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Research Article

Resveratrol attenuates high glucose-induced nucleus pulposus cell apoptosis and senescence through activating the ROS-mediated PI3K/Akt pathway

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Background: Diabetes mellitus is closely correlated with disc degeneration. Nucleus pulposus (NP) cell apoptosis and senescence are typical cellular features within the degenerative disc. Resveratrol is a newly identified phytoalexin that has protective effects on cartilaginous tissue.
Objective: To investigate the whether resveratrol can protect against high glucose-induced NP cell apoptosis and senescence, and the potential mechanism in this process.
Methods: Rat NP cells were cultured in either 10% FBS culture medium (control group) or 10% FBS with a high glucose concentration (0.2 M, experiment group) for 3 days. Resveratrol or the combination of resveratrol and LY294002 was added into the culture medium of experiment group to investigate the effects of resveratrol and the PI3K/Akt pathway.
Results: High glucose significantly promoted NP cell apoptosis and NP cell senescence compared with the control group. Resveratrol exhibited protective effects against high glucose-induced NP cell apoptosis and senescence. Further analysis showed that resveratrol suppressed reactive oxygen species (ROS) generation and increased the activity of the PI3K/Akt pathway under the high glucose condition. However, the LY294002 had no significant effects on ROS content in the resveratrol-treated high glucose group.
Conclusion: Resveratrol can attenuate high glucose-induced NP cell apoptosis and senescence, and the activation of ROS-mediated PI3K/Akt pathway may be the potential mechanism in this process.

Introduction

Intervertebral disc degeneration (IDD) is a worldwide disease with multipathogenesis [1]. It can lead to stenosis, spine instability, and neurothlipsis [2]. Due to these complications, IDD is a main contributor to low back and leg pain [3]. The existing treatments are mainly aimed to symptom relief but not to inhibiting and/or retarding disc degeneration. One key reason for the current situation is the lack of clear understanding of its pathogenesis.

Diabetes mellitus (DM) is regarded as a systematic disease that can cause many complications, such as cardiovascular disease, chronic renal failure, retinopathy, and neuropathy [4]. In the recent years, DM is proved to be an important etiological factor of disc degeneration [5-7]. Previous epidemiological investigations have reported that DM patients have a higher incidence of degenerative disc diseases than the patients without DM, and that DM patients with degenerative disc diseases are much younger than

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non-DM patients [8,9]. Based on these findings, many researchers have performed some basic studies to investigate the underlying mechanism of IDD in DM patients.

During disc degeneration, the nucleus pulposus (NP) region first exhibits degenerative changes, such as cellular apoptosis, cellular senescence, and matrix degradation [10-13]. Previously, several studies have used high glucose supply *in vitro* to imitate the cellular surrounding microenvironment in the DM patients [6,7,14-16]. These studies have demonstrated that a high glucose supply has obvious effects on disc cell biology, such as inducing cell apoptosis and cell senescence, and decreasing cellular biosynthesis. All these findings indicate that inhibiting high glucose-induced damage on disc cell biology may be a potential strategy to retard disc degeneration in DM patients.

Resveratrol is a natural phytoalexin found in plants including peanuts and grapes [17]. Recently, resveratrol is found to have a wide protective effect in different cell types, such as the anti-inflammatory, antiaging, and cartilage protection [18-20]. Therefore, in the present study, we mainly aimed to investigate whether resveratrol can attenuate high glucose-induced NP cell apoptosis and senescence, as well as the potential mechanism underlying this regulatory process.

Materials and methods
Ethical statement
All experiments in the present study were approved by the Ethics Committee at No. 89 Hospital of PLA.

NP cell isolation and culture
The lumbar discs (L1-L5) were harvested from healthy 22 Sprague-Dawley rats (male, 230–250 g and 7–8 weeks old) under sterile conditions as described previously [21]. Then, the central gelatinous NP tissue was carefully isolated under a dissecting microscope. Then, NP cell pellets were isolated by sequential digestion (0.25% trypsin (Gibco, U.S.A.) for 5 min and 0.25% type I collagenase (Sigma, U.S.A.) for 10–15 min) and centrifugation at 1000 rev/min for 5 min, as described previously [22]. Then, NP cells were expanded in the DMEM/F12 medium containing 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin–streptomycin (Gibco, U.S.A.) under standard conditions (37°C, 21% O_2 and 5% CO_2). When the NP cells grew to 80% confluence, they were split once. To avoid the cell passage on cell senescence and apoptosis, the passage 2 NP cells were used in the present study. Specifically, the passage 2 cells were placed in either 10% FBS medium (normal control) or 10% FBS medium with a high glucose concentration (0.2 M, experiment group) for 3 days. To investigate the role of resveratrol and the PI3K/Akt pathway, resveratrol (100 μM) [23,24] and LY294002 (10 μM) were added along with the culture medium in the experiment group.

Apoptosis assay
NP cell apoptosis was analyzed using the annexin V-FITC/PI staining method (Beyotime, China). Briefly, NP cells were trypsinized with 0.25% trypsin, washed with phosphate buffer solution (PBS), and then incubated with reaction mixture containing 195 μl of Annexin V-FITC binding buffer, 5 μl of Annexin V-FITC, and 10 μl of propidium iodide for 20 min under dark condition. Thereafter, NP cells were subjected to a flow cytometry machine to analyze the NP cell apoptosis ratio.

Caspase enzyme activity measurement
Caspase-3 activity was evaluated using commercial caspase-3 activity detection kit (Beyotime, China). Briefly, NP cells were washed with PBS for three times and incubated with 300 μl of lysis buffer for 15 min at 4°C. Then, the protein supernatant was obtained by centrifugation at 15000 *g* for 15 min. Thereafter, a reaction mixture system containing 40 μl of detection buffer solution, 50 μl of sample, and 10 μl of Ac-DEVD-*p*NA (2 mM) was subjected to an auto microplate reader. The blank control was also set according to the manufacturer's instructions. Finally, caspase-3 activity was calculated by detecting the absorbance value at a wavelength of 405 nm.

Senescence associated β-galactosidase activity
NP cell senescence was analyzed using a senescence associated β-galactosidase (SA-β-Gal) staining kit (Beyotime, China). Briefly, NP cells were washed with PBS for three times and fixed with fixative for 15 min, followed by rinsing with PBS for three times (3 min for each time). Thereafter, NP cells were incubated with staining working solution overnight at 37°C under seal condition. Finally, SA-β-Gal activity expressed as the staining-positive NP cells to the total NP cells was analyzed using the Image-Pro Plus software (Version 5.1, Media Cybernetics, Inc.).

Table 1 Primers of target genes

Gene	Accession number	Forward (5′-3′)	Reverse (5′-3′)
β-Actin	NM_031144.3	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Bcl-2	NM_016993.1	GGGGCTACGAGTGGGATACT	GACGGTAGCGACGAGAGAAG
Bax	NM_017059.2	GGCGAATTGGCGATGAACTG	CCCAGTTGAAGTTGCCGTCT
Caspase 3	NM_012922.2	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
P53	XM_008767773.1	CCTTAAGATCCGTGGGCGT	GCTAGCAGTTTGGGCTTTCC
P16	NM_031550.1	TACCCCGATACAGGTGATGA	TACCGCAAATACCGCACGA

Cell cycle assay

NP cell cycle was analyzed by flow cytometry using the PI staining method. Briefly, after NP cells were rinsed with PBS and fixation with 75% ethanol overnight at 4°C, they were incubated with PI dye (50 µg/ml, Beyotime, China) and RNase A (100 µg/ml, Beyotime, China) for 30 min. Finally, they were subjected to a flow cytometry machine (FACS Aria; BD Company). The cell cycle phases (G0/G1, G2/M, and S) were analyzed by multicycle software (Japan PHENIX Company).

Telomerase activity

Briefly, NP cells were incubated with RIPA lysis buffer (Beyotime, China) and centrifuged to collect the supernatant. Then, NP cell telomerase activity was measured according to the manufacturer's instructions of a commercial telomerase activity detection kit (Mlbio, China).

Reactive oxygen species content measurement

Intracellular reactive oxygen species (ROS) of the NP cells was measured using a reactive oxygen species assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Briefly, after washing with PBS for three times and incubation with DCFH-DA (10 µM, diluted by FBS) for 40 min, NP cells were washed with FBS again to remove the uncombined DCFH-DA solution. Thereafter, NP cells were collected by digestion with 0.25% trypsin and 10^5 cells in each group were used to measure the intracellular ROS that was expressed as fluorescence intensity at an excitation/emission wavelength of 490/585 nm.

Real-time PCR analysis

Expression of senescence markers (p16 and p53) and apoptosis-related molecules (Bcl-2, Bax, and caspase-3) was analyzed using real-time PCR. Briefly, total RNA was extracted using Trizol Reagent (Invitrogen, U.S.A.) and reverse-transcribed by a reverse transcription kit (Roche, Switzerland). Then, PCR was performed on a C1000^{TM} PCR machine using the SYBR Green Mix (TOYOBO, Japan). Primers of the target genes (Table 1) were designed and synthesized by a domestic biological company (Sangon, Biotech Co., Ltd., China). The relative gene expression was normalized to the reference gene β-actin according to the method of $2^{\triangle\triangle C}{}_{t}$.

Western blotting analysis

The activity of the PI3K/Akt pathway was analyzed using the Western blotting assay. Briefly, total protein was extracted using the RIPA lysis buffer (Beyotime, China) and the protein concentration was determined with a BCA kit (Beyotime, China). Then, equal amounts of protein sample was subjected to SDS/PAGE system and transferred to the PVDF membrane, followed by the incubation with primary antibodies at 4°C overnight, and then with HRP-conjugated secondary antibody at room temperature for 2 h. After protein bands were detected with ECL Plus reagent (Thermo, U.S.A.), gray value of protein bands was measured using ImageJ software (National Institutes of Health, U.S.A.). Activity of the PI3K/Akt pathway was expressed as the percentage of p-Akt expression to the total Akt expression.

Statistical analysis

All numerical data were presented as mean ± standard error of mean (SED) from the results of three independent experiments. After the homogeneity test for variance, intergroup difference was analyzed using a one-way analysis of variance (ANOVA) using the SPSS 17.0 software. The *post hoc* test was performed using the LSD method. A value of $P<0.05$ was considered as a statistically difference.
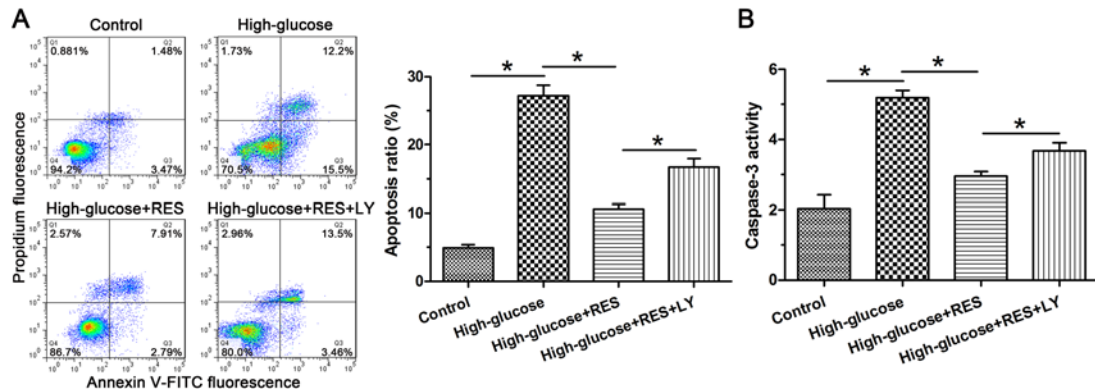
Figure 1. Evaluation of nucleus pulposus (NP) apoptosis

(**A**) Flow cytometry analysis of NP cell apoptosis. (**B**) Caspase-3 activity measurement. Data are presented as mean $\pm$ SD (n=3); * indicates a significant difference (P<0.05) between two groups.

Results
NP cell apoptosis ratio and caspase-3 activity

Results showed that NP cell apoptosis ratio in the experiment group was increased compared with the control group. On the other hand, addition of the resveratrol into the high glucose group attenuated NP cell apoptosis, whereas inhibition of the PI3K/Akt pathway by LY294002 counteracted the effects of resveratrol in the high glucose group (Figure 1A). Caspase-3 is a typical enzyme that mediates cell apoptosis. Results showed that high glucose increased caspase-3 activity compared with the control group, and that resveratrol decreased caspase-3 activity in the high glucose group whereas inhibitor LY294002 suppressed the protective effects of resveratrol against NP cell apoptosis (Figure 1B).

SA-β-Gal activity, cell cycle, and telomerase activity

Senescent cells often have increased SA-β-Gal activity, aggravated G0/G1 cell cycle arrest, and decreased telomerase activity [25-27]. SA-β-Gal staining and cell cycle analysis showed that high glucose increased SA-β-Gal staining-positive NP cells and G0/G1 phase fraction compared with the control group. Although resveratrol decreased SA-β-Gal staining-positive NP cells and G0/G1 phase fraction, the inhibitor LY294002 counteracted this trend in the high glucose group (Figure 2A and B). Telomerase activity analysis showed that telomerase activity decreased in the experiment group compared with the control group, whereas resveratrol increased the telomerase activity which was decreased by inhibitor LY294002 in turn in the high glucose group (Figure 2C).

Gene expression of apoptosis-related molecules and senescence markers

Results showed that gene expression of the proapoptosis molecules (Bax and caspase-3) was up-regulated and the antiapoptosis molecule (Bcl-2) was down-regulated by the high glucose. However, resveratrol partly decreased expression of the proapoptosis molecules (Bax and caspase-3) and increased expression of the antiapoptosis molecule (Bcl-2) in the high glucose group, whereas the inhibitor LY294002 partly reversed these effects of resveratrol in the high glucose group (Figure 3A).

For the expression of senescence markers (p16 and p53), results showed that they were significantly up-regulated by the high glucose compared with the control group. Similarly, resveratrol could down-regulate them but the inhibitor LY294002 could partly resist resveratrol's effects in the high glucose group (Figure 3B).

Intracellular ROS generation

Results showed that intracellular ROS generation in the high glucose concentration group significantly increased compared with the control group; meanwhile, the addition of resveratrol obviously decreased ROS content in the high glucose group, whereas the inhibitor LY294002 did not significantly affect the effects of resveratrol on ROS generation in the high glucose group (Figure 4).
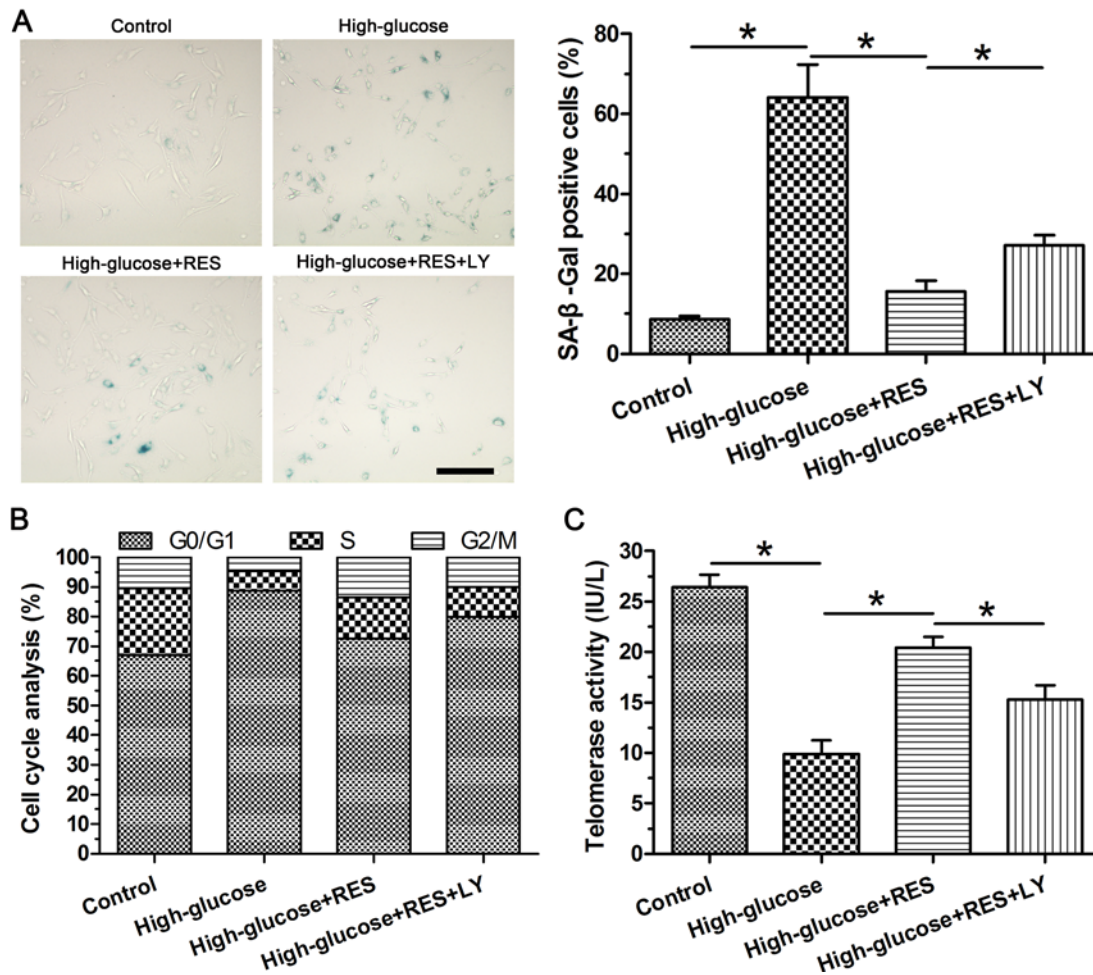
Figure 2. Evaluation of nucleus pulposus (NP) senescence
(**A**) SA-β-Gal staining assay and its quantification. (**B**) Quantification of cell cycle phases. (**C**) Telomerase activity measurement.
Data are presented as mean $\pm$ SD (n=3); * indicates a significant difference ($P<0.05$) between two groups.

Activity of the PI3K/Akt pathway

Results showed that high glucose significantly decreased the activity of the PI3K/Akt pathway compared with the control group. However, the addition of resveratrol into the high glucose group obviously increased the activation of the PI3K/Akt pathway. Expectantly, addition of the inhibitor LY294002 into the high glucose group significantly inhibited the resveratrol-mediated activation of the PI3K/Akt pathway (Figure 5).

Discussion

IDD is a common disease worldwide which brings enormous socioeconomic burden to the healthcare system [28]. To date, the pathogenesis of disc degeneration remains to be further investigated. DM is a major global public health problem. In the recent years, several epidemiological studies have demonstrated that DM is a potential etiological factor of IDD [5,6] and DM patients exhibit a higher IDD incidence than the non-DM patients [8,9]. Therefore, further exploration of the effects of DM-associated hyperglycemia on disc cell biology is important to better understand and development of corresponding therapeutic strategies of DM patient-associated IDD. In the present study, we demonstrated that resveratrol could alleviate high glucose-induced NP cell apoptosis and senescence, and the activation of ROS-mediated PI3K/Akt pathway may be the potential mechanism in this process.

During disc degeneration, the NP region first presents degenerative changes, such as cell number decline, cellular density decrease, and matrix degradation [10–12]. Previous studies have demonstrated that DM-associated hyperglycemia often exhibits negative effects on NP cell biology. For example, studies by Kong et al. [15] and Zhang et al. [29] have shown that high glucose can induce NP cell apoptosis and senescence respectively. It is well established that
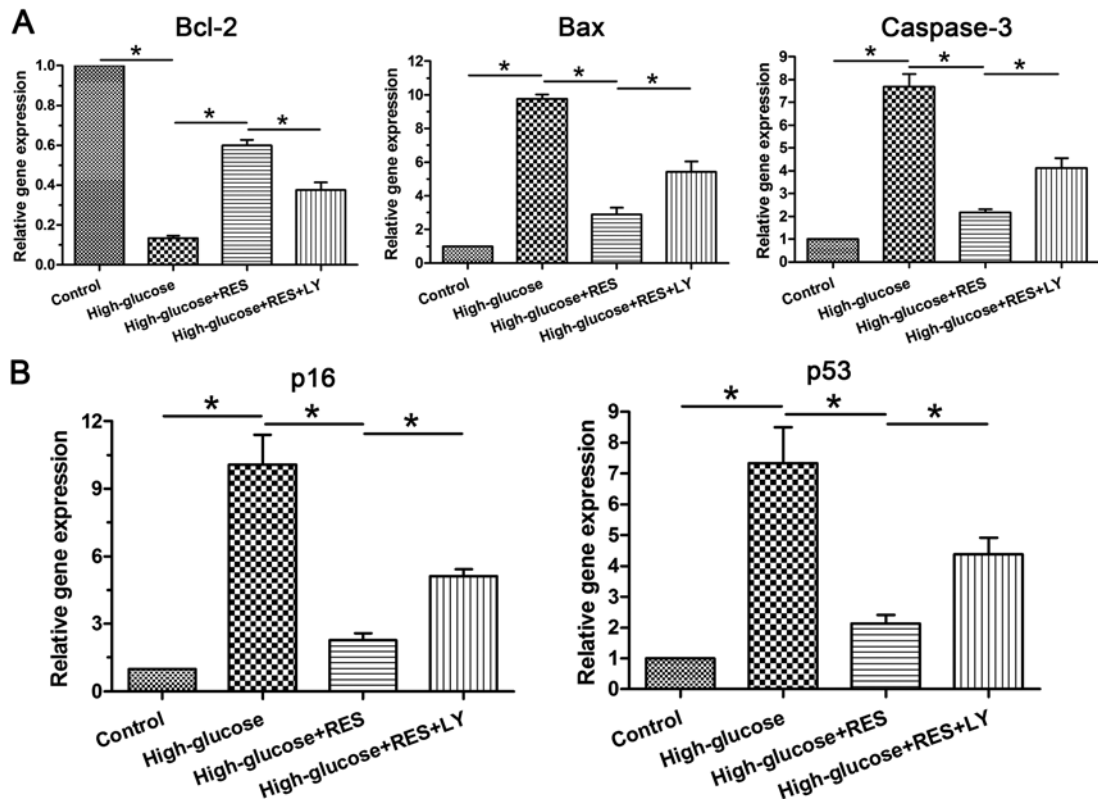
Figure 3. Real-time PCR analysis of apoptosis-related molecules and senescence markers
(**A**) Gene expression of the apoptosis-related molecules (Bcl-2, Bax, and caspase-3). (**B**) Gene expression of the senescence markers (p16 and p53). Data are presented as mean $\pm$ SD (n=3); * indicates a significant difference (P<0.05) between two groups.
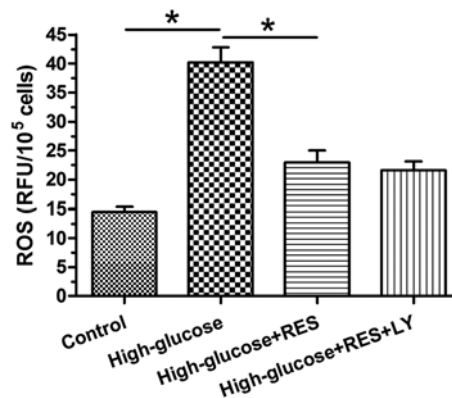

Figure 4. Intracellular reactive oxygen species (ROS) content measurement
Data are presented as mean $\pm$ SD (n=3); * indicates a significant difference (P<0.05) between two groups.

NP cell senescence and NP cell apoptosis are two common pathological features during disc degeneration [12,30]. Therefore, how to inhibit and/or alleviate high glucose-induced NP cell apoptosis- and senescence-like negative effects is becoming a research focus in this field. In the present study, we found that high glucose promoted NP cell apoptosis and senescence. This is in line with previous studies [15,29] and further confirms that high glucose is not helpful to maintain healthy disc NP cell biology.

Recently, resveratrol is found to have a wide protective effects in different cell types, such as the anti-inflammatory, anti-aging and cartilage protection [17]. In the present study, we found that resveratrol could partly alleviate high glucose (0.2 M)-induced NP cell apoptosis, NP cell senescence, and intracellular ROS generation. The present study
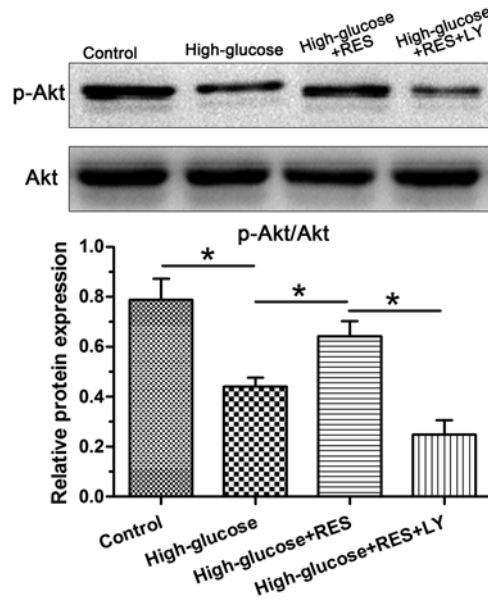
Figure 5. Western blotting analysis of PI3K/Akt pathway activity
Data are presented as mean $\pm$ SD (n=3); * indicates a significant difference (P<0.05) between two groups.

for the first time demonstrated that resveratrol plays a protective role against high glucose-caused NP cell apoptosis, senescence, and oxidative stress. In line with this to some extent, several previous studies have shown that resveratrol can play an important role in suppressing inflammatory cytokine-induced NP cell apoptosis and matrix degradation *in vitro* [23,24]. All these findings indicate that resveratrol may play a protective role in retarding and/or regenerating disc degeneration. Similar with the resveratrol, estradiol is another kind of phytoestrogen. Previously, several studies have demonstrated that estradiol is helpful to maintain NP cell viability and active biological functions under certain pathological conditions [31-34].

High glucose usually induces oxidative stress and thus damages cell biology through increasing the intracellular ROS generation [35]. In the present study, we found high glucose promoted intracellular ROS generation. This is in line with previous studies on disc cell or other cell lines [14,15], and indicates that excessive ROS generation may be responsible for the positive effects of high glucose on NP cell apoptosis and senescence. According to previous studies, the PI3K/Akt pathway participates in many cellular activities, such as cell senescence, cell apoptosis, and cell proliferation [36-38]. Here, we found that the activity of PI3K/Akt pathway was significantly decreased by the high glucose; however, resveratrol partly increased its activity in the high glucose group. Combined with the corresponding changes in NP cell apoptosis and senescence among these groups, it can be deduced that activation of the PI3K/Akt pathway is involved in the protective effects of resveratrol under high glucose condition. Additionally, we found that ROS generation and activity of the PI3K/Akt pathway showed contrary changes in the resveratrol-treated high glucose group. However, when the PI3K/Akt pathway was inhibited by LY294002 in the resveratrol-treated high glucose group, the NP cell apoptosis and senescence became aggravated again, but the intracellular ROS content did not show any obvious changes. Collectively, these results indicate that resveratrol may attenuate high glucose-induced NP cell apoptosis and senescence through activating ROS-mediated PI3K/Akt pathway.

The present study also has several limitations. First, NP cells were plate cultured under the normal oxygen condition, which is not to the physiological condition that NP cells were three-dimensionally surrounded by the native extracellular matrix in hypoxic condition. Second, there are no specific cellular markers to distinct the NP cells from notochordal cells, the isolated NP cells may contain some notochordal cells, which can affect the experimental results in the present study. Third, because the NP region first exhibits degenerative changes, we mainly focused on the response of NP tissue to mechanical compression in the present study. Fourth, the present study is just a beginning of our research plan, we will continue to study the dose-dependent effects of resveratrol on NP cell biology in the future study

In a word, we can draw the conclusion that resveratrol can attenuate high glucose-induced NP cell apoptosis and senescence, and the activation of ROS-mediated PI3K/Akt pathway may be responsible for its protective effects. The

present study provides that resveratrol may be a potential drug for regenerating and/or retarding disc degeneration in DM patients.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

Conception and design of this study: J.W., L.W., and H.T. Experiment performance: W.W., P.L, J.X., and X.W. Collection, analysis, and explanation of experimental data: Z.G., L.F., and R.S. Drafting and critically revising of this article: P.L., W.W., J.W., and H.T. All authors approved the final submission.

Abbreviations

DM, diabetes mellitus; IDD, intervertebral disc degeneration; NP, nucleus pulposus; ROS, reactive oxygen species.

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