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S-allyl cysteine protects retinal pigment epithelium cells from hydroquinone-induced apoptosis through mitigating cellular response to oxidative stress

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Abstract. – OBJECTIVE: Retinal pigment epithelium (RPE) degenerative death is an evident hallmark of advanced age-related macular degeneration (AMD). The present study aims to evaluate the protective effects of S-allyl L-cysteine (SAC), a bioactive component from aged garlic extracts, on the oxidative stress-related apoptosis of RPE cells and to investigate the potential underlying mechanisms.

MATERIALS AND METHODS: Cell Counting Kit-8 (CCK-8) assay, flow cytometry, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining were performed to evaluate the effects of SAC on the hydroquinone-treated human ARPE19 cells. The Reactive Oxygen Species (ROS) production was measured by virtue of flow cytometry or determined under an inverted fluorescence microscope. Furthermore, the expression of antioxidant factor Nrf2, as well as downstream antioxidant genes, including NQO1, SOD1, SOD2, and HO1 was assessed in hydroquinone stimulated ARPE19 cells, in the presence or absence of SAC pretreatment.

RESULTS: Hydroquinone incitement contributed to a marked decrease in cell viability, but enhanced cell apoptosis, whereas SAC addition did not cause significant alterations. When cells were pre-treated with SAC, cell proliferation was dramatically enhanced whereas apoptosis was mitigated, and the ROS generation induced by hydroquinone was also significantly suppressed, indicating a prominent function of SAC in preventing ARPE19 cells from oxidant-related apoptosis. The elevated expression levels of Nrf2 and other antioxidant genes driven by hydroquinone were downregulated by SAC addition.

CONCLUSIONS: These data suggest that SAC can effectively attenuate hydroquinone-induced oxidative damage in human RPE cells. Our work is the first to demonstrate that SAC modulates oxidative stress-induced RPE apoptosis, thereby potentially proving new insights into the treatment of AMD.

Key Words:

Age-related macular degeneration, Retinal pigment epithelium, Apoptosis, S-allyl cysteine, Oxidative stress.

Introduction

Age-related macular degeneration (AMD) is an acquired disease of the macula that represents the predominant cause of central visual loss among the population over 50 years old in developed countries[1]. During recent decades, the morbidity of AMD has been dramatically increased worldwide as a result of the rapidly growing aging population[2]. AMD can be clinically classified into either dry or wet form, with the former comprising ~85% of all diagnosed cases. Retinal pigment epithelium (RPE) is a single layer of non-dividing cells which play a crucial role in the maintenance of neuroretinal hemostasis and photoreceptor cell survival. RPE cell death caused by oxidative stress, mostly attributed to apoptosis, as well as necrosis, is significantly implicated in AMD pathogenesis[3-5]. Furthermore, during the process of aging RPE undergoes an array of pathological changes that will cause deposits of lipofuscin within the macula, which is known as drusen. Individuals presented with drusen and other ocular disturbances usually have an increased risk of progressing into advanced dry AMD or even vision loss[6].

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Although the etiology of AMD is complicated and heterogeneous, there are multiple established risk factors which are responsible for this disease, including aging, gene polymorphisms, as well as several environmental factors such as cigarette smoking, dietary habits, and phototoxic exposure[7]. Among them, active or passive smoking has been considered as an independent risk factor for the initiation and/or development of AMD[8,9]. Using experimental animal models, Fujihara et al[10] found that the exposure to smoke for a long term leads to RPE apoptosis and oxidative injury. Additionally, apoptotic RPE cell death was also induced by hydroquinone, a potent pro-oxidative component in cigarette tar[11,12]. Hydroquinone also suppresses the RPE expression of MCP1 and thereafter impedes the recruitment of scavenging macrophages, which ultimately accelerates the deposition of pro-inflammatory debris in the RPE[13].

S-allyl L-cysteine (SAC), as the most abundant ingredient derived from fermented black garlic, exhibits a variety of beneficial functions including anti-carcinoma[14,15], anti-oxidation[16-20], anti-diabetes[17,21], anti-inflammation[17,21], and neuroprotection[20,22]. Overwhelming lines of evidence demonstrate that SAC effectively alleviates cell apoptosis induced by oxidative stress by promoting the antioxidant defense system[16,23-26]. However, the effect of SAC on hydroquinone exposure-related RPE apoptosis remains elusive. We herein evaluated the protective function of SAC in RPE impairments induced by hydroquinone and investigated the underlying mechanisms.

Materials and Methods

Cell Culture

Human retinal pigment epithelial cell line ARPE-19 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and starved overnight with the serum-free DMEM/F12 medium until growing to 80% confluence, before they were subjected to further experiments.

Preparation of Agents

SAC (Sigma-Aldrich, St Louis, MO, USA) was diluted with phosphate-buffer solution (PBS) and stored at -20°C, with a stock concentration of 50 mM. Hydroquinone (Adamas, Shanghai, China) was freshly prepared for every experiment. It was dissolved in PBS and diluted to a final concentration of 200 μM.

Cell Counting Kit-8 (CCK-8) Assay

RPE cell viability was assessed using the Cell Counting Kit-8 (MeilunBio, Dalian, Liaoning, China) according to the manufacturer's protocols. In brief, the cells (5×10^3 cells/well) were plated into 96-well plates and grown to 80% confluence. After being quiescent overnight with the serum-free DMEM/F12 medium, the cells were pre-treated with SAC for 2 h followed by incubation with 200 μM hydroquinone for 48 h. Afterward, 10 μL of CCK-8 solution was added to each well, and the cells were incubated at 37°C for other 2 h. The absorbance of the samples was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Assay

Apoptotic cells were detected using the *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. Briefly, the cells on the coverslips were fixed in 4% PFA for 25 min before permeabilization for 2 min on ice with 0.1% citrate buffer supplemented with 0.1% Triton X-100. Afterward, the coverslips were incubated at 37°C in TUNEL reaction mix containing nucleotides and terminal deoxynucleotidyl transferase (TdT) for 1 h. DAPI reagent was added after three washes with PBS, and the coverslips were mounted to a slide. TUNEL staining-positive cells were visualized under a fluorescence microscope (Olympus, Melville, NY, USA).

Flow Cytometry

Annexin V-fluorescein isothiocyanate (FITC)/ Propidium Iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to assess the apoptosis rate of ARPE19 cells. The cells were harvested and washed twice with pre-chilled PBS, and resuspended in binding buffer, and then incubated with 5 μL of annexin V-FITC and 5 μL of PI for 15 min in the dark before they were subjected to analysis with a flow cytometer (FACScan, BD Biosciences, San Jose, CA, USA).

Reactive Oxygen Species (ROS) Analysis

The content of ROS in ARPE19 cells was measured using the specific ROS detection kit (MeilunBio, Dalian, China). In brief, the cells were pre-treated with 50 μM SAC for 2 h and then incubated with 200 μM hydroquinone for another 2 h, followed by the addition of 10 μM DCFH-DA and 1 hour-incubation at 37°C in the dark. After washing thoroughly with serum-free medium, the cells were observed under an inverted fluorescence microscope. ROS production was determined by fluorescence spectroscopy with excitation/emission at 488 nm / 525 nm.

Morphological Examination

The cells were seeded into 6-well plate and treated with 50 μM SAC for 2 h at 80% confluence, followed by exposure to 200 μM hydroquinone and incubation for 48 h. Cell morphological changes were observed under an inverted phase-contrast microscope (Olympus, Melville, NY, USA).

Western Blotting

The total proteins were extracted from cell lysates with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide) before being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were then blocked with 5% non-fat milk for at least 1 h and incubated overnight at 4°C with specific primary antibodies against HO-1, NQO-1, SOD1, SOD2 (Cell Signaling Technology, Beverly, MA, USA), Nrf2 (Abcam, Cambridge, UK), or anti-tubulin (OriGene Technologies, Rockville, MD, USA). After rinsing with Tris-Buffered Saline and Tween 20 (TBST), the membrane was then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG (CWBio, Beijing, China). After rinsing with TBST, the labeled proteins were visualized with enhanced chemiluminescence (ECL) substrates (Millipore, Billerica, MA, USA).

Statistical Analysis

Data are presented as means ± SD (standard deviation). All experiments were repeated 3 times independently. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). All statistical analyses were carried out using the Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Inc., Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

Results

SAC Reverses the Impaired Cell Viability Caused by Hydroquinone in a Dose-Dependent Manner

Human ARPE19 cells were treated with 200 μM hydroquinone before the CCK-8 assay was performed. As shown in Figure 1A, there was a remarkable decline in cell viability after treatment for at least 48 h. In order to evaluate the impact of SAC on cell viability, ARPE-19 cells were pre-treated with a concentration series of SAC before hydroquinone exposure. The effect of SAC on cell viability was barely detectable when it was employed alone (Figure 1B, $p>0.05$). Of note, the impaired cell viability caused by hydroquinone was reversed in the presence of SAC (Figure 1C, $p<0.05$), and this effect was quite evident when the concentration of SAC was only 2 μM. The cell viability was increased in parallel with the elevation of SAC concentration and reached a peak value when it was increased to 50 μM.

SAC Suppresses Hydroquinone-Induced RPE Cell Apoptosis

ARPE-19 cells were exposed to hydroquinone with or without pretreatment of SAC, after which the cells were examined for apoptotic status by using flow cytometry and TUNEL assay. According to the Annexin-V/PI flow cytometry analysis (Figure 2A), the proportion of apoptotic cells was dramatically elevated upon hydroquinone stimulation in the absence of SAC ($p<0.05$). The results also showed that the cell apoptosis rate was significantly hindered when ARPE-19 cells were treated with hydroquinone in combination with SAC. Likewise, under the fluorescence microscopy, we observed that SAC addition resulted in a decrease in fluorescein-labeled apoptotic cells when compared to the cells treated with hydroquinone alone (Figure 2B, $p<0.05$). These data indicate that pre-exposure to SAC mitigates hydroquinone induced RPE cell apoptosis while SAC *per se* has no effect on these cells.

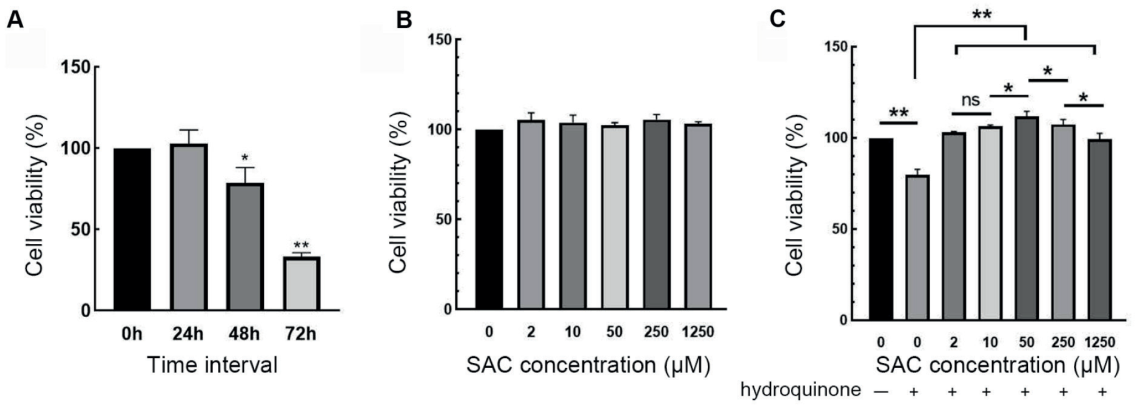

Figure 1. SAC protects RPE cells from hydroquinone-induced death. **A,** ARPE-19 cells were stimulated with 200 μM hydroquinone and cell viability was evaluated at different time intervals by using CCK-8 assay. **p*<0.05; ***p*<0.01 compared with the control group. n=3 independent experiments. **B,** The cells were treated with SAC of escalating concentrations for 48 h, followed by assessment of cell viability. **C,** The cells were pre-treated with SAC for 2 h, and then incubated with 200 μM hydroquinone for another 48 h. **p*<0.05; ***p*<0.01; and ns indicated that the difference is not significant. n=3 independent experiments.

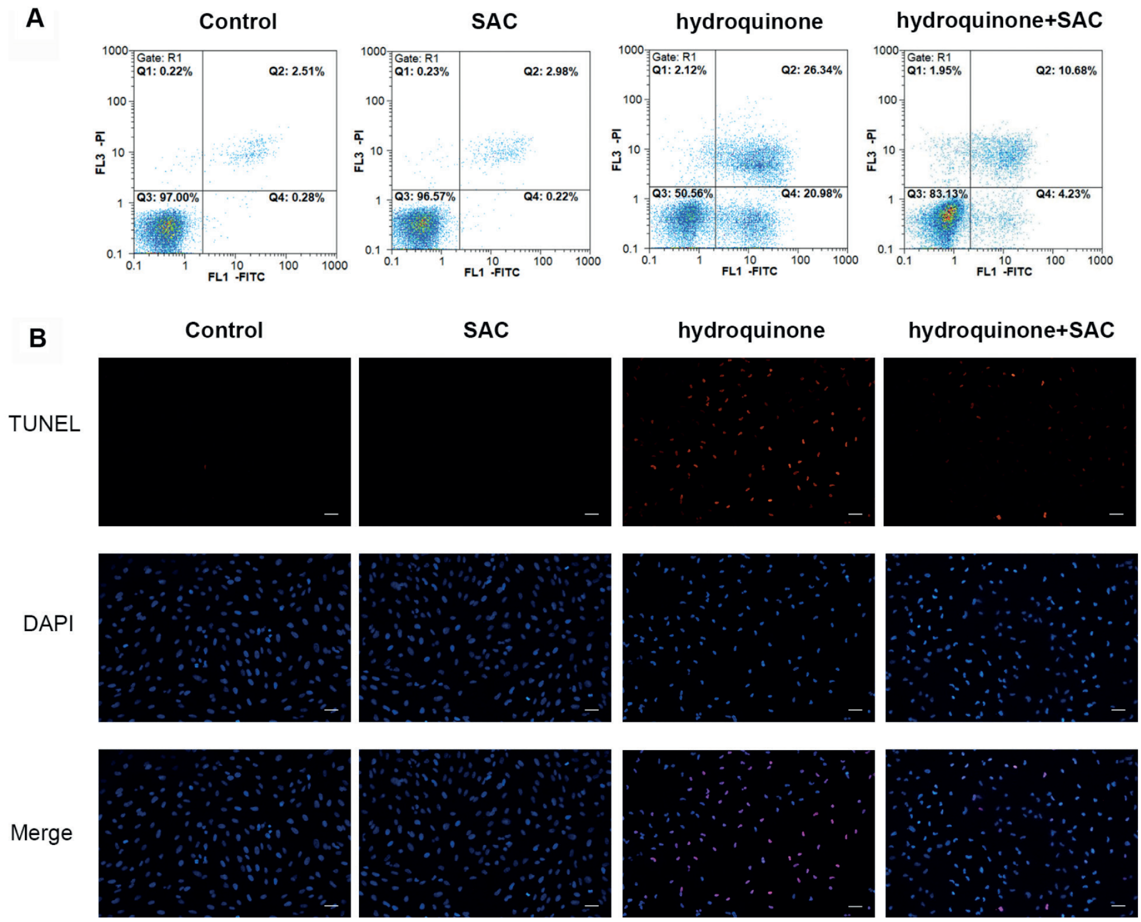

Figure 2. Hydroquinone induces apoptosis in ARPE-19 cells, which was attenuated by SAC. ARPE-19 cells were exposed to 200 μM hydroquinone for 24 h in the presence or absence of 2 h- pretreatment of 50 μM SAC. The apoptosis rate of ARPE-19 cells was detected by flow cytometric analysis (**A**) or TUNEL assay (**B,** scale bar: 100 μm). ***p*<0.01 compared to the control group; and ##*p*<0.01 compared with cells treated with hydroquinone alone. **C,** Representative images of the morphological changes of ARPE-19 cells in different groups (scale bar: 100 μm).

Figure continued

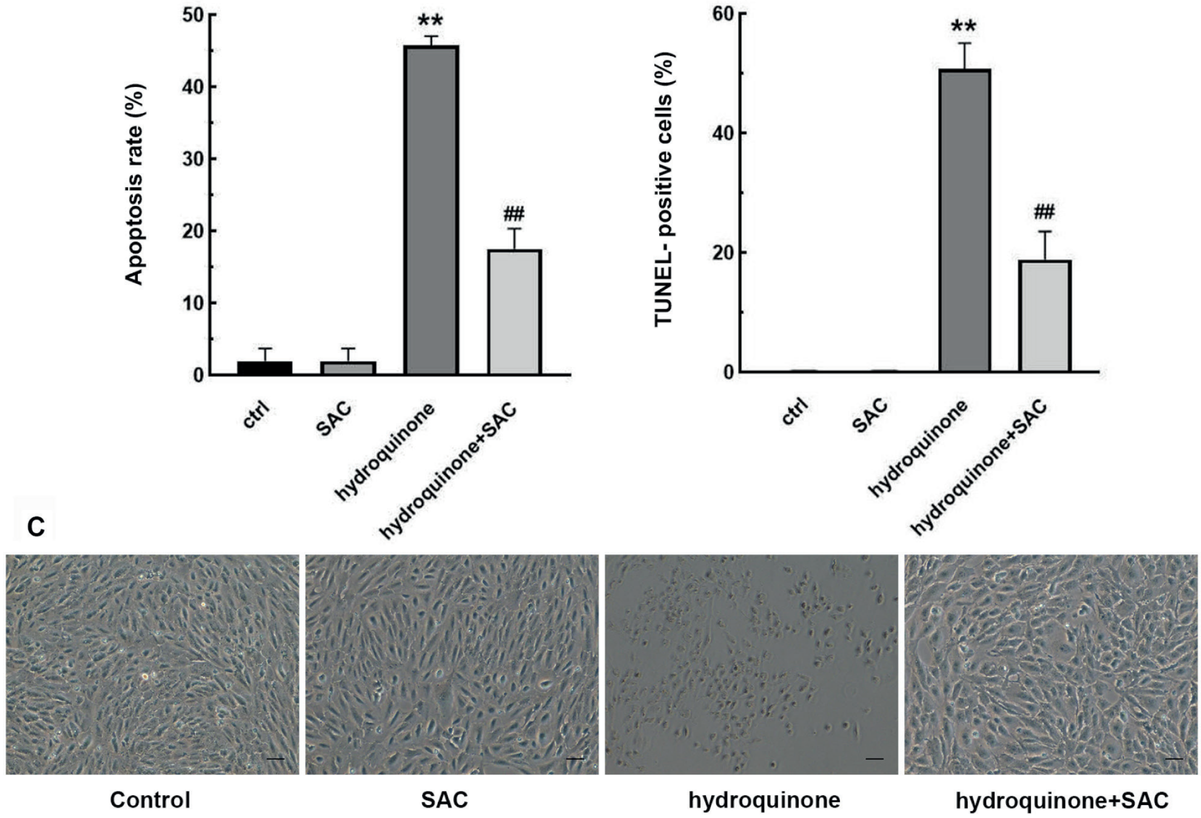

Figure 2. *(Continued).* Hydroquinone induces apoptosis in ARPE-19 cells, which was attenuated by SAC. ARPE-19 cells were exposed to 200 µM hydroquinone for 24 h in the presence or absence of 2 h- pretreatment of 50 µM SAC. The apoptosis rate of ARPE-19 cells was detected by flow cytometric analysis (**A**) or TUNEL assay (**B,** scale bar: 100 µm). $^{**}p<0.01$ compared to the control group; and $^{\#\#}p<0.01$ compared with cells treated with hydroquinone alone. **C,** Representative images of the morphological changes of ARPE-19 cells in different groups (scale bar: 100 µm).

SAC Attenuates the Morphological Changes of ARPE-19 Cells Treated With Hydroquinone

In addition, to verify the inhibitory effect of SAC on hydroquinone-induced cell apoptosis, morphological changes in ARPE-19 cells were observed under an inverted microscope. As shown in Figure 2C, normal RPE cells were in a typical spindle-shaped and elongated shape. Also, SAC exposure did not lead to apparent morphological changes in RPE cells. Following hydroquinone exposure, the number of adherent cells decreased, and the cells displayed characteristic apoptotic morphology, including cell shrinkage, chromatin condensation, and the formation of apoptosis body-like structures. However, the abovementioned apoptotic phenotype was not found in the hydroquinone-treated cells undergoing pretreatment with SAC.

SAC Decreases ROS Generation Induced by Hydroquinone

To determine whether hydroquinone-induced apoptosis is related to intracellular oxidative stress, the reactive oxygen species (ROS) level produced by ARPE-19 cells was assessed after hydroquinone treatment, in the presence or absence of SAC pretreatment. As shown in Figure 3A and 3B, hydroquinone stimulation contributed to a considerable increase in ROS generation in ARPE-19 cells ($p<0.05$), while the ROS production was not significantly altered in response to SAC treatment alone. Remarkably, after SAC pretreatment, the ROS level in hydroquinone-stimulated cells was evidently lower in comparison to the cells treated by hydroquinone alone ($p<0.05$). The results suggest that SAC probably protects ARPE-19 cells from oxidative-initiated apoptosis mainly by reducing ROS accumulation.

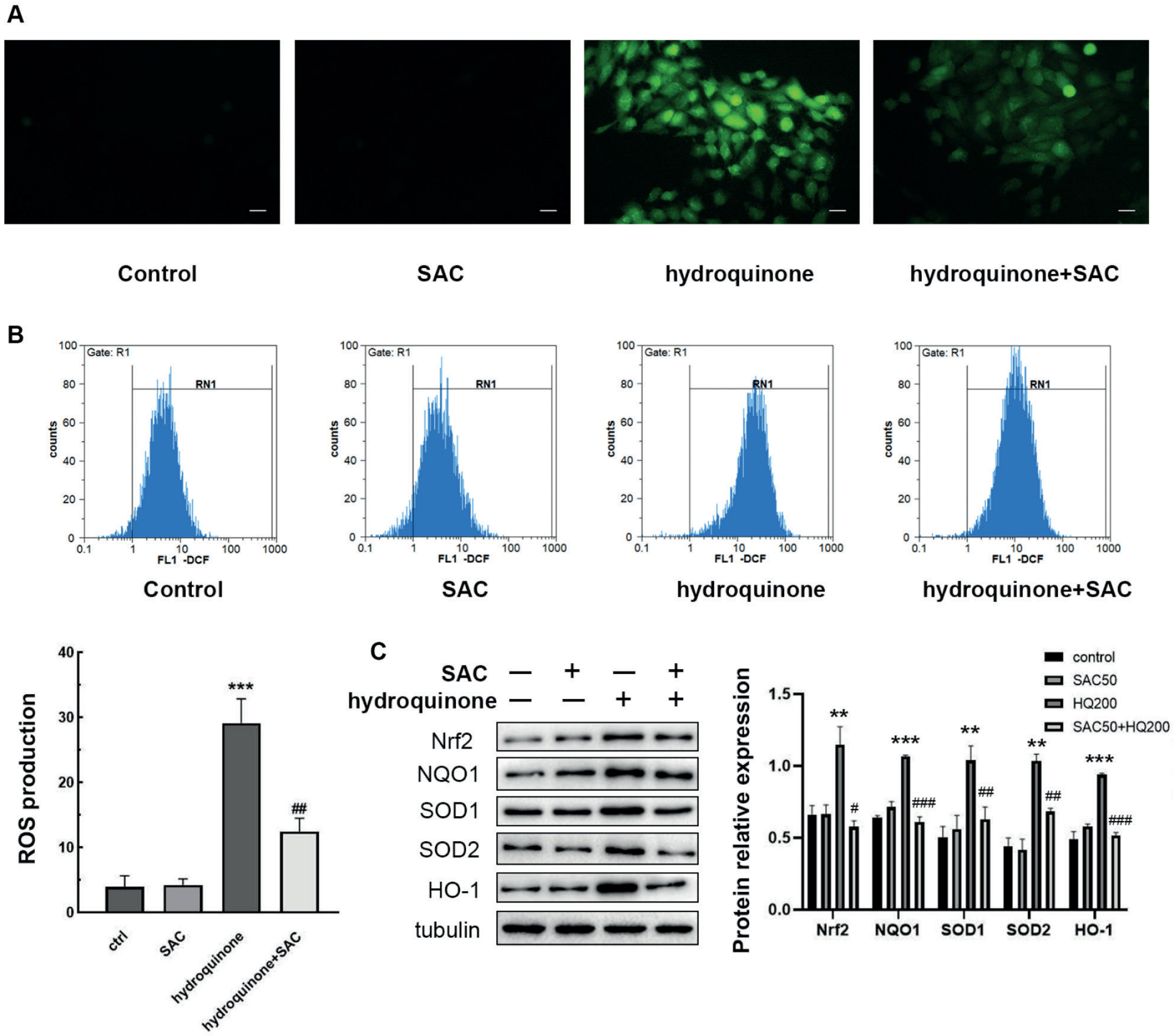

Figure 3. SAC mitigates hydroquinone-stimulated anti-oxidative response in RPE cells. ARPE-19 cells were exposed to 200 μM hydroquinone in the presence or absence of pretreatment with 50 μM SAC, and then **(A)** DCFH-DA-stained cells were observed under fluorescence microscope. Scale bar: 50 μm. **B,** ROS production was otherwise assessed by flow cytometry with excitation/emission at 488 nm/525 nm. ***p<0.001 compared to the control group; ##p<0.01 compared with cells treated with hydroquinone alone. **C,** The expression levels of antioxidant-related proteins were measured by Western blot analysis. Tubulin was used as an internal control. *p<0.05, **p<0.01, ***p<0.001 compared to the control group, and #p<0.05, ##p<0.01, and ###p<0.001 compared with cells treated with hydroquinone alone. n=3 independent experiments.

The Altered Expression Profiling of Anti-Oxidative Factors

Nrf2 is a key regulator of the cellular redox state and its activation promotes the transcription of several antioxidant mediators. As expected, treatment with low-dose SAC (50 μM) did not lead to significant alterations in the expression of Nrf2 compared to the control group (p>0.05, shown in Figure 3C). Otherwise, Nrf2 expression in ARPE-19 cells was significantly aggrandized after hydroquinone incitement (p<0.01), because the cellular antioxidant defense was induced in response to the oxidant hydroquinone. Also, the expression patterns of other antioxidant factors, including NQO1, SOD1, SOD2, and HO1 were examined through immunoblotting, where analogous results were observed. As shown in Figure 3B, SAC treatment did not change the expression of these proteins in ARPE-19 cells whereas their levels were evidently elevated after exposure to hydroquinone.

In parallel with SAC addition, intriguingly, the hydroquinone-induced augmented expression levels of the abovementioned antioxidant proteins were remarkably decreased in comparison with those incited with hydroquinone alone, indicating that SAC effectively reduces the cellular oxidative stress irritated by hydroquinone.

Discussion

So far, no effective therapeutic strategy has been developed for dry AMD, which ultimately progresses into irreversible visual impairments or loss. One of the principal pathological hallmarks of late-stage dry (atrophic) AMD is the malfunction and cell death of RPE[4]. Hydroquinone is a potent pro-oxidative component in cigarette smoke and it has been proposed to induce mitochondrion-mediated apoptotic RPE cell death[5,27]. Mitochondria are the primary source of cellular ATP and ROS production and, in addition, they contain a self-destructive arsenal of apoptotic factors that can be unleashed to promote cell apoptosis. As mentioned in previous study[5], cigarette smoke exposure increased the mitochondrial ROS accumulation, which disturbed intracellular oxidative homeostasis and reduced mitochondrial potential, thereby causing mitochondria fragmented and diffusely distributed in RPE cells and ultimately contributing to cell apoptosis. On the other hand, hydroquinone can also suppress RPE expression of MCP1, a potent chemokine to recruit scavenging immunocytes, such as macrophages, and thereby exacerbate the accumulation of inflammatory debris in RPE, which contributes to the degenerative damage of it[13]. Therefore, we used human RPE cells treated with hydroquinone in the present work to mimic the oxidative damage which plays a significant role in AMD development[28-31], and found that the viability of hydroquinone stimulated ARPE19 cells was significantly decreased (20.23% reduction from the control), whereas the apoptosis rate was higher than untreated cells (43.79% ± 1.04% *vs.* 2% ± 0.52%, p<0.05), and this was consistent with previous reports.

The antioxidant garlic derivative SAC was previously proved to exert neuroprotective effects on the kainite excitotoxicity-induced degenerative damage of retinal ganglion cells (RGCs), and thus prevent retinal ischemia which is responsible for the development of many ocular disorders[22]. Our results showed that pretreatment of SAC before the hydroquinone exposure resulted in a significant increase in RPE cell viability, and meanwhile, blunted the hydroquinone-induced cell apoptosis, as compared to those treated with hydroquinone alone. The cell viability reached a peak value when SAC concentration was increased to 50 μM, indicating that SAC exerts its protective effect on RPE cells even at a low concentration.

Envisioned as a sort of crucial signaling molecules, ROS participate in diverse physiological cell events, such as adaptation to hypoxia, autophagy, immunity, and differentiation[32]. However, redundant ROS may contribute to cell death through oxidation of membrane lipids and proteins, which is a fundamental pathomechanism of several oxidative stress-related diseases, such as diabetes mellitus and neurodegenerative diseases[33]. In the present study, the generation of ROS was found evidently aggrandized in ARPE19 cells treated with hydroquinone, in comparison to the untreated cells. In the presence of SAC, the augmented ROS production induced by hydroquinone was evidently abated, although treatment of SAC alone did not lead to significant changes in ROS level. These results suggest that SAC inhibits the hydroquinone-induced apoptosis of ARPE-19 cells by, at least in part, impinging on the oxidation-related signaling pathways.

In this context, an essential antioxidant mediator Nrf2 was dramatically upregulated after ARPE19 cells were treated with hydroquinone, which reflected increased oxidative stress upon hydroquinone stimulation. This agrees with previous study displaying that Nrf2 acts as a response factor to reduce oxidative stress and protect RPE cells from cigarette smoke-induced apoptosis[27,34-36]. Notably, when ARPE19 cells were pre-treated with SAC before hydroquinone stimulation, the enhanced expression of Nrf2 induced by hydroquinone was significantly suppressed. Furthermore, similar changes were observed in the expression of downstream antioxidant genes, including NQO1, SOD1, SOD2, and HO1. These data suggest that SAC protects ARPE-19 cells from apoptosis mainly by repressing hydroquinone-triggered oxidative stress.

To the best of our knowledge, it is the first time that SAC has been proved to alleviate the hydroquinone-initiated oxidative damage within human RPE cells, which is tightly related to AMD pathogenesis[37-39], thereafter driving the improvement of novel therapeutic strategies. However, our *in vitro* data are not adequate to conclude that SAC can directly ameliorate the development of AMD. The rats exposed to additional oxidative stresses, such as sodium iodate and cigarette smoke/hydroquinone, usually serve as representative animal models for AMD and they have helped to reveal the underlying pathological mechanisms of this disease. These animal models are applied in our on-going experiments to verify the protective role of SAC in the development of AMD, and further results will be published in the future.

Conclusions

In summary, our results demonstrate that SAC effectively attenuates hydroquinone-induced oxidative damage in human RPE cells, represented by the significantly reduced apoptosis and ROS production. Furthermore, the pretreatment of SAC downregulated Nrf2, which was induced otherwise by hydroquinone, and similar alterations of expression profiles were observed in the downstream antioxidant genes. These data suggest that SAC exerts its effects by suppressing cell response to the detrimental oxidative stress initiated by hydroquinone.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Acknowledgments

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