Upregulation of SIRT1 by Evodiamine activates PI3K/AKT pathway and blocks intervertebral disc degeneration

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Abstract. Intervertebral disc degeneration (IDD) is a major cause of a number of spinal diseases, resulting in serious public health problems. Evodiamine (Evo) is an indole quinazoline alkaloid extracted from *Evodia rutaecarpa*, which has antioxidant, anti-apoptosis and anti-inflammatory effects. The purpose of the present study was to investigate lipopolysaccharide (LPS)-induced IDD progression in human nucleus pulposus cells (NPCs) and its potential mechanism. The viability and apoptosis of NPCs were detected by Cell Counting Kit-8 (CCK-8) and TUNEL staining, respectively. Western blotting was used to detect the expression levels of proteins, cell transfection was performed to knockdown Sirtuin 1 (SIRT1) and the expression of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) was detected by enzyme-linked immunosorbent assay kits. The results showed that Evo effectively alleviated LPS-induced NPCs apoptosis and caspase-3 activation and Evo treatment reversed the upregulation of matrix metalloproteinase-13, as well as the downregulation of collagen type II (collagen II), Sry-type high-mobility-group box 9 and aggrecan and reduced the production of pro-inflammatory factors TNF-α and IL-6 in LPS-stimulated NPCs. In addition, treatment with Evo upregulated SIRT1 and activated the PI3K/Akt pathway, knockdown of SIRT1 inhibited the phosphorylation of Akt and PI3K in LPS-stimulated NPCs. In general, Evo upregulated SIRT1 and inhibited LPS-induced NPCs apoptosis, extracellular matrix degradation and inflammation by activating the PI3K/Akt pathway.

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Introduction

Intervertebral disc (IVD) degeneration (IDD) is a common natural aging process characterized by chronic back and low back pain (1,2). With the increasing aging population in the world, IDD has become the leading cause of spinal related disability worldwide, with an increasing incidence of IDD and few inexpensive and effective treatments (3). Therefore, there is an urgent need for effective treatment to alleviate the progression of IDD.

Intervertebral disc is an avascular organ composed of peripheral annulus and central nucleus pulposus (4), in which the nucleus pulposus cells (NPCs) are responsible for regulating the synthesis and decomposition of components of extracellular matrix (ECM) (5), including synthesis of collagen type II (collagen II), sry-type high-mobility-group box 9 (SOX-9) and proteoglycans (mainly aggrecan) and the decomposition of matrix metalloproteinases (MMPs) (6). It has previously been observed that the process of IDD is closely associated with apoptosis and inflammatory response of NPCs and these pro-inflammatory molecules are secreted by nucleus pulposa (7). In the process of degeneration, elevated levels of inflammatory factors promote ECM degradation, leading to cell phenotypic changes and a host of degenerative events (2). Therefore, finding effective drugs to inhibit NPCs apoptosis, ECM degradation and inflammation may be a feasible strategy for the prevention and treatment of IDD.

Evodiamine (Evo), a type of indole quinazoline alkaloid extracted from dried fruit of *Evodia rutaecarpa* (8). In traditional Chinese medicine, *Evodia rutaecarpa* is widely used for treating various infection-related diseases, such as diarrhea, ulcerative colitis and beriberi (9-11), showing strong anti-inflammatory activity. A previous study suggested that Evo can enhance NLRP3 inflammasome activation by inducing α-tubulin acetylation, thereby improving innate immunity to bacterial infections (11). It can also reduce the peripheral hypersensitivity and anxiety of mice with nerve injury or inflammation through TRPV1 (12) and regulate the TLR4/NF-κB signaling pathway to inhibit lipopolysaccharide (LPS)-induced HUVECs injury and promote cell proliferation (13). In addition, Shi *et al* (14) demonstrate that Evo possesses an important protective effect on LPS-induced acute kidney injury and cytotoxicity. However, the role and

mechanism of Evo in IDD have yet to be studied, to the best of the authors' knowledge.

Previous studies have shown that Sirtuin 1 (SIRT1) possesses a protective effect in IDD (15,16), while Evo regulates SIRT1 level in colorectal cancer and inhibits the migration and invasion of colorectal cancer (17). Another study also reported that Evo can induce apoptosis of human melanoma A375-S2 cells by regulating SIRT (18). Therefore, it was hypothesized that Evo might serve a role in the progression of IDD by regulating SIRT1. Notably, PI3K/Akt pathway also serves an important role in the pathogenesis of IDD (19,20). The activation of PI3K/Akt pathway can inhibit interleukin-1β (IL-1β)-induced NP cell apoptosis (21), possibly by increasing ECM content, preventing apoptosis and alleviating oxidative damage and inflammatory reaction (19). In Ren *et al* (22), Sirt1 as the upstream of PI3K/Akt can directly connect with PI3K/Akt and curcumin served a role in diabetic cardiomyopathy treatment by modulating the Sirt1-Foxo1 and PI3K/Akt pathways. Qi *et al* (23) demonstrate that tyrosol upregulates SIRT1, inhibits apoptosis and inflammation of IL-1β-stimulated NPCs and regulates ECM remodeling by activating the PI3K/Akt pathway. Based on the above studies, it was hypothesized that Evo could serve a protective role in IDD by regulating SIRT1 and PI3K/Akt pathway. Therefore, the aim of the present study was to investigate the effects of Evo on LPS-induced NPCs apoptosis, ECM degradation and inflammation and to preliminarily analyze its underlying mechanism.

Materials and methods

Cell culture. Immortalized human nucleus pulposus cells (NPCs; iCell Bioscience Inc.; cat. no. iCell-0028a) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. The cells were passaged once after three days. The cells cultured to logarithmic phase were used in following experiments.

Cell Counting Kit-8 (CCK-8) assay. Cells ($1x10^3$ cells/well) were seeded into 96-well plates and incubated at 37˚C with 5% CO_2. Cell proliferation was determined using CCK-8 reagent (Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Following incubation with 1 μg/ml LPS and Evo at different concentrations (5, 10 and 20 μM) for 24 h, 10 μl CCK-8 solution was added to each well for 4 h at 37˚C. Absorbance was measured at a wavelength of 450 nm using a microplate reader.

TUNEL assay. The effects of Evo on the apoptosis of LPS-induced NPCs cells were detected using TUNEL according to the manufacturer's protocol. In brief, cells were collected and washed three times with PBS. Following fixing with 4% paraformaldehyde at room temperature for 20 min, the cells were washed twice with PBS. Then, 0.2% Triton-X-100 was added to the cells at room temperature for 5 min. Subsequently, 50 μl TUNEL assay solution (Roche Diagnostics GmbH) was added to the cells and incubated at 37˚C in the dark for 60 min. 0.5 μg/ml of DAPI solution (Beyotime Biotechnology) was applied to stain cell nuclei for 3-5 min at room temperature. The detection solution was discarded and cells were washed three times with PBS. Subsequently, three fields of view were selected at random and then cells were sealed with anti-fluorescence quenched sealing solution for observation under a fluorescence microscope (Zeiss GmbH; magnification, x200).

Western blotting. The extraction of total proteins from NPCs cells was conducted by radioimmunoprecipitation (RIPA) lysing buffer (Beijing Solarbio Science & Technology Co., Ltd.) and the protein concentrations were quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). The samples were subjected to 12% gel with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes before being blocked with 5% non-fat milk for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4˚C with the following primary antibodies (all purchased from Abcam): Anti-Bcl2 (1:1,000; cat. no. ab32124), anti-Bax (1:1,000; cat. no. ab32503), anti-MMP13 (1:1,000; cat. no. ab219620), anti-Aggrecan (1:1,000; cat. no. ab3778), anti-Collagen II (1:1,000; cat. no. ab34712), anti-SOX9 (1:1,000; cat. no. ab185996), anti-SIRT1 (1:1,000; cat. no. ab110304), anti-phosphorylated (p-)P13K (1:1,000; cat. no. ab32503), anti-P13K (1:1,000; cat. no. ab32089), anti-p-AKT (1:1,000; cat. no. ab182651), anti-AKT (1:1,000; cat. no. ab191606) and anti-GAPDH (1:1,000; cat. no. ab181602). Following that, the membranes were incubated with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (1:5,000; cat. no. ab150077) for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence reagent (Cytiva). Protein expression levels were semi-quantified using ImageJ software (version 1.46; National Institutes of Health) with GAPDH as the loading control.

Detection kit. The levels of TNF-α (cat. no. PT518) and IL-6 (cat. no. PI330) in NPCs cells were quantified using ELISA kits (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The effect of Evo on the activity of caspase-3 in LPS-treated NPCs cells was detected using caspase-3 activity assay kit (cat. no. BC3830; Beijing Solarbio Science & Technology Co., Ltd.).

Cell transfection. Cells ($1x10^5$ cells/well) were seeded into 6-well plates and cultured for 24 h at 37˚C with 5% CO_2. siRNA targeting SIRT1 (si-SIRT1 forward: 5'-GGAUGAAAGUGAAAUUGAA-3', reverse: 5'-UUCAAUUUCACUUUCAUCC-3') and a control non-targeting siRNA (si-NC forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse: 5'-ACGUGACACGUUCGGAGAATT-3') were designed and synthesized by Shanghai GenePharma Co., Ltd. Subsequently, All plasmids were transfected using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells in the blank control group (Control) were untreated. At 48 h post-transfection, transfection efficiency was assessed via RT-qPCR.

Data analysis. The data were plotted with GraphPad Prism 8.0 software (GraphPad Software, Inc.). The measurement data are expressed as the mean ± standard deviation from ≥3 independent experiments. One-way ANOVA followed by Tukey's post hoc

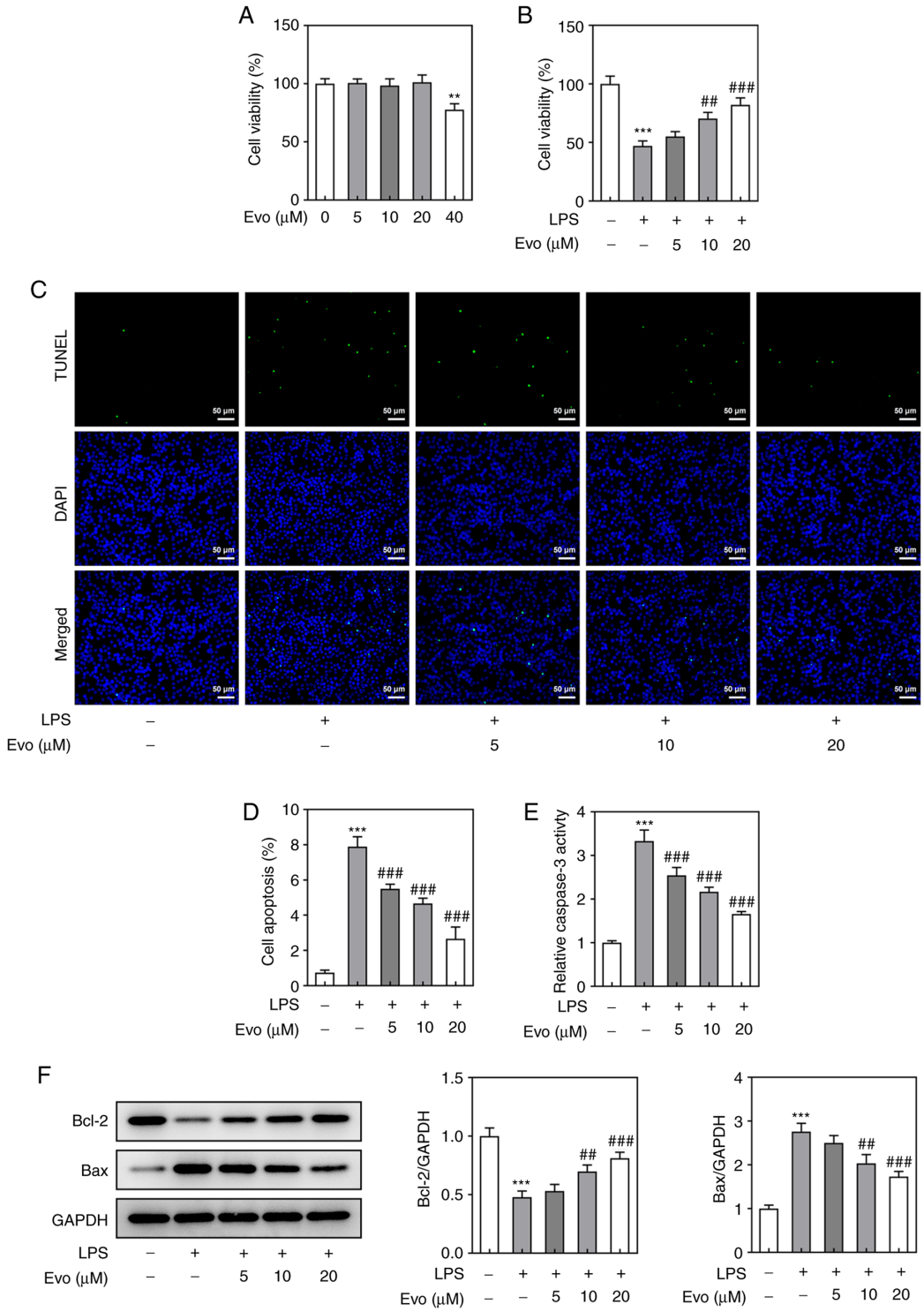

Figure 1. Evo inhibits LPS-induced NPCs apoptosis. (A) Cell viability of NPCs incubated with Evo at 5, 10, 20 and 40 μM for 24 h. (B) CCK-8 assay and (C and D) TUNEL staining were used to detect the effects of Evo on the viability and apoptosis of LPS-induced NPCs. (E) Caspase-3 activity was detected in NPCs and (F) the expression levels of apoptosis-related proteins (Bax and Bcl-2) were detected by western blotting. **P<0.01 and ***P<0.001 vs. Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. Evo, evodiamine; LPS, lipopolysaccharide; NPCs, human nucleus pulposus cells.

test was used for comparison between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Evo inhibits LPS-induced NPCs apoptosis. First, the effect of Evo on NPCs viability was detected. The results showed that Evo at 5, 10 and 20 μM had no significant effect on NPCs viability, but Evo at 40 μM caused damage on NPCs (Fig. 1A). Therefore, the maximum Evo concentration was 20 μM in the following experiments. Subsequently, CCK-8 assay (Fig. 1B) and TUNEL staining (Fig. 1C and D) showed significant apoptosis of NPCs induced by LPS. It should be noted that Evo enhanced NPCs viability in a concentration-dependent manner. In addition,

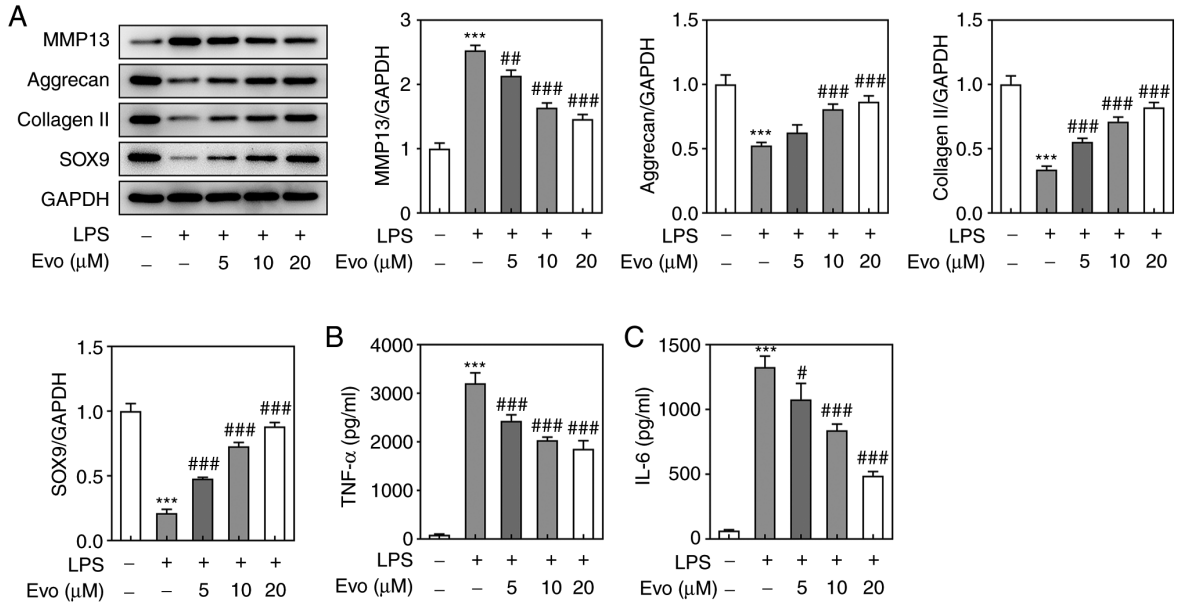

Figure 2. Evo effectively alleviated LPS-induced extracellular matrix degradation and inflammation in NPCs. (A) Western blotting was applied to examine the expression of MMP-13, Aggrecan, collagen II and SOX-9 proteins. The concentrations of (B) TNF-α and (C) IL-6 in NPCs were determined by ELISA. ***P<0.001 vs. Control. #P<0.05, ##P<0.01 and ###P<0.001 vs. LPS. Evo, evodiamine; LPS, lipopolysaccharide; NPCs, human nucleus pulposus cells; SOX-9, sry-type high-mobility-group box 9; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; IL, interleukin.

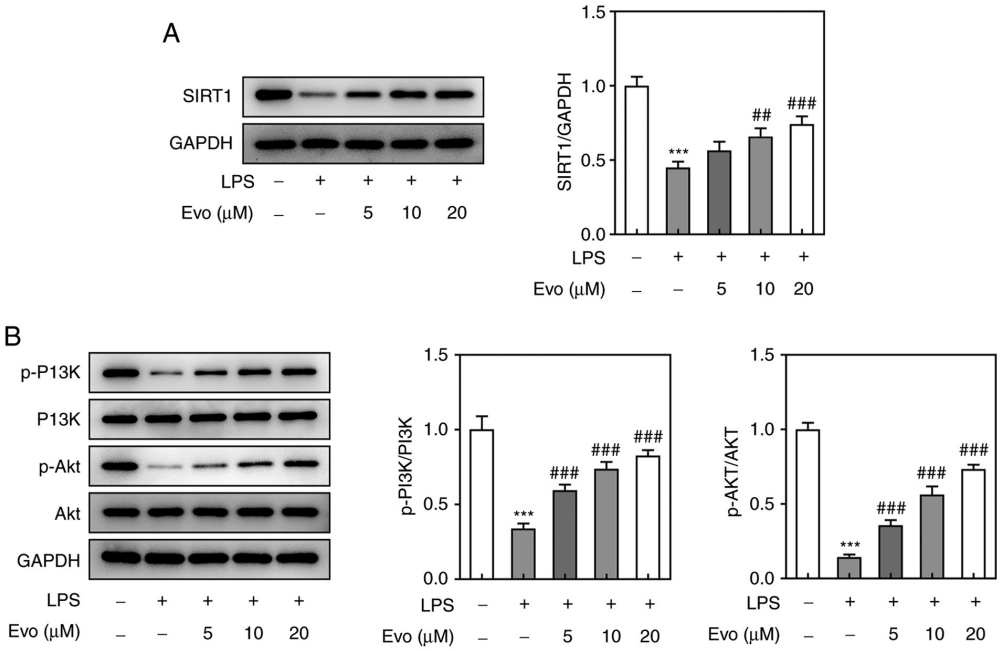

Figure 3. Evo activates the PI3K/AKT pathway by upregulating SIRT1. (A) Western blotting was used to detect the expression level of SIRT1 protein and (B) the effect of Evo on the P13K/AKT pathway-related proteins (P13K, AKT, p-P13K and p-AKT) in LPS-induced NPCs. ***P<0.001 vs. Control. ##P<0.01 and ###P<0.001 vs. LPS. Evo, evodiamine; SIRT1, Sirtuin 1; p-, phosphorylated; LPS, lipopolysaccharide; NPCs, human nucleus pulposus cells.

caspase-3 activity and the expression levels of apoptosis-related proteins in NPCs were also assessed (Fig. 1E and F). Following LPS induction, caspase-3 activity was increased, Bax protein was significantly upregulated and Bcl-2 protein was downregulated, indicating that LPS induced apparent apoptosis. Similarly, this change also could be reversed by Evo.

Evo effectively alleviates LPS-induced extracellular matrix degradation and inflammation in NPCs. Subsequently, the effects of Evo on LPS-induced ECM degradation and inflammation in NPCs was analyzed. The protein expression level of ECM catabolism gene MMP-13 was upregulated after LPS stimulation, but Evo treatment attenuated the effect of LPS; the protein expressions of ECM synthesis genes (Aggrecan, collagen II and SOX-9,) in NPCs were downregulated following LPS treatment, while Evo treatment reversed these changes (Fig. 2A). ELISA was used to measure the expression levels of pro-inflammatory cytokines (TNF-α

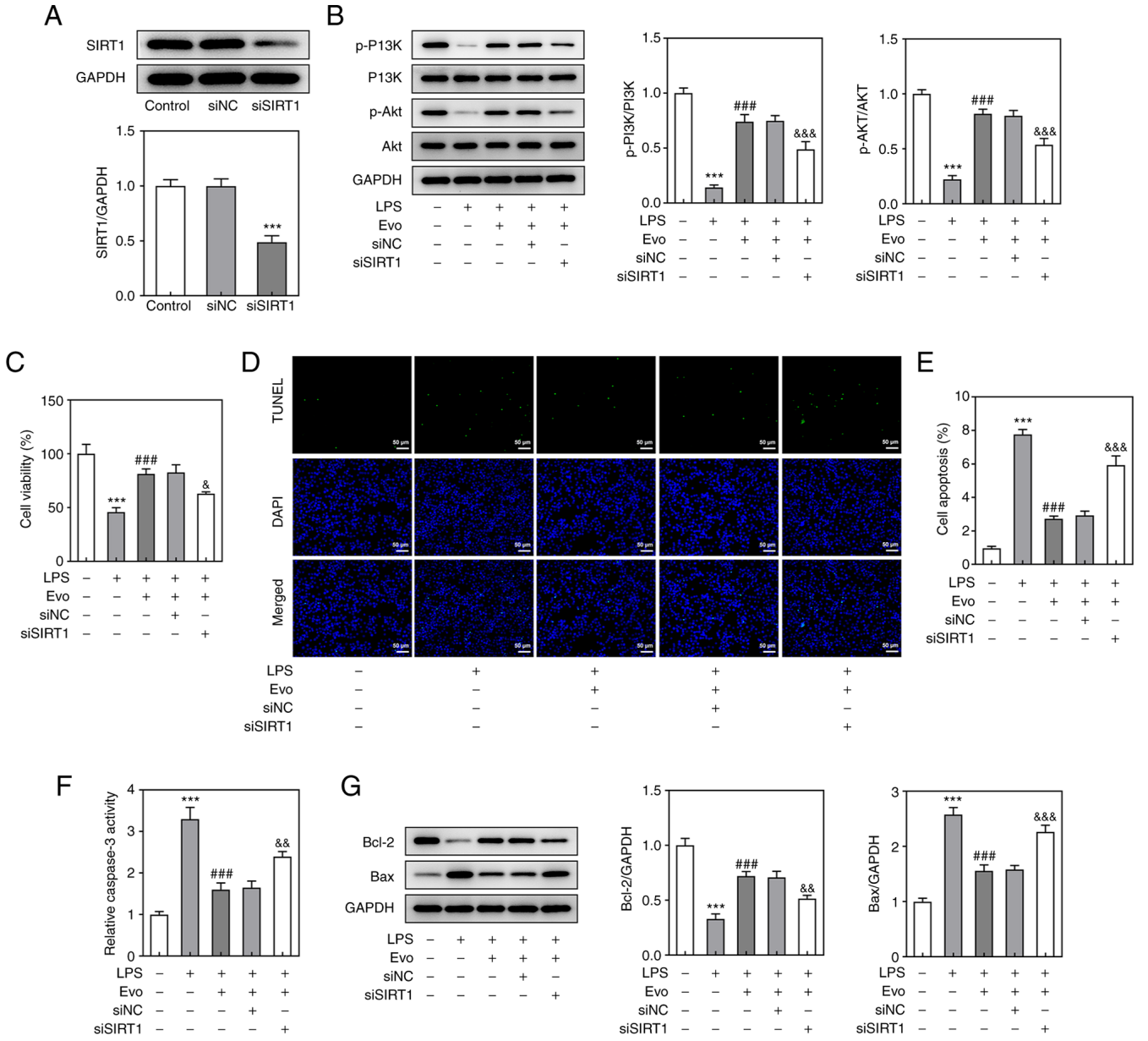

Figure 4. SIRT1 knockdown attenuates the inhibitory effects of Evo on LPS-induced apoptosis in NPCs. Western blotting was performed to detect the interference efficiency of (A) SIRT1 and (B) the expression levels of P13K, AKT, p-P13K and p-AKT protein. (C) CCK-8 assay and (D and E) TUNEL staining were conducted to test the viability and apoptosis level of NPCs and (F) Caspase-3 detection kit was used to detect the activity of caspase-3 in NPCs. (G) The expression level of Bax and Bcl-2 protein in NPCs. ***P<0.001 vs. Control. $^{###}$P<0.001 vs. LPS. $^{&}$P<0.05, $^{&&}$P<0.01 and $^{&&&}$P<0.001 vs. si-NC. SIRT1, Sirtuin 1; Evo, evodiamine; LPS, lipopolysaccharide; NPCs, human nucleus pulposus cells; p-, phosphorylated.

and IL-6) in cells. The results indicated that TNF-α and IL-6 levels were significantly increased following LPS treatment (Fig. 2B and C). Similarly, Evo treatment significantly downregulated the expression of TNF-α and IL-6 in cells in a concentration-dependent manner. These results suggested that Evo effectively alleviated LPS-induced ECM degradation and inflammation in NPCs.

Evo activates the PI3K/AKT pathway by upregulating SIRT1. To further investigate the mechanism of Evo's role in IDD cell model, western blotting revealed that Evo conspicuously alleviated LPS-induced downregulation of SIRT1 (Fig. 3A). A previous study established that SIRT1 acts as a key role in the survival of human NP degraded cells by regulating the Akt pathway (23). Therefore, the effect of Evo on the P13K/Akt pathway in LPS-induced NPCs was then examined. The expression of p-Akt and p-P13K was downregulated by LPS stimulation and this effect was also salvaged by Evo (Fig. 3B). To further verify whether Evo activates the P13K/AKT pathway by upregulating SIRT1, a SIRT1 interfering plasmid was constructed (Fig. 4A) for repeated experiments and it was found that in the absence of SIRT1, Evo's activation of P13K/AKT pathway was weakened compared with the si-NC group (Fig. 4B). These results demonstrated that Evo could upregulate SIRT1 and activate the PI3K/Akt pathway, but this positive effect was significantly weakened after SIRT1 silencing, indicating that Evo might activate the PI3K/Akt pathway by upregulating SIRT1.

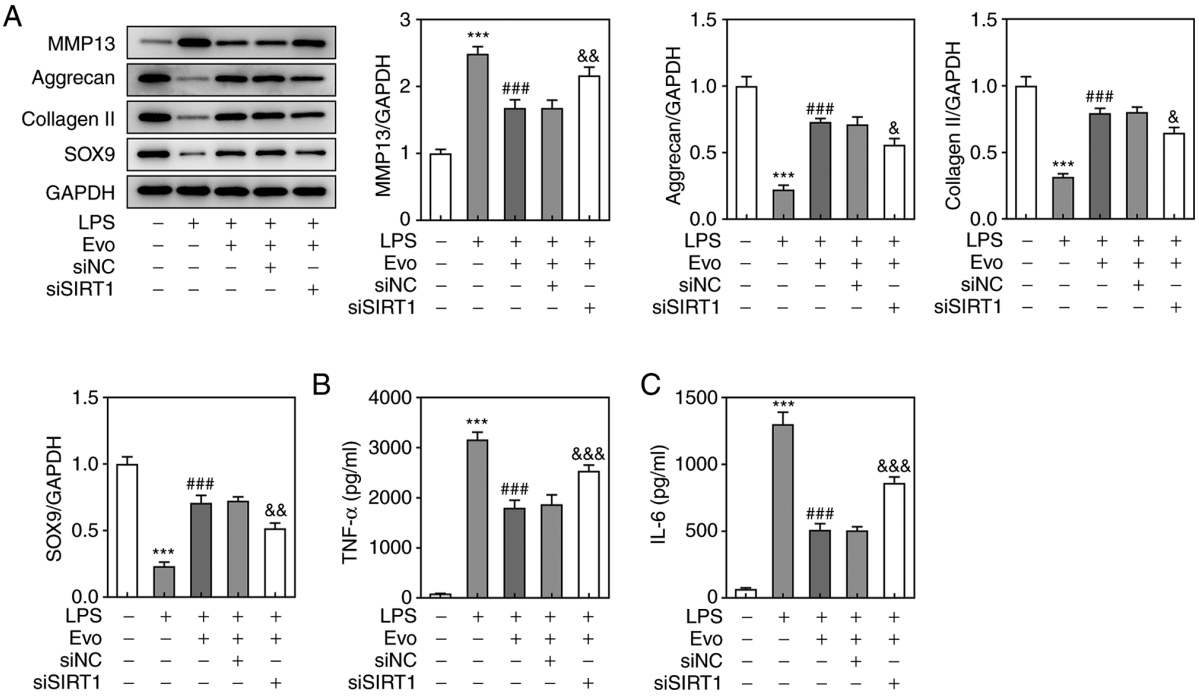

Figure 5. SIRT1 knockdown attenuates the inhibitory effects of Evo on LPS-induced inflammation and ECM degradation in NPCs. (A) Western blotting was applied to examine the expression of MMP-13, Aggrecan, collagen II and SOX-9 proteins. The concentrations of (B) TNF-α and (C) IL-6 in NPCs were determined by ELISA. ***P<0.001 vs. Control. $^{###}$P<0.001 vs. LPS. $^{&}$P<0.05, $^{&&}$P<0.01 and $^{&&&}$P<0.001 vs. si-NC. SIRT1, Sirtuin 1; Evo, evodiamine; LPS, lipopolysaccharide; ECM, extracellular matrix; NPCs, human nucleus pulposus cells; SOX-9, sry-type high-mobility-group box 9; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; IL, interleukin; si, short interfering; NC, negative control.

SIRT1 knockdown attenuates the inhibitory effects of Evo on LPS-induced apoptosis, inflammation and ECM degradation in NPCs. Considering that Evo serves a role by upregulating SIRT1, CCK-8 assay (Fig. 4C) and TUNEL staining (Fig. 4D and E) were used again to detect Evo's effect on LPS-induced apoptosis in NPCs following SIRT1 knockdown. The inhibitory effect of Evo on LPS-induced NPCs apoptosis and increased caspase-3 activity was weakened after SIRT1 knockdown (Fig. 4F). At the same time, the expression of apoptotic proteins detected by western blotting also confirmed this result (Fig. 4G). As shown in Fig. 5A, si-SIRT1 transfection partially eliminated the promotive effect of Evo on ECM synthesis. Furthermore, si-SIRT1 also increased the concentrations of TNF-α and IL-6 compared with Evo group (Fig. 5B and C), which indicated that SIRT1 knockdown attenuates the inhibitory effect of Evo on LPS-induced NPCs inflammation.

Discussion

IVD degeneration is a multifactorial pathological process associated with low back pain, which has been the leading cause of disability worldwide (1,24). Increasing studies have proved that the pathological changes of intervertebral disc disease are closely associated with the degradation of ECM, apoptosis and inflammation (25-27). The present study demonstrated for the first time, to the best of the authors' knowledge that Evo could effectively reduce LPS-induced NPCs apoptosis, ECM degradation and inflammation, possibly by upregulating SIRT1 and activating the PI3K/Akt pathway.

Evo, as an effective ingredient of Traditional Chinese medicine, has a variety of pharmacological effects such as antioxidant, anti-tumor, anti-ulcer and neuroprotection, especially in anti-infection and anti-apoptosis (10,11). Evo, for example, inhibits p2x7-dependent TNF-α expression and ERK1/2 phosphorylation, thereby inhibiting oxidative stress and inflammatory response (28). Another study found that Evo inhibits the secretion of interleukin (IL-10) and IL-2 by LPS-stimulated endothelial cells, inhibiting inflammatory responses (29). It also relieves DSS-induced ulcerative colitis by increasing *Lactobacillus acidophilus* levels and acetate production (10). In addition, Evo also regulates the TLR4/NF-κB signaling pathway to inhibit LPS-induced HUVECs injury and promote cell proliferation (13). In the present study, Evo was also shown to inhibit LPS-induced apoptosis of NPCs in a concentration-dependent manner and Evo treatment reversed the upregulation of MMP-13, as well as the downregulation of collagen II, SOX-9 and aggrecan in LPS-stimulated NPCs. Furthermore, compared with LPS group, Evo markedly reduced the production of pro-inflammatory factors TNF-α and IL-6, thus effectively alleviating the degradation of ECM, apoptosis and inflammatory pathological characteristics in the progression of IDD.

To further investigate the mechanism of Evo alleviating IDD pathological process, the present study focused on Sirt1, the most prominent and widely explored member of the sirtuin family, which has been shown to serve a role in various cellular processes including cell survival and apoptosis in a number of studies (30,31). It is noteworthy that SIRT1 serves a protective role in IDD. For example, SIRT1 enhances the proliferation of aging nucleus pulposus cells by inhibiting P16 and serves a protective role in disc degeneration in rodents (16). CircERCC2 can regulate the apoptosis, autophagy and ECM degradation

in tert-butyl hydroperoxide-induced NPCs by targeting miR-182-5p/SIRT1 (32). Notably, it has also been reported that Evo exerts a role in a variety of diseases by mediating SIRT1, Zhou *et al* (17) suggest that Evo inhibits migration and invasion of colorectal cancer by regulating SIRT1 level. The present study demonstrated that the expression of Sirt1 was downregulated in LPS-induced NPCs and increased in a concentration-dependent manner following Evo treatment. These results indicated that Evo serves a therapeutic role in IDD by upregulating sirt1.

PI3K/Akt pathway is a classic pathway involved in regulating cell proliferation, differentiation, apoptosis and other biological processes (33,34). Existing research has shown that activation of PI3K/Akt pathway can inhibit IL-1β-induced apoptosis of NPCs and enhance the adaptability of NPCs to hypoxia microenvironment (19). Furthermore, tyrosol performs an active role in IDD by upregulating SIRT1, which inhibits apoptosis and inflammation of IL-1β-stimulated NPCs by activating the PI3K/Akt pathway (23). The present study showed that Evo rescued LPS-induced inactivation of the PI3K/Akt pathway in NPCs. Notably, downregulation of Sirt1 reversed Evo's activation of Akt and PI3K phosphorylation, thereby partly eliminated Evo's inhibitory effect on NPCs apoptosis and promoting the degradation of ECM and inflammation in NPCs, Therefore, it was hypothesized that Evo may serve a protective role in IDD by upregulating Sirt1 and then activating PI3K/Akt.

In conclusion, the present study is, to the best of the authors' knowledge, the first to demonstrate that Evo has a protective effect on IDD, possibly by upregulating Sirt1 and then activating the PI3K/Akt pathway to inhibit apoptosis, ECM degradation and inflammation in LPS-stimulated NPCs. Although the present study confirmed that Evo protects IDD and its possible mechanism, the signaling pathway is complex and subject to multiple factors, which requires further study of the interaction of SIRT1/PI3K/Akt with other signaling mediators. In addition, further overexpression of Sirt1 to verify the underlying mechanism of EVO is a next research objective. In general, more research on the mechanism of Evo's role in IDD will contribute to the development of new biologic therapies for IDD.

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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

JK participated in the study design, conducted the experiments and participated in manuscript writing. NZ performed the data analysis and manuscript writing. JK and NZ confirm the authenticity of all raw data. Both authors read and approved the final manuscript.

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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