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SIRT3 retards intervertebral disc degeneration by anti-oxidative stress by activating the SIRT3/FOXO3/SOD2 signaling pathway

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Abstract. – OBJECTIVE: The objective of this paper is to determine whether SIRT3 could retard intervertebral disc degeneration and study the mechanism.

MATERIALS AND METHODS: We chose the 3-month mice to establish intervertebral disc degeneration model and study the effect of SIRT3 on the intervertebral disc by Western blotting, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), immunohistochemistry. Mouse nucleus pulposus cells were cultured to study the exact mechanism.

RESULTS: The expression of SIRT3 was decreased in degenerated human nucleus pulposus. Intervertebral discs of mice treated with theacrine expressed more collagen II and less collagen X. In addition, nucleus pulposus cells stimulated with interleukin-1β (IL-1β) expressed less SIRT3 than that in the control group and nucleus pulposus cells with SIRT3 overexpress vectors expressed more collagen II FOXO3a and superoxide dismutase 2 (SOD2), indicating that SIRT3 could improve the intervertebral disc degeneration by anti-oxidative stress.

CONCLUSIONS: SIRT3 is a protective factor for intervertebral discs and can reduce oxidative stress in the intervertebral disc.

Key Words:
Intervertebral disc degeneration, SIRT3, Oxidative stress.

Introduction

Discogenic lumbar and cervical pain, lumbar and cervical disc herniation and related motor dysfunction secondary to intervertebral disc degeneration (IVDD) are common in life[1]. According to statistics, about two-thirds of adults have experienced low back pain at some point in their lives[2]. Low back pain is one of the most important factors to influence lifespan in the world health organization's "lost years of healthy life due to disease" report. At the same time, low back pain has imposed a huge economic burden on the country. In The United States, low back pain caused $87.6 billion in direct economic losses in 2013[3]. The indirect and indirect economic costs of losing the workforce were even higher, at $100 billion a year[3]. Therefore, it is of great social significance to study the prevention and treatment of low back pain.

Currently, in addition to physical therapy, non-steroidal anti-inflammatory drugs are mainly used to relieve symptoms in the treatment of IVDD and surgical treatment is required if the disease progresses[4]. There is no effective way to intervene intervertebral disc degeneration in the early stage. Furthermore, the etiology of IVDD is not clear at present. Aging, oxidative stress, mechanical stress, smoking, infection, trauma and genetics may all promote IVDD[5]. The reduction of intervertebral disc cells and extracellular matrix degeneration caused by the death of intervertebral disc cells are the main characteristics of intervertebral disc degeneration and oxidative stress plays an important role in the above pathological process[6].

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SIRT3 is a member of the "sirtuin family". Sirtuins are a group of highly conserved NAD-dependent histone deacetylases in mammals, which have certain regulatory effects on cell survival, proliferation, metabolism, death and aging, as well as longevity of organs[7]. Sirtuins are important regulatory factors for the acetylation of non-histone proteins in the body. In addition to the activity of deacetylase and ADP ribosylation, sirtuins have the activity of desuccinylation, demannosylation, and other enzymes. Their enzyme activity is affected by the regulation of NAD^+ and NADH contents in cells. Studies have confirmed that the activity of sirtuins protease can be activated when the content of NAD^+ in cells is increased, whereas the activity of sirtuins protease can be inhibited when the content of NADH is increased[8]. So far, seven members of the sirtuins family have been identified: SIRT1-SIRT7, which have different cell localization. Among them, SIRT1, SIRT6 and SIRT7 are located in the nucleus, SIRT2 is located in the cytoplasm, and SIRT3, SIRT4 and SIRT5 are located in the mitochondria[9].

SIRT3 is currently recognized as a protein related to human longevity. It is involved in regulating cell energy metabolism, interacting with longevity genes such as FOXO3a and participating in scavenging reactive oxygen species to extend cell life[10]. Studies have confirmed that the content of reactive oxygen species (ROS) in cells that lack the SIRT3 gene will increase. On the contrary, in the cells that express SIRT3 highly, the ROS levels will be significantly lower. Its mechanism may be that SIRT3 can activate transcription factor FOXO3a and then promote the high expression of superoxide dismutase (MnSOD), increasing antioxidant pathways mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) and other factors to restrain and reduce the generation of ROS[11]. Therefore, we hypothesized that SIRT3 may retard intervertebral disc degeneration through its antioxidant stress effect on intervertebral discs.

In this work, we determined whether SIRT3 could retard IVDD through antioxidant stress. We established a model of IVDD in tail-suspended mice and studied the effect of SIRT3 on the intervertebral disc of mice through the intervention of SIRT3 agonist[12]. In addition, *in vitro* experiments, we used mouse nucleus pulposus cells (NPCs) to verify the effect of SIRT3 on NPCs and study its mechanism.

Materials and Methods

Animals and Grouping

This study was approved by the Animal Ethics Committee of Inner Mongolia Medical University Animal Center. A total of 80 C57BL/6 mice were used in this study. We selected mice aged 3 months and with an average weight of 25-35 g. The mice were raised in a standard environment (conventional food and drinking water, room temperature of 24°C, 12-hour artificial circadian cycle). Theacrine is used as a SIRT3 agonist to intervene in mice with disc degeneration[13]. The mice were randomly divided into three groups (control group, vehicle group and theacrine group). Control group mice were bred without specific intervention. Vehicle group was mice with suspended tails; theacrine group was mice with suspended tails and administrated with theacrine orally (10 mg/kg).

Operative Procedure and Treatment

After the mice were anesthetized with 2 percent paraformaldehyde, the tail of the mice was fixed with medical tape and suspended from the pulley above the special cage. The height of suspension was controlled to the extent that the hind legs of the mice were just off the ground. The tail suspension time was one month. Vehicle group mice were administrated with the same amount of saline daily. Theacrine group mice were administrated with theacrine solution daily (10 mg/kg).

Cell Culture and Treatment

NPCs were purchased from Shanghai Saibaikang Biotechnology (Shanghai, Chian) and were cultured with Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) medium (10% fetal bovine serum (FBS) and 1% penicillin/streptomycin; Gibco, Grand Island, NY, USA). After the cells grow to a suitable density, the cells are transfected with SIRT3 overexpressed plasmids to compare the differences between treatment groups. In addition, we used recombinant mouse interleukin-1β (IL-1β) to stimulate NPCs degeneration for the following experiments.

X-Ray Examination

After a one-month tail suspension, three mice were randomly selected from each group for X-ray examination. After anesthesia, mice were placed on a tray in a lateral position for X-ray examination. Radiographs were taken at a colli-

mator-to-film distance of 25 cm, an exposure of 25 mAs and penetration power of 160 kV. The degree of disc degeneration in mice was assessed by Disc Height Index (DHI). DHI is the ratio of the height of intervertebral height to the sum of the height of adjacent two vertebral bodies.

Western Blotting Technology

Intervertebral disc tissues of each group were separated. We used protein pyrolysis liquid containing protease inhibitors and phosphatase inhibitors to dissolve intervertebral disc tissues and take the supernatant after the centrifugal (13000 rpm, 4°C). The Bicinchoninic Acid protein concentration kit was used to measure the protein concentration. After the concentration of protein in each group was stabilized, the protein was added to each well for gel electrophoresis (10% sodium dodecyl sulfate-polyacrylamide gel). Then, the protein was transferred to polyvinylidene difluoride membrane at 4°C for 2 h. Non-specific antigen was blocked with 5% non-fatty milk Tris-Buffered Saline with Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After being washed with TPST three times, the membrane was incubated with primary antibody (Collagen II, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; Collagen X, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; Aggrecan Abcam, Rabbit, 1:3000; FOXO3a Abcam, Cambridge, MA, USA, Rabbit, and SOD2, Abcam, Cambridge, MA, USA, Rabbit, 1:3000) at 4°C overnight. The next day, after washing with TBST three times, the membrane was incubated with secondary antibody (Goat Anti-Rabbit IgG, Cell Signaling Technology, Danvers, MA, USA, 1:3000) for 2 h and washed again 3 times. The visualization of the purposed protein was draw supported from electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) on an exposure machine.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from nucleus pulposus tissue and NPCs using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After using spectrophotometry measure RNA concentration, we used PrimeScript™ RT Master Mix (TaKaRa, Otsu, Shiga, Japan) to reverse RNA to cDNA. The mRNA levels of SIRT3, collagen II, aggrecan, SOD2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified by qPCR. GAPDH was used for normalization. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. (Collagen II, Forward: 5'-GGGAATGTCCTCTGCGATGAC-3', Reverse: 5'-GAAGGGGATCTCGGGGTTG; collagen X, Forward: 5'-TTTCTGGGATGCCTGTTGTC-3', Reverse: 5'-AATGGGATGGGAGCACCTAC-3'; SIRT3, Forward: 5'-ACCCAGTGGCATTCCAGAC-3', Reverse: 5'-GGCTTGGGGTTGTGAAAGAAG-3'; FOXO3, Forward: 5'- CGGACAAACGGCTCACTCT-3', Reverse: 5'-GGACCCGCATGAATCGACTAT-3'; SOD2, Forward: 5'-CGTGACTTTGGTTCCTTTGAC-3', Reverse: 5'-ATTTGTAAGTGTCCCCGTTCC-3'; GAPDH, Forward: 5'- GCATTGCCCTCAACGACCAC-3', Reverse: 5'- CCACCACCCTGTTGCTGTAG -3').

Histology and Immunohistochemistry

After a month of tail suspension, the mouse spine was removed. The tissue was placed into 4% paraformaldehyde for 24 hours. The spinal tissue was then dehydrated with gradient alcohol to perform paraffin embedding and then we use rotary microtome to make paraffin slices. Immunohistochemistry staining was performed to observe the expression of collagen II and SIRT3 in mouse intervertebral disc nucleus pulposus tissue.

Flow Cytometry Analysis of Cell ROS Levels

The mouse intervertebral disc tissue was separated and cut into 1-2 mm^3 pieces. The tissue was washed by saline solution and then was digested by 0.2% collagenase for 30 min. Nylon sieves were used to remove tissue block and achieve the cells we need. Cells in each group were resuspended in Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) to make a single cell suspension. DCFH-DA (10 mol/L) was added and incubated in the dark for 15 to 30 min before Flow Cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was used to detect intracellular ROS levels.

Immunofluorescence

NPCs in the culture plate was washed three times by PBS and then soak in 4% paraformaldehyde for 15 min. After washing three times by PBS, the heterogenetic antigens of NPCs were blocked by goat serum for 30 min. Each glass added enough amount of primary and was put

into the wet box overnight at 4°C. After washing three times by PBS, each glass added the corresponding secondary antibody and incubated for 1 h. In addition, 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. The glass was sealed with the sealing liquid containing anti-fluorescence quenching agent and the images were observed and collected under the fluorescence microscope.

Patient Tissue Samples

Human disc tissue from the normal control group was obtained from surgical patients with congenital scoliosis undergoing deformity correction surgery (n=10, mean age; 23.3 years). Degenerative discs were obtained from patients undergoing discectomy and spinal fusion surgery (n=10, mean age; 45.3 years). The degree of IVDD was evaluated according to the Pfirrmann grading system. The discs of the control group patients were Grade I-II and the discs of the degenerative group were Grade III-IV.

Plasmid Transfection

NPCs were transiently transfected with a plasmid encoding SIRT3 or with empty vector, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h of transfection, the cells were treated or not with IL-1β (10 ng/mL) for 24 h and then harvested for subsequent experiments.

Statistical Analysis

We use Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) to analyze the experimental data. Measurement data were presented as $\bar{x} \pm s$. The difference between the two groups means was evaluated with the t-test. Comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Test level α=0.05. All experiments were repeated 3 times.

Results

Expression of SIRT3 in Human NP Tissues of Different Degrees of Degeneration

To study the role of SIRT3 in human intervertebral disc tissue, we detected the expression of SIRT3 in intervertebral discs with different degrees of degeneration. According to Western blotting (Figure 1A) and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR; Figure 1B, Figure 1C and Figure 1D), we found that the highly degenerative intervertebral disc tissue expressed less collagen II and more collagen X than that in the control group. In addition, corresponding to the degree of degeneration, SIRT3 expressed less in highly degenerative intervertebral discs than the intervertebral discs in the control group. This suggested that SIRT3 is important for human intervertebral discs.

SIRT3 Agonists Retarded Intervertebral Disc Degeneration in Mice

To determine the effect of SIRT3 on the intervertebral disc, we performed X-ray detection in three groups of mice. The DHI of mice in the vehicle group was significantly lower than that in the control group, while the DHI of mice in the theacrine group was markedly higher than that in the vehicle group (Figure 2A). Immunohistochemical results showed that the intervertebral disc nucleus pulposus of mice in the vehicle group expressed less collagen II and SIRT3, but theacrine could significantly reverse this result (Figure 2B). Western blotting (Figure 2C) and qRT-PCR (Figure 2D) results showed that theacrine remarkably improved disc degeneration and SIRT3, FOXO3a and SOD2 were markedly increased compared with the vehicle group. This suggested that SIRT3 can improve intervertebral disc degeneration and activate the SIRT3/FOXO3/SOD2 signaling pathway.

Expression of SIRT3 in NPCs in Response to IL-1β Stimulation

To study the expression of SIRT3 in NPCs, we stimulated NPCs degeneration with IL-1β. Cell immunofluorescence showed that, as the concentration of IL-1β increased, NPCs expressed less and less SIRT3 (Figure 3A). Western blotting (Figure 3B) and qRT-PCR (Figure 3C) results showed the expression of Collagen II decreased and Collagen X was the opposite. In addition, the expression of SIRT3, FOXO3a and SOD2 under the stimulation of IL-1β was reduced. This suggested that as NPCs degenerative degree increases, the expression of the SIRT3/FOXO3/SOD2 signaling pathway also decreases.

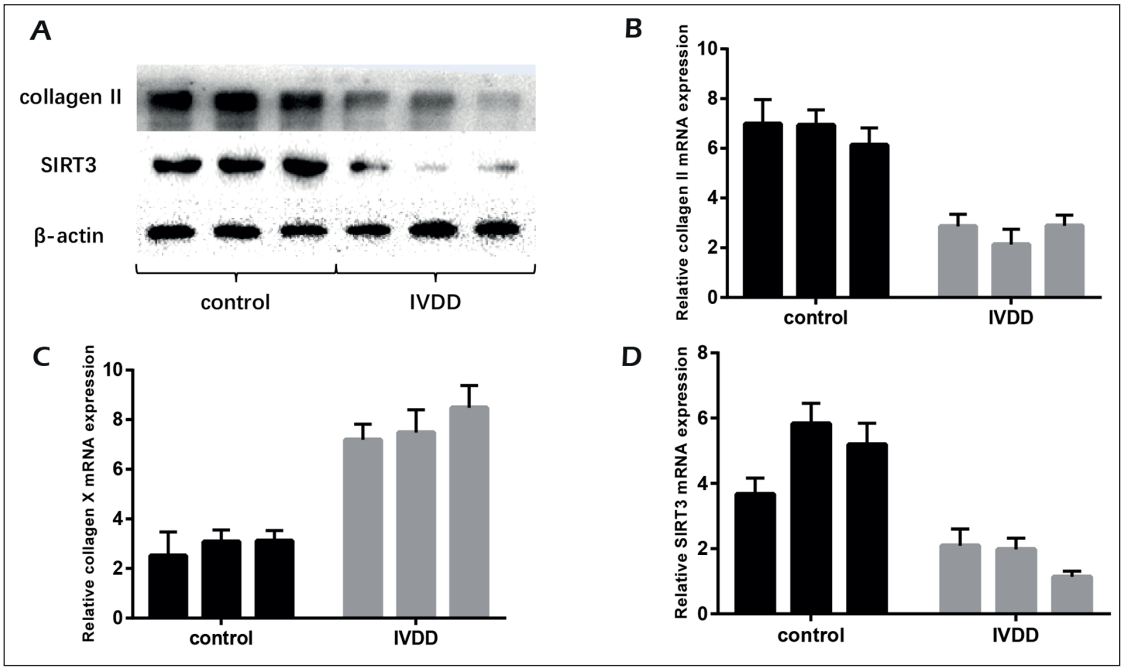

Figure 1. Expression of SIRT3 in the intervertebral disc of people with different degrees of IVDD. **A,** The results of the protein expression of collagen II and SIRT3 in two groups were determined by Western blotting. The results of mRNA expression of collagen II **B**, collagen X **C,** and SIRT3 **D,** in the two groups were determined by Western blotting.

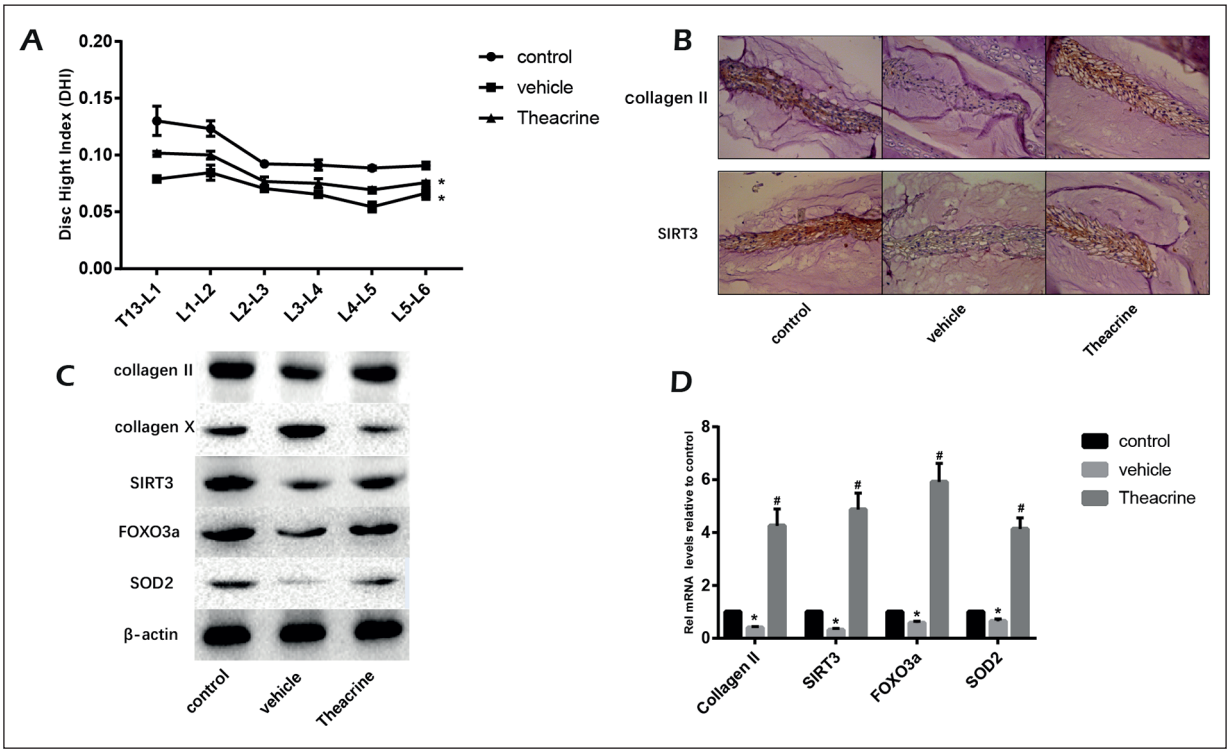

Figure 2. SIRT3 agonist retarded IVDD of mice. **A,** The results of the difference of DHI in three groups were calculated. **B,** The expression of collagen II and SIRT3 in the intervertebral disc of mice was detected by immunohistochemistry (magnification: 100×). **C,** The results of protein expression of collagen II, collagen X, SIRT3, FOXO3a and SOD2 were determined by Western blotting. **D,** The results of mRNA expression of collagen II, SIRT3, FOXO3a and SOD2 were determined by qRT-PCR; ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the vehicle group).

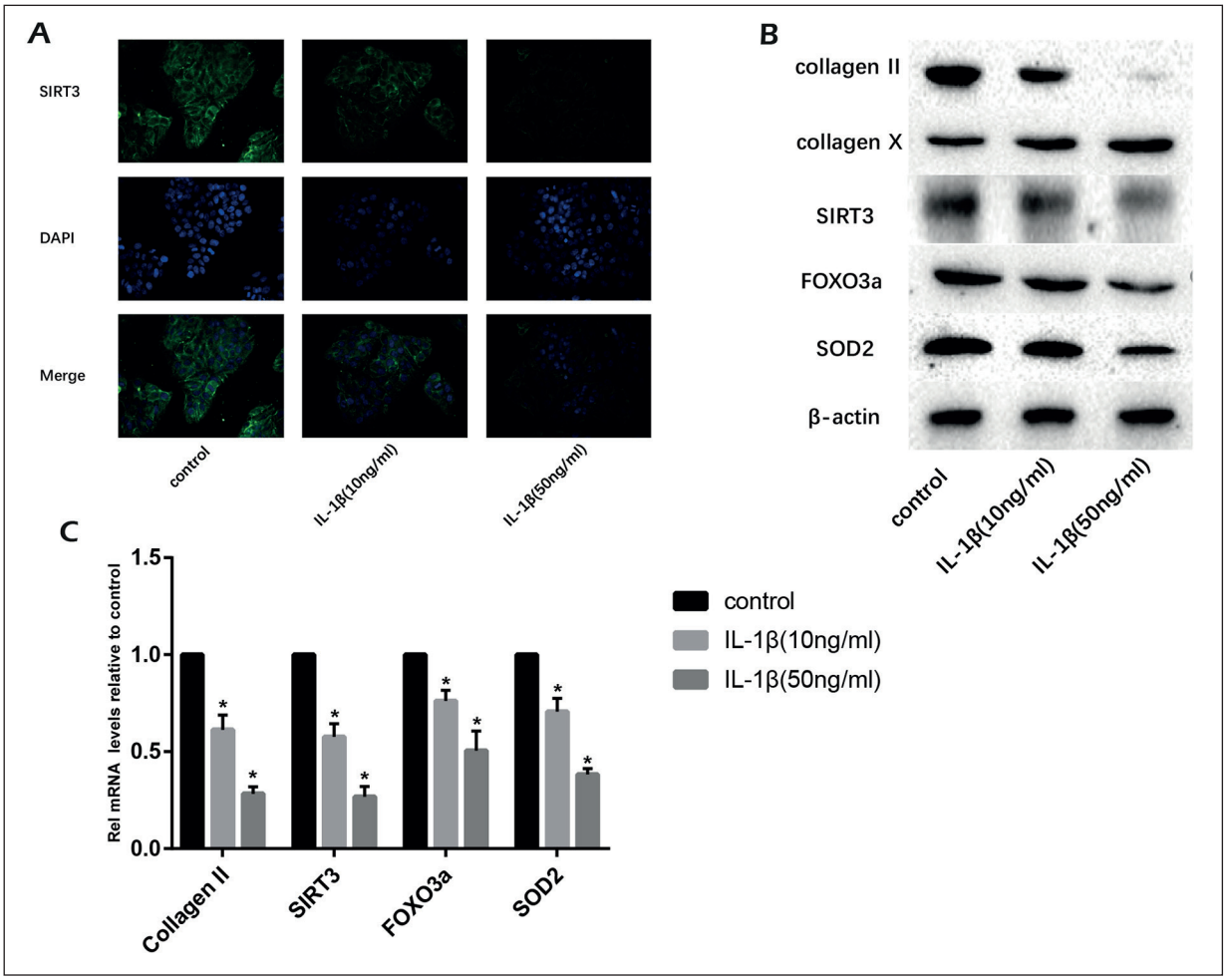

Figure 3. Expression of SIRT3 in NPCs in response to IL-1β stimulation. **A,** Cell immunofluorescence was performed to detect the difference of expression of SIRT3 in three groups (magnification: 40×). **B,** The results of the protein expression of collagen II, collagen X, SIRT3, FOXO3a and SOD2 in three groups were determined by Western blotting. **C,** The results of mRNA expression of collagen II, SIRT3, FOXO3a and SOD2 in three groups were determined by qRT-PCR; ("*" means there is a statistical difference with the control group).

Overexpression of SIRT3 Retarded IVDD by Activating the SIRT3/FOXO3/SOD2 Signaling Pathway

To study the specific mechanism of SIRT3 in improving intervertebral disc degeneration, we constructed SIRT3 overexpressed plasmids. The results of Western blotting (Figure 4A) and qRT-PCR (Figure 4B) showed that SIRT3 overexpressed plasmids had a good overexpression effect. Cell immunofluorescence results showed that the expression of collagen II was significantly decreased in the IL-1β group and the IL-1β + empty vector group, while the expression of collagen II was significantly increased after the overexpression of SIRT3 (Figure 4C). Western blotting (Figure 4D) and qRT-PCR (Figure 4E) showed decreased expression of collagen II, SIRT3, FOXO3a and SOD2 in the IL-1β group and IL-1β + empty vector group and increased expression in the SIRT3 overexpression group. The flow cytometry showed the oxidative stress level increased markedly in the IL-1β group and IL-1β + empty vector group, while decreased in the IL-1β + SIRT3 group (Figure 4F). This suggested that SIRT3 retarded intervertebral disc degeneration by activating the SIRT3/FOXO3/SOD2 signaling pathway.

Discussion

Oxidative stress plays a significant role in the process of IVDD. In this study, we found that the expression of SOD2 and other anti-oxidative

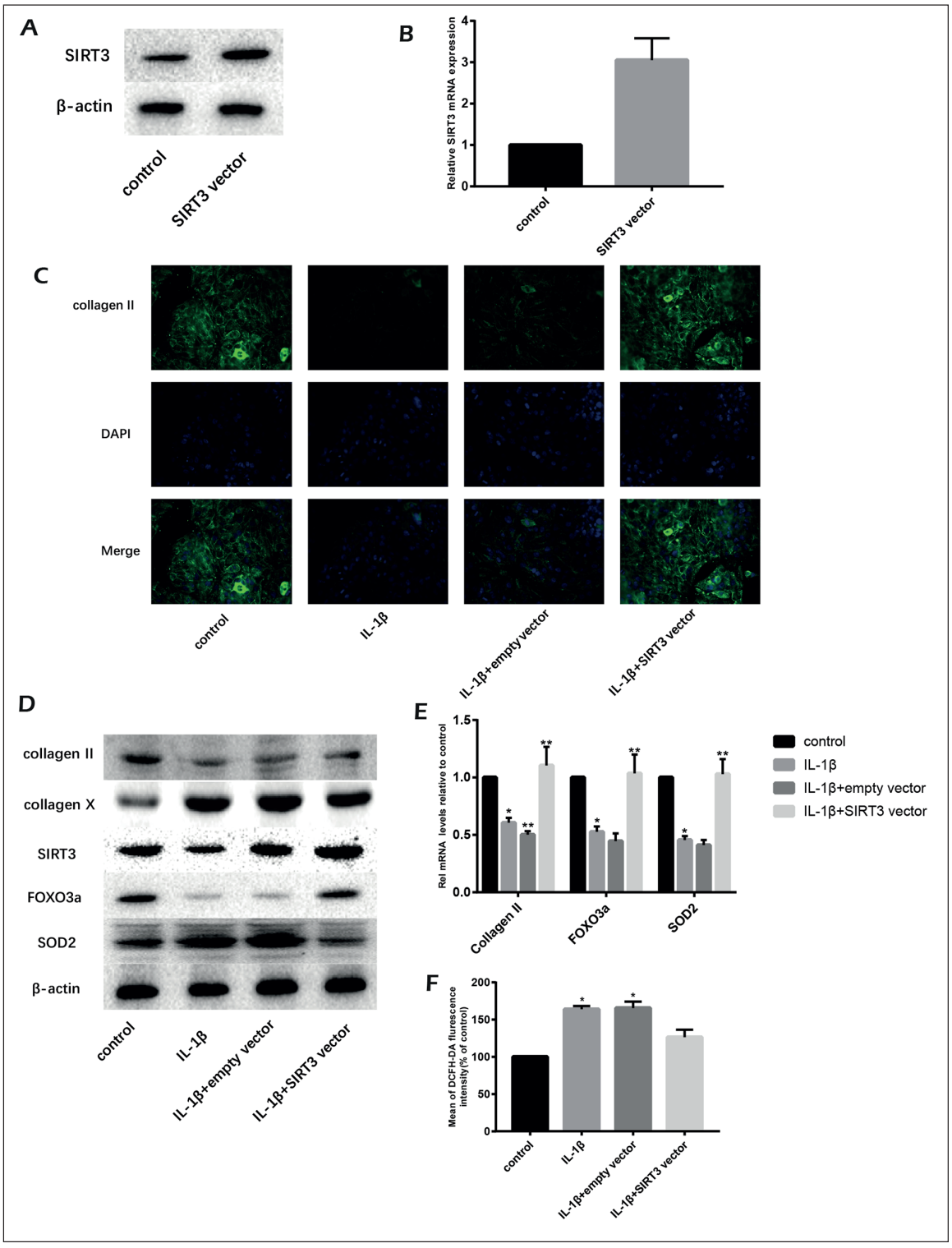

Figure 4. Overexpression of SIRT3 retarded IVDD by activating the SIRT3/FOXO3/SOD2 signaling pathway. Effect of SIRT3 overexpression vector on NPCs was detected by Western blotting **A,** and qRT-PCR **B**. **C,** Cell immunofluorescence was performed to detect the difference of expression of collagen II in four groups (magnification: 40×). **D,** The results of the protein expression of collagen II, collagen X, SIRT3, FOXO3a and SOD2 in four groups were determined by Western blotting. **E,** The results of mRNA expression of collagen II, SIRT3, FOXO3a and SOD2 in four groups were determined by qRT-PCR. **F,** The Flow cytometry was performed to detect the ROS levels in four groups; ("*" means there is a statistical difference with the control group and "**" means there is a statistical difference with the IL-1β group).

stress molecules were significantly reduced in the degenerative intervertebral disc. In addition, we found that the expression of SIRT3 in degenerative human nucleus pulposus tissues was lower than that in normal human nucleus pulposus tissues. We also found for the first time that SIRT3 can effectively retard IVDD through anti-oxidative stress. This suggests that SIRT3 may be an inhibitor of IVDD.

SIRT3, a member of the Sirtuins family located in mitochondria, is involved in many metabolic activities of organisms[14]. Moreover, SIRT3 is the mitochondrial sirtuins that have been studied most and it is highly expressed in tissues with high metabolisms such as muscle, brown fat, kidney, liver, brain and heart[15]. In mitochondria, SIRT3 can regulate metabolic enzymes to determine the rate of ATP production. When SIRT3 is missing, protein acetylation level is abnormal and ATP generation is reduced. When SIRT3 expression is increased in cells with SIRT3 knockout, ATP production can reach a normal level[16]. In the liver, SIRT3 is involved in urea circulation, synthesis and decomposition of fatty acids, carcass formation and other metabolic activities[17]. In the Sirtuins family, SIRT3 and SIRT5 located in the mitochondria have the function of regulating the intracellular urea circulation. When people were hungry, SIRT3 will be induced to be expressed in large quantities in the liver[18]. SIRT3 can enhance the activity of the rate-limiting enzyme K88 through deacetylation, further enhancing the urea cycle[18]. In the regulation of fatty acid metabolism, there are more free fatty acids in the liver of mice lacking SIRT3, which will accelerate the death rate of liver cells, but this situation can be improved when SIRT3 expression is increased[19]. Hirschey et al[20] found that during the fasting period of mice, if SIRT3 was absent, the mice would suffer from fatty acid oxidation disorder, at which time the ATP level of the body would be reduced and the mice would have an intolerant reaction to the cold environment. This experiment showed that SIRT3 played a role in regulating mitochondrial metabolism and fatty acid oxidation. Furthermore, SIRT3 played a certain role in slowing down aging and extending life. SIRT3 activated mitochondrial enzymes involved in fatty acid oxidation, amino acid metabolism, electron transport chain and antioxidant defense through deacetylation, to enhance mitochondrial metabolism, increase body metabolism and slow down aging[21]. In addition, SIRT3 can prevent apoptosis by reducing reactive oxygen species and inhibiting the components of mitochondrial permeability transition pores[22]. Therefore, SIRT3 plays an important role in various metabolic activities in organisms and we can explore SIRT3 as a drug to retard IVDD through anti-oxidative stress.

Extracellular matrix degeneration of intervertebral disc was mainly caused by an imbalance of extracellular matrix synthesis and catabolism[23]. The decrease of extracellular matrix synthesis and the increase of protease activity directly led to the increase of extracellular matrix catabolism. Early in intervertebral disc degeneration, collagen synthesis was increased overall, especially collagen II[24]. This may be caused by the body repair mechanism. When IVDD developed, collagen synthesis changed and the collagen II can be found more in the outer fiber ring, but the inner annulus fibrosus and nucleus pulposus increased the synthesis of collagen, which reduced the compressive ability of issues. In the intervertebral disc of serious degeneration, especially in the intervertebral disc around the crack, collagen X can be found and the secretion of collagen X is considered linked to oxidative stress[25].

In this study, we indicated the effect of SIRT3 on IVDD. SIRT3 acts on the anti-oxidant stress of intervertebral disc tissue to retard IVDD. This will provide a new idea for the clinical treatment of IVDD.

Conclusions

We revealed that SIRT3 activates the transcription factor FOXO3a, which in turn promotes the synthesis of SOD2 to inhibit oxidative stress in intervertebral discs. Therefore, SIRT3 can effectively retard IVDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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