Basic Study

6-gingerol protects nucleus pulposus-derived mesenchymal stem cells from oxidative injury by activating autophagy

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Abstract

BACKGROUND

To date, there has been no effective treatment for intervertebral disc degeneration (IDD). Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) showed encouraging results in IDD treatment, but the overexpression of reactive oxygen species (ROS) impaired the endogenous repair abilities of NPMSCs. 6-gingerol (6-GIN) is an antioxidant and anti-inflammatory reagent that might protect NPMSCs from injury.

AIM

To investigate the effect of 6-GIN on NPMSCs under oxidative conditions and the potential mechanism.

METHODS

The cholecystokinin-8 assay was used to evaluate the cytotoxicity of hydrogen peroxide and the protective effects of 6-GIN. ROS levels were measured by 2’7’-dichlorofluorescin diacetate analysis. Matrix metalloproteinase (MMP) was detected by the tetraethylbenzimidazolylcarbocyanine iodide assay. TUNEL assay
INTRODUCTION

Intervertebral disc degeneration (IDD) is a primary cause of lower back pain (LBP) and poses a tremendous burden on individuals and society. There is still a lack of effective treatments to repair the damaged structure of intervertebral discs (IVDs).

Mesenchymal stem cells (MSCs) have the potential to self-renew, proliferate, and differentiate into various types of cells in the body, including chondrocytes and osteoblasts. Therefore, MSCs are considered a promising candidate for treating IDD. However, there has been no effective treatment for IDD to date. Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) have the potential to alleviate LBP and protect the ECM from degeneration, representing a promising candidate for treating IDD.

Core Tip:

To date, there has been no effective treatment for intervertebral disc degeneration (IDD). Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) showed encouraging results in IDD treatment, but the overexpression of reactive oxygen species (ROS) impaired the endogenous repair abilities of NPMSCs. 6-gingerol efficiently decreased ROS levels, attenuated hydrogen peroxide-induced NPMSC apoptosis, and protected the ECM from degeneration, representing a promising candidate for treating IDD.

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RESULTS

6-GIN exhibited protective effects against hydrogen peroxide-induced injury in NPMSCs, decreased hydrogen peroxide-induced intracellular ROS levels, and inhibited cell apoptosis. 6-GIN could increase Becl-2 expression and decrease Bax and caspase-3 expression. The MMP, Annexin V-FITC/PI flow cytometry and TUNEL assay results further confirmed that 6-GIN treatment significantly inhibited NPMSC apoptosis induced by hydrogen peroxide. 6-GIN treatment promoted extracellular matrix (ECM) expression by reducing the oxidative stress injury-induced increase in MMP-13 expression. 6-GIN activated autophagy by increasing the expression of autophagy-related markers (Beclin-1 and LC-3) and decreasing the expression of p62. Autophagosomes were visualized by transmission electron microscopy. Pretreatment with 3-MA and BAF further confirmed that 6-GIN-mediated stimulation of autophagy did not reduce autophagosome turnover but increased autophagic flux. The PI3K/Akt pathway was also found to be activated by 6-GIN. 6-GIN inhibited NPMSC apoptosis and ECM degeneration, in which autophagy and the PI3K/Akt pathway were involved.

CONCLUSION

6-GIN efficiently decreases ROS levels, attenuates hydrogen peroxide-induced NPMSCs apoptosis, and protects the ECM from degeneration. 6-GIN is a promising candidate for treating IDD.

Key Words: Nucleus pulposus-derived mesenchymal stem cells; 6-Gingerol; Intervertebral disc degeneration; Oxidative stress; Autophagy; Apoptosis
differentiate into specific types of cells. Tao et al.[9] and Han et al.[10] confirmed that nucleus pulposus-derived MSCs (NPMSCs) can robustly adapt to the acidic and hyperosmotic microenvironments of degenerated IVDs. Long-term overload pressure, trauma, inflammation, and other pathological factors induce the overexpression of reactive oxygen species (ROS), causing apoptosis and senescence of NPMSCs, which eventually impairs their endogenous repair abilities[9,10]. Thus, studies should focus on strategies to protect NPMSCs from oxidative injury[10].

Autophagy is closely related to the senescence or apoptosis of MSCs and NP cells[11-13]. Ginger (Zingiber officinale roscoe, Zingiberaceae) is frequently used as a popular spice and medicinal plant worldwide. Ginger is regarded as a source of traditional medicine in curing flu, rheumatism, muscular aches, and infectious diseases. One of the major active constituents in ginger is 6-gingerol (6-GIN). 6-GIN can bear a pH range from 4-7 and is stable at 100 °C for 10 h. 6-GIN can affect the homeostasis of the human body after entering the blood without structural decomposition. 6-GIN (Figure 1) (C_{10}H_{16}O_{5}) has various pharmacological effects, including antioxidant, antitumor, and anti-inflammatory effects[11-13]. Wang et al.[14] reported that 6-GIN could inhibit cell apoptosis by inducing autophagy. In addition, PI3K/Akt signaling is closely related to NPMSC apoptosis[15]. Lv et al.[16] showed that 6-GIN could attenuate myocardial ischemia/reperfusion injury via the PI3K/Akt signaling pathway in cardiomyocytes.

We designed this study to explore the effect of 6-GIN on NPMSCs and the potential mechanism.

**MATERIALS AND METHODS**

**NPMSC isolation and culture**

Nucleus pulposus (NP) samples were collected from patients who underwent lumbar discectomy. Every volunteer provided informed consent before tissue collection. All procedures were approved by the Research Ethics Committee of Clinical Medical College of Yangzhou University (No. SBYY2019-023). The cells were isolated according to the Navaro et al.[17] method for NPMSC culture with minor modifications. Briefly, the collected samples were separated and washed carefully with normal saline. Then, the samples were cut into 1 mm × 1 mm × 1 mm pieces and incubated with collagenase type II for 12 h at 37 °C. Then, NP tissues were washed with normal saline and centrifuged at 1000 g for 4 min. Next, the cells were resuspended in complete MSC medium (Cyagen, United States) and cultured at 37 °C with 5% CO_2. The culture medium was refreshed every 3 d. Each primary culture was digested and subcultured at a ratio of 1:3 when the adherent cells reached 80% confluence. The cells were observed and photographed under a microscope (Olympus, Japan). At the third passage (P3), cells were harvested for subsequent experiments.

**Identification of immunophenotypes**

The immunophenotypes was detected by flow cytometry. For this purpose, P3 cells were trypsinized and approximately 10^6 cells were washed with phosphate buffer saline (PBS) and labeled with antibodies according to the International Society for Cellular Therapy (ISCT)-protocol, including CD73 PE (BD Pharmingen TM, catalog No. 550257, Untied States), CD90 FITC (Exbio, catalog No. 1F-652-T100, The Czech republic), CD105 PE (BD Pharmingen TM, catalog No. 560839, Untied States), CD45FITC-CD34PE (BD Pharmingen TM, catalog No. 341071, Untied States), and HLA-DR APC (BD Pharmingen TM, catalog No. 559868, Untied States) antibodies. After being cultured for 30 min at 37 °C, the cells were washed twice with PBS and resuspended in 500 μL of PBS. The labeled cells were analyzed by flow cytometry (FACSCalibur, BD, LSR II, Becton Dickinson, Untied States) according to standard procedures. FLOW Jo (version 6.2) software was used to analyze the expression rate of positive cells for each monoclonal antibody.

**Multilineage differentiation**

To assess the multilineage differentiation potential of the cells, we cultured the P3 cells with adipogenic, osteogenic and chondrogenic (Cyagen Biosciences, China) differentiation assay kits separately. Briefly, harvested NPMSCs were seeded onto 6-well plates (5 × 10^4/well). After reaching about 80% confluence, osteogenic, adipogenic, and chondrogenic differentiation kits were used according to the manufacturer’s instructions. After the required days of induction, the cells were rinsed with PBS and fixed with 4% paraformaldehyde for 25 min. Finally, the cells were
incubated with oil red O, alizarin red, and alcian blue. After being washed several times with PBS, the stained cells were observed and imaged under a microscope (Olympus, Japan).

**Treatment of NPMSCs with hydrogen peroxide and 6-GIN**

NPMSCs were incubated with multiple concentrations of 6-GIN (Solarbio, China, catalog No. SG8180) (0-120 μmol/L) to evaluate the protective effect of 6-GIN on hydrogen peroxide-induced injury. As shown in the “Results” section, NPMSCs were incubated with 30 μmol/L 6-GIN for 24 h and then 80 μmol/L hydrogen peroxide (China, Jian Cheng, catalog No. E004) for 6 h in the follow-up experiment. Cells were further incubated with 10 mmol/L 3-methyladenine (3-MA, MedChem, China, catalog No. HY-19312) prior to exposure to hydrogen peroxide for 2 h. NPMSCs were also preconditioned with bafilomycin A1 (Baf-A1) (100 nmol/L) prior to 6-GIN treatment to analyze autophagic flux. The cells were divided into four groups according to the different treatments: (1) CON group: blank control; (2) HYD group: 80 μmol/L hydrogen peroxide; (3) 6-GIN group: 80 μmol/L hydrogen peroxide + 30 μmol/L 6-GIN; and (4) 3-MA group: 80 μmol/L hydrogen peroxide + 30 μmol/L 6-GIN + 10 mmol/L 3-MA.

**Cell viability assay**

Cholecystokinin-8 (CCK-8) was used to evaluate the cytotoxicity of 6-GIN on NPMSCs according to the protocol. Briefly, P3 NPMSCs were seeded into 96-well plates (5 × 10^3 cells/well). When the cells reached 80% confluence, different interventions were conducted according to the previously described four groups. Then, the wells were washed with PBS, and 200 μL of DMEM/F12 containing 10% CCK-8 solution was added to each well. The optical density value (OD) was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, United States) after 2 h.

**5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide assay for mitochondrial membrane potential analysis**

A 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) assay was conducted to evaluate mitochondrial membrane potential (MMP). Briefly, the collected NPMSCs were washed with PBS and incubated with 2 μmol/L JC-1 dye for 20 min. The ratio of green-to-red fluorescence was analyzed by flow cytometry.

**ROS assay**

ROS levels were measured using the ROS-specific fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The collected NPMSCs were incubated with 10 μmol/L DCFH-DA for 30 min at 37 °C in the dark. The mean fluorescence intensity was measured by flow cytometry. Each analysis was repeated six times.

**Apoptosis evaluation**

The Annexin V-FITC/PI apoptosis detection kit was used to evaluate apoptosis. Briefly, NPMSCs were seeded into 6-well plates (5 × 10^4 cells/well), and different interventions were performed when the cells reached 80% confluence. These cells were then trypsinized, blocked with fetal bovine serum, washed twice with PBS, collected, and resuspended in 100 μL of binding buffer together with 5 μL of Annexin V-FITC. After the cells were incubated for 10 min at room temperature in the dark, another 400 μL of binding buffer was added, and the cells were then analyzed with FACS Calibur (BD Bioscience). The data were analyzed with Summit software version 4.3.
**TUNEL assay**

The terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay is often used to detect the level of DNA damage. The cells were fixed with 4% paraformaldehyde for 25 min at 37 °C, incubated with 0.1% Triton X-100 for 8 min, and washed with PBS twice between each step. The cells were incubated with TUNEL reagent (Beyotime, China, catalog No. C1088) according to the manufacturer’s instructions, and the nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma, United States, catalog No. D-9542) after the required time. The samples were observed and imaged under a fluorescence microscope (Olympus Europe GmbH, Germany).

**Immunofluorescence**

NPMSCs from different groups were collected and fixed with 4% paraformaldehyde for 15 min. The cells were washed twice with PBS containing 0.5% Triton X-100 for 6 min. Then, the cells were incubated with 10% bovine serum albumin for 1 h at 37 °C, rinsed with PBS, and incubated with primary antibodies against matrix metalloproteinase 13 (MMP-13) (Bioss, China, catalog No. bs-10250R) (1:200), collagen II (Bioss, China, catalog No. bs-10589R) (1:200), and LC3 (Bioss, China, catalog No. bs-8878R) (1:200) at 4 °C overnight. The plates were washed and incubated with FITC-conjugated secondary antibodies (Bioss, China, catalog no. bs-0293M-FITC) (1:400) for 1 h at room temperature. The nuclei were labeled with DAPI for 4 min, and the cells were observed under a fluorescence microscope.

**Transmission electron microscopy**

NPMSCs were collected and fixed with 2.5% glutaraldehyde overnight, then treated with 1% osmium tetroxide for 2 h, and stained with 2% uranyl acetate for 1 h. After dehydration in an ascending series of acetone, samples were embedded into Araldite and cut into semithin sections, which were stained with 1% uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (Hitachi, Japan).

**Western blot analysis**

Cellular proteins were collected with a total extraction sample kit according to the manufacturer’s instructions. Then, a BCA protein assay kit (Beyotime, China, catalog No. P0010) was used to measure the protein concentration. Equal aliquots of the obtained protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nonfat milk (5%) was used to block the membranes for 2 h at room temperature after electrotransfer onto PVDF membranes (Millipore, United States, catalog No. IPVH 20200), followed by incubation with primary antibodies overnight at 4 °C. The primary antibodies used were as follows: Beclin1 (1:500, Bioss, China, catalog No. bsm-33323M), Bax (1:500, ABclonal, Wuhan, China, catalog No. A12009), LC3 (1:500, Bioss, China, catalog No. bs-8878R), p-Akt (1:1000, Proteintech, United States, catalog no. 66444-1-Ig), β-actin (1:10000, ABclonal, Wuhan, China, catalog No. AC004), Akt (1:500, Bioss, China, catalog No. bsm-33282M), p62 (1:500, Abcam, China, catalog No. ab56416), Bcl-2 (1:500, ABclonal, Wuhan, China, catalog No. A11025), and Caspase-3 (1:500, ABclonal, Wuhan, China, catalog No. 1953). The membranes were then incubated with the secondary antibodies for 2 h at room temperature. The bands were observed and photographed with a computer imaging system. The expression level was semiquantified with ImageJ software (NIH, United States).

**Real-time polymerase chain reaction**

Quantification of the mRNA levels of collagen II, aggrecan, and MMP-13 was performed after different treatments. Total RNA was obtained from NPMSCs using TRizol reagent (Invitrogen, United States, catalog No. 15596-026) according to the manufacturer’s instructions. Prime Script-RT reagent kit (Vazyme Biotech, Nanjing, China, R123-01) and SYBR Premix Ex Taq (Vazyme Biotech, Nanjing, China, catalog No. Q111-02) were used for reverse transcription reactions of RNA to cDNA, and cDNA amplification assays were conducted according to the manufacturer’s instructions. The expression level was evaluated by the 2^(-ΔΔCt) method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the expression of the target genes. Primer 5.0 (Premier Biosoft, Palo Alto, CA, United States) software was used to design the primer sequences, which are shown in Table 1.
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Table 1 Primers used for reverse transcription-quantitative polymerase chain reaction analysis of gene expression

| Gene          | Primer sequence                  |
|---------------|----------------------------------|
| GAPDH         | Forward: TCAACGACCACTTTGTCAAGCTAGCT |
|               | Reverse: GGTTGTCAGGGGTCTTAC       |
| Aggrecan      | Forward: CTACCACTGGATCGGCTGAA     |
|               | Reverse: CGTGCCAGATCATCACCCACA    |
| Collagen II   | Forward: GGTAGTGCCGGCAAAGCTGTA   |
|               | Reverse: TGTTGTCTGGTGTCAGGTATA   |
| MMP-13        | Forward: CCACTTCAAGATGGCGATTG    |
|               | Reverse: GGCATCTCCCTCATAATTGGC   |

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MMP-13: Matrix metalloproteinase 13.

Statistical analysis
Each measurement was conducted in triplicate. The results are expressed as the mean ± standard deviation. All statistical analyses were conducted using SPSS 19.0 (IBM, Chicago, IL, United States). One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used to analyze data from multiple groups, and differences were regarded as statistically significant when $P < 0.05$.

RESULTS

Identification of NPMSCs
The isolated cells aggregated to form a sunflower shape and changed to exhibit long, spindle shapes after passage (Figure 2A and B). Based on the flow cytometric analysis of cell surface antigens shown in Figure 2C, the cells were positive for the MSC markers CD73, CD90, and CD105 but negative for hematopoietic stem cell markers CD34, CD45, and HLA-DR. After osteogenic differentiation induction, the cells showed visible calcium deposits (Figure 2D). The adipogenic differentiation results showed that oil droplets formed, as indicated by staining with oil red O (Figure 2E). The cells were stained with alcian blue after chondrogenic differentiation induction, and some cells were positively stained (Figure 2F). These results suggested that the cells isolated from NP tissue met the criteria of MSCs, as defined by the ISCT.

6-GIN alleviates hydrogen peroxide-induced decreases in NPMSC viability and intracellular ROS levels
As shown in Figure 3A, 6 GIN (0, 10, 20, 30, 60, and 120 μmol/L) enhanced NPMSC viability at certain concentrations after 24 h incubation. Hydrogen peroxide decreased NPMSC viability in a dose-dependent manner. Hydrogen peroxide at a concentration of 80 μmol/L led to appropriate inhibition of cell viability; thus, this concentration was chosen to induce oxidative injury in subsequent experiments (Figure 3B) ($P < 0.05$). However, different concentrations of 6-GIN obviously improved the cell viability decreased by hydrogen peroxide, and 6-GIN showed a maximum protective effect at a concentration of 30 μmol/L. Thus, this concentration was used for subsequent experiments (Figure 3C). The flow cytometry results showed that intracellular ROS levels in the HYD group were higher than those in the CON group. 6-GIN decreased ROS levels induced by hydrogen peroxide, but this effect was reversed by pretreatment with 3-MA (Figure 3D, $P < 0.05$).
Identification of nucleus pulposus-derived mesenchymal stem cells. A: Primary cultured nucleus pulposus-derived mesenchymal stem cells (NPMSCs) showed a rod-shape; B: NPMSCs presented a spindle-shape after passage; C: NPMSCs showed highly expression of CD73, CD90, and CD105 and low expression of hematopoietic stem cell markers CD34, CD45, and HLA-DR; D-F: Multipotential differentiation of NPMSCs.

6-GIN decreases hydrogen peroxide-induced NPMSCs apoptosis
A reduction in MMP reflects mitochondrial dysfunction, which is usually used to predict early apoptosis. A significant MMP loss was found in the HYD group and was partly recovered in the GIN group. However, the protective effect of GIN was inhibited when the cells were pretreated with 3-MA (Figure 4A). The cell apoptosis rate in the HYD group was higher than that in the CON group, and 6-GIN partially alleviated the degree of apoptosis, but this effect was reversed by pretreatment with 3-MA (Figure 4B and C, \( P < 0.05 \)). Consistent with the flow cytometry results, the positive TUNEL staining rate was upregulated after hydrogen peroxide treatment, and 6-GIN partially reversed this effect. Furthermore, this protective effect was weakened
6-Gingerol alleviates hydrogen peroxide-induced decrease of nucleus pulposus-derived mesenchymal stem cell viability and intracellular reactive oxygen species level. A: The viability of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) treated with different concentrations of 6-gingerol (6-GIN) was detected by cholecystokinin-8 (CCK-8); B: CCK-8 results of NPMSCs treated with different concentrations of hydrogen peroxide; C: Effect of 6-GIN on the NPMSC viability induced by hydrogen peroxide; D: Statistical analysis of the production of intracellular reactive oxygen species [NPMSCs treated with cell culture medium (CON group), 80 μmol/L hydrogen peroxide alone (HYD group), hydrogen peroxide and 30 μmol/L 6-GIN (6-GIN group), or hydrogen peroxide and 6-GIN combined with 10 mmol/L 3-methyladenine (3-MA group)]. All data are the mean ± SE of at least three independent experiments. *P < 0.05 vs 0 μmol/L 6-GIN group; †P < 0.05 vs 0 μmol/L 6-GIN; ‡P < 0.05 vs 0 μmol/L H₂O₂ group; ††P < 0.05 vs 80 μmol/L H₂O₂ group; ‡‡P < 0.05 vs CON group; ℡P < 0.05 vs HYD group; ❅P < 0.05 vs 6-GIN group. 6-GIN: 6-gingerol; ROS: Reactive oxygen species; H₂O₂: Hydrogen peroxide; 3-MA: 3-Methyladenine.

6-GIN regulates the expression of ECM-related genes and proteins

The expression of ECM-related genes and proteins by NPMSCs was also analyzed. Hydrogen peroxide treatment significantly inhibited the mRNA expression of collagen II and aggrecan and promoted the expression of MMP-13 (Figure 5A-5C P < 0.05). 6-GIN pretreatment showed a protective effect on NPMSCs, as the mRNA expression levels of collagen II and aggrecan were enhanced and MMP-13 expression was weakened. However, 3-MA pretreatment reversed this protective effect. Based on the qPCR results, we further verified the changes in collagen II and MMP-13 expression through an immunofluorescence assay. As shown in Figure 5D-5G the fluorescence intensity of collagen II in the HYD group was decreased compared with that of the CON group, but the fluorescence intensity of MMP-13 was increased. 6-GIN reversed this change, and this protective effect could be inhibited in the presence of the autophagy inhibitor 3-MA (P < 0.05). These results indicated that 6-GIN could modulate ECM metabolism by modulating autophagy in NPMSCs.

Role of autophagy in the protective effect of 6-GIN

To further verify the role of autophagy in this protective effect, we examined the expression of autophagy-related proteins (Beclin-1, LC3, and p62). The expression of Beclin1 and LC3II/I increased and p62 decreased significantly after 6-GIN treatment. However, the autophagy inhibitor 3-MA reversed the expression of these proteins, suggesting that 6-GIN could significantly enhance autophagy in hydrogen peroxide-induced NPMSCs (Figure 6A-6D P < 0.05). This result was further confirmed by LC3 immunofluorescence staining (Figure 6E-6F P < 0.05). Autophagosomes were visualized by transmission electron microscopy (Figure 6G). The inhibitor of autophagolyosomal degradation BAF was used to estimate autophagic flux. After
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Figure 4 6-Gingerol decreases hydrogen peroxide-induced nucleus pulposus-derived mesenchymal stem cell apoptosis. Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were treated with cell culture medium (CON group), 80 μmol/L hydrogen peroxide alone (HYD group), hydrogen peroxide and 30 μmol/L 6-gingerol (6-GIN group), or hydrogen peroxide and 6-GIN combined with 10 mmol/L 3-methyladenine (3-MA group). A: 6-GIN partially blocks hydrogen peroxide-induced matrix metalloproteinase loss. The data are expressed as the ratio of red over green fluorescence intensity assessed by flow cytometry; B-C: Representative pictures and statistical analysis of NPMSC apoptosis rate by Annexin-V/PI dual staining in different groups; D: TUNEL assay results of NPMSCs in different groups (bar = 200 μm); E: Quantitative analysis of fluorescence results; F-I: Western blot results and quantitative analysis of apoptosis related proteins (cleaved caspase-3, Bax, and Bcl-2) in different groups; J: Bcl-2/Bax ratio. All data are the mean ± SE of at least three independent experiments. *P < 0.05 vs CON group; †P < 0.05 vs HYD group; ‡P < 0.05 vs 6-GIN group. 6-GIN: 6-gingerol; H₂O₂: Hydrogen peroxide; 3-MA: 3-methyladenine.

BAF treatment, the expression levels of P62 and LC3-II increased compared with those of cells incubated with 6-GIN alone. These results demonstrated that 6-GIN-mediated autophagy induction was due to increased autophagic flux but not reduced autophagosome turnover (Figure 6H-J, P < 0.05).

**Activity of the PI3K/Akt pathway in the protective effect of 6-GIN**

The expression of the PI3K/Akt signaling pathway-related proteins Akt and p-Akt was detected by Western blot. The expression of p-Akt was decreased after hydrogen peroxide treatment, and 6-GIN inhibited this effect. This trend was consistent with the change in autophagy signaling and was closely related to NPMSC apoptosis. However, the expression of p-Akt did not change after 3-MA treatment (Figure 7, P < 0.05). These results indicated that the PI3K/Akt signaling pathway was involved in alleviating hydrogen peroxide-induced apoptosis of NPMSCs by stimulating autophagy to some extent.
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A

B

C

D

Collagen II  DAPI  Merge

Control

H₂O₂

6-GIN + H₂O₂

3-MA + 6-GIN + H₂O₂
Figure 5 6-gingerol regulates the expression of extracellular matrix proteins. Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were treated with cell culture medium (CON group), 80 μmol/L hydrogen peroxide alone (HYD group), hydrogen peroxide and 30 μmol/L 6-gingerol (6-GIN group), or hydrogen peroxide and 6-GIN combined with 10 mmol/L 3-methyladenine (3-MA group), bar = 100 μm. A-C: The mRNA expression of aggrecan, collagen II, and matrix metalloproteinase 13 (MMP-13); D-G: Representative immunofluorescence photomicrographs and quantitative analysis of fluorescence results of collagen II and MMP-13. All data are the mean ± SE of at least three independent experiments. *P < 0.05 vs CON group; †P < 0.05 vs HYD group; ‡P < 0.05 vs 6-GIN group. 6-GIN: 6-gingerol; H₂O₂: Hydrogen peroxide; 3-MA: 3-methyladenine.
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A. 

|           | H$_2$O$_2$ | - | + | + | + | 3-MA | - | - | + | MV (kDa) |
|-----------|------------|---|---|---|---|------|---|---|---|----------|
| 6-GIN     | -          |   |   |   |   |      |   |   |   | 60       |
| LC-3 I    | -          |   |   |   |   |      |   |   |   | 16       |
| LC-3 II   | -          |   |   |   |   |      |   |   |   | 14       |
| β-Actin   | -          |   |   |   |   |      |   |   |   | 42       |

B. 

The ratio of p62/β-Actin (% of control)

C. 

The ratio of Beclin 1/β-Actin (% of control)

D. 

The ratio of LC3I/LC3II (% of control)

E. 

Control  H$_2$O$_2$  6-GIN + H$_2$O$_2$  3-MA + 6-GIN + H$_2$O$_2$

LC3  DAPI  Merge

F. 

LC3I Fluorescence intensity (% of control)
DISCUSSION

MSC transplantation therapy may restore the structure and function of degenerated IVDs\cite{21,22}. The existence of MSC-like cells in degenerated lumbar nucleus pulposus has been reported to meet the standard of MSCs described by the ISCT\cite{22}. NPMSCs have aroused extensive interest, and many studies have shown that NPMSCs commonly exist in both rat and human NP tissues\cite{8,23,24}. NPMSCs can differentiate into NP cells and play an endogenous repair role in damaged IVDs. NPMSCs can better tolerate the harsh acidic and hyperosmolar microenvironment of degenerative IVDs than other types of MSCs\cite{5,6}. Furthermore, NPMSC transplantation has achieved encouraging results in IDD treatment in a limited animal model study\cite{25}. In conclusion, NPMSCs show exciting prospects for reversing the pathology and restoring degenerated IVDs over other kinds of MSCs\cite{22,26,29}. NPMSCs might be a better seed cell type for biological...
Figure 7 Activity of PI3K/Akt pathway in the 6-gingerol related protective effect. A-B: Western blot results and quantitative analysis of the expression of the PI3K/Akt pathway-related proteins p-Akt and Akt. Nucleus pulposus-derived mesenchymal stem cells were treated with cell culture medium (CON group), 80 μmol/L hydrogen peroxide alone (HYD group), hydrogen peroxide and 30 μmol/L 6-gingerol (6-GIN group), or hydrogen peroxide and 6-GIN combined with 10 mmol/L 3-methyladenine (3-MA group). All data are the mean ± SE of at least three independent experiments. *P < 0.05 vs CON group; †P < 0.05 vs HYD group; ‡P < 0.05 vs 6-GIN group. H₂O₂: Hydrogen peroxide; 6-GIN: 6-gingerol; 3-MA: 3-methyladenine.
autophagy effector and plays a vital important cross-talk role in the apoptosis pathway. BH3 domain connects Beclin 1 with Bcl-2 family members. The Beclin 1-Bcl-2 interaction plays important role in autophagy and apoptosis. Their expression levels can indicate whether cells are resistant to apoptosis or autophagy[40]. In this study, the expression levels of Beclin 1 and Bcl-2 were both upregulated in the 6-GIN group. 6-GIN increased Beclin 1 expression and weakened the interaction between Beclin 1 and Bcl-2. Therefore, 6-GIN could activate autophagy by increasing Beclin 1 and show an inhibitory effect on NPMSC apoptosis by increasing Bcl-2.

Aggrecan and collagen II degradation is a hallmark of IDD, and MMP-13 could regulate the metabolism of the above two proteins. Qi et al[41] showed that oxidative stress plays a critical role in high glucose-induced NPMSC injury and could inhibit the expression of aggrecan and collagen II. In the present study, hydrogen peroxide negatively regulated the expression of aggrecan and collagen II but upregulated the expression of MMP-13. In contrast, pretreatment with 6-GIN reversed these effects. The results of the present study showed that 6-GIN treatment promoted ECM expression by reducing the oxidative stress injury-induced increase in MMP-13 expression.

Autophagy is a physiological process that degrades large molecules and damaged organelles in cells under normal or stress conditions. Autophagy is involved in self-protective processes of the cell and maintains homeostasis during injury. Increasing evidence has shown that autophagy participates in many degenerative diseases, including osteoarthritis and IDD[42,43]. Recently, many studies have demonstrated that autophagy is closely related to changes in apoptosis or senescence in MSCs and NP cells[44,45]. A basal level of autophagy maintains the balance between the synthesis and metabolism of NP cells to avoid degeneration[45,46]. Ye et al[47] reported that autophagy was significantly increased in degenerative NP cells. In contrast, Jiang et al[48] found reduced levels of autophagy in human degenerative IVD. Collectively, these results did not agree with the effect of autophagy in degenerative IVD, indicating that autophagy may play different roles corresponding to different pathological stimulations in degenerative IVD. In this study, we found that 6-GIN activated autophagy by increasing the expression of autophagy-related markers (Beclin-1 and LC-3) and decreasing the expression of p62. These findings were consistent with those of previous studies showing that 6-GIN could stimulate autophagy and exert a protective effect to attenuate oxidative injury in mouse pancreatic β-cells, hepatocytes, and colon cells[49–52]. 3-MA is a specific autophagosome formation inhibitor that is widely used to detect autophagy levels. Pretreatment with 3-MA and BAF further confirmed that 6-GIN-mediated stimulation of autophagy did not reduce autophagosome turnover but increased autophagic flux. The PI3K/Akt pathway was also found to be activated by 6-GIN in the present study, but the activity of this pathway was not influenced by the autophagy inhibitor 3-MA. Thus, it can be concluded that 6-GIN inhibits NPMSC apoptosis and ECM degeneration by stimulating autophagy via the PI3K/Akt pathway.

CONCLUSION

In summary, this study evaluated the protective effect of 6-GIN on oxidative stress-induced injury in NPMSCs for the first time. As shown in Figure 8, we have successfully isolated human NPMSCs from NP tissues, and hydrogen peroxide induced oxidative damage in NPMSCs. 6-GIN could inhibit NPMSC apoptosis by activating autophagy and protect against ECM degeneration by stimulating autophagy via the PI3K/Akt pathway, which is beneficial for the repair of IDD. Additional studies to investigate the potential protective effect of 6-GIN on NPMSCs in an animal IDD model are required to better understand the potential use of 6-GIN to regenerate and/or slow IDD in the future.
Figure 8 Schematic of protective effects of 6-gingerol. 6-gingerol (6-GIN) inhibits nucleus pulposus-derived mesenchymal stem cell apoptosis and extracellular matrix degeneration by promoting autophagy via the PI3K/Akt pathway under oxidative damage induced by hydrogen peroxide alone (H\textsubscript{2}O\textsubscript{2} group). NP: Nucleus pulposus; 6-GIN: 6-gingerol; NPMSCs: Nucleus pulposus-derived mesenchymal stem cells; H\textsubscript{2}O\textsubscript{2}: Hydrogen peroxide; ROS: Reactive oxygen species.

ARTICLE HIGHLIGHTS

Research background
Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) can better tolerate the harsh acidic and hyperosmolar microenvironment of degenerative intervertebral discs (IVDs) than other types of mesenchymal stem cells (MSCs). NPMSCs can differentiate into nucleus pulposus cells and play an endogenous repair role in damaged IVDs.

Research motivation
To date, there has been no effective treatment for intervertebral disc degeneration (IDD). Overexpression of reactive oxygen species (ROS) causes apoptosis and senescence of NPMSCs, which eventually impairs their endogenous repair abilities. Thus, this study focuses on the strategies of how to protect NPMSCs from oxidative injury.

Research objectives
The present study investigated whether 6-gingerol (6-GIN) could protect NPMSCs from apoptosis induced by oxidative stress and the potential mechanism.

Research methods
The protective effects of 6-GIN against hydrogen peroxide-induced injury in NPMSCs were investigated by analyzing the expression of apoptosis-associated proteins, matrix metalloproteinase (MMP), Annexin V-FITC/PI flow cytometry, and TUNEL assay. Additionally, autophagy-related tests including the protein, TEM, LC-3 immunofluorescence, and PI3K/Akt signaling pathway-related proteins were evaluated. The expression of extracellular matrix (ECM) was evaluated by real-time polymerase chain reaction (RT-PCR) and immunofluorescence.

Research results
6-GIN could increase Bcl-2 expression and decrease Bax and caspase-3 expression. The MMP, Annexin V-FITC/PI flow cytometry, and TUNEL assay results further confirmed that 6-GIN treatment significantly inhibited NPMSC apoptosis induced by hydrogen peroxide. 6-GIN treatment promoted ECM expression by reducing the oxidative stress injury-induced increase in MMP-13 expression. Also, 6-GIN activated autophagy by increasing the expression of autophagy-related markers (Beclin-1 and LC-3) and decreasing the expression of p62.
Research conclusions
6-GIN inhibits NPMSC apoptosis and ECM degeneration. Autophagy and the PI3K/Akt pathway are involved in this process.

Research perspectives
We demonstrated the positive roles of 6-GIN in attenuating hydrogen peroxide-induced NPMSC apoptosis and protecting the ECM from degeneration. 6-GIN may be successfully applied to IDD therapy.

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