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Research Article
Ursolic Acid Protects Human Retinal Pigment Epithelial Cells Against Hyperglycemia-Triggered Oxidative Damage and Senescence

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Abstract

Background and Objective: Previous studies have manifested that oral administration of ursolic acid (UA) to type 2 diabetic rats prevents vascular aging. However, no study has yet revealed the effect of UA on retinal pigment epithelial (RPE) cellular impairment in diabetic retinopathy (DR). This study aimed to analyze the effect of UA on hyperglycemia-induced oxidative stress damage and senescence in RPE cells. **Materials and Methods:** In this study, RPE cells were used as research subjects, cultured in a hyperglycemia medium to establish an *in vitro* injury model of RPE and the cells were intervened with UA at a final concentration of 2 μM. After the intervention, Lactate Dehydrogenase (LDH) release, apoptosis rate, SA-β-gal positive cell rate, p21 and p16 protein levels, reactive oxygen species (ROS) content, superoxide dismutase (SOD), Glutathione (GSH) contents and the expression of Sirtuin 1 (SIRT1) were analyzed. The differences among groups were analyzed using one-way analysis of variance. **Results:** Compared with normal medium, hyperglycemia medium caused a significant increase in LDH release, apoptosis rate, SA-β-gal positive rate, P21 and P16 protein levels and ROS content of RPE cells and led to a decrease in SOD and GSH contents and SIRT1 expression; which were ameliorated by the addition of UA. The UA has no significant effect on the above indicators in hyperglycemia-treated RPE cells when SIRT1 was silenced. **Conclusion:** In summary, UA reduces oxidative stress injury-mediated apoptosis and senescence in RPE cells under hyperglycemia conditions by activating SIRT1 expression.

Key words: Diabetic retinopathy, ursolic acid, oxidative stress, senescence, SIRT1

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease with a high prevalence that damages many organs and tissues of the body[1]. The total number of DM patients in the world has exceeded 300 million, of which the incidence of DM in China is also increasing year by year[2]. Diabetic retinopathy (DR), which involves micro-vessels and nerves, is a common complication of diabetes mellitus and may lead to vision-threatening or even blinding retinal damage and it has now become a major irreversible blinding eye disease in adults worldwide[3]. A study predicts that the number of people with DM will reach 552 million by 2030 and more than one-third of these patients will have symptoms of DR[4]. The development of DR is a long-term, complex process that usually occurs in different successive stages. It ranges from non-proliferative DR with an initial increase in vascular permeability to the development of vascular occlusion and eventually to proliferative DR with neovascularization of the retina and posterior vitreous surface. The pathogenesis of DR has not yet been clarified and most scholars believe that the basis of DR is chronic hyperglycemia, mismanagement of hypertension, dyslipidemia and obesity[5]. In addition, hyperglycemia, hypertension, duration of diabetes mellitus, abnormal lipids and formation of glycosylation end products have been shown to be independent risk factors for DR[6]. Prolonged hyperglycemia may lead to thickening of the retinal microvascular basement membrane, loss of vascular cells, increased vascular permeability and generation of neovascularization. Further, it may cause an imbalance in the oxidative/antioxidative effects of the body. In the advanced stages of DR, multiple mechanisms such as glycosylation end products, Toll-like receptors and oxidative stress-induced inflammatory responses produce complex interactions culminating in varying degrees of pathology[7-9]. Therefore, clarifying the specific pathogenesis of DR is indispensable for developing its therapeutic options.

Almost all retinal cell types are affected by diabetes during DR lesions, including apoptosis of neurons within the retina, dysfunction of Müller cells and astrocytes, activation of microglia and retinal pigment epithelium (RPE) dysfunction or degeneration[10]. The RPE cells are located between the retinal nerve cell layer and the choroid and their tight junctions are involved in the formation of the blood-retinal barrier, which functions as an extraretinal barrier[11]. Besides, RPE cells metabolically support photoreceptor cells and possess a variety of important physiological functions. In early studies of DR, it was found that it may be related to the disruption of the outer barrier function of RPE cells, which are regularly arranged in a hexagonal shape and whose structural and functional integrity is essential for the maintenance of the normal function of the entire retina[12]. The RPE cells have no regenerative capacity and in the event of apoptosis, neighboring cells make up for the excess space that occurs. Additionally, it has been found that RPE cells are sensitive to oxidative stress injury and during the development of DR, the high concentration of sugar in the organism tends to induce the formation of oxygen free radicals and cause oxidative stress injury[13]. Thus, the RPE extracellular barrier function is highly susceptible to hyperglycemia, which in turn results in altered pathological function. Moreover, DR was previously thought to be a microvascular disease. However, abnormalities in retinal function such as reduced contrast sensitivity, delayed dark adaptation and visual field abnormalities have been found in patients with diabetes in the early stages of the disease when funduscopic examination did not reveal significant microvascular abnormalities. Nowadays researchers are aware of neuronal dysfunction and neurodegeneration in the early stages of DR. It notably states that senescence is the greatest risk factor for neurodegenerative disease, with increasing age leading to neuronal degeneration and decreased visual system function[14]. In fact, diabetes and senescence are closely linked and the multiple organ system dysfunctions caused by diabetes (cognitive impairment, cardiovascular disease, renal insufficiency, osteoporosis and visual impairment) are similar to those caused by senescence[15] and there is increasing evidence of parallel molecular mechanisms, including cellular senescence, immune-inflammation, aberrant homeostasis of proteins, aberrant processing of mRNAs and impaired repair of DNA damage. Currently, the main treatment modalities for DR are retinal laser photocoagulation, vitreous cavity injection (Compazine, Ranibizumab, etc.) and vitrectomy. Retinal laser photocoagulation is beneficial in preventing further damage of DR, however, it is not curative and complications such as visual field defects, loss of color vision and night blindness can occur after laser treatment[16]. Vitrectomy surgery also has the problems of how to correctly grasp the timing and indications of the operation, to reduce the possible complications during and after the operation, the cost is expensive and the patients who implanted silicone oil after the operation need to insist on the prone position to cooperate with the treatment, which is difficult to be accepted by the older patients and difficult to cooperate with them and so on[17]. Therefore, it is very important to seek effective drugs with few side effects and moderate price in the treatment of DR.

Ursolic acid (UA) is a weakly acidic pentacyclic triterpenoid compound present in many natural plants and it is a functional component of many natural products, which can be originated from many natural herbs such as chasteberry, white-flower snakebite, gardenia, forsythia, etc[18]. It has anti-neovascular, anti-inflammatory, blood sugar, blood lipid, anti-tumor and other biological effects, which are widely sourced and safe with low toxicity[19,20]. In previous studies, UA play several beneficial role in insulin secretion, glucose absorption, diabetic vascular dysfunction, retinopathy and so on[21]. Wu *et al.*[22] found that UA treatment improved diabetic nephropathy by preventing aberrant proliferation, aberrant apoptosis and overproduction of reactive oxygen species (ROS) in glomerular thylakoid cells under high-glycemic conditions, which may be associated with a reduction in inflammation, oxidative stress and renal fibrosis. Pordanjani *et al.*[23] showed that oral supplementation of 0.5% UA given to type 2 DM Wistar rats prevented vascular aging. However, no study has yet revealed the effect of UA on RPE cellular impairment in DR. Synthesizing the hints of biological links between UA, DR and cell senescence, the present study attempts to consider the efficacy of UA and the possible mechanisms from the perspective of RPE damage senescence in DR.

In this experiment, the changes of cell damage, apoptosis, senescence and oxidative stress in RPE cells were observed under hyperglycemia environment treated with UA *in vitro* compared with RPE cells without any drug treatment. Further, the protective effects and possible mechanisms of UA on RPE cells was explored under hyperglycemia environment *in vitro* to provide new clinical ideas for seeking the treatment of DR.

MATERIALS AND METHODS

Study area: This study was conducted at Nanchong Central Hospital from September 2021 to June 2022.

Cell culture: Human RPE cells ARPE-19 (#iCell-h020, icell bioscience, Shanghai, China) were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U L^{-1} penicillin, 100 ug L^{-1} streptomycin and 5 or 30 mmol L^{-1} glucose (Hyclone, USA) in a 5% CO_2 incubator at 37°C. The culture medium was changed for 2-3 days and the cell growth was fused to 80-90%, then passaged and washed twice with phosphate-buffered saline (PBS) solution. After adding an appropriate amount of 0.25% trypsin and leaving it to digest for about 1 min at room temperature. Under the microscope (Olympus, Tokyo, Japan), it can be seen that the inter-cellular space widens and the cellular morphology becomes rounded and then immediately add DMEM medium containing 10% FBS to stop the digestion. Cells were repeatedly blown and then passaged 1:2 or 1:3 at 1×10^7 cells/L and then continued to be placed in the incubator for incubation.

Cell transfection: The Small Interfering RNA (siRNA) designed by the full-length silent information regulator Sirtuin 1 (SIRT1) gene sequence (Gene ID: 23411) was obtained from Shanghai Jima Biotechnology Co., Ltd. (Shanghai, China). A total of three different siRNA sequences were designed to avoid off-target effects (Table S1). While 24 hrs before transfection, cells were digested and inoculated into 12-well plates with 4×10^4 cells per well. When the cells grew to 50~75%, si-SIRT1 was transfected into ARPE-19 cells using Lipofectamine 3000. About 50 nmol L^{-1} siRNA and 2 µL liposomes were dissolved in 100 µL serum-free DMEM medium, respectively and gently mixed within 5 min and allowed to stand at room temperature for 20 min. Subsequently, 1 mL of DMEM medium and the above mixtures were added to each well and incubated at 37°C overnight. After 72 hrs of transfection, a negative control group (si-NC) was set up for the following experiments.

Experimental grouping: The RPE cells cultured to 3-5 generations were randomly divided into 4 groups, including normal glucose (NG), NG+UA, high glucose (HG) and HG+UA. For the NG group, DMEM medium was used to culture ARPE-19 cells. For the NG+UA group, cells were continued to be cultured by introducing 2 µM of UA in DMEM medium. For the HG group, glucose was added to the DMEM medium to a final concentration of 30 mmol L^{-1}. For the HG+UA group, cells were continued to be cultured by introducing 2 µM of UA into the DMEM medium containing 30 mmol L^{-1} glucose. Subsequently, the cells were randomly divided into 4 groups including si-NC, si-NC+UA, si-SIRT1#2 and si-SIRT1#2+UA. For the si-NC group, si-NC was transfected into ARPE-19 cells after 4 days of incubation in HG environment for 48 hours. For the si-NC+UA group, si-NC was transfected into ARPE-19 cells cultured for 48 hrs after the introduction of HG+UA for 4 days. For the si-SIRT1#2 group, si-SIRT1#2 was transfected into ARPE-19 cells cultured for 48 hrs after 4 days of culture in HG environment. For the si-SIRT1#2+UA group, si-SIRT1#2 was transfected into ARPE-19 cells cultured for 48 hrs after the introduction of HG+UA culture for 4 days.

Cell viability assay: The RPE cells were digested and inoculated into 96-well plates as a suspension at a density of 1×10^5 cells/L when the cells reached the logarithmic growth phase. After 24 hrs, the cells were treated with different concentrations of UA. After 48 hrs, 10 µL of CCK-8 (ab228554, Abcam, UK) was added and the absorbance of the wells was measured at 450 nm for calculation of the cell survival rate.

Table S1: Primer sequences

Gene	Sequence	Primer
SIRT1	Forward	CATTTTCCATGGCGCTGAGG
	Reverse	GTCCCAAATCCAGCTCCTCC
GAPDH	Forward	GAGAAGGCTGGGGCTCATTT
	Reverse	AGTGATGGCATGGACTGTGG
si-SIRT1#1		TTCTGAAATATTCAATATCAA
si-SIRT1#2		TTTTCCTTCCTTATCTGACAA
si-SIRT1#3		TATGTTCTGGGTATAGTTGCG

Table S2: The antibodies used in this research

Antibody	Manufacturer	Cat. No
p21	BosterBio	M00145-2
p16	BosterBio	M00016-1
SIRT1	Bio-Rad	AHP2420
GAPDH	BosterBio	H00227

Lactate Dehydrogenase (LDH) assay: The cell culture supernatants from different treatments of each group were collected and the leakage of LDH in the cell culture supernatants was detected separately according to the procedure in the instructions of the LDH kits (#37291, Cell signaling Technology, USA).

Cell apoptosis: The RPE cells to be tested were washed twice with PBS and then stained with 5 μL of Annexin-V dye solution and 10 μL of propidium iodide dye solution (MedChemExpress, USA) for 30 min at 4°C in the dark. Subsequently, the apoptosis rate was measured using flow cytometry.

Senescence-associated protein β-galactosidase (SA-β-gal) staining: The drug-treated RPE cell suspension was inoculated into 6-well plates at 1×10^5/well. The RPE cells were washed once with PBS after aspirating the cell culture medium and then 1 mL of β-galactosidase staining fixative (Solarbio, Beijing, China) was added to each well and fixed at room temperature for 15 min. The RPE cells were washed three times with PBS after aspirating the cell fixative. The cells were washed with PBS for 3 times after removing the cell fixative. Then 1 mL of staining solution was added to each well after removing the PBS and incubated at 37°C overnight. The RPE cells were photographed under a 100× light microscope (Olympus, Tokyo, Japan) and the positively stained cells were counted in the field of view, with 3 subsequent fields of view in each well and 5 replicate wells were set up in each group.

DCFH-DA fluorescent probe: The drug-treated RPE cell suspension was inoculated at 1×10^4/well in 96-well plates and each well was incubated with 100 μL of DCFH-DA serum-free solution at a final concentration of 10 μmol L^{-1} for 20 min at 37°C. After termination of the incubation, the cells were washed three times with serum-free DMEM medium to remove the DCFH-DA that had not entered into the cells. The intensity of ROS red fluorescence was observed under an inverted fluorescence microscope (OLYMPUS, Japan).

ELISA: The cell culture supernatants from different treatments of each group were collected and the secretion of superoxide dismutase (SOD, KGT00150-1, KeyGEN BioTECH, Nanjing, China) and glutathione (GSH, C0055, YaJi Biological, Shanghai, China) in the cell culture supernatants were detected separately according to the procedure in the instructions of the ELISA kits.

Molecular docking: The three-dimensional structure of SIRT1 and UA was respectively obtained from the Protein Data Bank[24] and the PubChem chemical library. The Schrodinger suite 2021 analysis was performed to predict the potential binding between UA and SIRT1.

RT-qPCR: Trizol reagent was utilized to extract total RNA from the cells. The RNA was converted into cDNA using the reverse transcription kit instructions (Promega, USA), followed by conducting real-time PCR experiments. The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative expression of SIRT1 mRNA, with GAPDH serving as an internal reference. Table S1 displayed the primer sequences for RT-qPCR.

Western blot analysis: The RIPA lysate containing protease inhibitor and phosphatase inhibitor (50 mmol L^{-1} Tris, pH 7.4, 150 mmol L^{-1} NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, Thermo Fisher, USA) was added to the cells separately and the liquid was collected after lysis for 30 min on a shaker on ice. Then centrifuged at 4°C 14000 rpm for 30 min and collected the supernatant. The proteins were separated by 8, 10 and 12% SDS-PAGE gal electrophoresis and after electrophoresis, the proteins were transferred onto a PVDF membrane. After completing the membrane transfer, 5% bovine serum albumin (BSA) was used to close the membrane at room temperature for 2 hrs and the primary antibodies (p21, p16, SIRT1) were added and incubated at 4°C overnight. The information of antibodies was listed in Supplementary Table S2. On the next day, the membrane was washed with TBST and then horseradish peroxidase-labeled anti-rabbit secondary antibody was added and incubated at room temperature for 2 hrs. The results of the images were analyzed by Gel-Pro Analyzer software version 4.5.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 8.0 software and the data in this study were presented as the Mean±Standard error of mean (SEM).

To assess the significant disparity between the two groups, the t-test was employed. The differences among more than two groups were analyzed using One-way Analysis of Variance (ANOVA). A significance level of less than 0.05 ($p<0.05$) was deemed statistically significant.

RESULTS

UA alleviates hyperglycemia-induced injury in RPE cells: The chemical structural formula of UA was illustrated in Fig. 1a. The effects of different concentrations of UA on the viability of normally cultured RPE cells were first evaluated. In this experiment, the cell viability of the normal group was set to be 100% and then compared between different groups, the results showed that the RPE cell viability was gradually decreased after adding 1, 2, 5, 10 and 20 μM of UA to the normal culture medium (Fig. 1b). Notably, UA began to affect ARPE-19 cell activity when the UA concentration was greater than 2 μM, so 2 μM was chosen for subsequent experiments. When RPE cells grew to 70% density, they were changed to normal (5 mM glucose) or hyperglycemia (30 mM glucose) medium for another 48 hrs and then LDH assay and Annexin

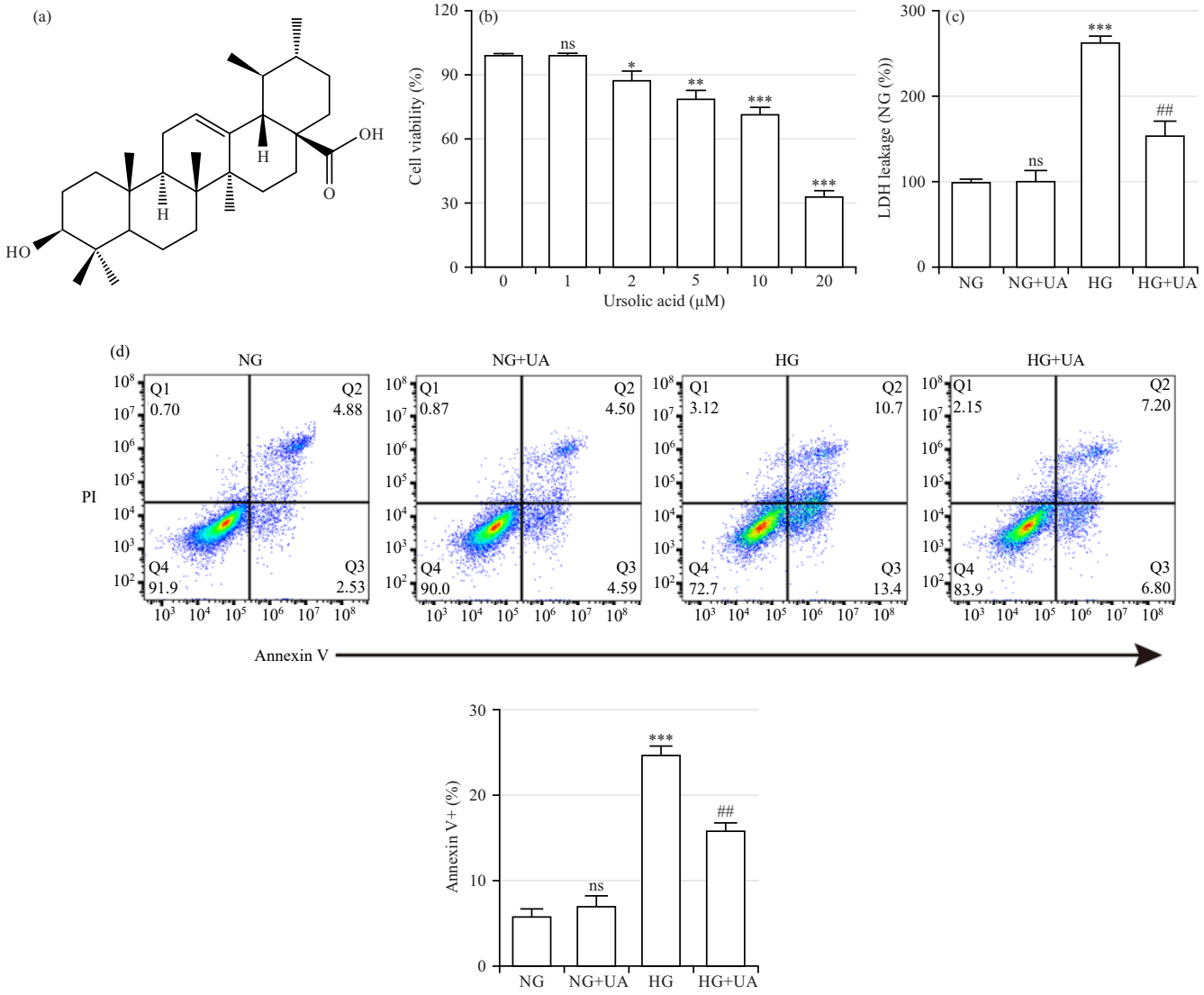

Fig. 1(a-d): UA alleviates hyperglycemia-induced injury in RPE cells, (a) Chemical structural formula of UA, (b) CCK-8 assay evaluation of the viability of RPE cells intervened with UA (1, 2, 5, 10 and 20 μM), (c) LDH assay kit evaluation of the LDH release in RPE cells intervened with UA under hyperglycemia culture and (d) Annexin V/PI double staining evaluation of the apoptosis rate of RPE cells intervened with UA under hyperglycemia culture

(b) $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs 0 μM UA group (c-d) $^{ns}p>0.05$, $^{***}p<0.001$ vs NG group, $^{\#\#}p<0.01$ vs HG group, Mean±SEM and n = 3

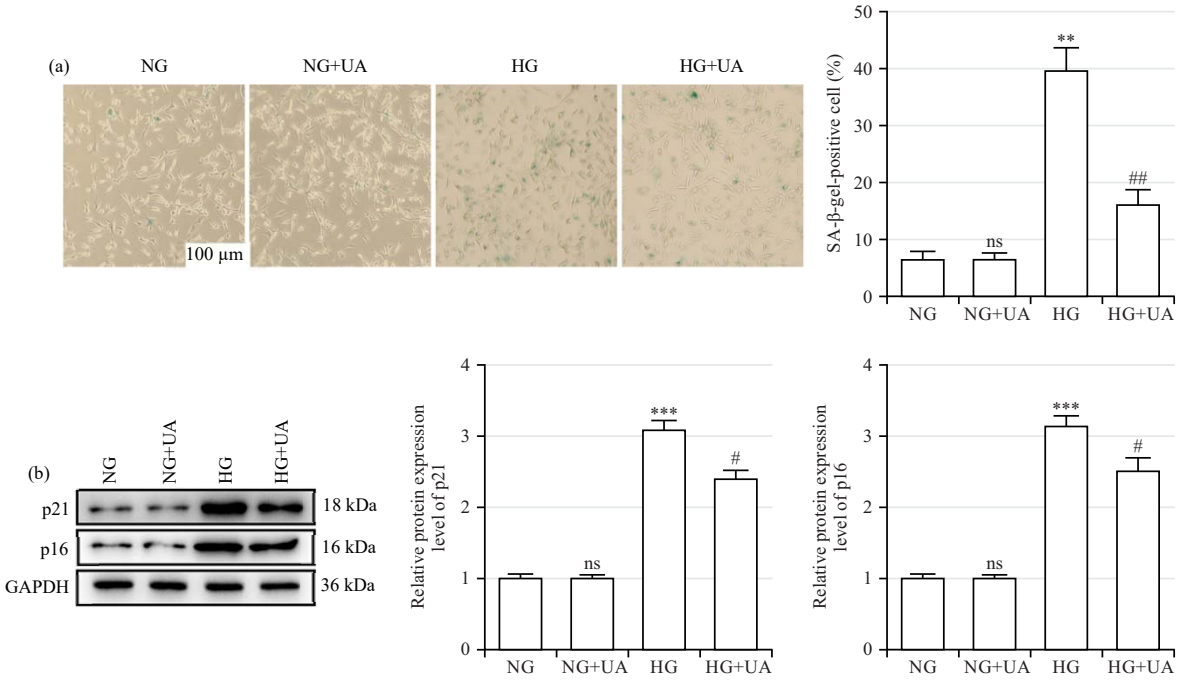

Fig. 2(a-b): UA alleviates hyperglycemia-induced RPE senescence, (a) SA-β-gal staining evaluation of the SA-β-gal-positive RPE cells intervened with UA under hyperglycemia culture and (b) Western blot analysis evaluation of the protein levels of p21 and p16 in RPE cells intervened with UA under hyperglycemia culture

Scale bar: 100 μm $^{ns}p>0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs NG group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs HG group, Mean±SEM and n = 3

V/PI double staining were introduced to assess cell damage and apoptosis. The results showed a significant increase in LDH release (Fig. 1c) and apoptosis (Fig. 1d) of RPE cells in hyperglycemia medium compared to normal medium, indicating that hyperglycemia-induced can cause RPE cell injury and ultimately lead to apoptosis. However, both cell damage and apoptosis were significantly improved by the addition of UA to the hyperglycemia medium. Notably, there was no significant change in the LDH release and apoptosis rate of RPE cells after the introduction of UA in normal medium. The above suggested that UA attenuates hyperglycemia-induced RPE cell injury.

UA alleviates hyperglycemia-induced RPE cell senescence: It is known from the literature that the activity of SA-β-gal was increased significantly during cellular senescence. Therefore, SA-β-gal staining was applied to detect the activity of SA-β-gal to differentiate between normal and senescent cells in this study. The results of β-galactosidase staining displayed that the number of β-galactosidase positive cells (senescent cells) was remarkably increased in RPE cells under hyperglycemia culture compared with the normal group, while UA intervention could reduce hyperglycemia-induced cell senescence (Fig. 2a). The P21 and P16 are well-known cellular senescence marker proteins and western blotting showed that the expression of senescent proteins P21 and P16 was preeminently up-regulated under hyperglycemia culture and the expressions of these two proteins were reversed by the UA intervention (Fig. 2b).

UA attenuates hyperglycemia-induced oxidative stress by SIRT1 activation in RPE cells: Cellular senescence is often accompanied by elevated levels of ROS and oxidative stress. In this experiment, ROS levels were assessed by loading the cellular fluorescent probe DCFH-DA to detect the average fluorescence intensity in RPE cells to further explore the relationship between hyperglycemia and oxidative stress with senescence. The results showed that the intracellular ROS level was significantly increased in RPE cells induced by hyperglycemia, as evidenced by a stronger ROS red fluorescence intensity, which was partially blocked by UA intervention (Fig. 3a). In addition, we also found that the contents of SOD and GSH were observably reduced under hyperglycemia induction, while UA intervention partially restored the synthesis of SOD and GSH in RPE cells (Fig. 3b-c). The SIRT1 belongs to the highly conserved Sirtuin family and

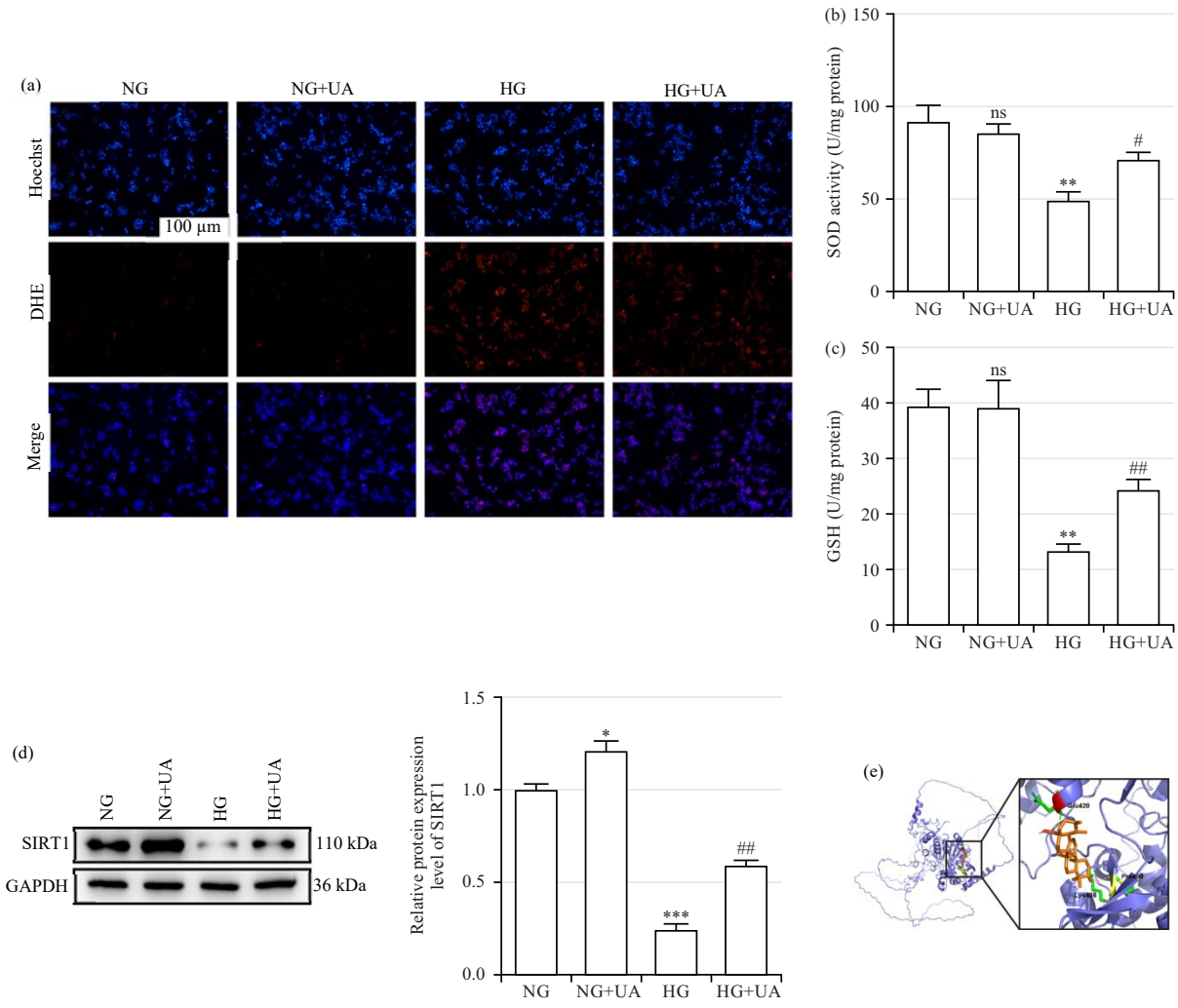

Fig. 3(a-e): UA attenuates hyperglycemia-induced oxidative stress by SIRT1 activation in RPE cells, (a) DCFH-DA fluorescent probe evaluation of the ROS levels in RPE cells intervened with UA under hyperglycemia culture, (b) ELISA evaluation of the SOD contents in RPE cells intervened with UA under hyperglycemia culture, (c) ELISA evaluation of the GSH contents in RPE cells intervened with UA under hyperglycemia culture, (d) Western blot analysis evaluation of the protein levels of SIRT1 in RPE cells intervened with UA under hyperglycemia culture and (e) Three-dimensional view of UA-SIRT1 molecular docking predictions

Scale bar: 100 μm, $^{ns}p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs NG group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs HG group and Mean±SEM and n = 3

is a well-known protein molecule for slowing down aging and reducing aging-related diseases. In this study, it was found that the protein expression of SIRT1 was significantly decreased under hyperglycemia culture, which was up-regulated after UA intervention (Fig. 3d). Accordingly, it was hypothesized that UA might play a role in the oxidative stress injury and senescence of RPE cells induced by high glucose, which may be related to the activation of SIRT1 protein. Next, the molecular docking model of UA-SIRT1 was further predicted which shows that UA was able to structurally dock with the three active sites of SIRT1 protein, including GLU420, PRO409 and Lys408 (Fig. 3e). In summary, it was hypothesize that UA catalyzes the synthesis of SIRT1 by binding to amino acids GLU420, PRO409 and Lys408 of the SIRT1 molecule, thereby attenuating hyperglycemia-induced oxidative stress and cellular senescence.

SIRT1 silence impairs the protection of UA in hyperglycemia-treated RPE cells: To further confirm the above conjecture, SIRT1-specific siRNA was introduced in this

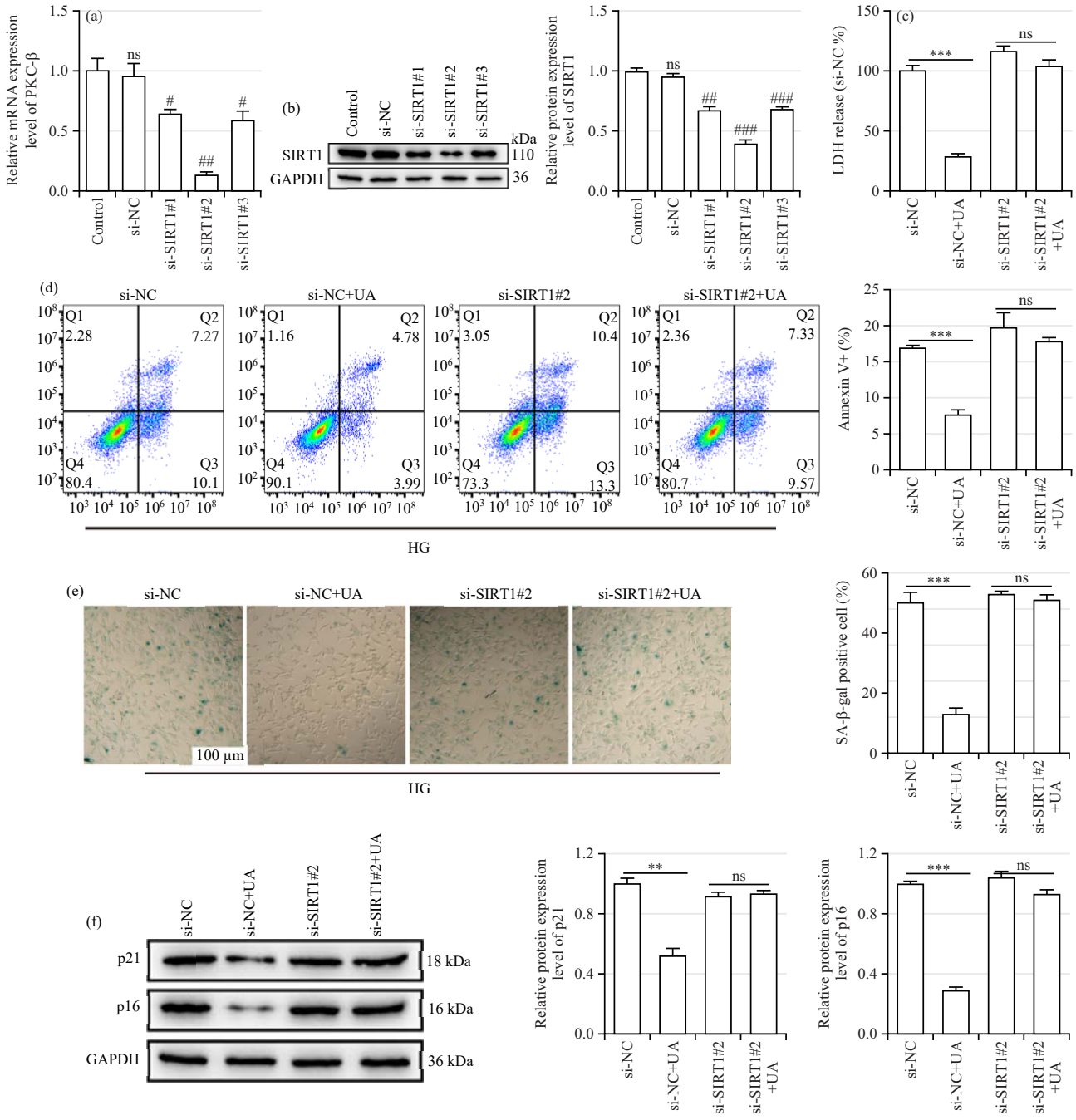

Fig. 4(a-f): SIRT1 silence impairs the protection of UA in hyperglycemia-treated RPE cells, (a) RT-PCR evaluation of the mRNA levels of SIRT1 in hyperglycemia-treated RPE cells, which transfected with si-NC, si-SIRT1#1, si-SIRT1#2, and si-SIRT1#3, (b) Western blot analysis evaluation of the protein levels of SIRT1 in hyperglycemia-treated RPE cells, which transfected with si-NC, si-SIRT1#1, si-SIRT1#2 and si-SIRT1#3, (c) LDH assay kit evaluation of the LDH release in hyperglycemia-treated RPE cells after the introduction of UA intervention on the basis of SIRT1 knockdown, (d) Annexin V/PI double staining evaluation of the apoptosis rate of hyperglycemia-treated RPE cells after the introduction of UA intervention on the basis of SIRT1 knockdown, (e) SA-β-gal staining evaluation of the SA-β-gal-positive RPE cells, which cultured with hyperglycemia after the introduction of UA intervention on the basis of SIRT1 knockdown and (f) Western blot analysis evaluation of the protein levels of P21 and P16 in hyperglycemia-treated RPE cells after the introduction of UA intervention on the basis of SIRT1 knockdown

Scale bar: 100 μm, $^{ns}p>0.05$ vs control group $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs si-NC group, $^{**}p<0.01$, $^{***}p<0.001$, Mean±SEM and n = 3

study and the knockdown efficiency of the three sequences was verified with the help of RT-PCR and western blotting. Figure 4(a-b) showed that si-SIRT1#2 had the highest silencing efficiency of SIRT1 mRNA and protein compared with si-SIRT1#1 and si-SIRT1#3 and thus si-SIRT1#2 was selected for the subsequent experiments. Next, LDH content assay, apoptosis assay, SA-β-gal staining and P21 and P16 protein detection were repeated to further verify whether SIRT1 knockdown had any effect on the efficacy of UA. The results exhibited that there were no significant changes in LDH release (Fig. 4c), apoptosis rate (Fig. 4d), SA-β-gal-positive cell rate (Fig. 4e) and the expression of senescence marker proteins P21 and P16 (Fig. 4f) in hyperglycemia-treated RPE cells after the introduction of UA intervention on the basis of SIRT1 knockdown. Accordingly, the above results confirmed that knockdown of SIRT1, the target of UA, might have resulted in its inability to exert a protective effect against hyperglycemia-induced PRE cell injury and senescence.

DISCUSSION

The present study, for the first time, explored the protective effect of UA on hyperglycemia-induced RPE cell injury and preliminarily uncovered the beneficial role of UA in RPE was mediated by the regulation of SIRT1 on cell oxidative damage and senescence. Our finding revealed the therapeutic potential of UA on DR. The DR is a common clinical fundus disease, long-term persistent hyperglycemia is the most basic pathophysiological state leading to retinal microangiopathy, common fundus manifestations such as the appearance of microaneurysms, hard exudates, soft exudates, neovascularization, fibrovascular proliferative strips, further vitreous hemorrhage, retinal detachment, etc[25]. The main pathological changes of DR are closely related to the decrease in the number and function of RPE. Many years of clinical research have shown that there are multiple independent risk factors for DR, including hyperglycemia, dyslipidemia and glycosylation end-product formation[26]. Therefore, *in vitro* experiments are often used to add one or more of these risk factors for modeling. In the present study, ARPE-19 cells were cultured with a hyperglycemia concentration medium to construct a model of hyperglycemia injury and to observe the protective effect of UA on ARPE-19 injury.

The UA is a pentacyclic triterpenoid derived from a variety of natural herbs such as chasteberry, white-flower snakeweed, gardenia, forsythia and so on. It has a variety of biological effects such as anti-neovascular, antioxidant, anti-inflammatory, blood glucose lowering, hypolipidemic, antitumor, etc. and its action is safe and low toxicity[18]. Current clinical treatments for DR are associated with a certain degree of side effects and expensive costs, so that the search for a highly effective, low-toxic and affordable drug against RPE cell damage is of particular importance. The results of this study discovered that the RPE cells in the hyperglycemia group leaked more LDH than the cells in the normal group and the apoptosis rate was conspicuously increased. The above suggests that RPE cells in the hyperglycemia group showed significant damage, which was consistent with the results of previous experiments[27]. Subsequently, the RPE cells introduced with UA were found to be resistant to high glucose-induced injury when UA was added to a high glucose medium and a normal medium for culture. Hyun *et al*.[28] showed antioxidant and anti-inflammatory effects of UA on hyperglycemia-induced hepatic inflammation in mice. Mazumder *et al*.[29] found significant reductions in blood glucose, glycated hemoglobin, creatinine, uric acid, blood urea nitrogen, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase levels and significant inhibition of the formation of AGEs in the kidneys, were observed in UA-treated mice, suggesting that UA proves to be an effective AGE inhibitor for the treatment of diabetic complications. Oh *et al*.[30] suggested that apoptotic features such as DNA breakage, impairment of mitochondrial function and regulation of apoptosis marker proteins were significantly restrained in UA-pretreated cells after exposure to high glucose, suggesting that UA may play an important role in regulating hyperglycemia-induced apoptosis by scavenging ROS.

Many experimental studies as well as clinical trials at home and abroad have shown that oxidative stress-induced RPE cell injury has become one of the major causes of the development of DR. The RPE cells between the photoreceptor cell layer and the choroidal capillary layer require large amounts of oxygen for normal retinal function and are therefore sensitive to changes in oxygen content[31]. Recent studies have shown that chronic hyperglycemia levels in patients with DR increased the oxygen consumption of the retina. Chronic hypoxia could increase the oxidative stress in RPE cells, resulting in the generation of large amounts of ROS, which can damage mitochondrial function by disrupting the structure and function of the cell membrane and activate lysosomes to initiate apoptotic cell death[32]. Tang *et al*.[33] discovered that astragaloside-IV was able to reduce apoptosis by increasing the antioxidant capacity of RPE cells, which in turn inhibited the pathological process of DR. Du *et al*.[34] demonstrated that kaempferol protected human RPE cells from hyperglycemia-induced oxidative cell damage and apoptosis by restraining ROS activity and encouraging SOD

and GSH synthesis. This study detected ROS levels and the contents of SOD and GSH in the RPE cells by loading the cellular fluorescent probe DCFH-DA and ELISA and identified that hyperglycemia-induced ROS levels were significantly elevated and the contents of SOD and GSH were decreased in RPE cells and the UA intervention could partially reverse the above changes.

Oxidative stress is not only an important pathogenetic mechanism of DR, but also occupies an equally important place in the numerous doctrines of senescence mechanisms[35]. Studies have shown that under the influence of a variety of stimulating factors, including high glucose, the body generates too many oxygen free radicals, the antioxidant defence system scavenging is reduced, oxidation and antioxidant imbalance, resulting in a large number of oxygen free radicals accumulate so that a variety of intracellular reactions induced by the damage, which ultimately leads to cellular senescence[36]. Cell senescence is the basis of tissue and organ aging and is a state of cellular damage before the cell enters into permanent cycle arrest (i.e., death), which is characterized by changes in form and function. Studies have found that people with type 2 diabetes have faster organ senescence compared to people with type 1 diabetes[37]. Chiu *et al.*[38] suggested that prolonged high glucose exposure induced an increase in senescence-related proteins p53 and p21 as well as SA-β-gal-positive cells in RPE cells, suggesting that prolonged high glucose exposure makes RPE cells more sensitive to premature senescence. The SA-β-gal staining is a key indicator for assessing cellular senescence and its positivity is manifested by the blue-green staining of cells and the staining is only targeted to senescent cells, so the cellular senescence status can be assessed by calculating the positive rate of the staining[39]. Further, cells can generate ROS through signaling pathways such as P21 and P16 to induce and maintain the cellular senescence process[40]. In the present study, results of SA-β-gal staining and p21 and p16 protein expression explained that the rate of SA-β-gal-positive cells and the expression of p21 and p16 proteins were elevated under the induction of hyperglycemia, which was consistent with the results of previous studies. Further, this study discovered that all cellular senescence indexes were reduced after the administration of UA intervention, indicating that UA could delay hyperglycemia-induced RPE cellular senescence. Previous studies have confirmed the anti-senescence efficacy of UA. The study by Bahrami and Bakhtiari[41] illustrated that UA modulates the aging process and attenuates mitochondria-related diseases by enhancing anti-aging biomarkers (SIRT1 and SIRT6) and PGC-1β in the hypothalamus. In addition, a study displayed that UA blocked the expression of biomarkers of vascular senescence in DM rats[23]. The above findings further confirm our conclusions.

The SIRTs are involved in the regulation of cellular metabolism, inflammation, oxidative stress and senescence and are known as longevity proteins that are beneficial to both senescence and metabolic diseases[42]. In mammals, there are seven homologs in the SIRTs family. Among them, SIRT1 is the first family member to be discovered and is the most studied one. The SIRT1 exists in the nucleus and cytoplasm and can regulate the occurrence and development of metabolic diseases, cardiovascular diseases, aging and senile degenerative diseases. It is considered to be a potential target for the treatment of human diseases such as DR[43]. Previous studies have reported that senescence increases tissue susceptibility, whereas SIRT1 inhibits cellular senescence and a variety of senescence-related cellular processes, including SIRT1/Keap1/Nrf2/HO-1 and SIRTI/PI3K/Akt/GSK-3β-mediated oxidative stress and quercetin has further been found to activate SIRT1 to counteract senescence-related diseases[44]. Xu *et al.*[45] demonstrated that coumestrol suppressed oxidative stress, inflammation and apoptosis in hyperglycemia-treated human retinal microvascular endothelial cells through activation of SIRT1 expression, thereby improving DR. The results of Lee *et al.*[46] suggested that GW501516, an activator of PPARδ, represses hyperglycemia-induced senescence of RPE cells by upregulating PPARδ-promoted SIRT1 signaling. Accordingly, the above findings suggest that SIRT1 may serve as a target for certain drugs to ameliorate hyperglycemia-induced cellular senescence and oxidative stress injury. In the present study, SIRT1 protein expression was reduced in RPE cells under a hyperglycemia environment, which was upregulated after further introduction of UA. Interestingly, the LDH release, apoptosis rate, SA-β-gal-positive cell rate and the expression of senescence marker proteins P21 and P16 were not changed in hyperglycemia-treated RPE cells after the introduction of UA intervention based on knockdown of SIRT1. The molecular docking model of UA-SIRT1 and observed that UA was able to structurally dock to three active sites of the SIRT1 protein, including GLU420, PRO409 and Lys408, which further confirmed current experimental results. Accordingly, the above results confirmed that SIRT1 indeed exerts anti-hyperglycemia-induced RPE cell senescence as a target of UA.

CONCLUSION

In summary, UA reduces oxidative stress injury-mediated apoptosis and senescence in RPE cells under hyperglycemia conditions by activating SIRT1 expression. Therefore, UA can inhibit the oxidative damage and senescence of RPE cells

induced by hyperglycemia and protect the normal structure and function of RPE cells, thus providing a new idea for the treatment of DR. However, this experiment is an *in vitro* cell culture assay that does not completely mimic the pathological changes in the retina of DR patients. Therefore, this experiment still has some limitations and further *in vivo* experiments are needed to verify the accuracy of the experimental conclusions. Moreover, the mitigation of cellular oxidative stress and senescence by SIRT1 is often accompanied by complex downstream pathways, which will be a pressing issue for this study in the future.

SIGNIFICANCE STATEMENT

In this paper, results elucidated that UA has the potential to ameliorate hyperglycemia-induced RPE cell injury. Mechanistically, UA reduced apoptosis and senescence of RPE cells mediated by oxidative stress injury under hyperglycemic conditions by activating the expression of SIRT1 and protected the normal structure and function of RPE cells. This study identified for the first time the ability of UA to regulate RPE cell senescence, which gives us a new understanding of the occurrence and development of DR and provides new ideas for the treatment of DR.

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