



Basic Study

Quercetin ameliorates oxidative stress-induced senescence in rat nucleus pulposus-derived mesenchymal stem cells via the miR-34a-5p/SIRT1 axis

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Abstract

BACKGROUND

Intervertebral disc degeneration (IDD) is a main contributor to low back pain. Oxidative stress, which is highly associated with the progression of IDD, increases senescence of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) and weakens the differentiation ability of NPMSCs in degenerated intervertebral discs (IVDs). Quercetin (Que) has been demonstrated to reduce oxidative stress in diverse degenerative diseases.

AIM

To investigate the role of Que in oxidative stress-induced NPMSC damage and to elucidate the underlying mechanism.

METHODS

In vitro, NPMSCs were isolated from rat tails. Senescence-associated β -galactosidase (SA- β -Gal) staining, cell cycle, reactive oxygen species (ROS), real-time quantitative polymerase chain reaction (RT-qPCR), immunofluorescence,

and western blot analyses were used to evaluate the protective effects of Que. Meanwhile the relationship between miR-34a-5p and Sirtuins 1 (SIRT1) was evaluated by dual-luciferase reporter assay. To explore whether Que modulates tert-butyl hydroperoxide (TBHP)-induced senescence of NPMSCs *via* the miR-34a-5p/SIRT1 pathway, we used adenovirus vectors to overexpress and downregulate the expression of miR-34a-5p and used SIRT1 siRNA to knockdown SIRT1 expression. *In vivo*, a puncture-induced rat IDD model was constructed, and X rays and histological analysis were used to assess whether Que could alleviate IDD *in vivo*.

RESULTS

We found that TBHP can cause NPMSCs senescence changes, such as reduced cell proliferation ability, increased SA- β -Gal activity, cell cycle arrest, the accumulation of ROS, and increased expression of senescence-related proteins. While abovementioned senescence indicators were significantly alleviated by Que treatment. Que decreased the expression levels of senescence-related proteins (p16, p21, and p53) and senescence-associated secreted phenotype (SASP), including IL-1 β , IL-6, and MMP-13, and it increased the expression of SIRT1. In addition, the protective effects of Que on cell senescence were partially reversed by miR-34a-5p overexpression and SIRT1 knockdown. *In vivo*, X-ray, and histological analyses indicated that Que alleviated IDD in a puncture-induced rat model.

CONCLUSION

In summary, the present study provides evidence that Que reduces oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway, suggesting that Que may be a potential agent for the treatment of IDD.

Key Words: Quercetin; Nucleus pulposus-derived mesenchymal stem cells; Oxidative stress; Senescence; Intervertebral disc degeneration; miR-34a-5p/SIRT1 pathway

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Core Tip: In our article, we provide the evidence that quercetin (Que) can prevent oxidative stress induced senescence of nucleus pulposus-derived mesenchymal stem cells *via* miR-34a/SIRT1 signaling pathway. Moreover, Que could ameliorate the progression of intervertebral disc degeneration (IDD) in rat model. Thus, Que can be considered as a potential agent for the treatment of IDD.

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INTRODUCTION

As a widely known musculoskeletal disorder, low back pain (LBP) is the leading cause of disability and results in a huge economic burden on the family and society[1,2]. Intervertebral disc degeneration (IDD) is a main contributor to LBP, but the etiology of IDD is multifactorial[3]. Therefore, elucidating the underlying molecular mechanisms of IDD will help develop solutions for the prevention and treatment of IDD.

Several previous studies have indicated that oxidative stress and reactive oxygen species (ROS) are highly associated with the progression of IDD[4-7]. While ROS production is inevitable during the metabolic processes of disc cells, the progression of IDD is considered to be the result of disc cell senescence caused by ROS accumulation[7,8]. Recently, stem cell-based endogenous repair has been shown to play a significant role in the repair and regeneration of degenerative intervertebral discs (IVDs)[9,10]. Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were first reported by Risbud *et al*[11] in 2007, which rendered a basis for the endogenous repair of IDD[11]. We also previously found NPMSCs in normal and degenerated IVDs[12-15]. Wu *et al*[16] indicated that the regenerative potential of NPMSCs decreases with aging and degeneration of IVDs[16]. In addition, Ma *et al*[17] proposed that the decrease in endogenous NPMSCs caused by adverse microenvironments, such as oxidative stress, low nutrition, and inflammation, is one of the main reasons for the failure of endogenous repair of IDD[17]. Therefore, inhibition of oxidative stress-induced senescence of NPMSCs may be of great significance in alleviating IDD.

Quercetin (Que), a natural flavonoid widely present in various plants, has antioxidative stress, anti-inflammatory, and antiaging properties. Que has been used as a senolytic to prevent diverse degenerative diseases[18-20]. Zhu *et al*[21] found that Que reduces senescent cells by selectively killing senescent human epithelial cells and mouse bone marrow mesenchymal stem cells[21]. Feng *et al*[22] reported that Que attenuates oxidative stress-induced apoptosis in rat

chondrocytes and prevents the progression of osteoarthritis in a rat model[22]. Previous clinical studies have also demonstrated that Que reduces the expression levels of senescence-related markers[23].

MicroRNAs (miRNAs) are small double-stranded noncoding RNAs that are approximately 20 nucleotides in length and interfere with RNA translation by binding to the 3'UTR sequence of the target gene RNA. Abnormal expression of miRNAs has been observed in degenerative IVD, indicating that miRNAs play an important role in the pathophysiological process of IDD[24,25]. A previous study has also demonstrated that the expression of miR-34a-5p is significantly upregulated in degenerated IVDs and that miR-34a-5p increases extracellular matrix degradation and cell apoptosis. Sirtuins 1 (SIRT1), the target of miR-34a, is a member of the sirtuin family and plays a significant role in cancer, age-related diseases, and degenerative diseases[26-28]. Several studies have reported that SIRT1 delays the senescence of IVD cells by decreasing oxidative stress and inflammation as well as by improving mitochondrial function[29,30]. However, the protective effect of Que on NPMSCs is still unknown.

In the present study, tert-butyl hydroperoxide (TBHP) was used to trigger oxidative stress in NPMSCs, which is widely accepted as an apoptosis and senescence *in vitro* model. The effects of Que on senescence in NPMSCs under oxidative stress and the role of the miR-34a-5p/SIRT1 pathway were investigated. Finally, the therapeutic effects were also evaluated in a puncture-induced rat IDD model.

MATERIALS AND METHODS

Isolation and culture of NPMSCs

All the procedures performed in this study were approved by the Ethical Committee of the Clinical Medical College of Yangzhou University. Sprague-Dawley (SD) rats (2-4 mo old and weighing 200-300 g) were obtained from Yangzhou University, No. SYXK (Su) 2017-0044. The separation of nucleus pulposus (NP) tissues and the isolation of NPMSCs were performed as previously described[14]. Briefly, the NP tissues obtained from SD rats were carefully separated by a microscope under sterile conditions, and the NP tissue was mechanically fragmented into 1 mm³ pieces and digested with 0.2% collagenase type II (Gibco, United States) at 37 °C in 5% CO₂ for 12 h. Cells were filtered through a 75-µm cellular filter, centrifuged at 1000 rpm for 3 min, washed with phosphate-buffered saline (PBS), resuspended in Mesenchymal Stem Cell Complete Medium (Cyagen, United States) with 10% fetal bovine serum (FBS; HyClone, United States) and 1% antibiotics (Gibco, United States), and cultured in an incubator at 37 °C with 5% CO₂. NPMSCs were subcultured at a ratio of 1:3 when they reached 80% confluence. The third passages of NPMSCs were used for the subsequent experiments.

Surface marker identification of NPMSCs

NPMSCs were cultured in a 12-well plate with a 25 mm diameter cell slide in complete medium. Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. Subsequently, cells were blocked with 10% bovine serum albumin for 1 h at room temperature and incubated with the following primary antibodies (1:100) overnight at 4 °C according to the International Society for Cellular Therapy (ISCT): CD34 (ABclonal, China), CD45 (ABclonal, China), CD73 (ABclonal, China), CD90 (ABclonal, China), CD105 (Proteintech, China), and tyrosine kinase with Ig and EGF homology domains 2 (Tie2, a disc NP progenitor marker) (Biodragon, China). Cells were then incubated with FITC- or Cy3-conjugated secondary antibodies (1:200) at room temperature for 1 h in the dark. After treatment with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, the cell slides were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Cell treatment and cell viability assay

To establish an oxidative stress-induced model of NPMSCs, NPMSCs were cultured in complete medium with different concentrations (0, 25, 50, 100, 200, and 400 µM) of TBHP (Sigma-Aldrich, United States) for 12 h. Cells were pretreated with different concentrations (0, 10, 20, 40, 80, and 160 µM) of Que (MedChem Express, China) for 24 h before the addition of a suitable concentration of TBHP to investigate the suitable dose of Que. A cell counting kit-8 (CCK-8, Beyotime, China) was used to evaluate cell viability according to the manufacturer's instructions. Briefly, 2 × 10³ cells/well were seeded in 96-well plates and cultured overnight at 37 °C in 5% CO₂. After the cells were treated as described above, a mixture of 10 µL of CCK-8 reagent and 100 µL of fresh medium was added to each well for 1 h at 37 °C. The optical density (OD) value of each well was measured at 450 nm by a microplate reader (Bio-Rad, United States). Cells were divided into the following groups: Control group (untreated), TBHP group (treated with 100 µM TBHP) and Que + TBHP group (treated with 20 µM Que and 100 µM TBHP).

Cell proliferation assay

Cell proliferation was evaluated by an 5-ethynyl-2'-deoxyuridine (EdU) assay (Beyotime, China). Cells (5 × 10³ cells/well) were seeded in 12-well plates and cultured at 37 °C in 5% CO₂. According to the manufacturer's instructions, NPMSCs were incubated with EdU for 2 h, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with Click Reaction Mixture for 30 min. After washing with PBS, cells were counterstained with Hoechst 33342 in the dark for 10 min. Finally, cells were observed under a fluorescence microscope and analyzed by ImageJ software (NIH, United States).

Senescence-associated β-galactosidase (SA-β-Gal) staining

NPMSCs were seeded in a 6-well plate at a density of 4 × 10⁵ cells/well at 37 °C in 5% CO₂. Cells were stained with SA-β-

Gal using a SA- β -Gal staining kit (Beyotime, China) according to the manufacturer's instructions. Cells were fixed with SA- β -gal fixing solution for 15 min at room temperature and then incubated overnight with an SA- β -Gal working solution at 37 °C without CO₂. Cells were observed under a microscope and analyzed by ImageJ software.

ROS assay

According to the manufacturer's instructions provided by a ROS detection fluorescent probe-DHE kit (Keygen, China), NPMSCs were incubated with 20 μ M DHE for 30 min at 37 °C. Cells were then observed under a fluorescence microscope and analyzed by ImageJ software.

JC-1 assay for mitochondrial membrane potential

A JC-1 Detection Kit (Keygen, China) was used to measure mitochondrial membrane potential (MMP). Briefly, NPMSCs were cultured in medium containing 2 μ M JC-1 dye, a cationic dye, for 20 min. After being washed twice with incubation buffer, cells were cultured in complete medium, observed by fluorescence microscopy, and analyzed by ImageJ software.

Cell cycle assay

The cell cycle phases of TBHP-induced senescent NPMSCs were determined by flow cytometric analyses using a Cell Cycle Detection Kit (Keygen, China). Briefly, NPMSCs were harvested from 6-well plates, washed twice with ice-cold PBS, fixed with 75% ethanol at 4 °C for 2 h, and incubated with a mixed solution of propidium iodide (PI) and RNase A for 30 min in the dark. Flow cytometry (BD Company, USA) was used to analyze the cell cycle phases.

Cell transfection

Adenovirus vectors containing miR-34a-5p mimic, miR-34a-5p inhibitor, and their corresponding negative controls were obtained from GeneChem (Shanghai, China). SIRT1 siRNA and scrambled siRNA were obtained from Gene Pharma (Shanghai, China). NPMSCs (5 \times 10⁵/well) were seeded in 6-well plates and then transfected with miR-34a-5p mimic or miR-34a-5p inhibitor using Lipofectamine 2000 (Thermo Fisher, UT, USA) for 12 h at 37 °C according to the manufacturer's protocol. After replacing the medium with fresh complete medium, NPMSCs were cultured for an additional 24 h. Cells were then harvested for subsequent experiments.

Dual-luciferase reporter assay

The TargetScan Human database (https://www.targetscan.org/vert_80/) was used to predict the complementary binding site of miR-34a-5p in the 3'-UTR of SIRT1 mRNA. According to the Lipofectamine 2000 transfection protocol, SIRT1 3'-UTR wild-type plasmids or mutant plasmids were cotransfected with miR-34a-5p mimic and mimic control into cells. After 48 h, a dual-luciferase reporter assay kit was used to detect luciferase activity.

qRT-PCR

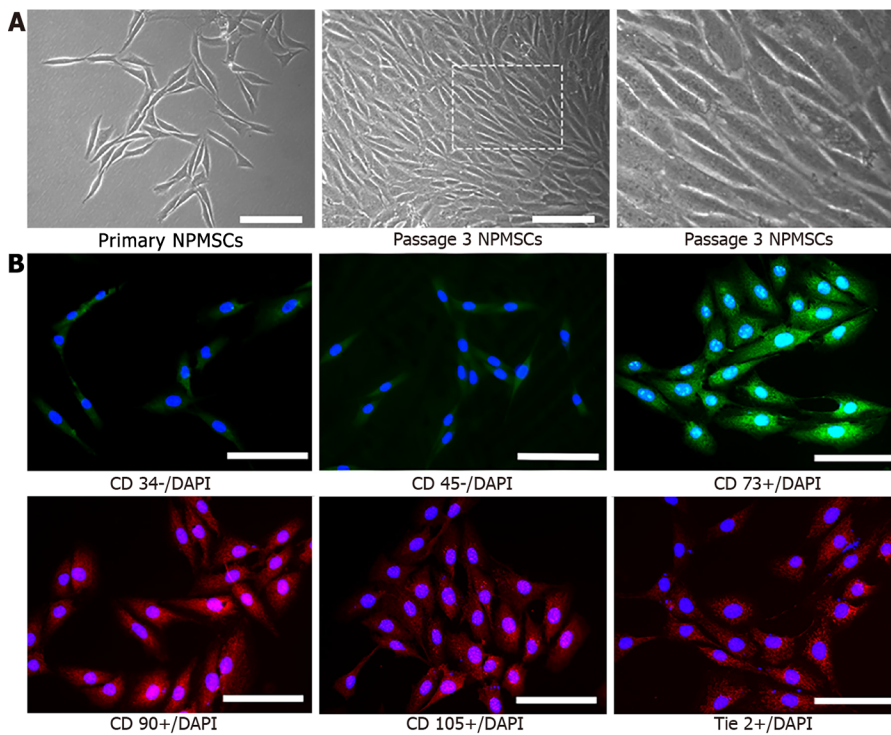
TRIzol reagent (Invitrogen, United States) was used to extract total RNA from NPMSCs. To measure the expression levels of mRNA, RNA was reverse-transcribed into complementary DNA (cDNA) by a Prime Script-RT reagent kit (Vazyme Biotech, China) according to the manufacturer's protocol. cDNA was amplified by SYBR Premix Ex Taq (Vazyme Biotech, China). To measure the expression levels of miR-34a, reverse transcription of RNA and amplification of cDNA were performed using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme Biotech, China) and miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech, China). The 2^{- $\Delta\Delta$ CT} method was used to calculate the expression level of target genes. The expression levels of mRNA or miRNA were normalized to Gapdh or U6. The primers for the target genes used in this study are listed in Table 1.

Western blot analysis

The total protein of NPMSCs was extracted by a Whole Cell Lysis assay (Keygen Biotech, China), and the protein concentration was determined by a BCA protein assay kit (Beyotime, China). Each sample containing 30 μ g of protein was separated by 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, United States). After being blocked in 5% skim milk for 2 h at room temperature, the membranes were incubated overnight with the following primary antibodies at 4 °C: P16 (1:1000; Proteintech, China), p21 (1:1000; Proteintech, China), p53 (1:5000; Proteintech, China), SIRT1 (1:1000; ABclonal, China), IL-1 β (1:1000; ABclonal, China), IL-6 (1:1000; ABclonal, China), matrix metalloproteinase-13 (MMP-13, 1:1000; ABclonal, China) and Gapdh (1:1000; Servicebio, China). The membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (1:5000; Abcam). The protein bands were observed with an enhanced chemiluminescence system, and the expression of protein was analyzed by ImageJ software.

Puncture-induced rat IDD model

In total, 15 male SD rats (2-4 mo old and weighing 200-300 g) were randomly divided into the following three groups: Control group ($n = 5$), IDD group ($n = 5$), and Que group ($n = 5$). The IDD model of SD rats was established as previously described[13]. Briefly, the rats were anesthetized with pentobarbital, and the tail skin was sterilized by povidone iodine. A 21 G needle was used to puncture the coccygeal IVD (Co 6-7), and the needle was rotated 180° and kept in the disc for 5 s at a depth of 5 mm. After surgery, the Que group was treated with Que (100 mg/kg, 5 mg/mL of Que suspended in 0.5% sodium carboxymethyl cellulose solution) every other day by intragastric administration for 4 wk. The control group and IDD group were treated with 0.5% sodium carboxymethyl cellulose solution for 4 wk.



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Figure 1 Identification of nucleus pulposus derived mesenchymal stem cells. A: Primary nucleus pulposus-derived mesenchymal stem cells (NPMSCs) presented with elongated spindle shape and grew in flower formation; B: NPMSCs exhibited low fluorescent expression of CD34 and CD45, but a high fluorescent expression of CD73, CD90, and CD105. Scar bar = 100 μ m.

Radiographic evaluation and histological analysis

At 4 wk postinjury, all rats were placed in a prone position after anesthetization with pentobarbital, and X-ray images were acquired. ImageJ software was used to calculate the disc height index (DHI) as previously described[31]. The tails were harvested after the rats were euthanized with an overdose of sodium pentobarbital. The specimens were fixed in 4% paraformaldehyde for 48 h, decalcified in EDTA for 1 mo, dehydrated in gradient alcohol, and embedded in paraffin. The specimens were cut into 5 μ m sections, and the sections were stained with hematoxylin-eosin (HE), Alcian blue, and Safranin O-fast Green. Histological scores were evaluated as previously described[31,32] based on the following scale: 5, normal discs; 6-11, moderately degenerated discs; and 12-14, severely degenerated discs.

Immunofluorescent staining

After the IVD specimens were prepared, a freezing microtome (Leica, Wetzlar, Germany) was used to cut the specimens into 5- μ m sections. The sections were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. Subsequently, the sections were blocked with 10% bovine serum albumin for 1 h at room temperature and incubated with the following primary antibodies (1:100) at 4 °C overnight: Collagen type II (ABclonal, China) and aggrecan (ABclonal, China). The sections were then incubated with FITC- or Cy3-conjugated secondary antibodies (1:200) at room temperature for 1 h in the dark. Fluorescence microscopy and ImageJ software were used to observe and analyze the sections.

Statistical analysis

All data were analyzed by GraphPad Prism 8 (GraphPad, La Jolla). Data are expressed as the mean \pm SD. Student's t test and one-way analysis of variance (ANOVA) was used to analyze the data of two groups and multiple independent groups, respectively. The Kruskal-Wallis H test was used to analyze the histological score. A *P* value < 0.05 was considered significant.

RESULTS

Characterization of NPMSCs

Elongated spindle-shaped NPMSCs were successfully isolated and cultured from rat IVDs (Figure 1A). Based on the immunofluorescence staining analysis, the MSC-associated markers, namely, CD73, CD90, and CD105 were highly expressed, whereas the expression of CD34 and CD45 was low (Figure 1B). Moreover, the expression of Tie2, a disc NP progenitor marker, was also high in NPMSCs (Figure 1B). These results indicated that NPMSCs isolated from IVD

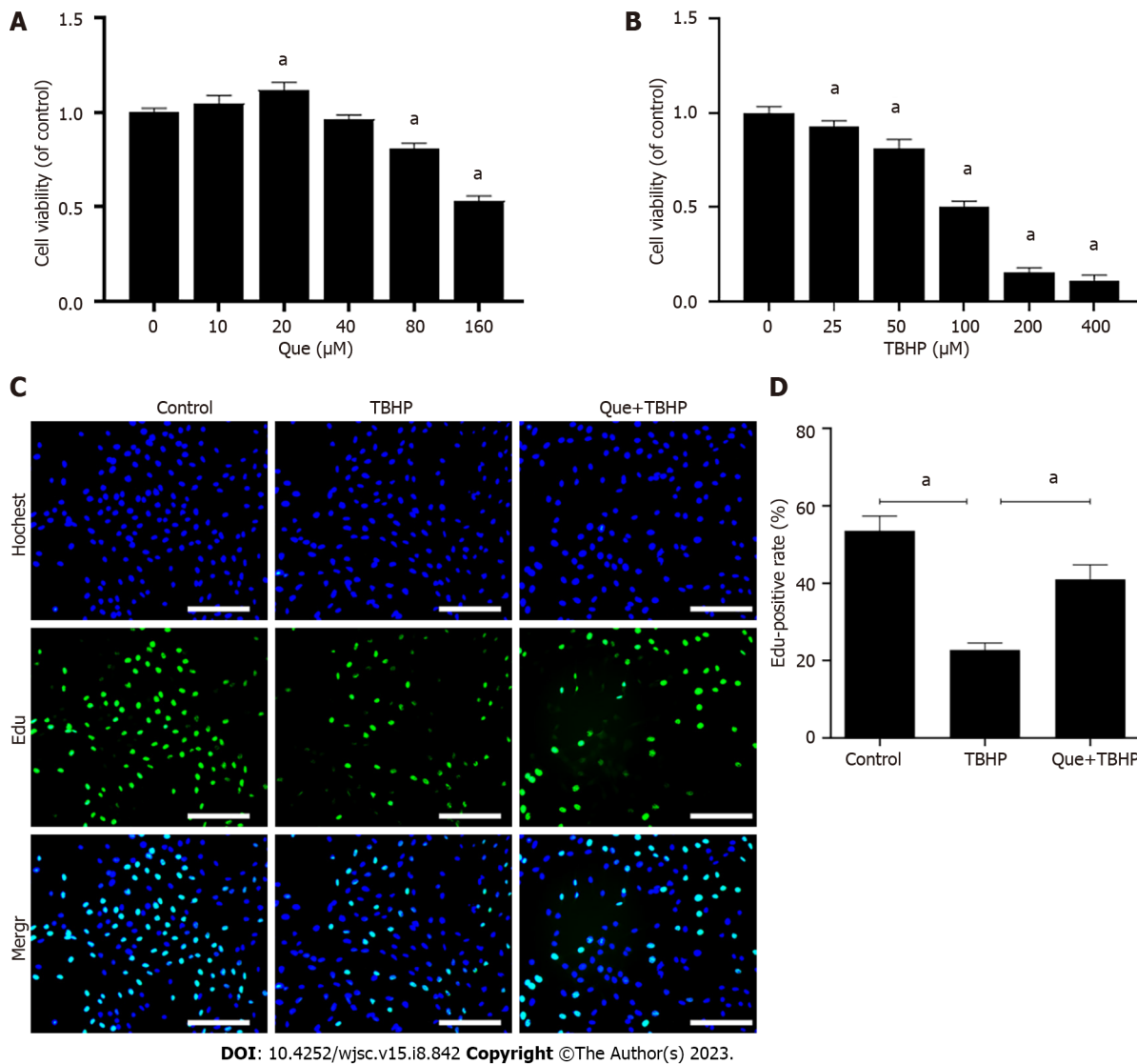


Figure 2 Cell viability assay and cell proliferation assay. A: Cell counting kit-8 (CCK-8) results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) treated with different concentrations of quercetin for 24 h; B: CCK-8 results of NPMSCs treated with different concentrations of tert-butyl hydroperoxide (TBHP) for 24 h; C: EdU assay results of NPMSCs in different groups. Green fluorescence represents cells in a proliferating state, and blue fluorescence represents cell nucleus (scale bar = 200 μm); D: Quantitative analysis of EdU results. Data are represented as mean ± SD. ^a $P < 0.05$ compared with control group, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

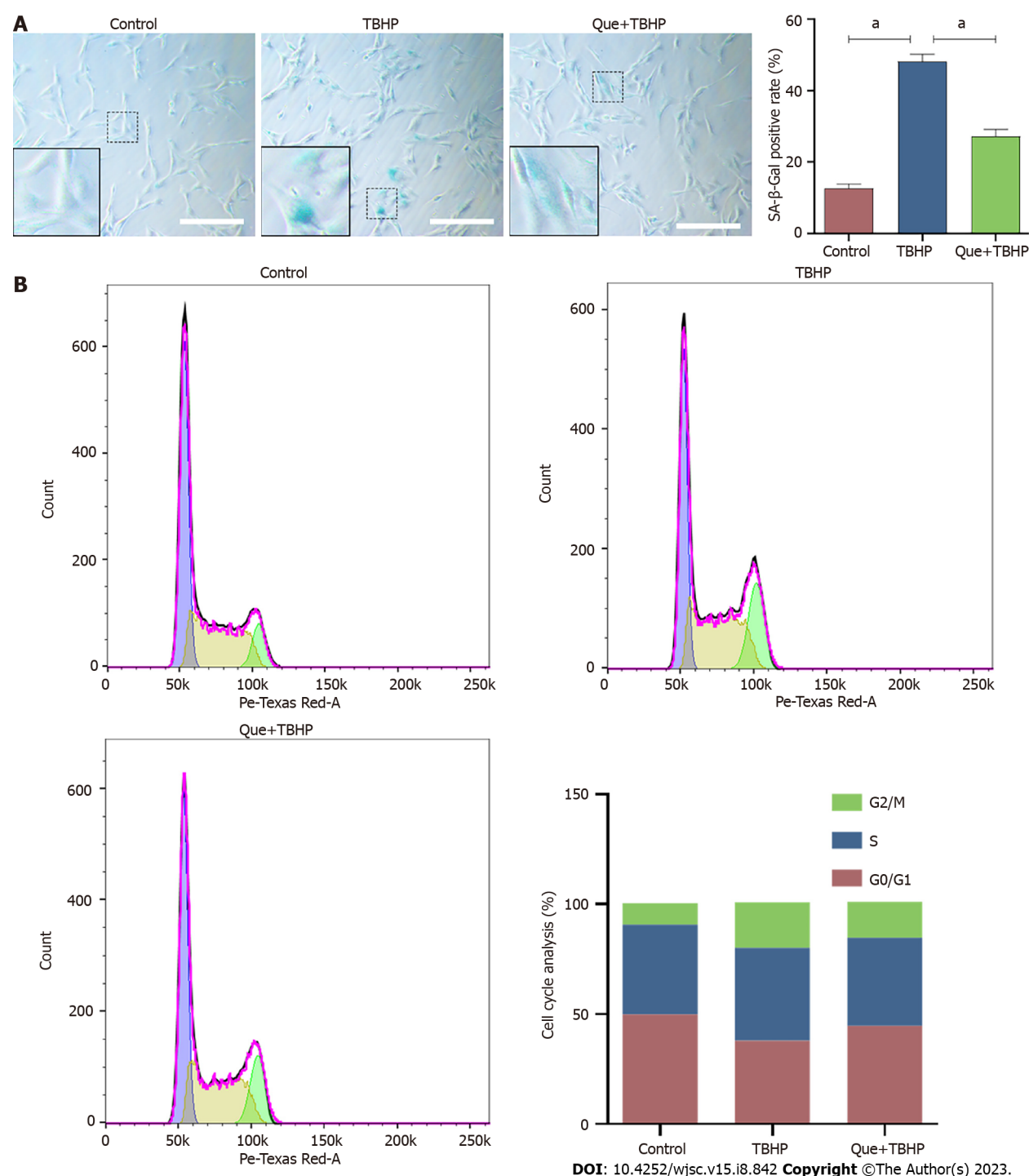
correspond to the standards of stem cells described by ISCT.

Effects of Que and TBHP on the viability and proliferation of NPMSCs

A CCK-8 assay was used to evaluate the effect of Que and TBHP on NPMSC viability. As shown in Figure 2A, Que showed no significant cytotoxic effect at concentrations up to 20 μM for 24 h. Therefore, a concentration of 20 μM Que was selected for the subsequent experiments. NPMSC viability was decreased after treatment with TBHP in a dose-dependent manner (Figure 2B), and 100 μM TBHP was selected for use in subsequent experiments. The effect of Que on NPMSC proliferation was detected by EdU staining. Compared to the control group, the positive rate of EdU in the TBHP group was significantly lower ($P < 0.05$), and the positive rate of EdU was partially increased in the Que + TBHP group ($P < 0.05$) (Figure 2C and D).

Effects of Que on SA-β-Gal staining and the cell cycle in NPMSCs

SA-β-Gal accumulation is an indicator of cellular senescence. Compared to the control group, the number of SA-β-Gal-positive senescent NPMSCs in the TBHP group was significantly higher ($P < 0.05$), while the number of SA-β-Gal-positive cells was decreased in Que + TBHP group (Figure 3A). Compared to the control group, the percentage of NPMSC in the G2/M phase in TBHP group was higher, which indicated cell cycle arrest, but treatment with Que decreased the percentage of NPMSCs arrested in G2/M phase (Figure 3B).



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Figure 3 Senescence-associated β-Galactosidase staining assay and cell cycle assay. A: Senescence-associated β-Galactosidase (SA-β-gal) staining results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) in different groups. Senescent cells exhibit high expression of SA-β-gal in blue staining; B: Cell cycle results of NPMSCs in different groups. Scar bar = 200 μm. Data are represented as mean ± SD. Significant differences between groups are indicated as * $P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

Effects of Que on MMP and ROS generated in TBHP-treated NPMSCs

When the MMP is high, JC-1 exists in the mitochondria as J-aggregates of polymers, which produce red fluorescence. When the MMP is low, JC-1 is released from the mitochondria matrix and exists in the cytoplasm in the form of monomers, which produce green fluorescence. As shown in Figure 4A and B, the MMP was decreased after TBHP treatment, and this effect was partially reversed by pretreatment with Que. Figure 4C and D show that NPMSCs in the TBHP group had higher ROS levels compared to the control group, and pretreatment with Que partially reversed the effect of TBHP on ROS generation.

Que inhibits oxidative stress-induced senescence of NPMSCs

Cell senescence is broadly characterized by cell cycle arrest and the production of SASP. To explore the effect of Que in

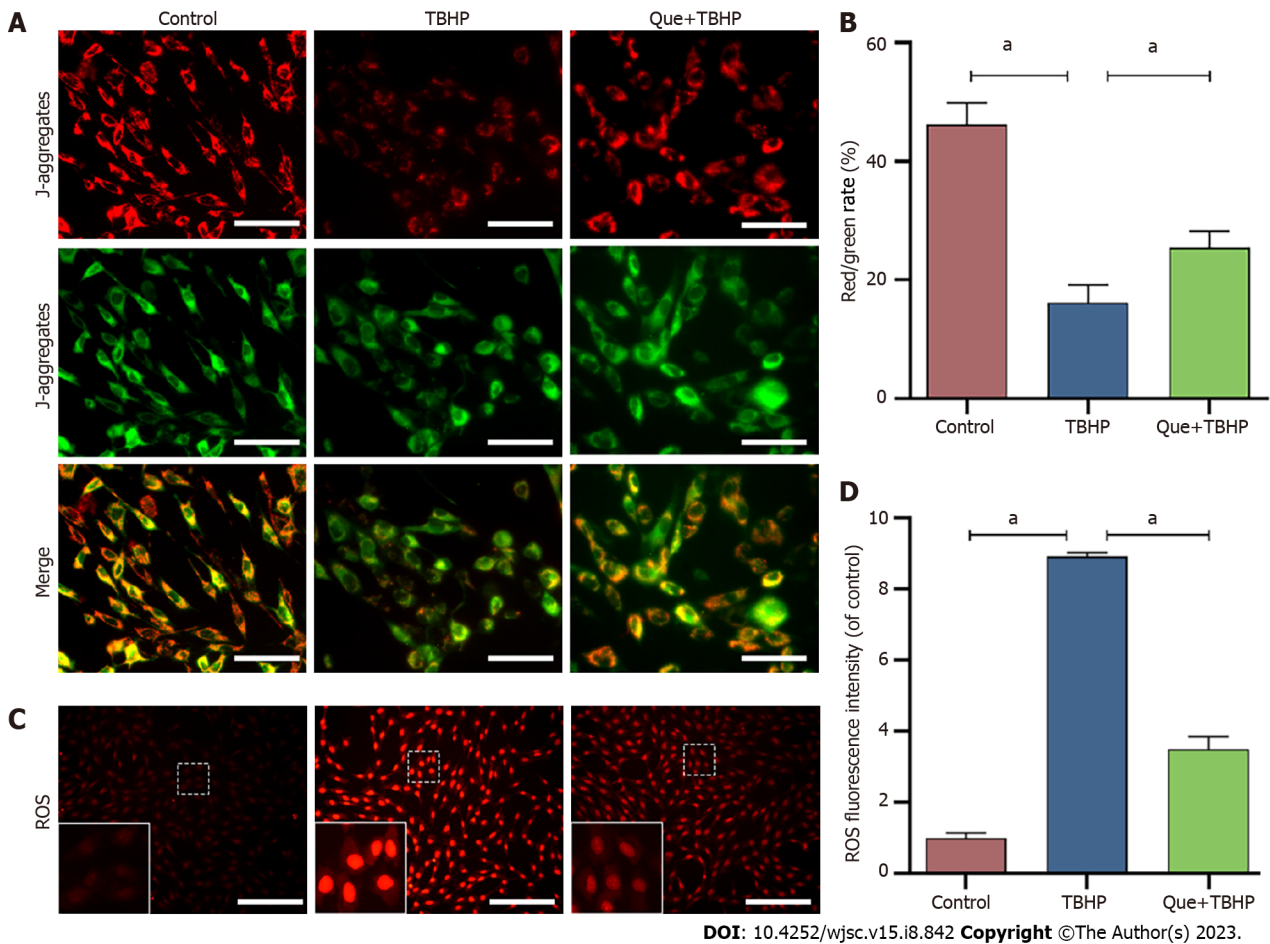


Figure 4 Mitochondrial membrane potential assay and reactive oxygen species assay. A: Results of Mitochondrial membrane potential (MMP) in different groups detected by fluorescence. Red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1. Scale bar = 100 μ m; B: Quantitative analysis of MMP results; C: Results of ROS in different groups detected by fluorescence. Red fluorescence represents high level of reactive oxygen species assay (ROS). Scale bar = 200 μ m; D: Quantitative analysis of ROS results. Data are represented as mean \pm SD. Significant differences between groups are indicated as $^*P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin; ROS: Reactive oxygen species assay.

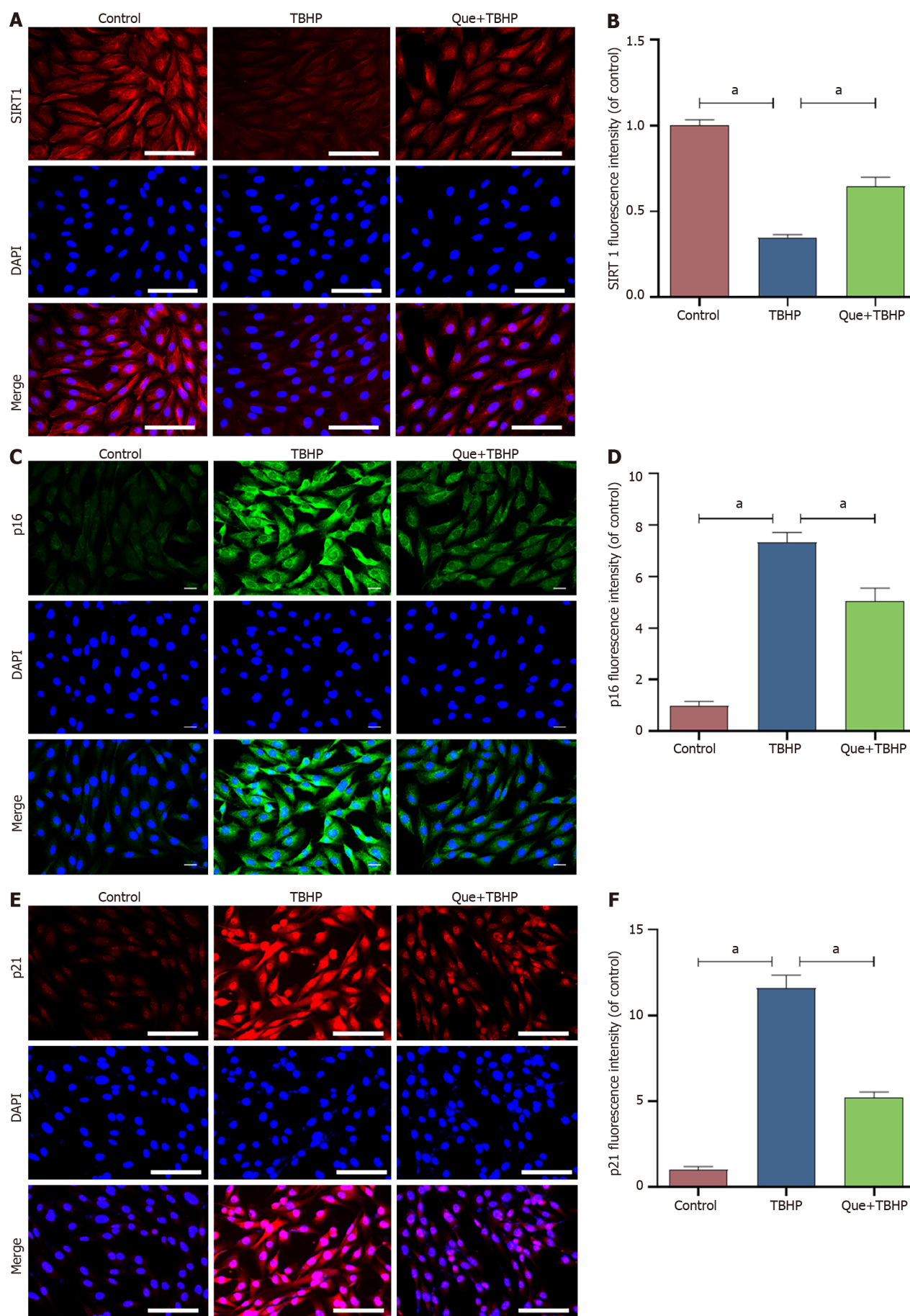
TBHP-induced NPMSCs, western blot analysis and immunofluorescence staining were used to evaluate the expression of SIRT1, cell senescence-related proteins (p16, p21, and p53), and SASP (IL-1 β , IL-6, and MMP-13). The expression of SIRT1 was downregulated after TBHP treatment, and this effect was partially reversed by Que pretreatment ($P < 0.05$; Figure 5). Moreover, Que treatment significantly decreased the upregulation of p16, p21, p53, IL-1 β , IL-6, and MMP-13 expression induced by TBHP ($P < 0.05$; Figures 5 and 6). Taken together, these results indicated that Que may have a protective role in TBHP-induced oxidative stress injury of NPMSCs.

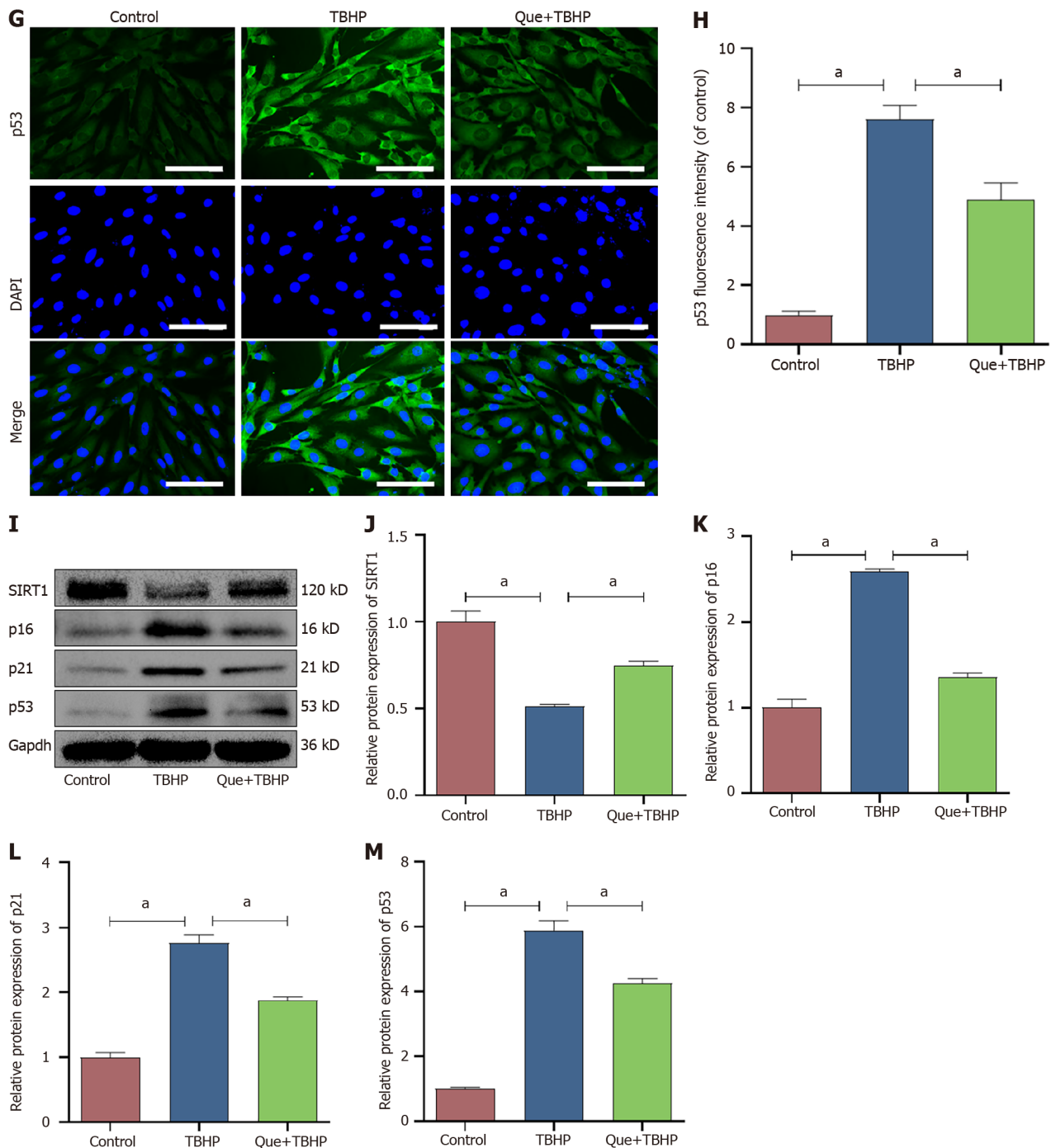
MiR-34a-5p represses SIRT1 expression by targeting the 3'-UTR of SIRT1

According to the TargetScan online prediction database, SIRT1 is a potential target of miR-34a-5p (Figure 7A). A dual-luciferase reporter assay revealed that the miR-34a-5p mimic significantly inhibited the relative luciferase activity in the wild-type SIRT1 reporter vector (WT-SIRT1), whereas there was no significant change in the luciferase activity in the mutant SIRT1 reporter vector (MUT-SIRT1) (Figure 7B). These data indicated that miR-34a-5p inhibits SIRT1 expression by directly binding to the 3'UTR of SIRT1. In addition, the mRNA and protein expression levels of SIRT1 were significantly inhibited by overexpression of miR-34a-5p (Figure 7C-E). Conversely, downregulation of miR-34a-5p increased the expression level of SIRT1 (Figure 7F-H). Moreover, the expression of SIRT1 was successfully downregulated by siRNA (Figure 7I-K).

MiR-34a-5p enhances oxidative stress-induced senescence of NPMSCs

According to the qRT-PCR analysis, the expression of miR-34a-5p was increased by TBHP treatment, and it was suppressed and promoted by the miR-34a-5p inhibitor and miR-34a-5p mimic, respectively (Figures 8A and 9A). In contrast, the expression of SIRT1 was suppressed after TBHP treatment, and it was promoted and suppressed and by miR-34a-5p inhibitor and miR-34a-5p mimic, respectively (Figures 8 and 9). The TBHP-enhanced senescence-related proteins (p16, p21, and p53) and SASP (IL-1 β , IL-6, and MMP-13) were significantly suppressed after miR-34a-5p knockdown (Figure 8). In contrast, the expression levels of senescence-related proteins and SASP were increased when cells were treated with the miR-34a-5p mimic (Figure 9).



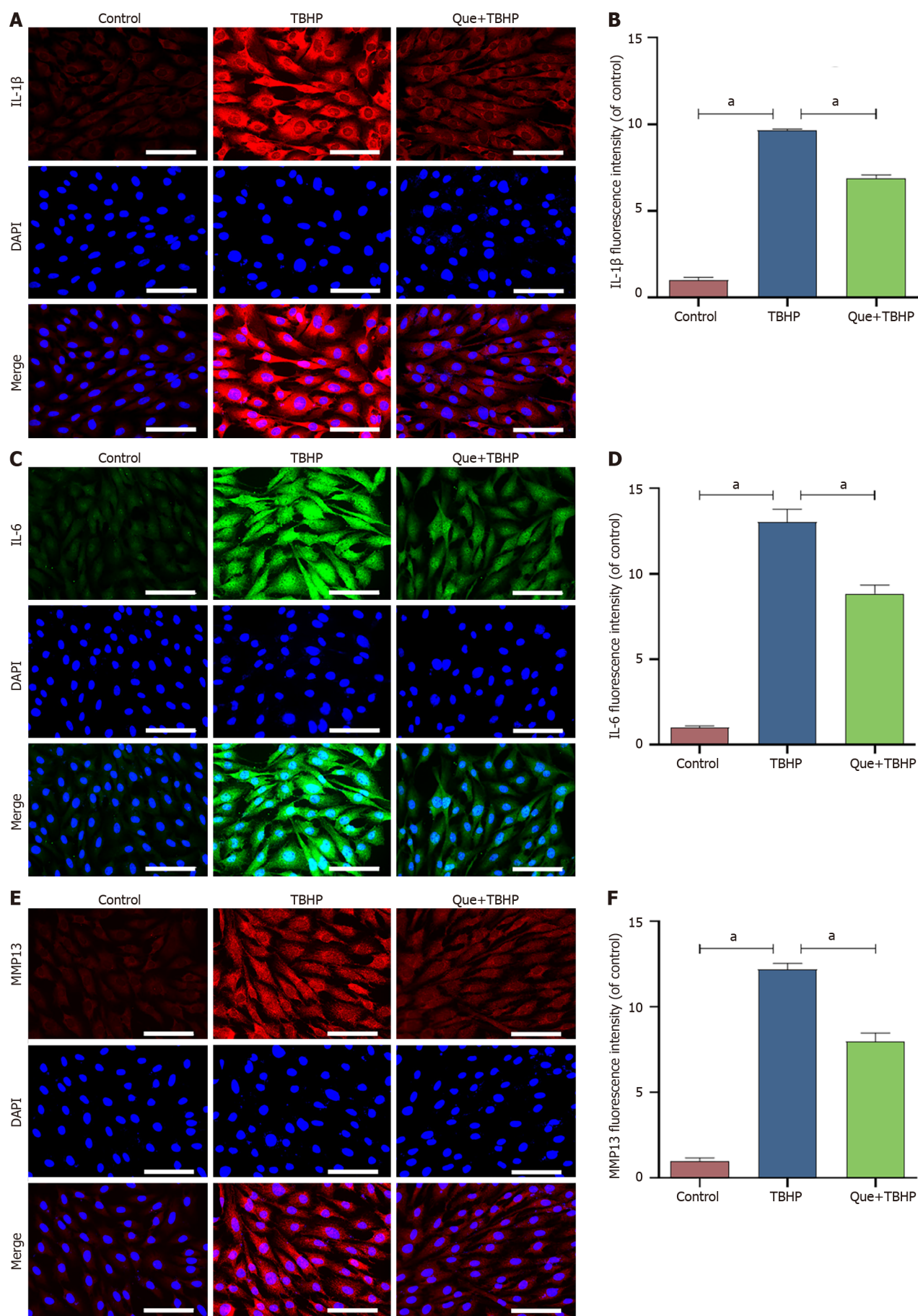


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Figure 5 Quercetin upregulates the expression of SIRT1 and downregulates the expression of p16 and p21 and p53. A and B: Immunofluorescence staining and quantitative analysis of SIRT1; C and D: Immunofluorescence staining and quantitative analysis of p16; E and F: Immunofluorescence staining and quantitative analysis of p21; G and H: Immunofluorescence staining and quantitative analysis of p53; I-M: The protein expressions and quantitative analysis of SIRT1, p16, p21 and p53 in the different groups. Scale bar = 100 μ m. Data are represented as mean \pm SD. Significant differences between groups are indicated as $^*P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

Que modulates TBHP-induced senescence of NPMSCs via the miR-34a-5p/SIRT1 pathway

To explore the relationship between miR-34a-5p/SIRT1 and oxidative stress-induced senescence in Que-treated NPMSCs, the miR-34a-5p mimic and siSIRT1 were used to investigate whether overexpression of miR-34a-5p and knockdown of SIRT1, respectively, counteracts the effects of Que on the expression of senescence-related proteins (p16, p21, and p53), SASP (IL-1 β , IL-6, and MMP-13), and SIRT1. As shown in Figures 10 and 11, Que suppressed the expression of senescence-related proteins and SASP but promoted the expression of SIRT1, whereas overexpression of miR-34a-5p and knockdown of SIRT1 impaired this protective effect in TBHP-treated NPMSCs. These results suggested that Que may protect NPMSCs from TBHP-induced senescence *via* the miR-34a-5p/SIRT1 pathway.



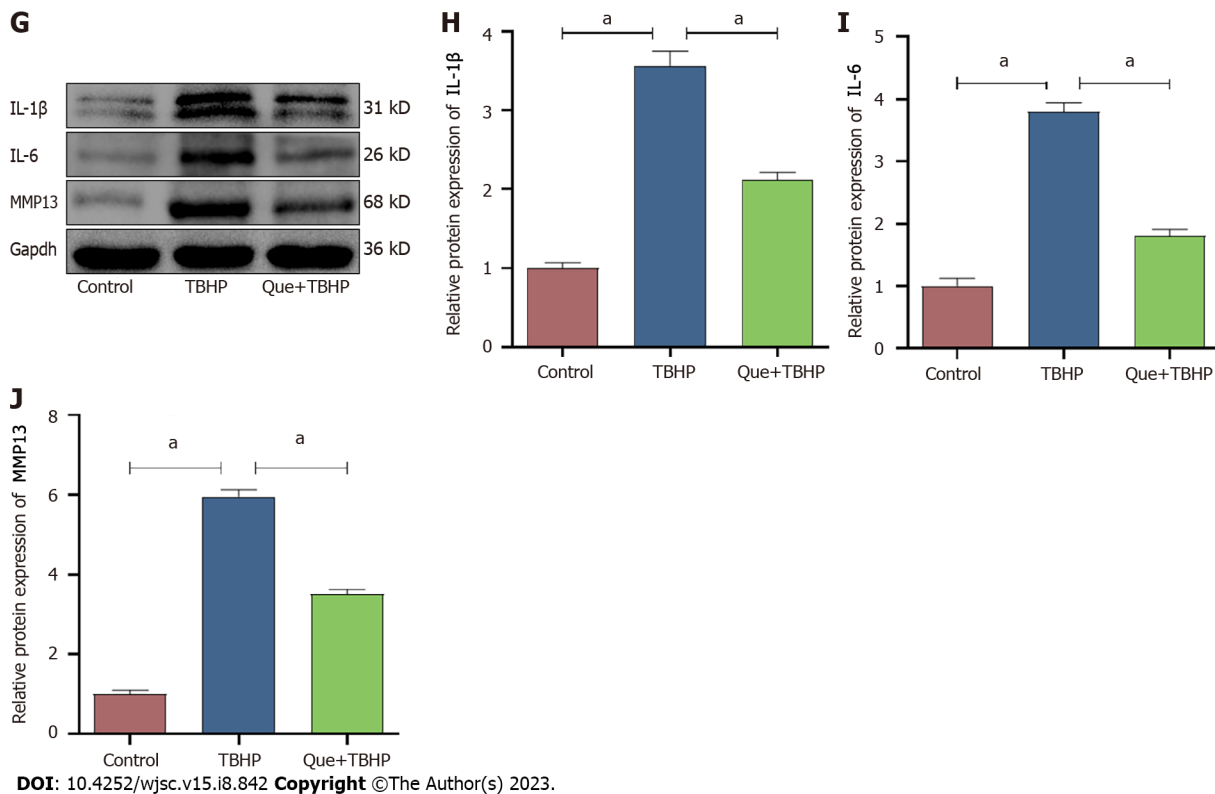


Figure 6 Quercetin downregulates the expression of senescence associated secreted phenotype: IL-1 β , IL-6 and MMP13. A and B: Immunofluorescence staining and quantitative analysis of IL-1 β ; C and D: Immunofluorescence staining and quantitative analysis of IL-6; E and F: Immunofluorescence staining and quantitative analysis of MMP-13; G-J: The protein expressions and quantitative analysis of IL-1 β , IL-6 and MMP-13 in different groups. Scale bar = 100 μ m. Data are represented as mean \pm SD. Significant differences between groups are indicated as $^aP < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

Que ameliorates disc degeneration in a rat model

A puncture-induced rat model was established to investigate the effect of Que in IDD *in vivo*, and disc height was measured by X-ray images at Week 0 and Week 4 after puncture (Figure 12A). Compared to the control group, the DHI of the IDD group was significantly decreased (Figure 12B-E). Interestingly, the DHI of the Que group was significantly higher than that of the IDD group ($P < 0.05$) (Figure 12B-E). According to the Hematoxylin & eosin staining, NP tissues occupied most of the area in the discs, and NP cells were well dispersed in the matrix. The annulus fibrosus (AF) was well organized in the remaining area. In the IDD group, the NP tissues almost disappeared, and the border between NP and AF was severely disrupted (Figure 12F). However, Que treatment alleviated the degeneration and morphological changes in the NP and AF (Figure 12F). The histological score was higher in the IDD group compared to the control group ($P < 0.05$) (Figure 12G). Both Safranin-O Fast Green and Alcian blue staining showed that the proteoglycan matrix area was lower in the IDD group compared to the control group (Figure 12F). However, Que treatment alleviated the proteoglycan decrease compared to the IDD group (Figure 12H). Immunofluorescence was also used to assess the expression of collagen II and aggrecan in the disc tissue. Compared to the control group, the expression of collagen type II and aggrecan was significantly lower in the IDD group, and Que treatment significantly attenuated the decrease in collagen type II and aggrecan compared to the IDD group (Figure 12I and J). Taken together, these results suggested that Que alleviates the IDD process in a puncture-induced rat model.

DISCUSSION

In the present study, the results demonstrated that Que ameliorates oxidative stress-induced senescence in NPMSCs *via* the miR-34a-5p/SIRT1 axis. The *in vivo* results also demonstrated that Que inhibits the expression of senescence-related proteins (p16, p21, and p53) and SASP (IL-1 β , IL-6, and MMP-13) as well as promotes the expression of SIRT1, suggesting that it has a therapeutic effect in the IDD rat model.

Several previous studies have found that oxidative stress is highly associated with the progression of IDD[4,7]. Oxidative stress is triggered in the microenvironment of degenerative IVD when the balance between generation and elimination of ROS is broken[33]. ROS not only trigger oxidative damage of the extracellular matrix of IVD but also induce oxidative damage to DNA, proteins, and mitochondria[33]. Moreover, ROS promote the production of ROS in IVD cells, forming a positive feedback loop[5-7,34]. The discovery of NPMSCs provides a theoretical basis for the endogenous repair of IDD[9,11]. The endogenous repair ability of NPMSCs in degenerative IVD is achieved by differentiation into NP

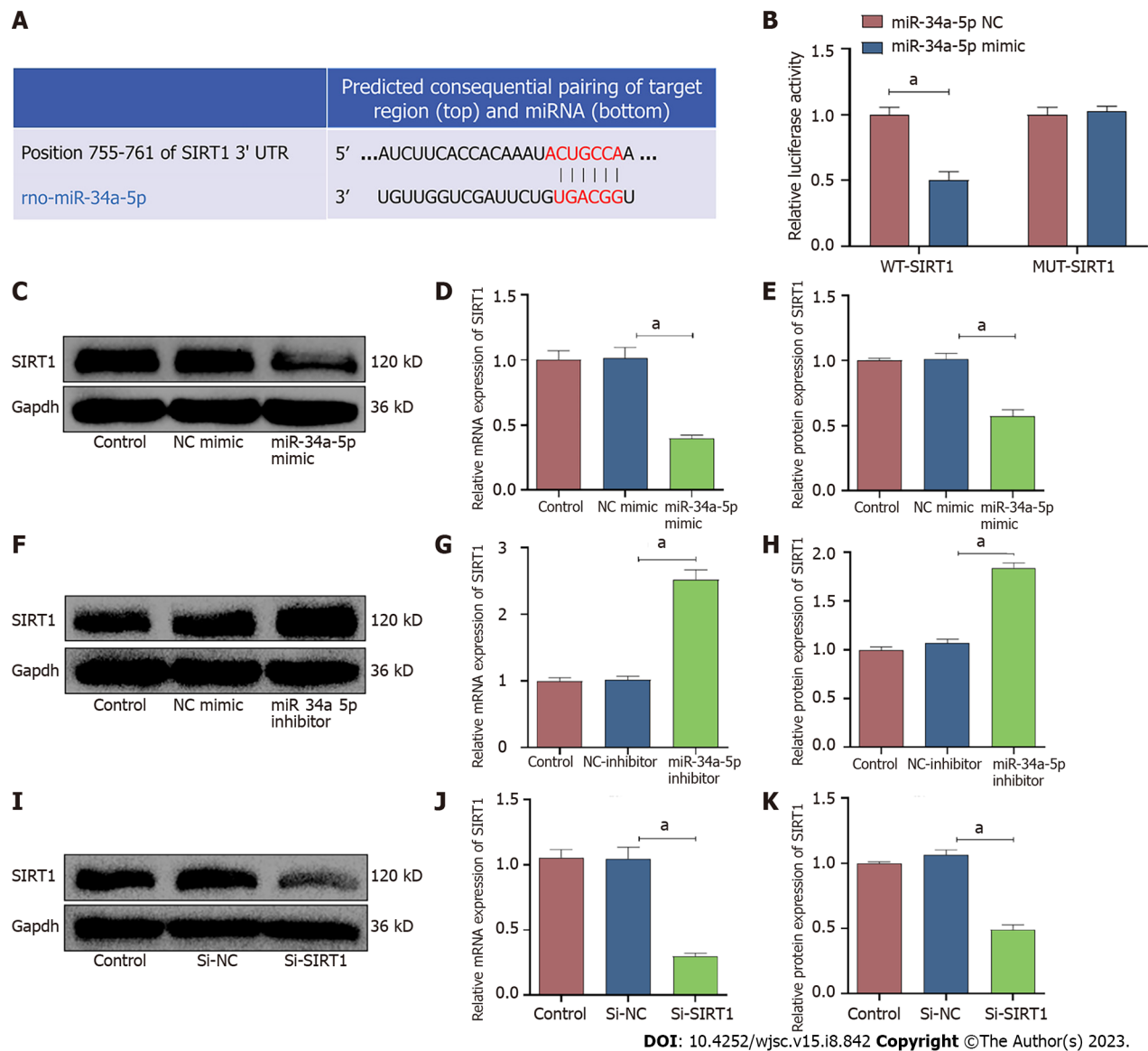


Figure 7 miR-34a-5p represses SIRT1 expression by targeting the 3'-UTR of SIRT1. A: Schematic of the predicted miR-34a-5p binding sites in the 3'-UTR of SIRT1 mRNA from TargetScan online database; B: Luciferase reporter activity of SIRT1 co-transfected with miR-34a-5p nc or miR-34a-5p mimic; C-E: The protein and mRNA expression level of SIRT1 in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) transfected with miR-34a-5p mimic; F-H: The protein and mRNA expression level of SIRT1 in NPMSCs transfected with miR-34a-5p inhibitor; I-K: The protein and mRNA expression level of SIRT1 in NPMSCs transfected with Si-SIRT1. Data are represented as mean \pm SD. Significant differences between groups are indicated as ^a $P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide.

cells and inhibiting cell apoptosis and/or senescence[10,35]. Oxidative stress in the microenvironment of IVD induces NPMSC senescence, and senescent NPMSCs decrease multipotency and self-renew ability, thus decreasing the regenerative potential and endogenous repair ability of NPMSCs[17,36-38]. In the present study, we found increased expression of senescence-related proteins (p16, p21, and p53), increased SASP (IL-1 β , IL-6, and MMP-13), increased SA- β -Gal-positive senescent cells, decreased expression of SIRT1, decreased proliferation, and decreased viability after TBHP treatment in NPMSCs. Therefore, it is important to understand how to maintain the quantity and quality of NPMSCs under adverse microenvironments in degenerative IVD, such as oxidative stress, hypoxia, nutrient deficiency, and compression.

As a potent antioxidant, Que scavenges ROS, scavenges free radicals, inhibits lipid peroxidation, and inhibits xanthine oxidase activity[18-20,39]. Previous studies have reported that Que is an effective activator of SIRT1[40-42]. Feng *et al*[22] reported that Que attenuates oxidative stress-induced apoptosis of rat chondrocytes *via* the SIRT1/mitogen-activated protein kinase (AMPK) pathway and prevents the progression of osteoarthritis in a rat model[22]. Lou *et al*[43] confirmed that Que inhibits HepG2 cell apoptosis through the p53/miRNA-34a/SIRT1 pathway[43]. To evaluate the effect of Que in TBHP-induced NPMSCs, we pretreated cells with Que (20 μ M) for 24 h before the addition of TBHP (100 μ M) in the present study. Pretreatment with Que decreased ROS generation, decreased SA- β -Gal activity, and partially restored cell proliferation in oxidative stress induced-NPMSCs. Further, Que significantly reduced the expression of senescence-related proteins (p16, p21, and p53), reduced SASP (IL-1 β , IL-6, and MMP-13), and increased the expression of SIRT1. Therefore, these results indicated that Que may have a protective effect against TBHP-induced oxidative stress in

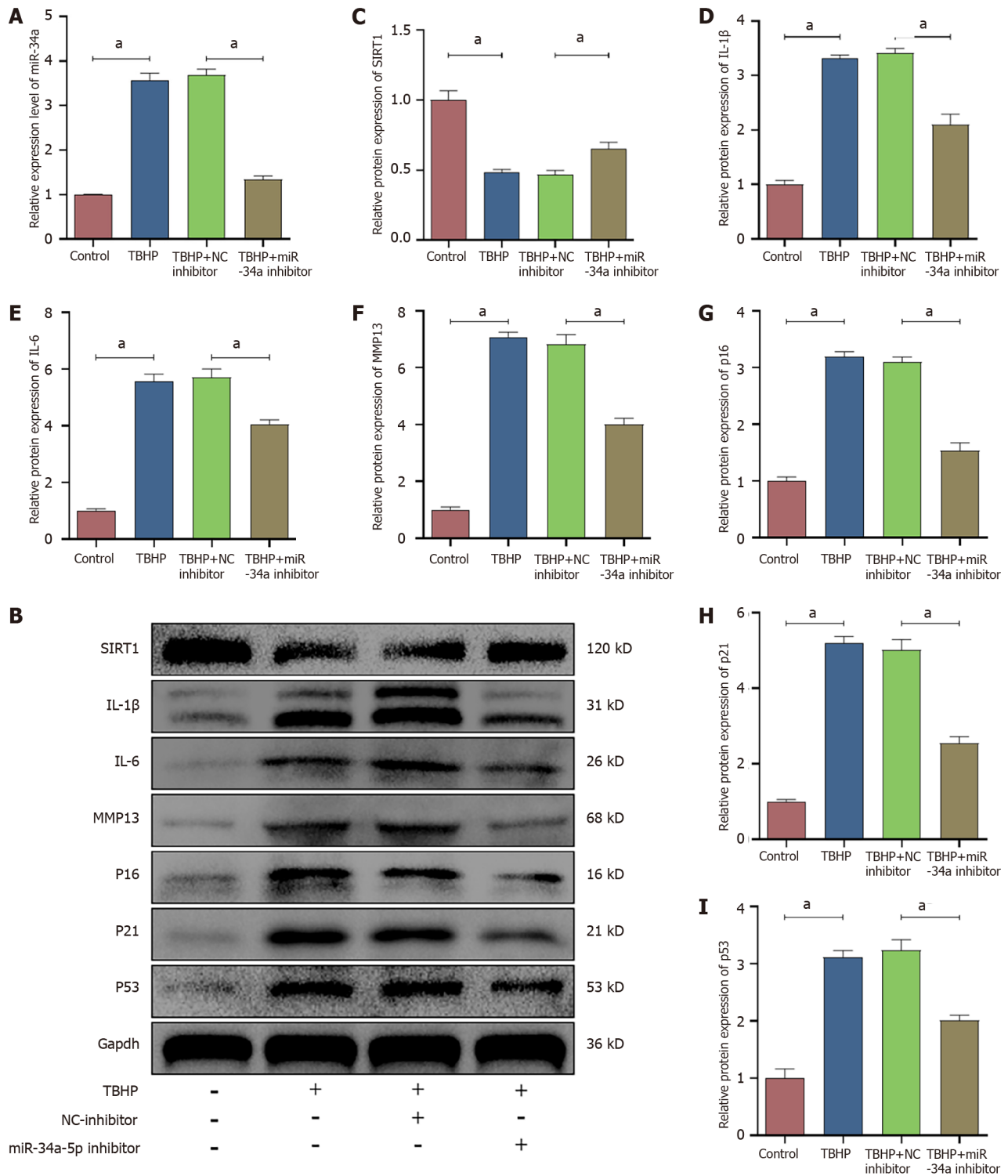
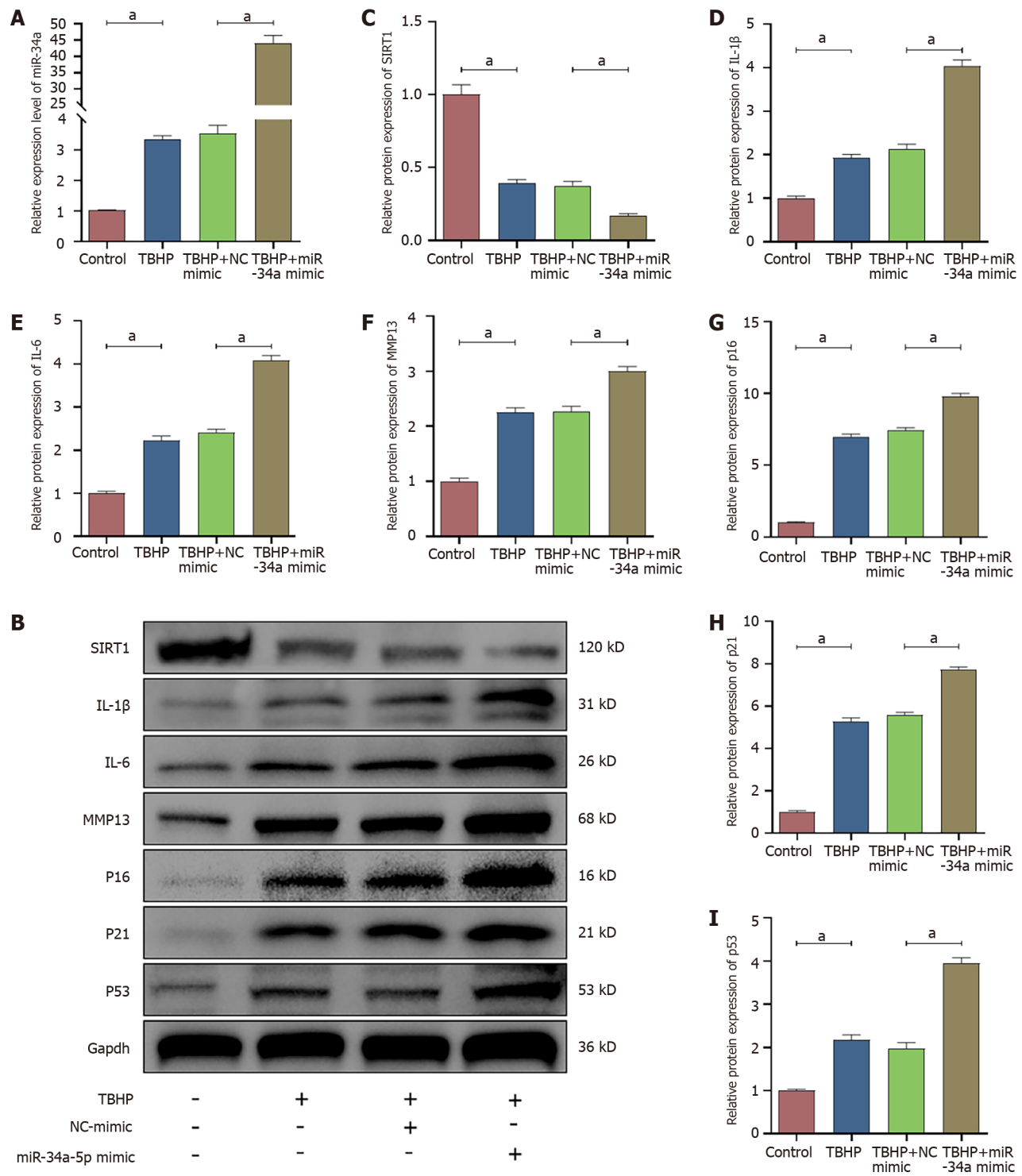


Figure 8 Downregulation of miR-34a-5p alleviated oxidative stress-induced nucleus pulposus-derived mesenchymal stem cells senescence. **A:** The RNA levels of miR-34a-5p in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were transfected with miR-34a-5p inhibitor and treated with tert-butyl hydroperoxide; **B:** The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in NPMSCs transfected with miR-34a-5p inhibitor; **C-I:** Quantitative analysis of protein expression in the different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as ^a $P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide.

NPMSCs.

A previous study has also demonstrated that the expression of miR-34a-5p is significantly upregulated in degenerative IVD and that miR-34a-5p increases extracellular matrix degradation and cell apoptosis. Chen *et al*[44] reported that miR-34a increases apoptosis in degenerated cartilage endplate (CEP) chondrocytes. In addition, inhibition of miR-34a reduces cell apoptosis, while upregulation of miR-34a has an opposite effect in CEP cells[44]. In the present study, TBHP significantly upregulated miR-34a-5p expression. Overexpression miR-34a aggravated the oxidative stress damage



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Figure 9 Overexpression of miR-34a-5p enhanced oxidative stress-induced nucleus pulposus-derived mesenchymal stem cells senescence. A: The RNA levels of miR-34a-5p in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were transfected with miR-34a-5p mimic and treated with TBHP; B: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in NPMSCs transfected with miR-34a-5p mimic; C-I: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as ^a*P* < 0.05, *n* = 3. TBHP: Tert-butyl hydroperoxide.

induced by TBHP in NPMSCs, including increased the expression of senescence-related proteins and SASP as well as and decreased the expression of SIRT1, whereas knockdown of miR-34a had an opposite effect in NPMSCs. Accumulating studies have demonstrated that SIRT1 exerts important function in the progression of IDD. The expression level of SIRT1 in IVD cells decreases with the progression of IDD[45], and SIRT1 expression is negatively correlated with Pfirrmann grade in IDD[46]. It has been reported that SIRT1 decreases the oxidative stress-induced senescence in CEP cells through the p53/p21 pathway[47]. H₂O₂-induced oxidative stress reduces the expression of SIRT1 as the expression of SIRT1 is downregulated with the increasing concentration of H₂O₂, and SIRT1 activation alleviates oxidative stress-induced

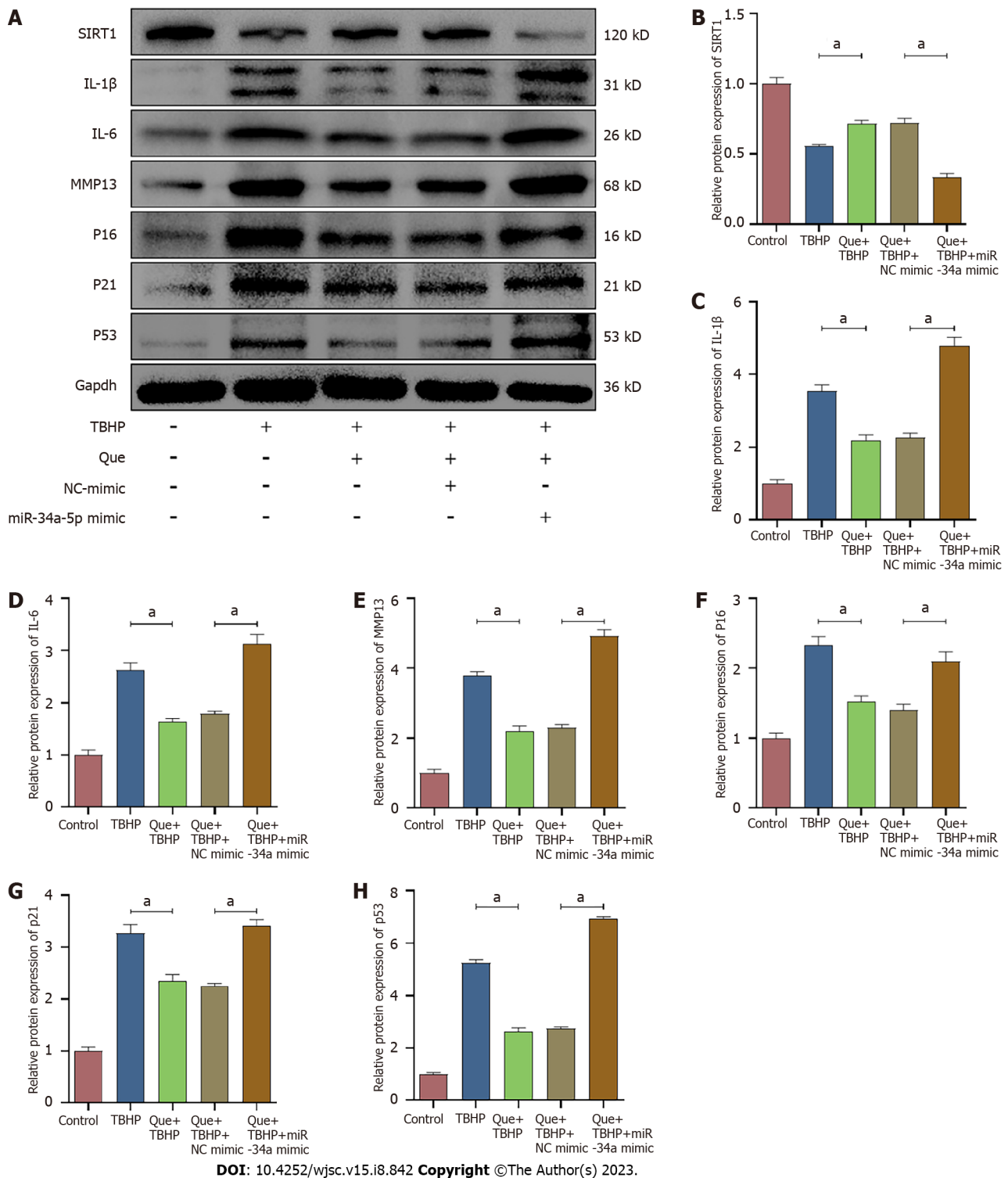
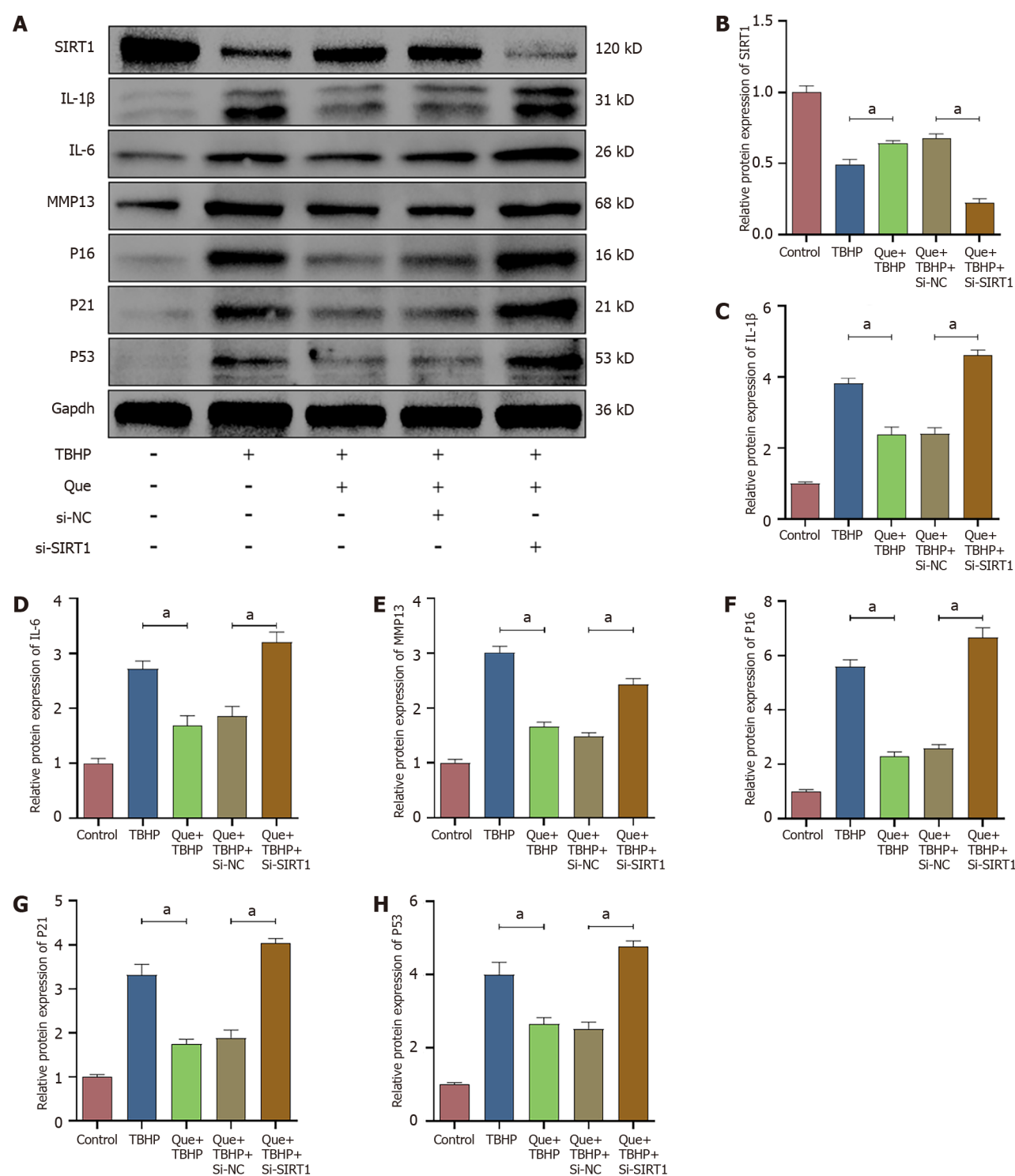


Figure 10 Quercetin alleviates TBHP-induced senescence via miR-34a-5p/SIRT1 axis. A: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in different groups; B-H: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as ^a $P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

senescence in NP cells[29,48]. Xia *et al*[49] found that the expression of p16 is downregulated by activating SIRT1 in rat IDD model[49]. The present study found that SIRT1 expression was downregulated in TBHP induced-NPMSCs, which was rescued by Que and inhibiting miR-34a-5p. These results suggested that SIRT1 plays a protective role in TBHP induced-NPMSCs. miR-34a-5p overexpression and SIRT1 downregulation in NPMSCs were utilized to investigate whether Que reduces oxidative stress damage *via* the miR-34a/SIRT1 pathway to delay NPMSC senescence. The results demonstrated that the protective effect of Que is reversed by miR-34a-5p overexpression and SIRT1 knockdown.

In the present study, Que was also administered to IDD animal models to evaluate the protective effect of Que *in vivo*. X-ray analysis indicated the loss of DHI in the IDD model, but Que treatment ameliorated the decreased DHI. Moreover, histological analysis using HE, Alcian blue, and S-O staining as well as immunofluorescence analysis of collagen type II

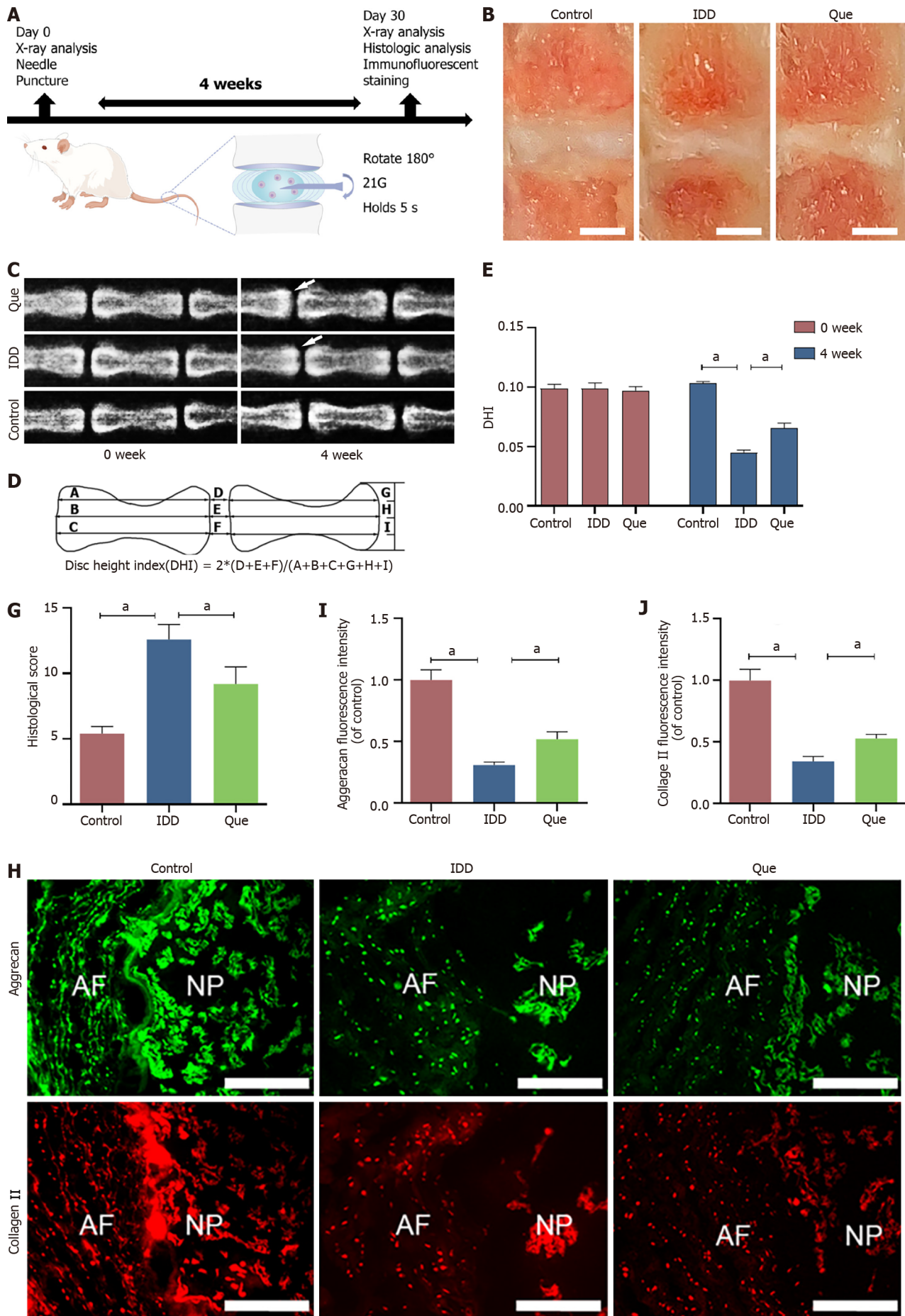


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Figure 11 Quercetin alleviates TBHP-induced senescence via miR-34a-5p/SIRT1 axis. A: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in different groups; B-H: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as ^a $P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

and aggrecan confirmed that Que had a positive effect on delaying the degree of IDD.

The present study had several limitations. Previous studies have shown Que has pleiotropic properties. In addition to the activation of SIRT1, Que has anti-inflammatory and antioxidative effects *via* activating nuclear factor erythroid 2-related factor 2 (NRF2)[39], mitogen-activated protein kinase (MAPK)[39], and phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT)[50]. Hence, it remains unknown whether Que plays a role in NPMSC senescence induced by oxidative stress *via* other signaling pathways, suggesting that further study is needed to explore the other mechanisms underlying the protective effect of Que on NPMSCs. Moreover, future clinical studies are needed to evaluate the effect of Que on IDD progress.



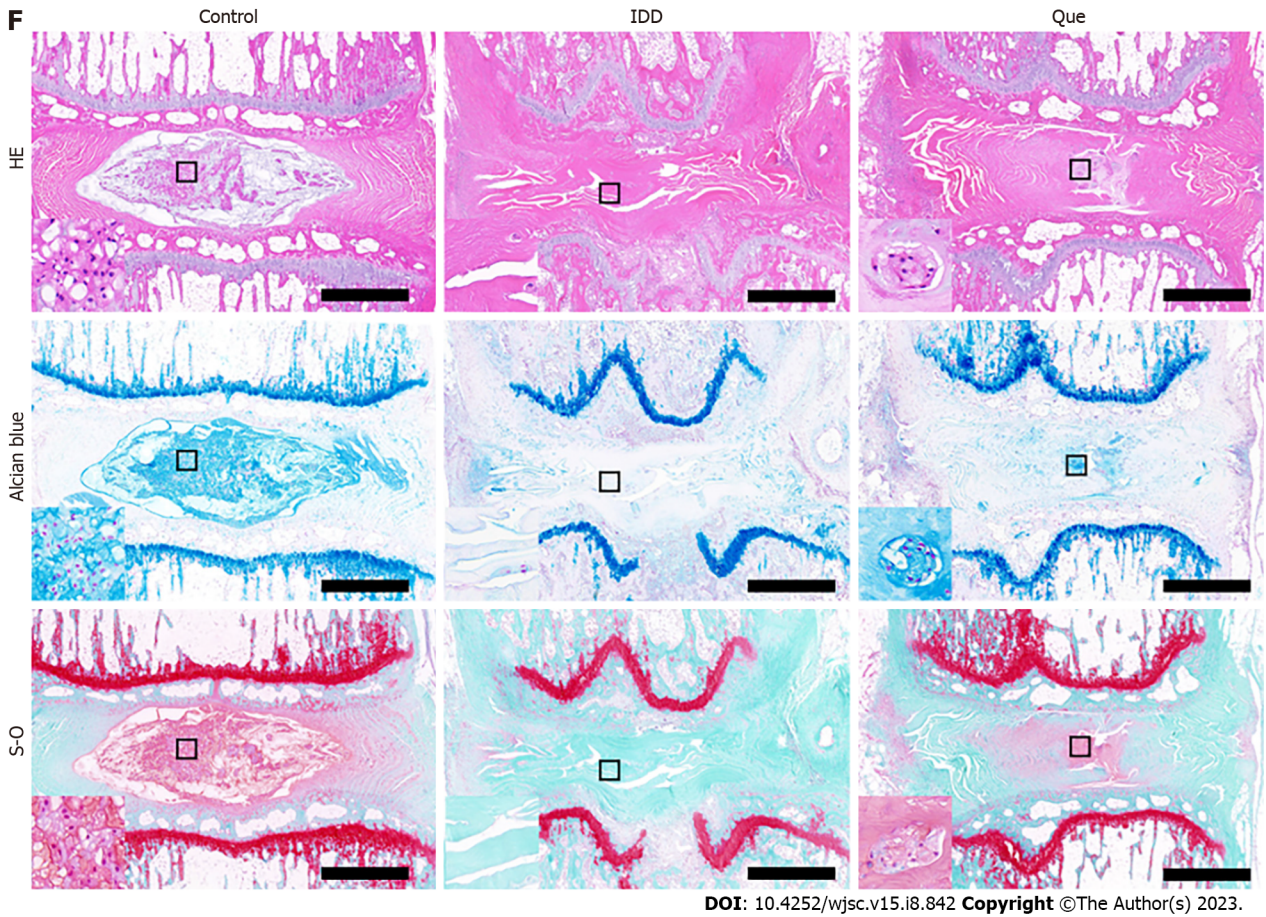


Figure 12 Quercetin ameliorates IVDD *in vivo*. A: Schematic of the basic process of *in vivo* experiment (By Figdraw); B: Macroscopic appearances of rat tail intact disc mid-sagittal sections in control group, intervertebral disc degeneration group and quercetin group (Scar bar = 2 mm); C: The X-ray in different groups at 0 wk and 4 wk after puncturing; D: Measurements of intervertebral disc height index (DHI); E: Quantitative analysis of DHI; F-G: Hematoxylin-eosin staining and quantitative analysis of histological score at 4 wk after puncture in different groups (scale bar = 1mm); H: The expression of aggrecan and collagen type II in different groups (scale bar = 400 μ m); I-J: Quantitative analysis of aggrecan and collagen type II in different groups. Data are represented as mean \pm SD. Significant differences between groups are indicated as $^aP < 0.05$, $n = 3$. HE: Hematoxylin-eosin; NP: Nucleus pulposus; AF: Annulus fibrosus; IDD: Intervertebral disc degeneration; TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

CONCLUSION

In summary, the present study demonstrated that Que prevents oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway (Figure 13). Moreover, Que ameliorates the progression of IDD in a rat model. These findings suggested that Que may be a potential agent for the treatment of IDD.

Table 1 Sequences of primers used for real-time polymerase chain reaction	
Gapdh	F: CTGGAGAAACCTGCCAAGTATG R: GGTGGAAGAATGGGAGTTGCT
SIRT1	F: TGACCTCCICATTGTTATTGGG R: GGCATACTCGCCACCTAACCT
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
miR-34a-5p	F: ACACTCCAGCTGGGTGGCAGTGCTTAGCT R: TGGTGTCTGGAGATCG

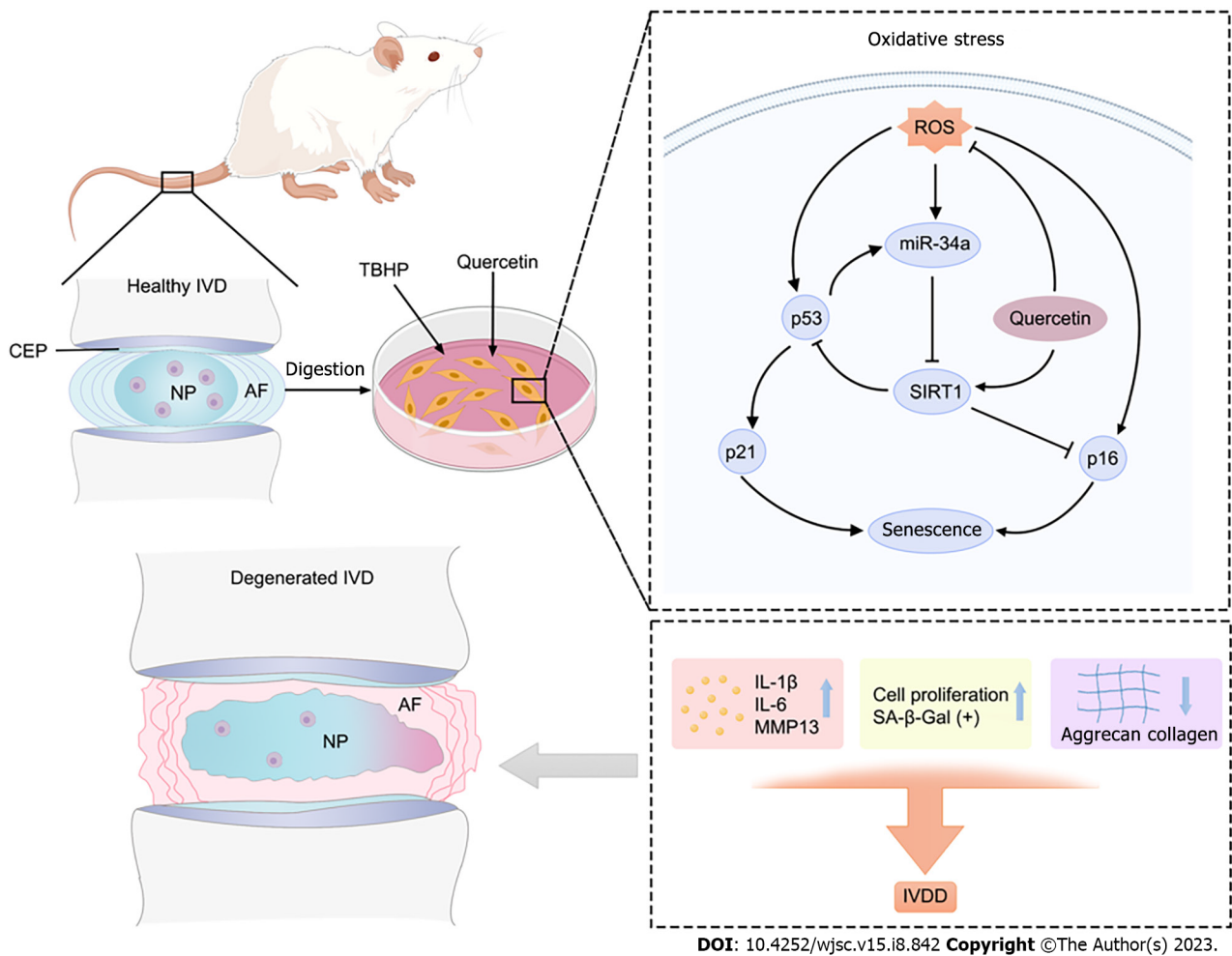


Figure 13 Schematic representation of the mode of action of quercetin. Quercetin ameliorates oxidative stress-induced senescence in nucleus pulposus-derived mesenchymal stem cells *via* miR-34a-5p/SIRT1 axis. HE: Hematoxylin-eosin; NP: Nucleus pulposus; AF: Annulus fibrosus; IVD: Intervertebral disc; TBHP: Tert-butyl hydroperoxide; ROS: Reactive oxygen species assay. By Figdraw.

ARTICLE HIGHLIGHTS

Research background

Intervertebral disc degeneration (IDD) is a main contributor to low back pain. Oxidative stress, which is highly associated with the progression of IDD, increases senescence of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) and weakens the differentiation ability of NPMSCs in degenerated intervertebral discs (IVDs). Quercetin (Que) has been demonstrated to reduce oxidative stress in diverse degenerative diseases.

Research motivation

An adverse microenvironments of degenerative intervertebral disc such as oxidative stress, low nutrition, and inflammation leads to increased senescence NPMSCs, which severely affects endogenous repair. Therefore, inhibition senescence of NPMSCs may be of great significance in alleviating IDD.

Research objectives

The present study aimed to investigate the role of Que in oxidative stress-induced NPMSC damage and to elucidate the underlying mechanism.

Research methods

In vitro, NPMSCs were isolated from rat tails. Senescence-associated β -galactosidase (SA- β -Gal) staining, cell cycle, reactive oxygen species (ROS), real-time quantitative polymerase chain reaction (RT-qPCR), immunofluorescence, and western blot analyses were used to evaluate the protective effects of Que. Meanwhile the relationship between miR-34a-5p and Sirtuins 1 (SIRT1) was evaluated by dual-luciferase reporter assay. To explore whether Que modulates tert-butyl hydroperoxide (TBHP)-induced senescence of NPMSCs *via* the miR-34a-5p/SIRT1 pathway, we used adenovirus vectors to overexpress and downregulate the expression of miR-34a-5p, and used SIRT1 siRNA to knockdown SIRT1 expression. *In vivo*, a puncture-induced rat IDD model was constructed, and X rays and histological analysis were used to assess

whether Que could alleviate IDD *in vivo*.

Research results

We found that TBHP can cause NPMSCs senescence changes, such as reduced cell proliferation ability, increased SA- β -Gal activity, cell cycle arrest, the accumulation of ROS, and increased expression of senescence-related proteins. While abovementioned senescence indicators were significantly alleviated by Que treatment. Que decreased the expression levels of senescence-related proteins (p16, p21, and p53) and senescence-associated secreted phenotype (SASP), including IL-1 β , IL-6, and MMP-13, and it increased the expression of SIRT1. In addition, the protective effects of Que on cell senescence were partially reversed by miR-34a-5p overexpression and SIRT1 knockdown. *In vivo*, X-ray, and histological analyses indicated that Que alleviated IDD in a puncture-induced rat model.

Research conclusions

In summary, the present study provides evidence that Que reduces oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway, suggesting that Que may be a potential agent for the treatment of IDD.

Research perspectives

We demonstrated Que ameliorates oxidative stress-induced senescence of NPMSCs and delays the progression of IDD. Que may be a potential agent for the treatment of IDD.

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FOOTNOTES

Author contributions: Zhao WJ and Liu X contributed to data curation, Writing-Original draft preparation, contributed equally to this work; Hu M and Zhang Y contributed to Visualization, Validation; Shi PZ and Wang JW performed Investigation; Lu XH, Cheng XF and Tao YP performed conceptualization, methodology; Feng XM, Wang YX and Zhang L performed supervision, writing- reviewing, editing and share corresponding author.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals, Yangzhou University, No. SYXK (Su) 2017-0044.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article. Figures 12A and 13 were created using Figdraw (www.figdraw.com).

Data sharing statement: All data during the study are available from the corresponding author by request at zhangliang6320@sina.com.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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