• Basic Research •

SIRT1 inhibits apoptosis of human lens epithelial cells through suppressing endoplasmic reticulum stress *in vitro* and *in vivo*

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Abstract

● **AIM:** To explore the effect of silent information regulator factor 2-related enzyme 1 (SIRT1) on modulating apoptosis of human lens epithelial cells (HLECs) and alleviating lens opacification of rats through suppressing endoplasmic reticulum (ER) stress.

● **METHODS:** HLECs (SRA01/04) were treated with varying concentrations of tunicamycin (TM) for 24h, and the expression of SIRT1 and C/EBP homologous protein (CHOP) was assessed using real-time quantitative polymerase chain reaction (RT-PCR), Western blotting, and immunofluorescence. Cell morphology and proliferation was evaluated using an inverted microscope and cell counting kit-8 (CCK-8) assay, respectively. In the SRA01/04 cell apoptosis model, which underwent siRNA transfection for SIRT1 knockdown and SRT1720 treatment for its activation, the expression levels of SIRT1, CHOP, glucose regulated protein 78 (GRP78), and activating transcription factor 4 (ATF4) were examined. The potential reversal of SIRT1 knockdown effects by 4-phenyl butyric acid (4-PBA; an ER stress inhibitor) was investigated. *In vivo*, age-related cataract (ARC) rat models were induced by sodium selenite injection, and the protective role of SIRT1, activated by SRT1720 intraperitoneal injections, was evaluated through morphology observation, hematoxylin and eosin (H&E) staining, Western blotting, and RT-PCR.

● **RESULTS:** SIRT1 expression was downregulated in TM-induced SRA01/04 cells. Besides, in SRA01/04 cells, both cell apoptosis and CHOP expression increased with the rising doses of TM. ER stress was stimulated by TM, as evidenced by the increased GRP78 and ATF4 in the SRA01/04 cell apoptosis model. Inhibition of SIRT1 by siRNA knockdown increased ER stress activation, whereas SRT1720 treatment had opposite results. 4-PBA partly reverse the adverse effect of SIRT1 knockdown on apoptosis. *In vivo*, SRT1720 attenuated the lens opacification and weakened the ER stress activation in ARC rat models.

● **CONCLUSION:** SIRT1 plays a protective role against TM-induced apoptosis in HLECs and slows the progression of cataract in rats by inhibiting ER stress. These findings suggest a novel strategy for cataract treatment focused on targeting ER stress, highlighting the therapeutic potential of SIRT1 modulation in ARC development.

● **KEYWORDS:** silent information regulator factor 2-related enzyme 1; endoplasmic reticulum stress; apoptosis; human lens epithelial cells; cataract

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INTRODUCTION

Age-related cataract (ARC) is a common ophthalmological disease that affects millions of adults aged 50y and older worldwide[1]. According to the latest of global and regional data, ARC remains the leading cause of global blindness, accounting for approximately 45.5% of cases (around 15.2 million patients in 2020)[2]. At present, the only available treatment for cataract is surgical removal and replacement with an artificial intraocular lens (IOL). However, this surgical procedure requires a skilled surgeon with expertise and access to a well-equipped surgical system to ensure successful outcomes and effectively handle any potential complications that may arise during or after the surgery. Given the limitations and requirements associated with cataract surgery, there is a pressing need to develop pharmacological interventions

that can delay the onset of cataracts and slow down their progression.

It has been reported that ARC can be caused by several risk factors such as lens aging, oxidation, calcium imbalance, hydration, and modifications in crystallin, which lead to the destabilization and aggregation of lens proteins, ultimately increasing the incidence of cataracts[3-7]. The endoplasmic reticulum (ER) is a complex network of membrane-bound structures inside cells that plays a crucial role in protein synthesis and folding. However, various cellular stresses on cells can disrupt the balance of ER, leading to a condition known as ER stress[8]. Recent evidence suggests that ER stress is intricately linked to the activation of apoptotic pathways, with prolonged or severe ER stress resulting in cell death, a mechanism that is increasingly recognized as a contributing factor in the pathology of ARC[9-11]. These apoptotic processes induced by ER stress have been identified as potential targets for therapeutic intervention in ARC.

Silent information regulator factor 2-related enzyme 1 (SIRT1), belongs to nicotinamide adenine dinucleotide (NAD^+) dependent class III histone deacetylases. It plays a crucial role in various physiological processes, including inflammation modulation, metabolism regulation, cellular senescence, and aging[12-13]. Notably, studies have shown that SIRT1 has positive effects on inhibiting ER stress-induced cell death[14]. Furthermore, SIRT1 has been demonstrated to protect lens epithelial cells (LECs) from cellular stresses and prevent cataract formation in rats[15]. Recent studies have suggested a potential link between SIRT1 and ER stress. It has been found that SIRT1 plays a crucial role in modulating the response to ER stress and maintaining ER homeostasis[16]. Based on these findings, we hypothesize that SIRT1 may be involved in the regulation of cataract formation by suppressing ER stress-induced apoptosis. To the best of our knowledge, rare previous studies have reported the relationship between SIRT1 and ER stress-induced apoptosis in ARC.

In this study, we aimed to study whether SIRT1 possessed the ability to suppress ER stress and apoptosis in the human lens epithelial cells (HLECs) line (SRA01/04 cell) apoptosis model. Specifically, we adopted siRNA transfection for SIRT1 knockdown, SRT1720 treatment for SIRT1 activation and 4-phenyl butyric acid (4-PBA) treatment for ER stress inhibition to access the effect of SIRT1 on tunicamycin (TM)-induced SRA01/04 cells. Furthermore, we conducted experiments in a rat ARC model, exploring the potential underlying molecular mechanisms that SIRT1 could prevent ER stress and attenuate lens opacification.

MATERIALS AND METHODS

Ethical Approval All rat experiments in this study were approved by the Animal Care and Ethics Committee at Chinese PLA General Hospital (permit No. 2022-X18-55) and the animals were treated in accordance with the National Institutes of Health and Nutrition Guidelines for the care and use of laboratory.

Reagents The SIRT1 agonist SRT1720 (Beyotime, China) and ER stress inhibitor 4-PBA (Sigma, USA) were utilized in this research.

Cell Culture and Transfection Immortalized SRA01/04 cells (Cellcook, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin and streptomycin (Gibco, USA) and were kept at 37℃ in a humidified atmosphere containing 5% CO_2. To evaluate the effects of different TM (YangGuangBio, China) dose on SIRT1 expression and define the optimal concentration of TM that would induce apoptosis, SRA01/04 cells were exposed to 0, 1, 3, 10, and 30 μg/mL of TM for 24h and harvested for analysis on day 2. The siRNA for human SIRT1 was designed and purchased from Hanbio Biotechnology Co. Ltd. (China). According to the manufacturers' instructions, SRA01/04 cells were transfected with 50 nmol/L SIRT1 knockdown siRNA or control siRNA by using lipofectamine 3000 (Thermo Fisher Scientific, USA). The transfection medium was replaced with fresh 10% fetal bovine serum medium 6h post-transfection. Cells were incubated for 48h, and then continued to subsequent experiments.

Cell Counting Kit-8 Assay SRA01/04 cells were seeded into 96-well plates at a density of 5000 cells/well. Phosphate buffered saline (PBS; Beyotime, China) was utilized to wash cells after exposure to different treatments. Then, the cells were incubated with CCK-8 reagent (Dojindo, Japan) at 37℃ for 3h. Finally, the absorbance of each well was recorded at 450 nm with a Microplate Reader (Multiskan SkyHigh, Thermo Fisher Scientific, USA) instrument.

Real-Time Quantitative Polymerase Chain Reaction Total RNA of each sample was extracted using TRIzol reagent (Thermo Fisher Scientifc, USA) and transcribed into cDNA using HiScriptIII RT SuperMix for quantitative polymerase chain reaction (qPCR) kit (Vazyme, China). The PCR was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China). The relative gene expression data were performed with QuantStudio5 System (Thermo Fisher Scientifc, USA) and calculated by the $2^{-\Delta\Delta CT}$ method with β-actin as the housekeeping gene. The sequences of forward and reverse primer of target genes were presented in Table 1.

Immunofluorescence Staining The treated SRA01/04 cells in each confocal dish were fixed with 1 mL 4% paraformaldehyde (PFA) for 25min, permeated with 0.1% Triton X-100 in PBS for 15min, and blocked with 5% bovine serum albumin (BSA) for 60min at 37℃. Then, the primary antibodies against

Table 1 The primer sequences of target genes

Gene name	Primer	Sequence (5′ to 3′)
SIRT1	Forward	CTCTGGCATGTCCCACTATCAC
	Reverse	CAAAGGAGCAGATTAGTAGGCG
GRP78	Forward	TTAGGCCAGCAATAGTTCCAG
	Reverse	CTGCCATGGTTCTCACTAAAATG
ATF4	Forward	GGCTGCTTATTAGTCTCCTGGAC
	Reverse	CTCCGGGACAGATTGGATGTT
CHOP	Forward	CTGCTTGAGCCGTTCATTCTC
	Reverse	GGAAACAGAGTGGTCATTCCC
β-actin	Forward	CTCCTTAATGTCACGCACGAT
	Reverse	CATGTACGTTGCTATCCAGGC

SIRT1 (1:150, Abcam, ab32441, USA) and CHOP (1:1000, ProteinTech, 15204-1-AP, USA) were added to the plate and incubated for 24h at 4℃. Subsequently, Alexa Fluor594-conjugated second antibody was added at a 1:500 dilution in PBS and incubated free of light for 90min. Ultimately, DAPI Fluoromount-G (SouthernBiotech, USA) solution was added to each group and incubated for 10min in the dark at ambient temperature. A confocal laser scanning microscopy (CLSM, Nikon, Japan) was applied to detect and analyze the positive staining cells in each confocal dish, and the fluorescence intensity in each group was assessed by the Image J software (version 21, NIH, USA).

Western Blotting The whole protein of each sample was extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Solarbio, China) containing protease inhibitor cocktail (Beyotime, China) and phosphatases (Solarbio, China). After centrifugation at 12 000 rpm for 15min at 4℃, the supernatant from each group was collected. A BCA protein assay kit (Beyotime, China) was used to measure the protein concentration of SRA01/04 cells in each group. 20 μg of total cellular protein from different groups was separated by SDS-polyacrylamide gel electrophoresis and transferred to poly-vinylidene difluoride (PVDF) membranes (Bio-Rad, USA). After blocking with 5% skim milk in Tris-buffered saline Tween (TBST; Beyotime, China) at room temperature for 90min, the bands were incubated overnight at 4℃ with primary antibodies against the following proteins for Western blotting analysis, including SIRT1 (1:5000, Abcam, ab32441, USA), glucose regulated protein 78 (GRP78; 1:5000, ProteinTech, 11587-1-AP, USA), activating transcription factor 4 (ATF4; 1:500, ProteinTech, 28657-1-AP, USA), CHOP (1:200, Abcam, 15204-1-AP, USA), GAPDH (1:1000, OriGene, OTI2D9, USA) and β-actin (1:5000, Abcam, ab8226, USA). Then, the bands were washed with TBST three times and incubated with secondary antibodies for 60min at ambient temperature. After that, the membranes were washed with TBST for three times again, followed by the ECL Western blotting substrate (Solarbio, China) and the UVITEC Alliance MINI HD9 system (UVITEC, Britain) applied to detect and analyze the density of each band.

Age-Related Cataract Model Fifty Sprague Dawley (SD) rat pups were purchased from SPF Biotechnology (China) and raised under standard conditions. All animals randomly divided into five groups, with ten animals in each group: the untreated normal control group (NC group), the model control group (MC group), the low-dose SRT1720 treated group (LD group), the medium-dose SRT1720 treated group (MD group), and the high-dose SRT1720 treated group (HD group). To induce cataracts[17], on the 10th day after birth, the rat pups in the MC, LD, MD, and HD group received a single subcutaneous injection of sodium selenite at a dose of 30 nmol/g body weight. The NC group was left untreated. From day 10 to 23, the LD, MD, and HD group each received intraperitoneal injections of SRT1720 at doses of 25, 50, and 100 mg/kg, respectively, once a day. On day 10, four hours before the subcutaneous injection of sodium selenite, the LD, MD, and HD group received their SRT1720 injections. On day 24, all rat pups were anesthetized with 10% chloral hydrate to examine their eyes for the presence of cataracts. After evaluating the development of cataracts, the animals were sacrificed by cervical dislocation. Subsequently, lens capsule membrane was isolated and preserved at -80℃ for later analysis.

Histological Analysis Fresh eyeballs were fixed in FAS Eyeball Fixative Solution (Servicebio, China) and embedded in paraffin. Serial sections were cut at 3 μm thickness through the mid-section of the lens. Slides were stained with hematoxylin and eosin (H&E) staining and imaged using an upright microscope (ZEISS Axio Imager 2, Germany).

Statistical Analysis At least three replicates were assessed independently for each group of SRA01/04 cells. The average of the values was presented as mean±standard error of the mean (SEM). The comparison of rate data among different groups was performed using unpaired Student's *t*-test for two groups and one-way analysis of variance (ANOVA) followed by Tukey's test for more than two groups. Statistical analyses were performed using SPSS software (version 20, IBM Corp, USA) or GraphPad Prism (version 9.0, GraphPad Software Inc., USA). $P<0.05$ in two-tailed tests was considered statistically significant.

RESULTS

SIRT1 Levels in SRA01/04 Cell Apoptosis Model Induced by Tunicamycin To explore the expression of SIRT1 in TM-induce cell apoptosis, SRA01/04 cells were exposed to different concentrations of TM for 24h. SIRT1 expression was significantly decreased, and exhibited a downward trend with increasing doses of TM both at the transcriptional and translational level (Figure 1A-1C). Compared with the control

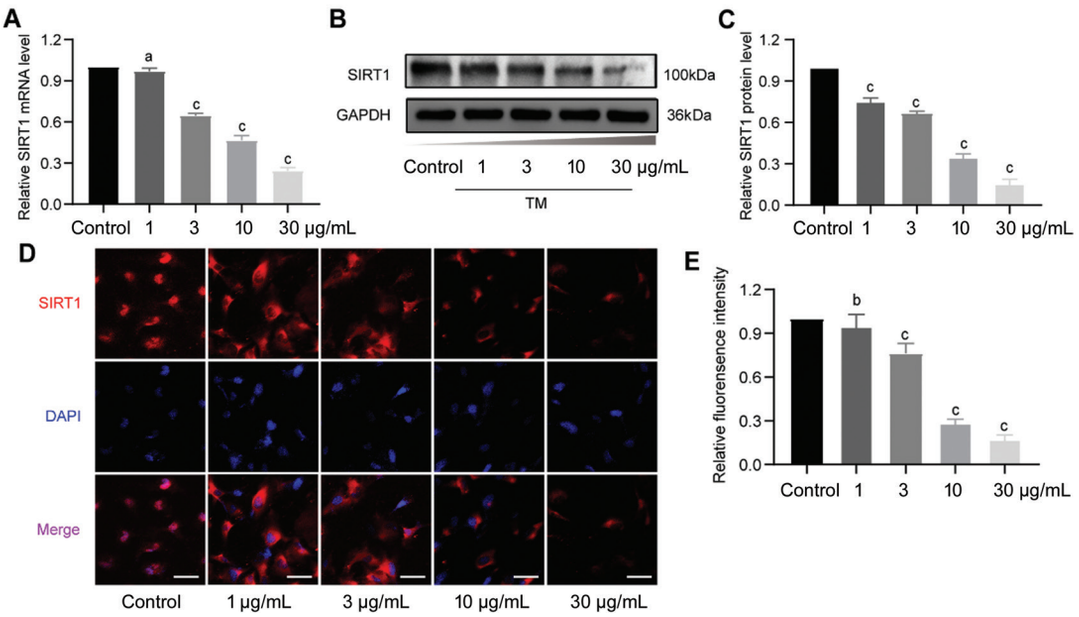

Figure 1 Different level of SIRT1 expression induced by various concentrations of TM in SRA01/04 cells A: The different level of relative SIRT1 mRNA expression induced by increased TM concentration; B-C: Western blotting analysis of SIRT1 induced by increased TM concentration; D-E: Immunofluorescence staining analysis of SIRT1 induced by increased TM concentration. Scale bar: 50 μm. All experiments were repeated at least three times. Data were presented as mean±SEM. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. SIRT1: Silent information regulator factor 2-related enzyme 1; TM: Tunicamycin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DAPI: 4′,6-diamidino-2-phenylindole; SEM: Standard error of the mean.

group, the relative mRNA expression of SIRT1 was decreased ($P<0.05$) in 1 μg/mL TM group and significantly decreased ($P<0.001$) in more than 1 μg/mL TM groups. Western blotting analysis showed a significantly decreased ($P<0.001$) in the protein level of SIRT1 in all TM treatment group. Moreover, the results of immunofluorescence staining indicated that TM stimulation led to a decrease ($P<0.01$) of SIRT1 (the red fluorescence) in 1 μg/mL TM group and a more significant decrease ($P<0.001$) in more than 1 μg/mL TM groups, while simultaneously promoting the translocation of SIRT1 into the cytoplasm. In contrast, under basic conditions, SIRT1 remained confined to the nuclear (Figure 1D, 1E).

Effects of Different Tunicamycin Concentration on SRA01/04 Cells Apoptosis After 24h of treatment with different doses of TM, the morphological features of cell apoptosis were observed under an inverted microscope. The dead cells were easily identified since they floated on the surface of the cell culture, appearing as small bright spots. The remained surviving cells exhibited irregular shapes, with elongated bifurcations, in contrast to cobblestone morphology of normal cells. We also observed a proportional increase in the number of dead cells with increasing TM concentrations. Specifically, the group exposed to the highest TM concentration (30 μg/mL), showed high death rates of SRA01/04 cells, resulting in clear spaces in 6-well plates filled with numerous cellular debris (Figure 2A). The CCK-8 assay revealed similar results, demonstrating that TM preferentially reduced the viability of SRA01/04 cells in a dose-dependent manner. The group treated with 30 μg/mL of TM showed only 30% viability compared with the control group ($P<0.001$; Figure 2B). The CHOP, known as DNA damage-inducible transcript 3, is a key activator of pro-apoptotic cascade[18]. In our study, SRA01/04 cells displayed a significant increase in the expression of CHOP with increasing TM doses (Figure 2C-2E). Compared with the control group, the relative mRNA expression and protein expression of CHOP were significantly increased ($P<0.001$ and $P<0.001$) both in 10 and 30 μg/mL TM groups. The results of immunofluorescence staining indicated that TM stimulation resulted in an increase in CHOP expression, represented by the red fluorescence (Figure 2F, 2G). Remarkably, the dose of 30 μg/mL resulted in severe deformation and massive cell death, while dose of 3 μg/mL did not show significant statistical differences compared with the control group, as determined by the CCK8 assay ($P>0.05$) and RT-PCR ($P>0.05$). Based on the above results, we believed that a concentration of 10 μg/mL TM specifically induced apoptosis in SRA01/04 cells. Therefore, it was chosen for use in the following experiments.

SIRT1 Suppresses Tunicamycin-induced SRA01/04 Cells Apoptosis Through ER Stress Pathway To investigate the role of SIRT1 in mediating ER stress-induced apoptosis, we first silenced SIRT1 in SRA01/04 cells. The results of both RT-PCR (Figure 3A, 3B) and Western blotting (Figure 3C, 3D) confirmed successful knockdown of SIRT1 in si-SIRT1 treated SRA01/04 cells, with less than 30% expression compared with normal cells (both $P<0.001$). The reduction of SIRT1 was

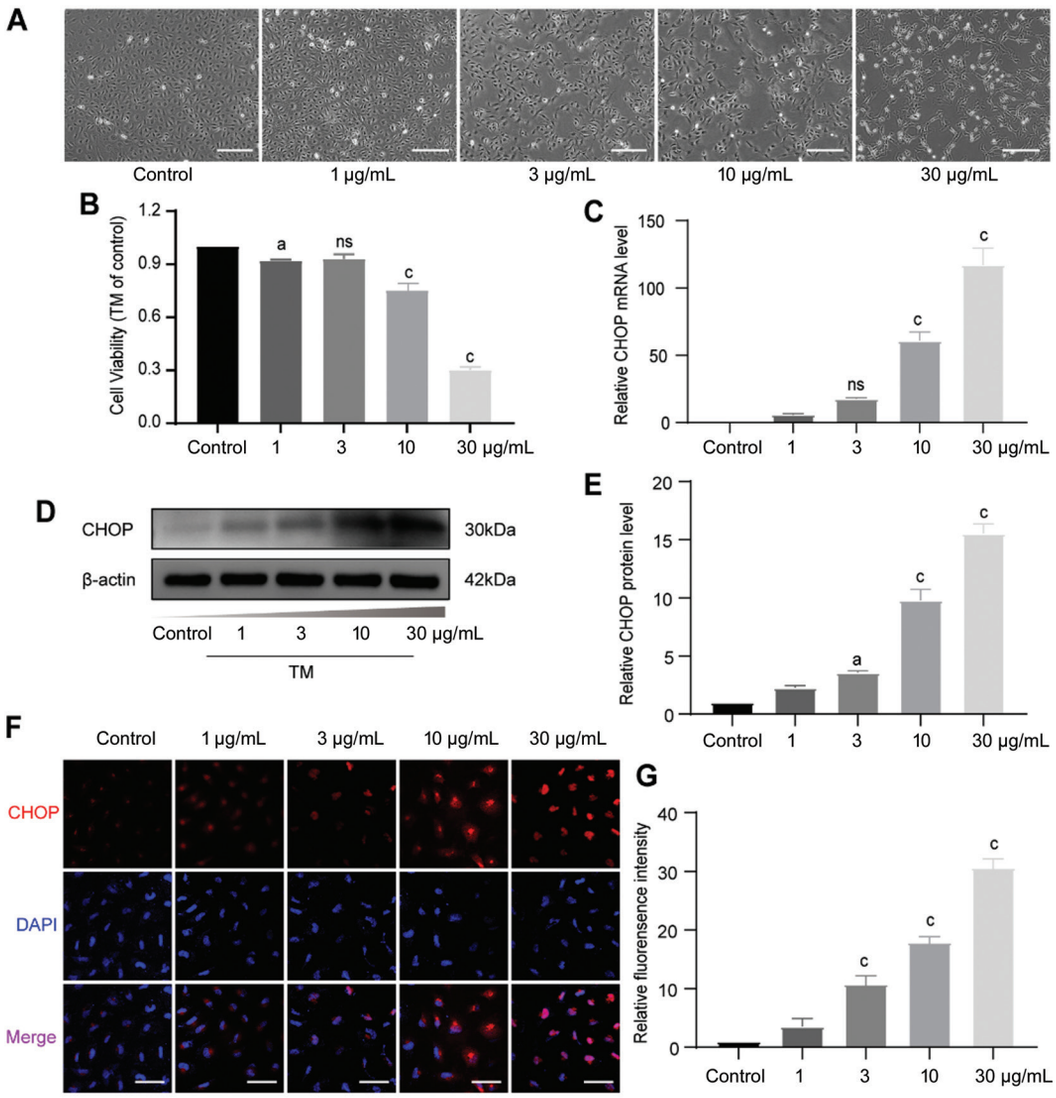

Figure 2 Effects of different TM concentration on SRA01/04 cells apoptosis A: The pictures of cellar morphology exposed to different doses of TM under an inverted microscope. Scale bar: 100 μm. B: The CCK8 assay was performed to detect the effect of different TM doses on SRA01/04 cells. C: The relative mRNA level of CHOP after SRA01/04 cells were treated with different TM doses. D-E: Western blotting analysis of CHOP induced by different TM doses. F-G: Immunofluorescence staining analysis of CHOP induced by different TM doses. Scale bar: 50 μm. All experiments were repeated at least three times. Data were presented as mean±SEM. $^{a}P<0.05$, $^{c}P<0.001$, ns: Not significant. TM: Tunicamycin; CCK8: Cell counting kit-8; CHOP: C/EBP homologous protein; DAPI: 4′,6-diamidino-2-phenylindole; SEM: Standard error of the mean.

further enhanced in the si-SIRT1+TM group compared with TM group at the translational level ($P<0.001$; Figure 3C, 3D). The SIRT1 immunofluorescence staining (the red fluorescence) got the accordant results that its staining and cytoplasm translocation induced by TM was weakened when SIRT1 was knocked down ($P<0.05$). We further explored the relationship between SIRT1 and ER stress by examining the levels of classical ER stress markers, including GRP78 and ATF4[19]. In the presence of si-SIRT1, both the mRNA and protein levels of GRP78 (both $P<0.001$) and ATF4 (both $P<0.001$) significantly increased compared with control group, indicating that SIRT1 was the negative regulator of them (Figure 3B-3F). These increases were further enhanced after TM stimulation and SIRT1 knockdown in the si-SIRT1+TM group (Figure 3B-3F). We also visualized structural changes in the ER using ER tracker imaging, which showed significantly enhanced staining in SRA01/04 cells with TM stimulation. CHOP expression trend in SRA01/04 cells was consistent with the changes in ER markers observed in RT-PCR (Figure 3B), Western blotting (Figure 3C, 3G), and immunofluorescence staining (the red fluorescence; Figure 3H, 3I), providing evidence of the involvement of ER stress in cell apoptosis.

Next, we utilized SRT1720, a synthetic and effective SIRT1 agonist known for its strong anti-aging and anti-apoptosis properties, to mimic the overexpression of SIRT1 in our in vitro experiments[20]. To investigate the protective effect of SRT1720 on SRA01/04 cells, we treated the cells with 10 μg/mL TM for 24h, with or without 2.5 μmol/L SRT1720 for an additional

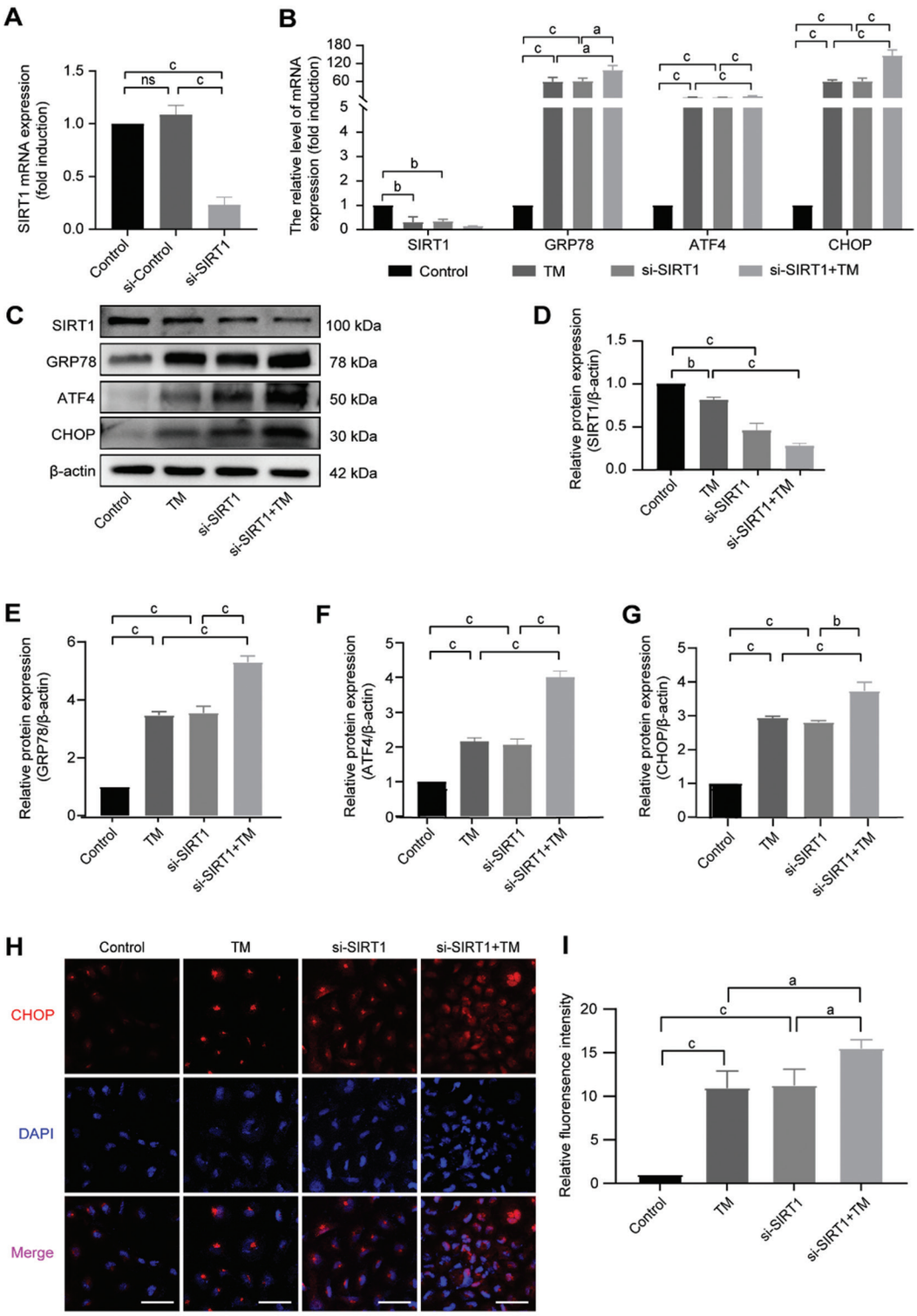

Figure 3 SIRT1 silencing affect TM-induced SRA01/04 cells apoptosis through ER stress pathway A: The relative SIRT1 mRNA expression when SIRT1 knocked down in SRA01/04 cells; B: The relative mRNA expression of SIRT1, GRP78, ATF4, and CHOP were detected by RT-PCR in different groups; C-G: The protein expression of SIRT1, GRP78, ATF4, and CHOP were detected by Western blotting in different groups; H-I: Immunofluorescence staining analysis of CHOP in different groups. Scale bar: 50 μm. All experiments were repeated at least three times. Data were presented as mean±SEM. [a]P<0.05, [b]P<0.01, [c]P<0.001, ns: Not significant. SIRT1: Silent information regulator factor 2-related enzyme 1; TM: Tunicamycin; GRP78: Glucose regulated protein 78; ATF4: Activating transcription factor 4; CHOP: C/EBP homologous protein; RT-PCR: Real-time quantitative polymerase chain reaction; DAPI: 4',6-diamidino-2-phenylindole; SEM: Standard error of the mean.

2h. CCK-8, RT-PCR and Western blotting demonstrated that SRT1720 treatment explicitly reversed the change in SIRT1 expression back to that of the control group. The effect of SRT1720 post-treatment (TM+SRT1720 group) was found to be better than SRT1720 pre-treatment (SRT1720+TM group) in the results of CCK8 assay (P<0.05) and Western blotting.

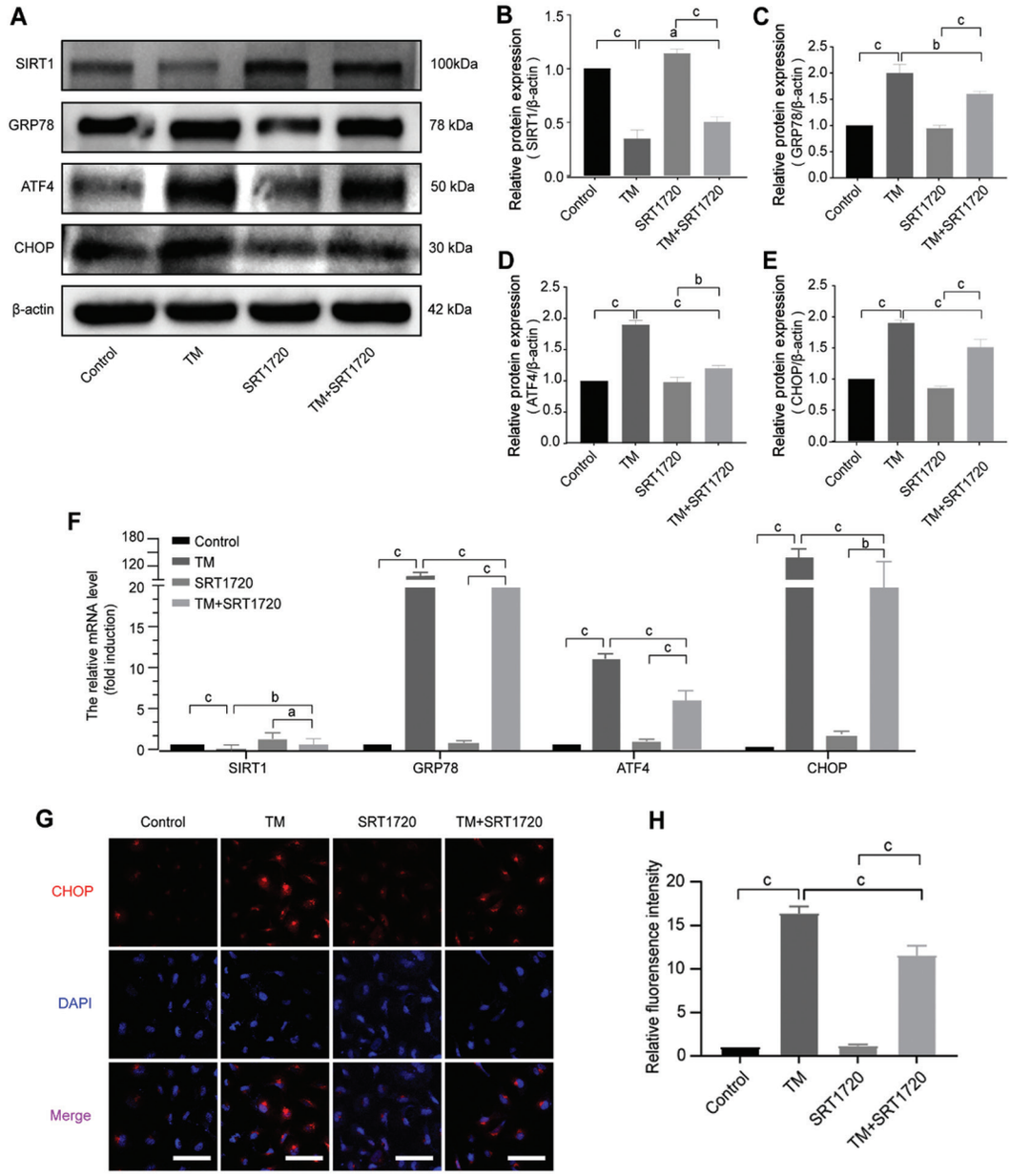

Figure 4 SIRT1 overexpression affect TM-induced SRA01/04 cells apoptosis through ER stress pathway A-E: The protein expression of SIRT1, GRP78, ATF4, and CHOP were detected by Western blotting in different groups; F: The mRNA expression of SIRT1, GRP78, ATF4, and CHOP were detected by RT-PCR in different groups; G-H: Immunofluorescence staining analysis of CHOP in different groups. Scale bar: 50 μm. All experiments were repeated at least three times. Data were presented as mean±SEM. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. SIRT1: Silent information regulator factor 2-related enzyme 1; ER: Endoplasmic reticulum; TM: Tunicamycin; GRP78: Glucose regulated protein 78; ATF4: Activating transcription factor 4; CHOP: C/EBP homologous protein; RT-PCR: Real-time quantitative polymerase chain reaction; DAPI: 4′,6-diamidino-2-phenylindole; SEM: Standard error of the mean.

Immunofluorescence staining of SIRT1 showed similar results. Therefore, SRT1720 post-treatment was adopted in our subsequent experiments. In contrast to the intervention of si-SIRT1, both the mRNA and protein levels of GRP78 (both $P>0.05$) and ATF4 (both $P>0.05$) showed no significant difference from control group (Figure 4A, 4C, 4D, 4F). While SRT1720 treatment in TM+SRT1720 group decreased the mRNA and protein levels of GRP78 ($P<0.001$ and $P<0.01$) and ATF4 (both $P<0.001$) compared with TM group (Figure 4A, 4C, 4D, 4F). Immunofluorescence staining of CHOP showed similar results, the red fluorescence of TM+SRT1720 group was weaker than that of TM group ($P<0.001$; Figure 4G, 4H). These solid data clearly demonstrated that SIRT1 hindered the apoptosis of SRA01/04 cells and alleviated the damage caused by ER stress.

Inhibition of ER Stress Partly Reverse the Adverse Effects of SIRT1 Knockdown on Apoptosis To further explore the protective function of SIRT1 in ER stress and apoptosis, we used ER stress inhibitor 4-PBA to restore apoptosis induced by TM[21]. In the treated groups, the SRA01/04 cells were divided

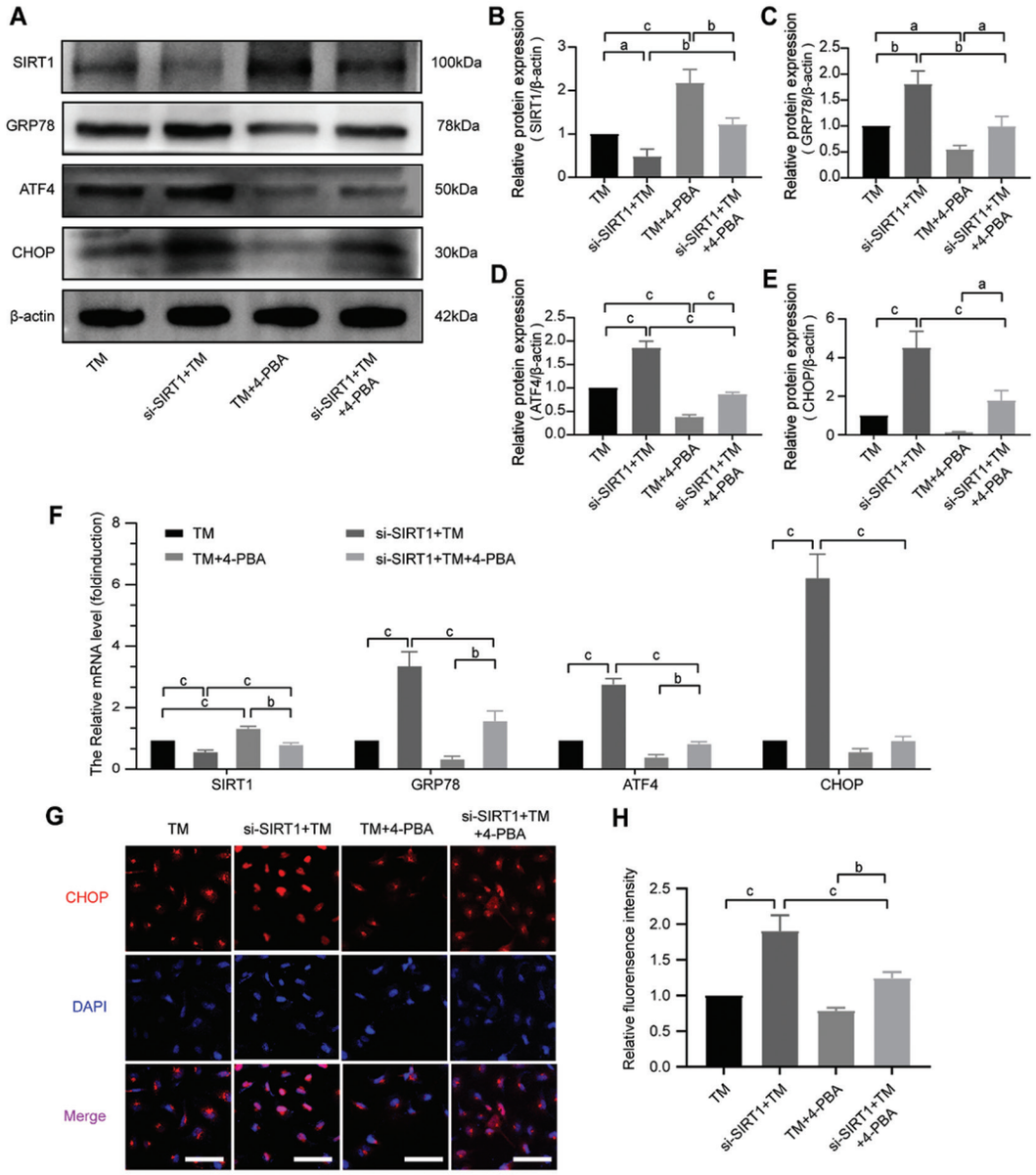

Figure 5 4-PBA partly reverse the adverse effects of SIRT1 knockdown on apoptosis through inhibiting ER stress A-E: The protein expression of SIRT1, GRP78, ATF4, and CHOP were detected by Western blotting in different groups; F: The mRNA expression of SIRT1, GRP78, ATF4, and CHOP were detected by RT-PCR in different groups; G-H: Immunofluorescence staining analysis of CHOP in different groups. Scale bar: 50 μm. All experiments were repeated at least three times. Data were presented as mean±SEM. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. 4-PBA: 4-phenyl butyric acid; SIRT1: Silent information regulator factor 2-related enzyme 1; ER: Endoplasmic reticulum; TM: Tunicamycin; GRP78: Glucose regulated protein 78; ATF4: Activating transcription factor 4; CHOP: C/EBP homologous protein; RT-PCR: Real-time quantitative polymerase chain reaction; DAPI: 4',6-diamidino-2-phenylindole; SEM: Standard error of the mean.

into different groups: TM group, si-SIRT1+TM group, TM+4-PBA group, and si-SIRT1+TM+4-PBA group. The dosage and administration method of 4-PBA were determined based on the cell viability results obtained from different concentrations of 4-PBA, as well as the SIRT1 expression in SRA01/04 cells under the impact of 4-PBA. The experimental results showed that compared with TM group, 4-PBA treatment reduced the protein expression levels of GRP78 and ATF4 in TM+4-PBA group ($P<0.05$ and $P<0.001$; Figure 5A, 5C, 5D). When SIRT1 was specifically knocked out in SRA01/04 cells, the severity of ER stress and apoptosis significantly increased, as evidenced by the increased expression of GRP78, ATF4 and CHOP in Figure 4. However, in comparison with the si-SIRT1+TM group, 4-PBA treatment of the si-SIRT1+TM+4-PBA group led to a downregulation of GRP78 ($P<0.01$ and $P<0.05$), ATF4 ($P<0.01$ and $P<0.001$) and CHOP ($P>0.05$ and $P<0.05$) detected by RT-PCR and Western blotting (Figure 5A-5F). Immunofluorescence staining of CHOP (the red fluorescence) showed more obvious downward trend ($P<0.01$; Figure 5G, 5H). Thus, these findings provided strong evidence that 4-PBA partially counteract the detrimental effects of SIRT1 knockdown.

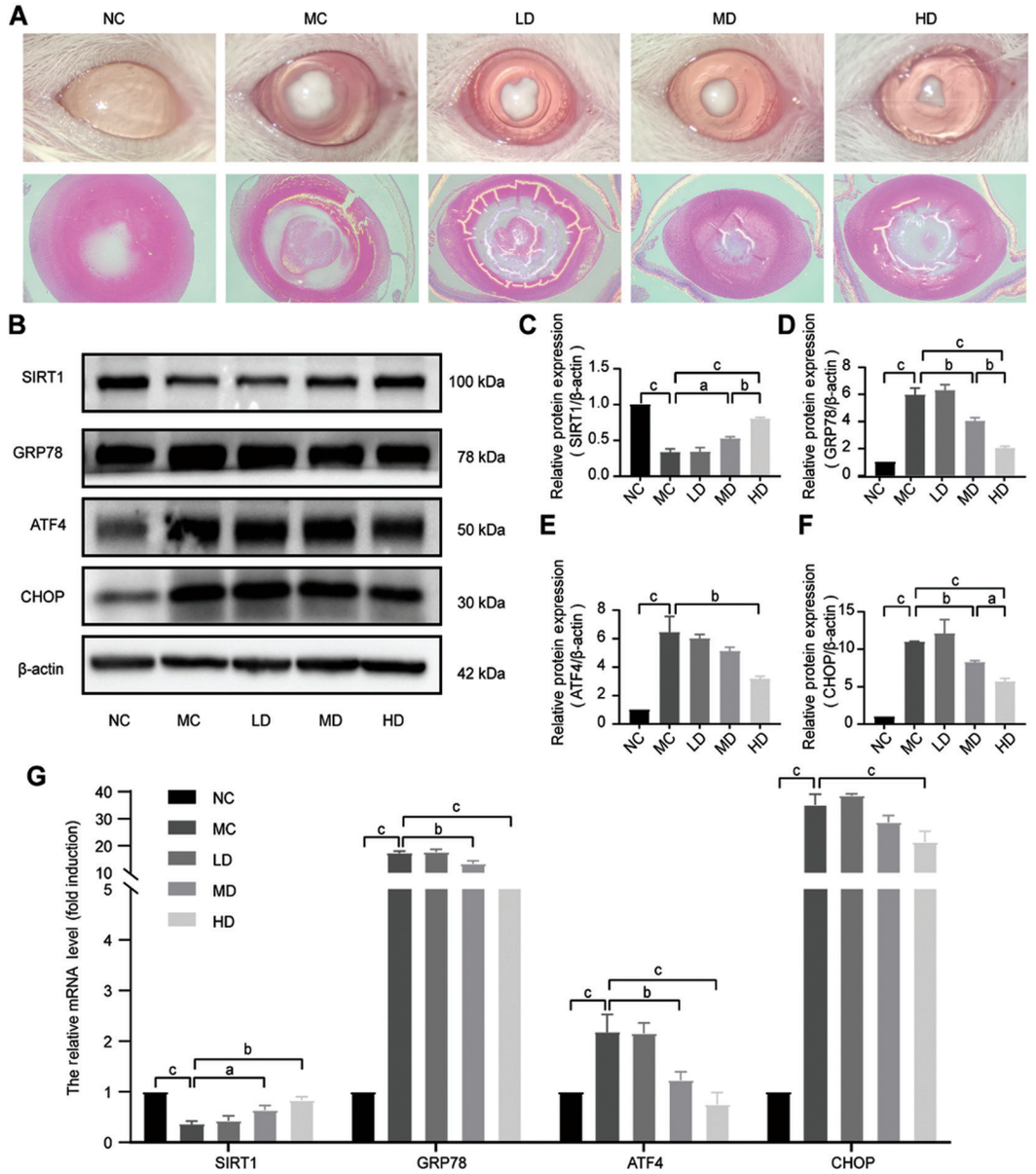

Figure 6 The impact of SIRT1 to ameliorate lens opacification and inhibit ER stress in rat ARC model A: The change of lens opacification observed by anterior segment photographs and H&E staining in different groups; B-F: The protein expression of SIRT1, GRP78, ATF4, and CHOP were detected by Western blotting in different groups; G: The mRNA expression of SIRT1, GRP78, ATF4, and CHOP were detected by RT-PCR in different groups. All experiments were repeated at least three times. Data were presented as mean±SEM. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. NC: Normal control group; MC: Model control group; LD: Low-dose SRT1720 treated group; MD: Medium-dose SRT1720 treated group; HD: High-dose SRT1720 treated group; SIRT1: Silent information regulator factor 2-related enzyme 1; ER: Endoplasmic reticulum; ARC: Age-related cataract; H&E: Hematoxylin and eosin; GRP78: Glucose regulated protein 78; ATF4: Activating transcription factor 4; CHOP: C/EBP homologous protein; RT-PCR: Real-time quantitative polymerase chain reaction; SEM: Standard error of the mean.

SIRT1 Inhibit ER Stress and Ameliorate Lens Opacification in Rat ARC Model To investigate the protective function of SIRT1 on ARC *in vivo*, SD rat ARC models were established by sodium selenite subcutaneous injection. On day 24, the lens in the MC group exhibited significant opacification compared to the NC group, indicating the successful induction of cataract. The extent of lens opacification in the MD and HD group was visibly less than that in the MC group (Figure 6A). In H&E staining of the NC group, the lens fibers remained intact with no significant pathology observed. In contrast, the MC group showed obvious changes in the lens structure, including degeneration or liquefaction of lens fibers (Figure 6A). Administration of 50 mg/kg or 100 mg/kg SRT1720 ameliorated these lens alterations to some extent. The results of Western blotting (Figure 6B, 6C) and RT-PCR (Figure 6G) revealed the SIRT1 expression of MC group lower than NC group ($P<0.001$ and $P<0.001$), MD group (both $P<0.05$) and HD group ($P<0.001$ and $P<0.01$), which were consistent with the results of cell experiments in this study. We also observed the decreased protein expression of GRP78

(P<0.01 and P<0.001), ATF4 (P<0.01 and P<0.001), and CHOP (P<0.01 and P<0.001) in both MD group and HD group in comparison with MC group (Figure 6B, 6D-6F). In summary, our results showed that SIRT1, activated by SRT1720, had a positive effect on inhibiting the expression of GRP78, ATF4 and CHOP, and ameliorating lens opacification in ARC rat models.

DISCUSSION

Growing evidence suggests that changes in lens protein conformation are linked to ER stress, which is a key mechanism in the development of cataracts[3]. In response to ER stress, HLECs activate the unfolded protein response, a protective system involving some sensors to maintain protein homeostasis. Normally, these sensors remain inactive due to their association with GRP78[22]; however, they are activated by the accumulation of misfolded proteins to restore ER balance by enhancing chaperone activity, reducing protein synthesis, and degrading abnormal proteins[23]. Prolonged ER stress can overwhelm these protective responses, leading to the activation of transcription factors such as ATF4, and ultimately causing cell apoptosis[24]. TM is known to induce ER stress, as evidenced by the elevation of GRP78 and ATF4, markers that signal apoptosis and the potential for cataract formation[9,25]. Herein, we demonstrate that SRA01/04 cell apoptosis induced by TM is mediated through the activation of ER stress pathways, as evidenced by the increased expression of GRP78 and ATF4, followed by a rise in the downstream protein CHOP. CHOP is a pro-apoptotic protein closely related to ER stress. Data from mutagenesis experiments have shown that the deletion of CHOP functional domains induced cell apoptosis[18]. Further research has shown that the apoptosis of CHOP-deficient cells was related to ER stress[26]. Thus, our findings support the hypothesis that excessive ER stress leading to cell apoptosis is a crucial mechanism underlying the development of cataracts.

To date, the mammalian SIRT family includes seven members, SIRT1-7[13]. SIRT1, closest to the Sir2 ortholog, participates in numerous physiological and pathological processes involving in ER stress, apoptosis as well as autophagy[27-28]. Evidently, SIRT1 serves as an anti-aging agent against ocular diseases, like cataracts and age-associated macular degeneration[29-30]. Data suggests that aging or failing cellular functions, particularly chaperone system decline, exacerbate protein aggregation and misfolding[31]. SIRT1, as a potent cellular stress inhibitor, guards against these stimuli[9,32]. We used TM to establish an apoptosis model in SRA01/04 cells, confirming ER stress's role in ARC development. Increased TM doses upregulated GRP78 and ATF4 expression, with corresponding ER Tracker immunofluorescence, indicating in vitro ER stress activation. CHOP expression matched GRP78 and ATF4. We applied si-SIRT1 to simulate SIRT1-knockout and SRT1720 for SIRT1-overexpression. RT-PCR and Western blot confirmed si-SIRT1 reduced SIRT1 expression below 30%. SRT1720, a potent SIRT1 activator, has been extensively studied for its potential in treating various age-related diseases in humans. Unlike the natural SIRT1 activator resveratrol, synthetic SIRT1 activators have shown much higher potency. For example, SRT1720 can increase SIRT1 activity by 750% at a concentration of 10 μmol/L[33]. In alignment with existing literature, our study found that activating SIRT1 with SRT1720 can effectively reverse the ER stress and apoptosis induced by TM in SRA01/04 cells. Additionally, we used 4-PBA to investigate whether suppressing ER stress could mitigate the negative effects of SIRT1 in SRA01/04 cells. 4-PBA, an ER stress antagonist and FDA-approved drug for urea cycle disorders, stabilizes proteins to alleviate ER stress[34]. Our findings demonstrated that 4-PBA could effectively lower ER stress and TM-induced apoptosis. Moreover, when SIRT1 was knocked down in TM-treated SRA01/04 cells, 4-PBA ameliorated the damage caused by SIRT1 deficiency and TM cytotoxicity, indicating a partial reversal of the effects resulting from SIRT1 knockdown. Thus, these findings offer compelling evidence that SIRT1 plays a role in regulating apoptosis in SRA01/04 cells by modulating ER stress.

To evaluate the effectiveness of SIRT1 in inhibiting cataractogenic stresses *in vivo*, we utilized the selenite cataract rat model and administered SRT1720. As expected, both doses of 50 and 100 mg/kg SRT1720 were effective in reducing lens opacification induced by sodium selenite in SD rats. Microscopic examination revealed a decrease in the area of opacity, and H&E staining showed a reduction in lens fiber degeneration. The changes in SIRT1 and ER stress marker proteins in rat LECs were consistent with our in vitro findings. These results collectively suggest the protective effect of SIRT1 against cataracts. Thus, it is plausible that SIRT1 could prevent sodium selenite-induced LECs apoptosis and cataract formation by regulating the ER stress.

There are some limitations of our study. Firstly, the accumulation of unfolded proteins in the ER induces the dissociation of GRP78 from PERK, ATF6, and IRE1α, activating downstream signaling pathways. The mechanisms by which SIRT1 affects the ATF6 and IRE1α pathways of ER stress are currently unknown and need further exploration in subsequent studies. Secondly, SIRT1 is a highly conserved deacetylase that can profoundly deacetylate various signaling molecules, transcription factors, histones, and non-histone proteins[35]. However, it has not been examined whether the deacetylation of SIRT1 plays an important role in resisting cataractogenic stresses. Therefore, the exact mechanisms and potential biological consequences need to be investigated in future research.

In conclusion, we demonstrated that TM-induced cell apoptosis led to ARC by regulating ER stress-associated pathways. As a powerful regulator, SIRT1 could attenuate ER stress and apoptosis induced by TM through mediating the expression of GRP78, ATF4 and CHOP. When si-SIRT1 and SRT1720 were applied to SRA01/04 cells respectively, the intensity of ER stress and apoptosis worsened or was mitigated. The 4-PBA treatment inhibited ER stress and partly reversed the detrimental effects of SIRT1 knockdown in SRA01/04 cells. Furthermore, the intraperitoneal injection of SRT1720 attenuated rat lens opacification induced by sodium selenite via regulating ER stress pathway. These findings highlight the anti-ER stress capacities of SIRT1, suggesting its potential as a therapeutic target for the treatment of ARC.

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