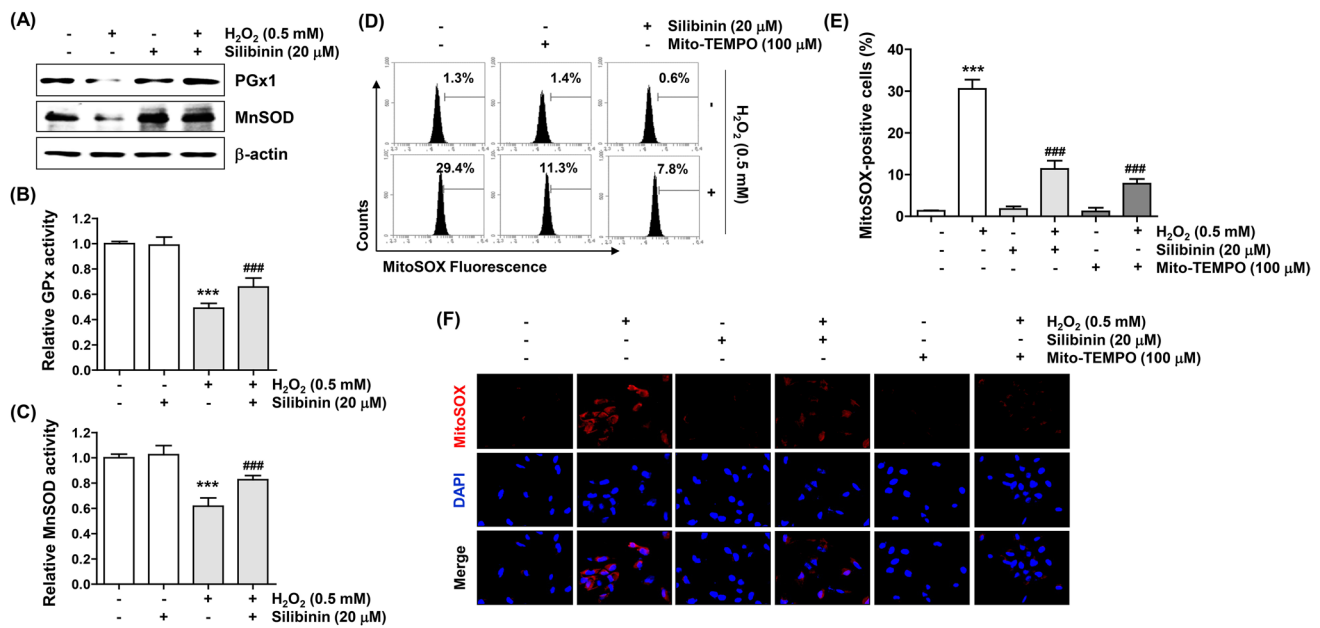


Fig. 6 Elimination of H_2O_2 -induced mtROS production by silibinin in ARPE-19 cells. Before treating cells with H_2O_2 for 24 h (A–C) or 1 h (D–F), cells were cultured in a medium with or without silibinin or Mito-TEMPO for 1 h. **A** Changes in expression of PGx1 and MnSOD proteins were obtained through immunoblotting using antibodies corresponding to each protein. **B** and **C** PGx and MnSOD activities of cells were estimated compared to the control group using

the corresponding assay kit. **D** and **E** To quantitatively analyze mitochondrial superoxide levels, flow cytometry was performed after staining with MitoSOX. Representative histogram (**D**) and quantitative analysis results (**E**) were shown. (**F**) After staining the cells using MitoSOX (red) and DAPI (blue), the stained cells were visualized by fluorescence microscopy and representative fluorescence images were shown

Discussion

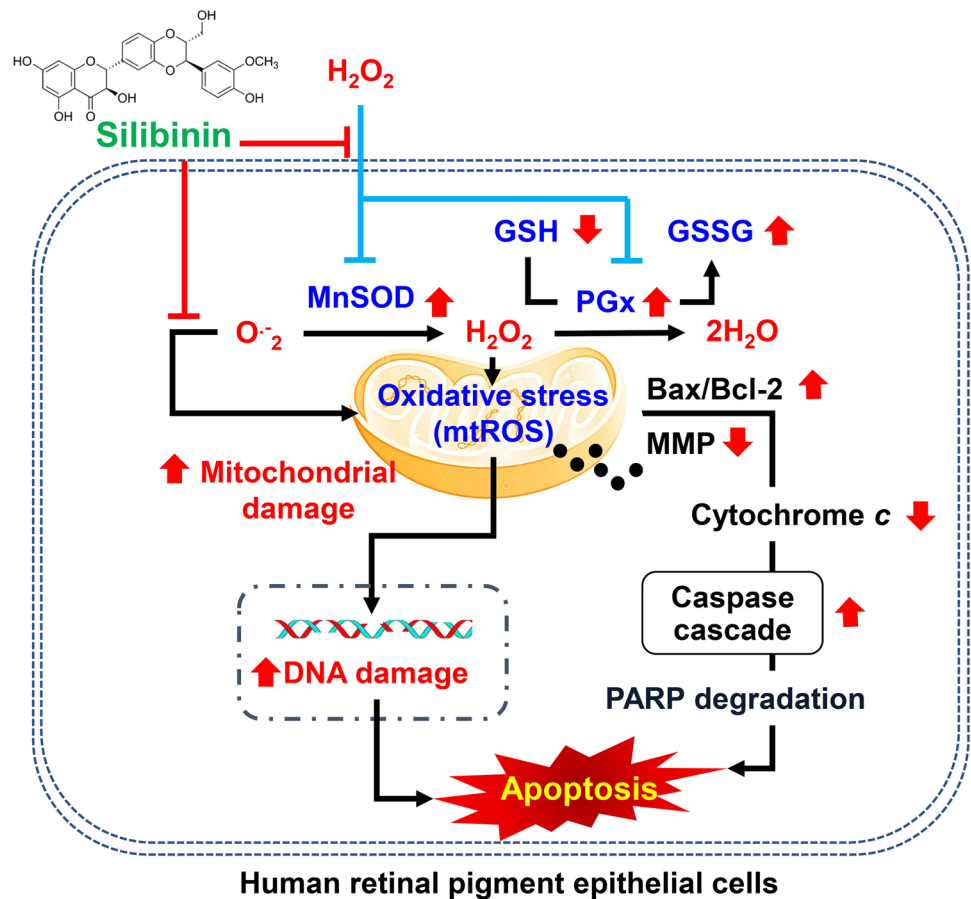
Several previous studies have reported the applicability of silibinin to block ocular diseases (Pooja et al. 2022; Shen et al. 2019; Goswami et al. 2018; Chen et al. 2017; Lin et al. 2013), but its efficacy against oxidative damage to RPE cells has not yet been properly evaluated. Therefore, in the current study, it was investigated whether silibinin could reduce oxidative damage elicited by H_2O_2 in RPE ARPE-19 cells. According to the results of this study, oxidative stress caused mitochondrial damage, leading to DNA damage and apoptosis in ARPE-19 cells, but silibinin maintained cell stability by inhibiting mtROS generation (Fig. 7).

There is growing evidence that DNA and mitochondrial damage induced by oxidative stimuli in RPE cells is closely associated with the induction of ROS-dependent apoptosis. Indeed, many previous results have shown that the cytotoxic effects of H_2O_2 , known as the most stable ROS, on RPE cells are mostly related to mitochondrial dysfunction and apoptosis associated with damage to intracellular macromolecules including DNA (Park et al. 2023; Clementi et al. 2022; Hernandez et al. 2021). According to results of the present study, silibinin could attenuate the process of H_2O_2 damaging the viability and cell morphology of ARPE-19 cells. And the cytotoxic inhibitory ability of silibinin against H_2O_2 was also clearly shown in the analysis of LDH release,

an indicator of damage to cell membrane integrity. However, in cells treated with silibinin alone, there was no significant difference in cell viability, cell shape, LDH release, etc. compared to the control group. Marazita et al. (2016) have shown that oxidative damage in RPE cells is critically involved in the initiation of AMD by causing DNA damage and promoting premature cellular senescence. In addition, H_2O_2 -induced apoptosis of ARPE-19 cells was accompanied by DNA damage, and silibinin was able to significantly block this, as evidenced by suppression of DNA tail formation and γ H2AX expression, which are hallmarks of DNA double-strand breaks (Cordelli et al. 2021; Kopp et al. 2019). Silibinin also normalized the level of 8-OHdG, a widely used biomarker for oxidative stress in nucleic acids (Hahm et al. 2022), increased by H_2O_2 treatment. Therefore, the current results document that silibinin abolished oxidative stimuli-mediated DNA damage in RPE cells as well as in other cells.

Many previous studies have already proven that oxidative stress has a pro-apoptotic effect on RPE cells through mitochondrial dysfunction, which is a major cause closely related to the degeneration of RPE cells (Zhang et al. 2023; Tong et al. 2022). As is well known, excessive accumulation of ROS caused by oxidative stress contributes to mitochondrial membrane depolarization, resulting in MMP loss, indicative of mitochondrial damage. Mitochondrial depolarization can

Fig. 7 Schematic diagram showing the blocking effect of silibinin on oxidative damage in ARPE-19 cells. Silibinin counteracted mitochondrial dysfunction, genotoxicity, and apoptosis by inhibiting H_2O_2 -induced mtROS production in ARPE-19 cells



trigger cytoplasmic release of cytochrome *c*, which in turn stimulates the activation of the caspase cascade, the central mediating pathway of apoptosis, and leads to initiation of mitochondria-mediated apoptotic pathway, ultimately resulting in degradation of target proteins of effector caspases such as PARP for the induction of apoptosis (Bock and Tait 2020; Kiraz et al. 2016). Consistent with several previous findings (Zou et al. 2023; Park et al. 2023; Clementi et al. 2022), H_2O_2 exposure significantly induced apoptosis in ARPE-19 cells, accompanied by accumulation of intracellular total ROS, as assessed using annexin V/PI and DCF-DA assays. In addition, H_2O_2 administration decreased the intensity of MitoTracker Red, consistent with the loss of MMP according to the JC-1 assay, indicating disruption of mitochondrial membrane stability. Moreover, in ARPE-19 cells treated with H_2O_2 , an increase of the Bax/Bcl-2 protein expression ratio, cytosolic translocation of cytochrome *c*, and cleavage of PARP by activation of caspase-3, a key contributor to the apoptosis process, were observed. In the mitochondria-mediated apoptotic pathway, pro-apoptotic proteins including Bax are involved in mitochondrial pore formation, which disrupts mitochondrial membrane stability, while anti-apoptotic proteins such as Bcl-2 play the opposite role (Lalier et al. 2022; Kiraz et al. 2016). Therefore, when

the Bax/Bcl-2 ratio increases, mitochondrial membrane permeability is increased and cytochrome *c* release from mitochondria to the cytosol is enhanced. However, all of these effects due to H_2O_2 were apparently eliminated in the presence of silibinin. These findings may be causally linked to the blockade of the caspase-3-dependent apoptotic pathway by oxidative stress following repair of H_2O_2 -mediated MMP loss by silibinin.

The level of ROS within cells is tightly regulated by a series of oxidative defense systems. Among them, GSH, which acts as a cofactor for intracellular antioxidant enzymes, removes ROS and electrophiles, so the ratio of GSH to GSSG can be used as a measure of the redox state of cells (Lou 2022; Enns and Cowan 2017). Indeed, even in RPE cells, accumulating evidence have shown that increasing the ratio of GSH/GSSG can reduce H_2O_2 -induced cytotoxicity, whereas lowering this ratio can increase H_2O_2 -induced apoptosis. According to results of this study, the GSH/GSSG ratio was reduced in ARPE-19 cells cultured in medium supplemented with H_2O_2 . However, as observed in several previous studies (Tie et al. 2022; Guo et al. 2020), such altered GSH/GSSG ratio was apparently restored by silibinin, which was related to the quenching of ROS production. Silibinin also increased the expression and activity of

GPx involved in H₂O₂ detoxification, as shown in previous results (Aamani et al. 2022; Kalemci et al. 2015), suggesting that the preventive effect of silibinin against oxidative damage was mediated by attenuation of reactive oxygen intermediates. Mitochondria are organelles vulnerable to ROS and are also main sources of ROS such as H₂O₂ and superoxide in eukaryotic cells (Zia et al. 2022; Demine et al. 2019). Moreover, H₂O₂-induced mtROS release mediates mitochondrial depolarization (Anupama et al. 2023; Park and Choi 2012), contributing to the activation of the mitochondria-mediated apoptotic pathway. Among SODs, MnSOD, which is localized in the mitochondrial matrix, acts as an enzyme that removes mitochondrial superoxide to protect cells from oxidative damage (Islam et al. 2022; Palma et al. 2020). The antioxidant activity of MnSOD comes from its ability to decompose two molecules of superoxide anion (O₂⁻) into oxygen (O₂) and H₂O₂ (Liu et al. 2022; Palma et al. 2020). Although the possibility that MnSOD is involved in the antioxidant activity of silibinin has been suggested (Zapavigna et al. 2019), it is not yet clear whether it is related to mitochondrial function and accumulation of intracellular ROS. According to the present results, the expression and activity of MnSOD were reduced in ARPE-19 cells treated with H₂O₂, but silibinin clearly reversed this. In addition, the results of flow cytometric analysis using MitoSOX showed that the level of mitochondrial superoxide was significantly weakened in cells pretreated with silibinin before treatment with H₂O₂, and this was further confirmed through fluorescence microscopy. Since superoxide is converted to membrane permeable H₂O₂, they can readily diffuse into cells (Yan et al. 2020; Munro and Treberg 2017), these results indicate that the ROS scavenging activity of silibinin is due to the reduction of mitochondrial superoxide generation by preserving MnSOD activity. These data therefore suggest that mtROS generation is upstream of mitochondrial dysfunction. This notion may be further supported by the results showing that NAC or Mito-TEMPO abrogated H₂O₂-induced intracellular ROS generation and mitochondrial superoxide, respectively. Although the molecular mechanisms for the correlation of activities of GSH, GPx and MnSOD require further investigation, these results indicate that silibinin can protect ARPE-19 cells from genotoxicity under conditions of oxidative stress while exerting mtROS scavenging activity. However, it is imperative to investigate whether silibinin can modulate oxidative damage-mediated ocular diseases *in vivo* in further studies.

Conclusion

The present study showed that silibinin could eliminate H₂O₂-induced genotoxicity and apoptosis while alleviating mitochondrial damage in ARPE-19 cells. Moreover, silibinin

blocked H₂O₂-induced intracellular ROS production while preserving GSH content and GPx activity, and inhibition of mitochondrial superoxide generation was associated with restoration of MnSOD activity. These results demonstrate that blocking mtROS generation by silibinin may act as an upstream event in the induction of DNA damage and mitochondria-mediated apoptosis (Fig. 7). This is the first report showing that silibinin plays a critical role in the inhibition of oxidative stress-induced RPE cell demise. Collectively, these findings support the preventive potential of silibinin, acting as a mtROS scavenger, in oxidative damage-related ocular disease.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Yung Hyun Choi declares that he has no conflict of interest.

Ethical approval The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

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