

Luteolin suppresses TNF- α -induced inflammatory injury and senescence of nucleus pulposus cells via the Sirt6/NF- κ B pathway

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Received March 11, 2022; Accepted May 4, 2022

DOI: 10.3892/etm.2022.11396

Abstract. Luteolin (3',4',5,7-tetrahydroxy flavone) is a flavonoid, which is widely distributed in various plants including flowers, vegetables, and medicinal herbs and spices. Luteolin can be applied in the treatment of various diseases due to its multiple biological activities, such as anti-inflammatory, anticancer, and antioxidative activity. However, its role in intervertebral disc degeneration has not been previously reported. Therefore, the purpose of the present study was to explore the effects of luteolin on Tumor necrosis factor (TNF)- α -induced inflammatory injury and senescence of human nucleus pulposus cells (HNPCs), as well as the underlying mechanisms of action of this compound. Cell viability and apoptosis were assessed by MTT assay and TUNEL staining, respectively. ELISA kits were applied to detect the levels of inflammatory cytokines and the activity of telomerase. Senescence β -galactosidase staining was used to detect the activity levels of β -galactosidase in the cells. Cell transfection was performed to achieve interference of sirtuin 6 (Sirt6). The protein expression levels were detected by western blot analysis. TUNEL staining and western blot analysis were performed to assess the expression levels of apoptosis-related proteins. The results indicated that TNF- α induced a significant decrease in HNPC viability and an increase in inflammatory factor levels, while the application of luteolin effectively increased cell viability and decreased intracellular interleukin (IL)-1 β and IL-6 expression levels. Furthermore, luteolin decreased apoptosis compared with the TNF- α groups in a dose-dependent manner. In addition, the results of the detection kits suggested that luteolin reversed TNF- α -induced senescence. Notably, interference with Sirt6 partially reduced the protective effect of luteolin on TNF- α -induced HNPC senescence via the Sirt6/NF- κ B pathway. In summary, the data indicated that

luteolin suppresses TNF- α -induced inflammatory injury and senescence of HNPCs via the Sirt6/NF- κ B pathway.

Introduction

The intervertebral disc (IVD) is a key connective tissue between vertebrae. Its degeneration can lead to morphological and physiological changes in the disc, lower back pain, and subsequently to impaired load-bearing capacity (1). Intervertebral disc degeneration (IDD) is a common musculoskeletal disorder with multifactorial pathology. It is caused by several factors, such as genetic susceptibility, inflammation, cellular senescence, non-physiological mechanical stresses, and apoptosis (2). Currently, treatments for IDD are mainly targeted to alleviate the symptoms, and no effective measures can reverse the pathological changes in the IVD (3). Therefore, it is necessary to explore the exact cause and pathogenesis of IDD and develop more effective conservative methods for IVD therapy.

Extracellular matrix (ECM) degradation and nucleus pulposus (NP) cell loss are two major pathological features of IDD (4). NP cells play an essential role in maintaining the homeostasis and structural integrity of the ECM; their degradation can further destruct the normal function of IVDs (5). Apart from ECM degradation, inflammation is also considered to be a critical event during IDD, which distinguishes asymptomatic from symptomatic disc degeneration (6). During IDD progression, NP cells increasingly secrete pro-inflammatory molecules, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-17, among which TNF- α is the most prominent and has been reported to be highly upregulated in disc tissue (7). These proinflammatory molecules trigger a range of inflammatory responses, and further influence disc cell senescence, death, autophagy, and apoptosis (8). Therefore, targeting TNF- α -induced inflammation and NP cell senescence may be promising for the treatment of IDD.

Luteolin, or 3',4',5,7-tetrahydroxy flavone, is a flavonoid, which is widely distributed in various plants including flowers, vegetables, and medicinal herbs and spices (9). It has been demonstrated that luteolin possesses various biological activities, such as anti-inflammatory (10), anticancer (11), antioxidant (12), neuroprotective (13), and antidiabetic (14) activity. Accumulating evidence indicates that the anti-inflammatory effects of luteolin are associated with the downregulation of

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Key words: luteolin, intervertebral disc degeneration, inflammatory injury, senescence, Sirt6/NF- κ B pathway

the expression levels of proinflammatory molecules, including TNF- α , nitric oxide, cyclooxygenase-2 (COX-2), and IL-1 β (15). A recent study reported that luteolin significantly inhibited IL-1 β -induced degradation of collagen II and attenuated the progression of osteoarthritis (16). In addition, Yang *et al* (17) demonstrated that luteolin prevented the production of proinflammatory factors of neutrophils and diminished inflammatory tissue injury. However, whether luteolin can mediate the inflammatory processes in IDD and improve disc impairment has not been previously investigated.

The sirtuin (Sirt) family comprises nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylases, which participate in multiple biological processes including inflammation, aging, cellular redox homeostasis, and metabolic homeostasis (18-20). Sirt6 is a member of the Sirt family, which is mainly enriched in the nucleus (21). A recent study demonstrated that the expression levels of Sirt6 were significantly decreased during the progression of IDD, while upregulation of Sirt6 expression prevented IL-1 β -induced NP ECM degradation and ameliorated IDD development (22). Moreover, Chen *et al* (23) demonstrated that Sirt6 prevented NP cell senescence and death by triggering autophagy. These findings indicate that Sirt6 plays a crucial role in the progression of ID. However, its detailed functions in the pathogenesis of IDD remain poorly understood. Notably, luteolin has been reported to act as a Sirt6 activator and trigger its expression by binding to the Sirt6-specific acyl binding channel (24).

Based on previous findings, the purpose of the present study was to investigate whether luteolin exerts protective effects on NP cells and inhibits IDD progression. Moreover, the mechanistic pathway of Sirt6 involvement in IDD was explored.

Materials and methods

Cell culture. Immortalized human nucleus pulposus cells (HNPCs) were obtained from AcceGen Biotechnology (cat. no. ABI-TC102D). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with F12 nutrient mixture (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Hyclone; Cytiva), and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C in the presence of 5% CO₂. The cells in the logarithmic growth phase were used for subsequent experiments.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) cell viability assay. MTT was first prepared as a stock solution of 5 mg/ml in phosphate-buffered saline (PBS, pH 7.2) and filtered. The cell suspension was transferred to a 96-well plate at a density of 5x10³ cells/well. The cells were treated with different concentrations of luteolin (1, 2, and 4 μ M; Sigma-Aldrich; Merck KGaA) or co-treated with luteolin (1, 2, and 4 μ M) and 50 ng/ml TNF- α for 24 h. To investigate the effect of Sirt6 knockdown on TNF- α -induced HNPC viability in the presence of luteolin (4 μ M), the Sirt6 knockdown HNPCs were co-treated with 5 ng/ml TNF- α and luteolin (4 μ M) for 24 h. Subsequently, 10 μ l MTT solution was added to each well. Following incubation for 4 h at 37°C, 100 μ l dimethyl sulfoxide (Adamas-Beta, Ltd.) was added to each well. The 96-well plate was shaken gently, and the

absorbance (abs) of each sample was read by a microplate reader at 570 nm to determine cell viability. The viable cells produced a dark blue formazan product, whereas this type of staining was not formed in the non-viable cells. The percentage of the viable cells was calculated using the following formula: (%)=[100x (sample abs)/(control abs)].

TUNEL assay. The induction of apoptosis was detected by TUNEL using *In Situ* Cell Death Detection kit (cat. no. 11684817910; Roche Diagnostics GmbH) according to the manufacturer's protocol. Briefly, the cells were washed with PBS three times and subsequently fixed at room temperature (20-25°C) with 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 15 min. Subsequently, 0.15% Triton X-100 was added to the cells at room temperature for an additional 5 min. FITC-deoxyuridine triphosphate solution (Roche Diagnostics GmbH) was added to the cells and incubated at 37°C for 60 min in the dark. The detection solution was discarded and the cells were washed three times with PBS. An inverted fluorescence microscope (Olympus Corp.) was used to measure the excitation and emission wavelengths in the range of 450-500 nm and 515-565 nm (green fluorescence), respectively.

Detection kit. ELISA kits (Beyotime Institute of Biotechnology) were applied to detect the expression levels of inflammatory cytokines, including IL-6 (cat. no. P1326) and IL-1 β (cat. no. P1305). Senescence β -galactosidase staining kit (Beyotime Institute of Biotechnology, cat. no. C0602) was used to detect the activity levels of β -galactosidase in the cells. ELISA kit (SenBeiJia Biological Technology Co., Ltd.; cat. no. SBJ-H1920) was used to detect the activity of telomerase. The effect of luteolin on TNF- α -induced Sirt6 activity was detected by the SIRT6 activity assay kit (Abcam, cat. no. ab156068).

Bioinformatics website. The Protein Data Bank (PDB) database (<https://www.rcsb.org/>) was used to obtain the three-dimensional structure of Sirt6 protein (PDB ID:3k35), and molecular docking was performed using Autodock (version 4.2; The Scripps Research Institute). The pose with the lowest free energy (-8.3 kcal/mol) was selected for visualization.

Cell transfection. The cells (3x10⁵ cells/well) were incubated in 6-well plates and cultured for 24 h at 37°C with 5% CO₂. Following incubation, the cells were transfected with Sirt6-targeting short interfering RNA (si-Sirt6#1, 5'-TTCTTCCACAAACATGTT C-3'; si-Sirt6#2, 5'-TCTTCCACAAACATGTTCC-3') and short interfering negative control (si-NC, 5'-ACGTGACACGTTCCG AGAATT-3') at a concentration of 25 nM. All plasmids were synthesized by Shanghai GenePharma Co., Ltd. and transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Blank control group cells were not treated. Following transfection, the cells were cultured for 48 h and the transfection efficiency was assessed by western blot analysis.

Western blot analysis. The total proteins from the cells were extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology) and determined using a BCA protein

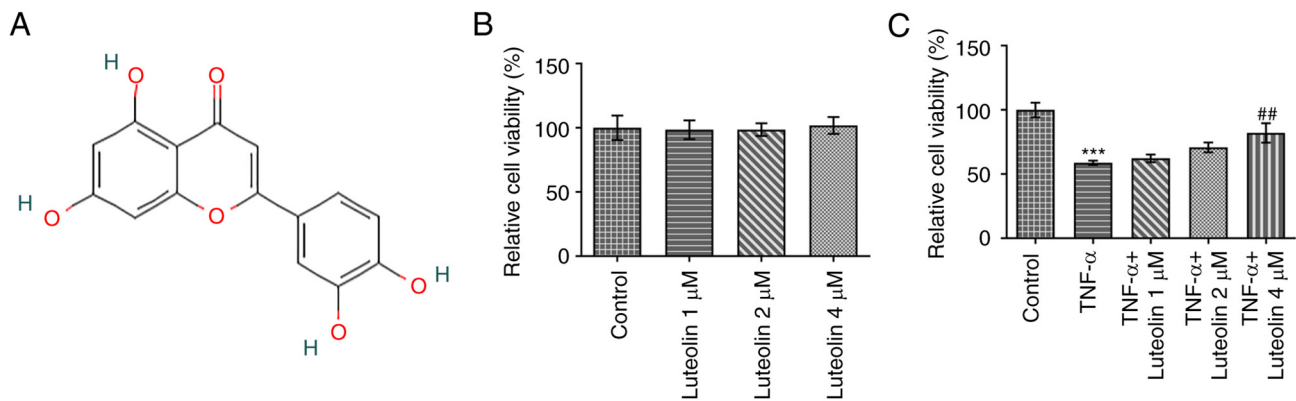


Figure 1. Luteolin enhances the viability of TNF- α -induced HNPCs. (A) Chemical structure of luteolin. (B) Effect of different concentrations (1, 2 and 4 μ M) of luteolin on HNPC viability. (C) Effect of luteolin on TNF- α -suppressed HNPC viability. *** P <0.001 vs. control. ** P <0.01 vs. TNF- α . TNF- α , tumor necrosis factor- α ; HNPCs, human nucleus pulposus cells.

assay kit (Thermo Fisher Scientific Inc.). The proteins were transferred on 12% SDS gels and analyzed with PAGE. Subsequently, they were transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 4 h at room temperature. Subsequently, the membranes (Abcam) were incubated overnight at 4°C with the following primary antibodies: anti-Bcl-2 (1:1,000; cat. no. ab182858), anti-Bax (1:1,000; cat. no. ab32503), anti-cleaved caspase 3 (1:1,000; cat. no. ab32042), anti-p16 (1:1,000; cat. no. ab51243), anti-p21 (1:1,000; cat. no. ab109520), anti-Sirt6 (1:1,000; cat. no. ab289970), anti-p-NF- κ B p65 (1:1,000; cat. no. ab239882), anti-NF- κ B p65 (1:1,000; cat. no. ab207297), anti-p53 (1:1,000; cat. no. Ab32389), anti-H3K9ac (1:500; cat. no. Ab32129), anti-histone H3 (H3; 1:1,000; cat. no. Ab1791) and anti-GAPDH (1:1,000; cat. no. ab8245; all from Abcam). Following primary antibody incubation, the membranes were incubated with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:5,000; cat. no. ab150077, Abcam) at room temperature for 4 h. Finally, the images of the protein bands were visualized using ECL reagents (MilliporeSigma). The protein expression levels were semi-quantified using ImageJ software (version 1.46; National Institutes of Health) with GAPDH or histone H3 as the loading control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction was performed from HNPCs using RNAzol RT (Sigma-Aldrich; Merck KGaA), followed by reverse transcription of the RNA into cDNA with a cDNA reverse transcription kit (Qiagen GmbH), according to the manufacturer's protocol. Subsequently, qPCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen GmbH), according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. The following primers (GenScript) were used for qPCR analysis: Sirt6 forward, 5'-TCCCGACTTCAGGGGTC-3' and reverse, 5'-GTTCTGGCTGACCAGGAAGC-3'; and GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAAATCC-3'. The mRNA expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method (25) and normalized to the expression levels of the internal reference gene GAPDH.

Statistical analysis. The measured data were expressed as mean \pm standard deviation from ≥ 3 independent experiments and GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used to plot the figures. One-way ANOVA followed by Tukey's post hoc test was used for comparison between multiple groups. P <0.05 was considered to indicate a statistically significant difference.

Results

Luteolin enhances the viability of TNF- α -induced HNPCs. The viability of HNPCs was detected by the MTT assay. The results indicated that luteolin (Fig. 1A) exerted no apparent reduction in the viability of HNPCs at the concentration range of 1-4 μ M, demonstrating optimal biocompatibility (Fig. 1B). Subsequently, the effects of luteolin were examined on TNF- α -induced HNPC viability. Treatment of the cells with luteolin indicated a concentration-dependent increase in HNPC viability (Fig. 1C).

Luteolin inhibits TNF- α -induced HNPC inflammatory injury. ELISA was performed to detect the levels of specific inflammatory factors produced by TNF- α -induced HNPCs. The results demonstrated that TNF- α induced a significant increase in IL-1 β (Fig. 2A) and IL-6 (Fig. 2B) levels, while luteolin treatment decreased the expression levels of these inflammatory factors in a concentration-dependent manner. Subsequently, TUNEL staining was used to detect the induction of apoptosis of HNPCs, as shown in Fig. 2C. Compared with the TNF- α group, luteolin decreased TNF- α -induced apoptosis in a concentration-dependent manner. To further verify this result, the expression levels of specific apoptosis-related proteins were examined by western blot analysis (Fig. 2D). The results demonstrated that TNF- α induction increased the expression levels of the pro-apoptotic proteins Bax and cleaved caspase 3, whereas it reduced the expression level of the anti-apoptotic protein Bcl-2. However, treatment of the cells with luteolin reversed these effects. The effects noted were positively correlated with the treatment concentration of luteolin.

Luteolin suppresses TNF- α -induced senescence of HNPCs. HNPC senescence was detected by the senescence-associated β -galactosidase (SA- β -Gal) staining kit. In senescent

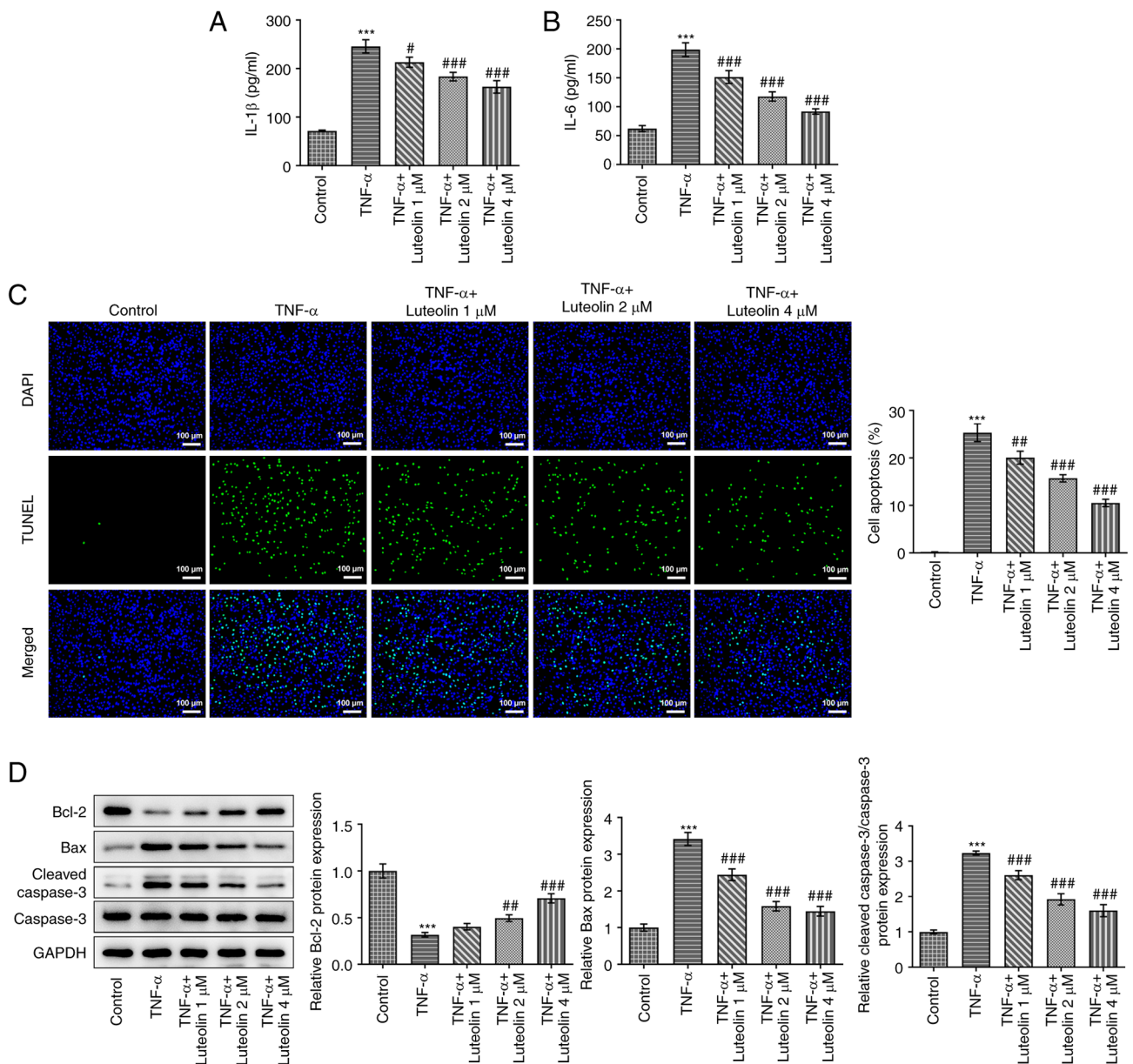


Figure 2. Luteolin inhibits TNF- α -induced HNPC inflammatory injury. (A and B) Expression levels of inflammatory cytokines IL-1 β (A) and IL-6 (B) were detected by ELISA. (C) TUNEL staining was used to detect the effect of luteolin on TNF- α -induced apoptosis. (D) Expression levels of Bcl-2, Bax and cleaved caspase-3 protein were detected by western blot analysis. *** P <0.001 vs. control. # P <0.05, ## P <0.01 and ### P <0.001 vs. TNF- α . TNF- α , tumor necrosis factor- α ; HNPs, human nucleus pulposus cells.

cells, a dark blue product was generated following the catalysis of senescence-specific β -galactosidase (26). TNF- α -induced HNPC senescence indicated a marked dark-blue color production, which was alleviated by treatment of the cells with different concentrations of luteolin (Fig. 3A). Telomerase activity is reduced in senescent cells (27); therefore, the effect of luteolin was examined on intracellular telomerase activity stimulated by TNF- α . Similar results to those of the senescent assay were obtained (Fig. 3B). Subsequently, the expression levels of aging-related proteins were examined, and the results indicated that TNF- α promoted the expression levels of p16 and p21 proteins, while luteolin treatment reversed these effects in a concentration-dependent manner (Fig. 3C). In addition, we also examined the effect of TNF- α induction

on the expression of p53, an aging-related protein. The results showed that TNF- α induction increased the expression of p53, while luteolin treatment annulled this effect in a concentration-dependent manner (Fig. 3D).

Luteolin regulates the Sirt6/NF- κ B pathway. To further investigate the mechanism of action of luteolin, western blot analysis was used to detect the expression levels of Sirt6/NF- κ B pathway proteins. The results indicated that TNF- α induced downregulation of Sirt6 protein expression and upregulation of phosphorylated (p)-NF- κ B p65 protein expression, while luteolin treatment reversed these effects in a concentration-dependent manner (Fig. 4A). Furthermore, the effect of TNF- α on histone acetylation was examined. TNF- α increased the histone acetylation-related H3K9ac

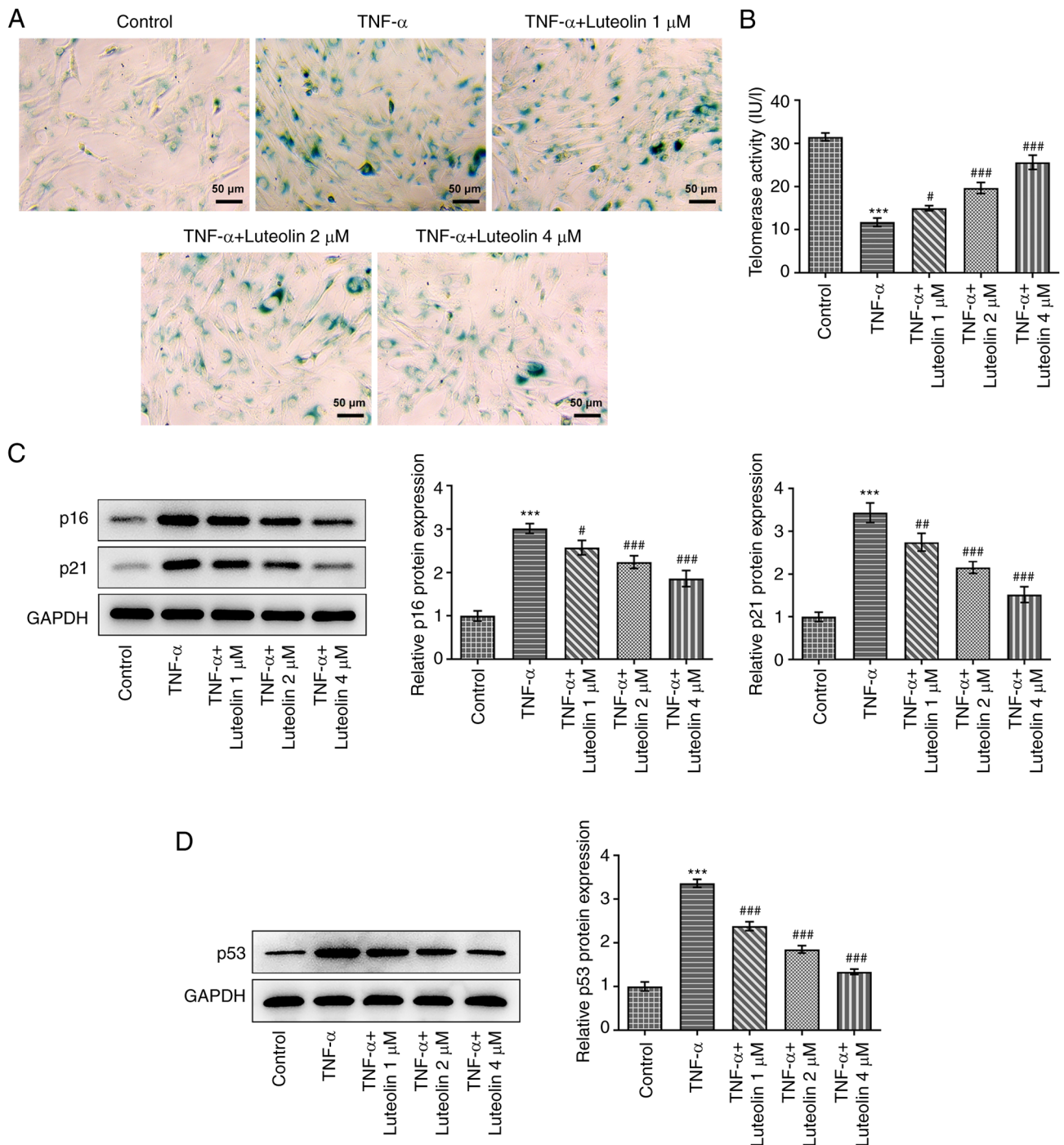


Figure 3. Luteolin suppresses TNF- α -induced senescence of HNPCs. (A) Senescence β -galactosidase staining kit was used to detect the activity level of β -galactosidase in HNPCs. (B) ELISA kit was performed to detect the activity of telomerase. (C) The expression levels of senescence related proteins (p16 and p21) were examined via western blot analysis. (D) Expression of p53 was detected by western blot analysis. ***P<0.001 vs. control. #P<0.05, **P<0.01 and ***P<0.001 vs. TNF- α . TNF- α , tumor necrosis factor- α ; HNPCs, human nucleus pulposus cells.

expression level, while luteolin reversed this effect in a concentration-dependent manner (Fig. 4B). The effect of luteolin on TNF- α -induced Sirt6 activity was detected by SIRT6 activity assay kit, as shown in Fig. 4C. Luteolin at 4 μ M significantly abrogated the TNF- α -induced decrease in Sirt6 activity. The three-dimensional structure of Sirt6 protein (PDB id: 3k35) was also obtained using the PDB database (<https://www.rcsb.org/>), and molecular docking was carried out using Autodock. The data indicate that Sirt6 interacts with luteolin (Fig. 4D).

Knockdown of Sirt6 expression partially reverses the protective effect of luteolin on TNF- α -induced HNPCs. Western blot and RT-qPCR analyses were used to assess the knockdown efficiency of Sirt6. The Sirt6 protein (Fig. 5A) and mRNA (Fig. 5B) expression levels were lower in the si-Sirt6#2 group compared with those noted in the si-Sirt6#1 group, and therefore si-Sirt6#2 was selected for subsequent experiments. Subsequently, MTT (Fig. 5C) and ELISA assays (Fig. 5D and E) were employed to detect the effects of Sirt6 knockdown on cell viability and expression of inflammatory

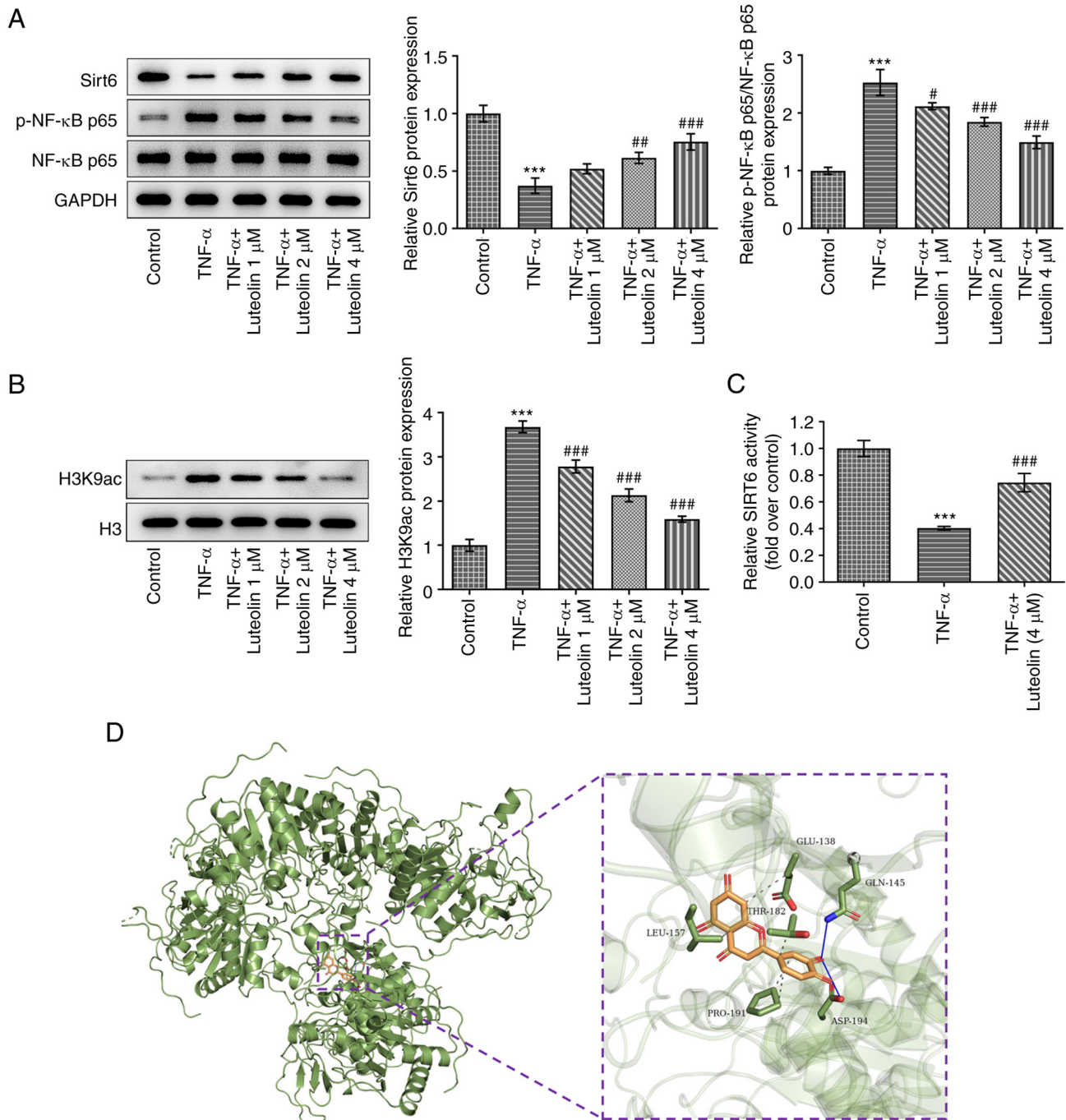


Figure 4. Luteolin regulates the Sirt6/NF-κB pathway. (A) The expression levels of Sirt6/NF-κB pathway-related proteins (Sirt6, p-NF-κB and p-NF-κB p65) were examined by western blot analysis. (B) Western blot analysis was used to examine histone acetylation-related H3K9ac expression level. (C) Effect of luteolin on TNF-α-induced Sirt6 activity was detected by SIRT6 activity assay kit. (D) Interaction between Sirt6 and luteolin was predicted by molecular docking. *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. TNF-α. TNF-α, tumor necrosis factor-α; HNPCs, human nucleus pulposus cells.

factors, respectively. Compared with the TNF-α + luteolin + si-NC group, the TNF-α + luteolin + si-Sirt6 group demonstrated significantly decreased cell viability and upregulation of IL-1β and IL-6 expression levels. Subsequently, TUNEL staining (Fig. 5F) and western blot (Fig. 5G) analyses were used to detect cell apoptosis. Knockdown of Sirt6 expression reversed the inhibitory effects of luteolin on TNF-α-induced apoptosis of HNPCs. Additional investigation of the knock-down effects of Sirt6 on cell senescence yielded similar results and this effect manifested as an increase in the blue product

of β-galactosidase (SA-β-Gal) staining (Fig. 6A), a decrease in telomerase activity (Fig. 6B), and an upregulation in the expression levels of senescence-related proteins (p16, p21 and p53) (Fig. 6C and D).

Discussion

Intervertebral disc degeneration (IDD) is the main cause of low back pain, which leads to the loss of labor capacity and causes a serious burden to human society (28). However, until

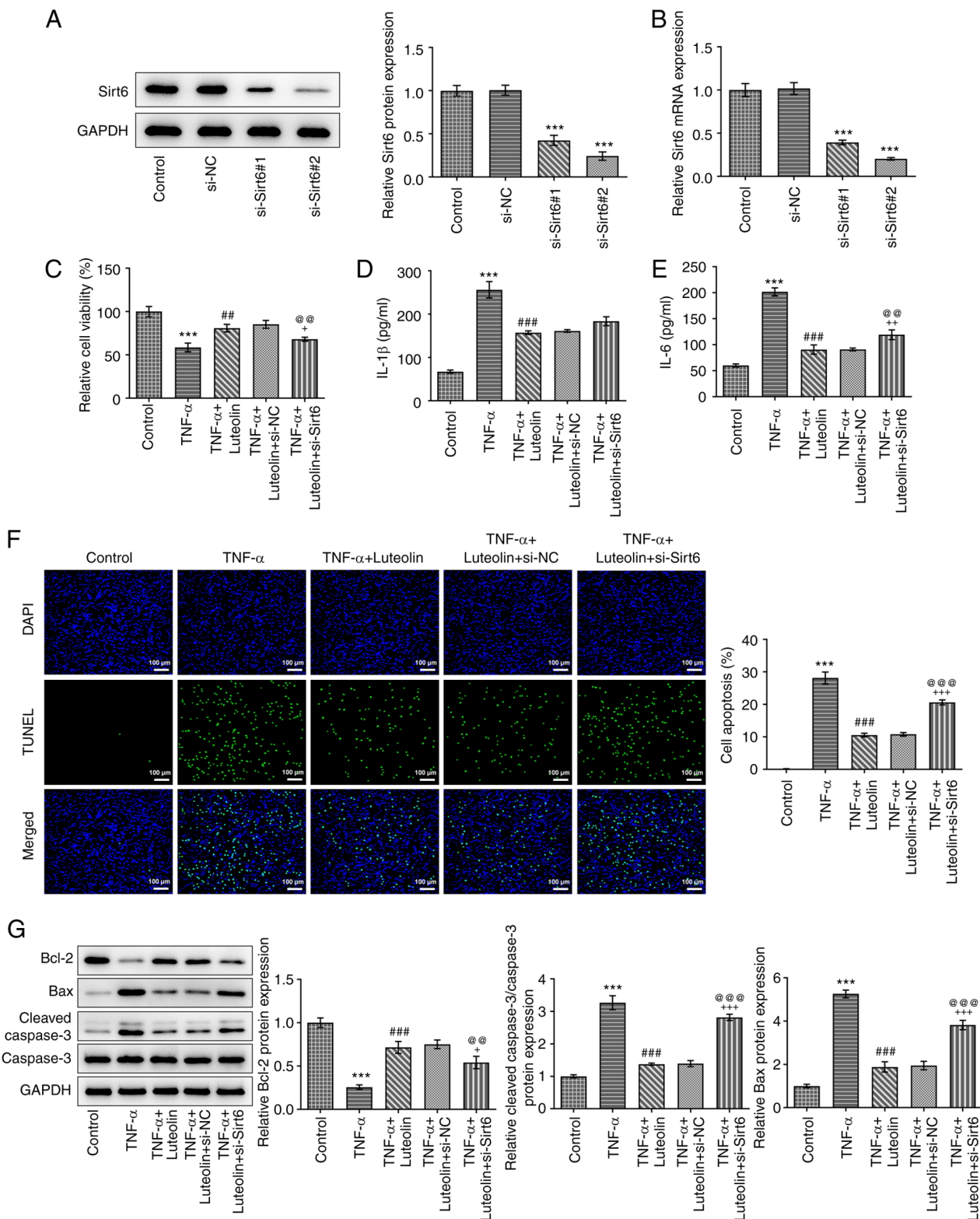


Figure 5. Sirt6 knockdown partially reverses the inhibitory effect of luteolin on TNF- α -induced inflammatory damage of HNPCs. (A and B) The Sirt6 protein (A) and mRNA (B) expression levels were detected by western blot analysis and RT-qPCR, respectively. (C-E) Effects of Sirt6 knockdown on cell viability (C) and intracellular IL-1 β (D) and IL-6 (E) expression levels. (F) TUNEL staining was used to detect the effect of Sirt6 knockdown on HNPC apoptosis. (G) Expression levels of Bcl-2, Bax and cleaved caspase 3 protein were detected by western blot analysis. *** $P < 0.001$ vs. control or si-NC. ** $P < 0.01$ and *** $P < 0.001$ vs. TNF- α . * $P < 0.05$ ** $P < 0.01$ and *** $P < 0.001$ vs. TNF- α + luteolin. @ $P < 0.01$ and @@@ $P < 0.001$ vs. TNF- α + luteolin + si-NC. TNF- α , tumor necrosis factor- α ; HNPCs, human nucleus pulposus cells; Sirt6, sirtuin 6.

recently no effective drugs and treatment methods have been reported for IDD progression. Recently, traditional Chinese medicine has attracted considerable attention due to its high

efficiency, safety, and low toxicity (29). Luteolin is a flavonoid with significant anti-inflammatory activity in various disease types (30). However, it is still unknown whether luteolin

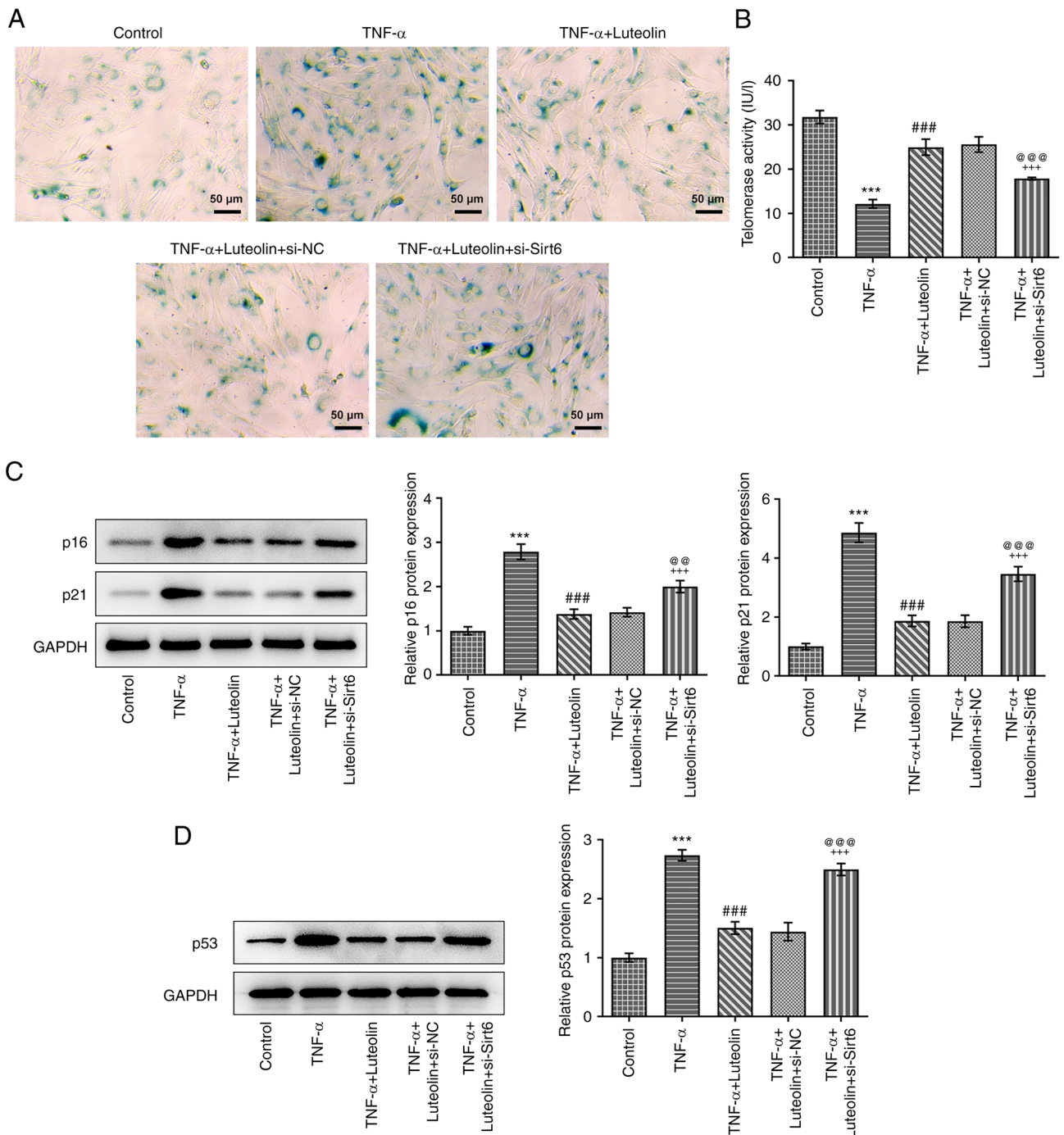


Figure 6. Knockdown of Sirt6 partially reverses the inhibitory effect of luteolin on TNF- α -induced senescence of HNPCs. (A) The activity level of β -galactosidase in HNPCs was assayed by senescence β -galactosidase staining. (B) ELISA kit was performed to detect the activity of telomerase. (C) Expression levels of senescence-related proteins (p16 and p21) were examined via western blot analysis. (D) Expression of p53 was detected by western blot analysis. ***P<0.001 vs. control. ###P<0.001 vs. TNF- α . +++P<0.001 vs. TNF- α + luteolin. @P<0.01, @@@P<0.001 vs. TNF- α + luteolin + si-NC. TNF- α , tumor necrosis factor- α ; HNPCs, human nucleus pulposus cells; Sirt6, sirtuin 6.

possesses protective effects in IDD. Therefore, the effects of luteolin on intervertebral disc (IVD) degeneration and the underlying mechanism of action were investigated. The present study demonstrated that luteolin could ameliorate TNF- α -induced nucleus pulposus (NP) cell senescence and apoptosis by targeting the Sirt6/NF- κ B pathway.

NP cellular senescence and apoptosis are the major biochemical changes that contribute to the development of IVD degeneration (31). Notably, inflammation and oxidative

stress may accelerate this chronic process. In addition, a relevant study has reported that ECM stiffness aggravated oxidative stress-induced senescence and apoptosis in human NP cells (32). Tumor necrosis factor (TNF)- α is one of the TNF superfamily ligands, which possesses proinflammatory activity and is considered to be linked to the development of multiple diseases (33). Recent research has indicated that TNF- α can lead to inflammatory disc degeneration (34). In a TNF- α -injected porcine model, Kang *et al* (35) demonstrated

that TNF- α resulted in early-stage disc degeneration as shown by NP cell cluster, ECM degeneration, vascularization, and IL-1 β secretion. These results indicate that TNF- α serves as a crucial early pathogenetic driver in IDD (35). In the present study, 50 ng/ml TNF- α was used to establish the inflammatory cell damage model and to mimic the pathophysiology of IDD *in vitro*. TNF- α induced the senescence of human nucleus pulposus cells (HNPCs), while luteolin treatment reduced the number of SA- β -Gal-positive cells and the expression of senescence-related proteins, such as p16 and p21. Moreover, it is worth mentioning that cellular senescence is closely related to the inflammatory response, interrupted proliferation, as well as damaged NP tissue self-repair (36). A previous study has reported that IL-1 β and IL-6 can upregulate the levels of matrix-degrading proteases, such as MMP-13 and thus directly promote matrix degradation (37). In the present study, after TNF- α induction, it was shown that the expression levels of IL-1 β , IL-6, and Bax cleaved caspase-3 in HNPCs were markedly increased. This finding was accompanied by decreased Bcl-2 level. However, luteolin administration significantly increased cell viability, inhibited cell apoptosis, and decreased the expression levels of specific cytokines. Similarly, Xia *et al* (38) highlighted that luteolin could target TNF- α -induced cell inflammation injury, whereas the anti-inflammatory effect of luteolin was linked to the selective inhibition of the NADPH oxidase 4/reactive oxygen species (ROS)/NF- κ B and MAPK pathways. In terms of the toxicity of luteolin, Ali and Siddique reported about the bio-availability of luteolin in the plasma, and human clinical trials indicated no dose limiting toxicity when administered at a dose of 100 mg/day (39). Taken together, these findings support the hypothesis that luteolin at a certain concentration range exerts protective effects against TNF- α -induced NP cell senescence and inflammation injury.

Sirt6, a member of the Sirt proteins, has been implicated in various cellular processes including inflammation, aging, energy metabolism, stress resistance, and cancer (40). Sirt6 plays a negative regulatory role in the regulation of cellular senescence. Loss of Sirt6 is known to be associated with cellular senescence and apoptosis (41). For example, Sirt6 can suppress the senescence of smooth muscle cells and thus reduce atherogenesis (42). Sirt6 has also been shown to protect nerve cells from amyloid- β 1-42 oligomer-induced cellular senescence in Alzheimer's disease (43). Kang *et al* (22) demonstrated that overexpression of Sirt6 could prevent ECM degradation in human NP cells and thus inhibit the progression of IDD, indicating that Sirt6 exerts protective effects on NP cells. The results of the present study indicated that Sirt6 expression was significantly downregulated in TNF- α -treated HNPCs. However, luteolin promoted the expression of Sirt6 in HNPCs. Luteolin has also been shown to upregulate Sirt6 levels under *in vitro* diabetic conditions (44). These results indicate that the protective effects of luteolin on IDD may be associated with the regulation of Sirt6.

It has been reported that the NF- κ B signaling pathway can promote ECM degradation by increasing the activity of matrix-degrading proteases in NP cells (45). Hyperactive NF- κ B signaling contributes to premature and normal aging (46). The interference with NF- κ B signaling is regarded as a targeted therapeutic strategy for IDD (47). Moreover,

Li *et al* (48) indicated that Sirt6 could directly target NF- κ B p65 to reduce the activation of NF- κ B and therefore prevent the NF- κ B-mediated expression of specific inflammatory cytokines. Similarly, Kang *et al* (22) reported that Sirt6 overexpression inhibited NF- κ B signaling and therefore prevented matrix degradation in IDD. These results suggest that Sirt6 plays a negative regulatory role in NF- κ B activation in IDD. P65 was found to be expressed simultaneously in the nucleus and cytoplasm of NP cells after induction of TNF- α for 24 h, which increased the nuclear translocation and phosphorylation of P65 (49). Consistent with this report, the present study demonstrated that downregulation of Sirt6 expression in TNF- α -treated HNPCs was accompanied by the phosphorylation of NF- κ B (p65 subunit), while luteolin inhibited NF- κ B p65 subunit phosphorylation. These results suggest that the protective role of luteolin is linked to the regulation of the Sirt6/NF- κ B pathway. Luteolin has also been shown to exhibit anti-inflammatory activity in various diseases, such as intracerebral hemorrhage (50), osteoarthritis (16), and diabetic cardiomyopathy (14). This type of activity is mediated by inhibiting the activation of the NF- κ B pathway. In addition, the present study demonstrated that downregulation of Sirt6 led to decreased cell viability, cell senescence, and increased secretion of IL-1 β and IL-6, indicating that inhibition of Sirt6 could partially abrogate the protective effects of luteolin in IDD. However, the present study still has the following deficiencies. First of all, the present study was based solely on *in vitro* data. Therefore, the mechanisms of luteolin in IDD require further assessment in future *in vivo* studies. Second, since luteolin has been shown to affect cellular senescence, its effect on longevity needs to be further validated in animal studies. In addition, the mechanism of luteolin effect on the NF- κ B/Sirt6 pathway remains to be further explored.

In summary, the present study indicated that luteolin attenuates TNF- α -mediated NP cell inflammation injury and senescence by targeting Sirt6 and inhibiting the activation of the downstream NF- κ B signaling pathway. These findings provide novel molecular insight into the anti-inflammatory effects of luteolin, which can be considered a potential candidate for IDD therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from Wuhan Municipal Health Commission (grant no. WZ15B09).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX and JY conceived and designed the study. LM, PL and RP performed the experiments. JY and LM wrote the paper. TX, PL and RP reviewed and edited the manuscript. TX, LM

and RP confirm the authenticity of the data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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