

Original Article

Protective effects of puerarin on injury of nucleus pulposus cells induced by hydrogen peroxide through inhibiting inflammatory response, oxidative stress and anti-apoptotic pathway

Weiwei Zheng, Min Yang, Cheng Wu, Xianlin Su

Department of Orthopedics and Traumatology, Yijishan Hospital, Wannan Medical College, Wuhu, Anhui Province, PR China

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Abstract: Puerarin, an isoflavanone C-glycoside extracted from the roots of *Pueraria lobata* (Willd) Ohw and *P. thomsonii* Benth, has been widely used in China for many years. During the last three decades, puerarin has become known for its profound pharmacological effects and strong medicinal activities. Puerarin injection was commonly used in treatment of cardiovascular disease such as arrhythmia, myocardial ischemia, and high blood pressure. It was reported that puerarin could protect hydrogen peroxide (H_2O_2)-induced injury through inhibiting the oxidative stress, inflammatory response and anti-apoptotic pathway. In this study, we constructed hydrogen peroxide (H_2O_2)-induced injury model by exposing nucleus pulposus cells (NP cells) to 200 μM H_2O_2 for 6 hours, which could cause a significant viability loss and an increase of apoptotic rate. The results indicated that co-treatment with puerarin (10, 20, and 50 μM) for 24 hours could protect the damaged cells through suppression of H_2O_2 -induced cytotoxicity and cell apoptosis of NP cells. We also found that puerarin has protective effects against H_2O_2 -induced oxidative response through inhibiting MDA, GSH activities and up-regulating SOD and CAT activities. Moreover, puerarin was found to inhibit the inflammatory cytokines of NF- κB , TNF- α , IL-1 β and IL-6 expression and production induced by H_2O_2 in NP cells. In addition, the expression levels of cleaved caspase-3 and Bax were up-regulated in the H_2O_2 group, which were significantly reversed by puerarin. These results suggest that puerarin may protect NP cells against H_2O_2 -induced injury by inhibiting oxidative stress, inflammatory and caspase-3/Bax expression.

Keywords: Puerarin, H_2O_2 -induced injury, oxidative stress, inflammatory response, anti-apoptotic

Introduction

Discogenic lower back pain, a common disease in orthopedic and pain clinic, interferes with the daily lives of patients, eventually decreasing their quality of life and ability to work [1]. Discogenic lower back pain mainly show clinical manifestations of internal disc disruption (IDD). Apoptosis of NP cell plays a key role in IDD, and thus, the inhibition of cell apoptosis may provide a novel treatment option for IDD diseases [2, 3]. Therefore, inhibition of apoptosis, oxidative damage and inflammatory response in disc nucleus pulposus cells (NP cells) may be a critical point for mitigating discogenic lower back pain. Research has demonstrated that puerarin (**Figure 1**), a kind of phytoestrogen, has protective effect on the cardiovascular system,

nervous system, osteoporosis, liver injury, and inflammation in vivo and in vitro [4], which suggested that these phytoestrogens such as puerarin may possess the potential to prevent discogenic diseases. However, few researches have been done on the effects of H_2O_2 -induced oxidative damage on NP cells derived from SD rats' disc in vitro, and the underlying mechanisms remain to be fully elucidated.

In this study, a cell model for oxidative damage was established, and puerarin intervention was initiated to investigate the effects of puerarin on hydrogen peroxide-induced injury and to determine the protective mechanisms through the detection of oxidative stress, inflammatory response, caspase-3, Bax protein and anti-apoptotic pathway.

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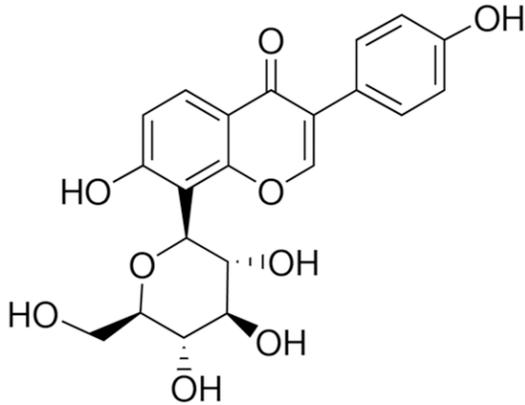


Figure 1. The chemical structure of puerarin.

Material and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12), fetal bovine serum (FBS) were provided by Hyclone (St Louis, MO, USA). Trypsin, collagenase II, CCK-8 dye and DCFH-DA were supplied by Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). V-FITC Apoptosis Detection Kit, Nuclear factor kappa B (NF- κ B), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) were acquired from KeyGEN (Nanjing, China).

Cell culture

Health male Sprague-Dawley rats (8 weeks old, Central Laboratory of Yijishan Hospital, Wannan Medical College, Wuhu, China) were euthanized by an overdose of pentobarbital (100 mg/kg body weight). The L1-L6 lumbar intervertebral discs were collected from the spinal column. Under aseptic conditions, a dissecting microscope was used to separate the gel-like nucleus pulposus (NP) tissue samples. The NP tissue samples were immediately transferred into small pieces in 10% FBS containing DMEM/F-12, and antibiotics (1% penicillin/streptomycin). Then NP tissue samples were digested with 0.01% trypsin (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) at temperature for 10-30 min. The trypsin was then removed, and NP tissue samples was washed with phosphate-buffered saline (PBS) and digested with 0.2% collagenase II (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) at 37°C for 4 h. The NP cells were harvested with 200-

μ m mesh strainer and incubated in 10% FBS containing DMEM/F-12 and antibiotics (1% penicillin/streptomycin) at 37°C in a humidified atmosphere. Cell culture media was changed every two days. The NP cells were washed with PBS and incubated with fresh medium containing 200 μ M H₂O₂ for 6 h.

Cell cytotoxicity

The NP cells were seeded into 96-well plate (5 \times 10³ cells/well) and cultured (0, 10, 20 and 50 μ M) for another 24 h [5] at 37°C in a humidified atmosphere. Afterwards, 10 μ L of CCK-8 dye (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) was added to each well and incubated at 37°C for 2 h. Optical density was examined with multi-mode microplate reader (Synergy2, Bio Tek, Winooski, VT) at 450 nm.

Detection of apoptosis by flow cytometry

The NP cells were seeded into 6-well plate (1 \times 10⁶ cells/well) and cultured (0, 10, 20 and 50 μ M) for another 24 h [5] at 37°C in a humidified atmosphere. Afterwards, NP cells were harvested and washed twice with cold PBS, and resuspended using 500 μ L of binding buffer (KeyGEN, Nanjing, China). 5 μ L of Annexin V-FITC were then added in the dark for 30 min at room temperature. After detection, 5 μ L of PI were added in the dark and were outright analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Oxidative stress measurement

The NP cells were seeded into 6-well plate (1 \times 10⁶ cells/well) and cultured (0, 10, 20 and 50 μ M) for another 24 h [5] at 37°C in a humidified atmosphere. A commercial ELISA kit was used to measure the activity levels of MDA, SOD, CAT and GSH according to the manufacturer's instruction (KeyGEN, Nanjing, China).

Inflammatory measurement

The NP cells were seeded into 6-well plate (1 \times 10⁶ cells/well) and cultured (0, 10, 20 and 50 μ M) for another 24 h [5] at 37°C in a humidified atmosphere. A commercial ELISA kit was used to measure the serum levels of NF- κ B, TNF- α , IL-1 β and IL-6 according to the manufacturer's instruction (KeyGEN, Nanjing, China).

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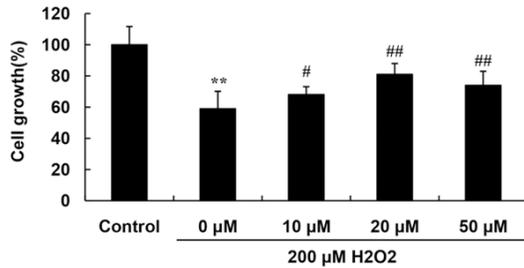


Figure 2. The effects of puerarin on the proliferation of NP cells following H₂O₂-induced oxidative damage. (n=3, mean ± standard deviation). **P<0.01, compared with the control; #P<0.05, compared with 0 μM puerarin group; ##P<0.01, compared with 0 μM puerarin group. Control, control group; 0 μM, no puerarin-treated group; 20 μM, puerarin (20 μM)-treated group and 50 μM, puerarin (50 μM)-treated group. 200 μM H₂O₂ and bars, pre-treated with 200 μM H₂O₂.

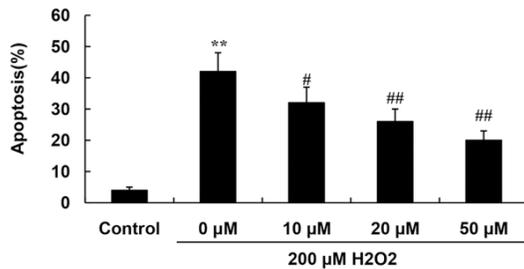


Figure 3. The effects of puerarin on the apoptotic rate of NP cells following H₂O₂-induced oxidative damage. (n=4, mean ± standard deviation). **P<0.01, compared with the control; #P<0.05, compared with 0 μM puerarin group; ##P<0.01, compared with 0 μM puerarin group. Control, control group; 0 μM, no puerarin-treated group; 20 μM, puerarin (20 μM)-treated group and 50 μM, puerarin (50 μM)-treated group. 200 μM H₂O₂ and bars, pre-treated with 200 μM H₂O₂.

Caspase-3 measurement

The NP cells were seeded into 6-well plate (1×10⁶ cells/well) and cultured (0, 10, 20 and 50 μM) for another 24 h [5] at 37°C in a humidified atmosphere. The caspase-3 activity in fluorescence was detected at the wavelength of 405 nm with the caspase-3 colorimetric assay kits (Beyotime, Nanjing, China).

Western blot analysis

The NP cells were seeded into 6-well plate (1×10⁶ cells/well) and cultured (0, 10, 20 and 50 μM) for another 24 h [5] at 37°C in a humidified atmosphere. Afterwards, NP cells were har-

vested and washed twice with cold PBS, and resuspended using lysis buffer (RIPA Lysis Buffer; Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) for 30-60 min on ice. After centrifugation at 10,000×g for 10 min at 4°C, the protein concentration was determined using bicinchoninic acid protein assay (KeyGEN, Nanjing, China). Equal amounts of protein were loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride (PVDF, Bio-Rad, MA, USA, 0.22 mm). The PVDF membrane was blocked for 1 h in 5% (w/v) bovine serum albumin, and incubated with anti-Bax (1:1000, American Diagnostica Inc. Stamford, CT, USA) and anti-β-actin (1:5000, BestBio Inc, Shanghai, China) overnight at 4°C. Next day, the PVDF membrane was incubated with secondary antibodies for 2 h at room temperature. The relative quantity of the protein was measured using AlphaEase FC (FluorChem FC2) software (Cell Biosciences Inc, Santa Clara, CA, USA).

Statistical analysis

ANOVA or Student's t test were used. Statistical analysis expressed as means ± SD deviation and was performed using SPSS (version 17; SPSS Inc. Chicago, IL, USA). A value of P<0.05 was statistically significant.

Results

Protective effect of puerarin on H₂O₂-induced cytotoxicity in NP cells

When puerarin-treated at 24 h, cell growth of NP cells was effectively inhibited by 200 μM H₂O₂ as compared to the control group (**Figure 2**). Treatment with puerarin (10, 20 and 50 μM) effectively increased the growth of NP cells as compared to the H₂O₂-induced-no puerarin group (0 μM puerarin) (**Figure 2**).

Protective effect of puerarin on H₂O₂-induced apoptosis in NP cells

We found that 200 μM H₂O₂ effectively induced cell apoptosis of NP cells as compared to the control group. As shown in **Figure 3**, puerarin treatment (10, 20 and 50 μM) effectively reduced H₂O₂-induced apoptosis in NP cells as compared to the H₂O₂-induced-no puerarin group (**Figure 3**).

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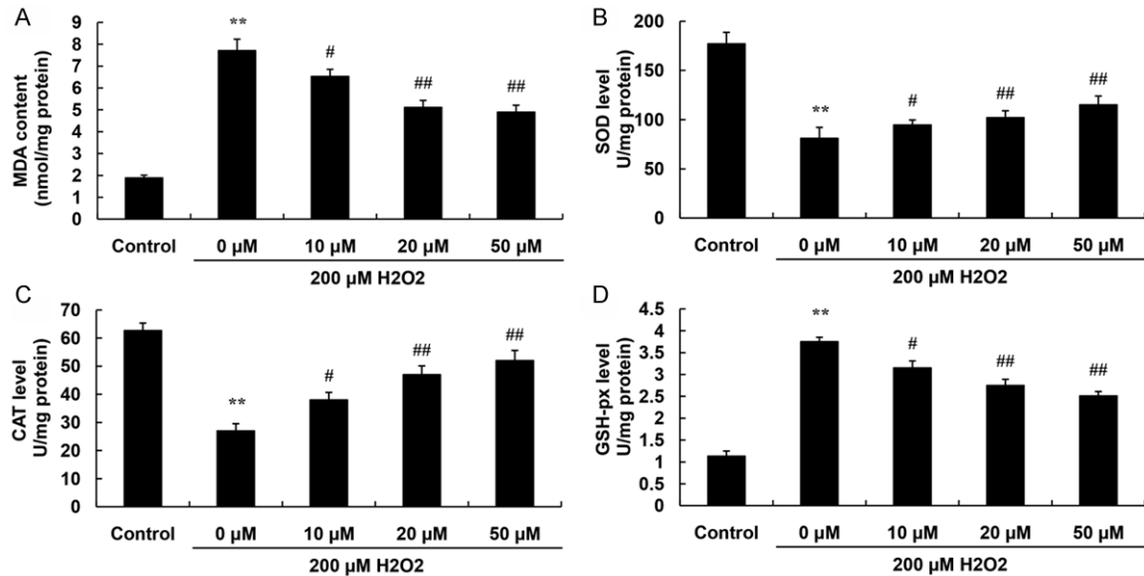


Figure 4. Effects of puerarin on the concentration of (A) MDA and on the activities of antioxidant enzymes (B) SOD, (C) CAT and (D) GSH following H_2O_2 -induced oxidative damage. (n=4, mean \pm standard deviation). **P<0.01, compared with the control; #P<0.05, compared with 0 μ M puerarin group; ##P<0.01, compared with 0 μ M puerarin group. Control, control group; 0 μ M, no puerarin-treated group; 20 μ M, puerarin (20 μ M)-treated group and 50 μ M, puerarin (50 μ M)-treated group. 200 μ M H_2O_2 and bars, pre-treated with 200 μ M H_2O_2 . SOD, superoxide dismutase; GSH, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde.

Protective effect of puerarin on inhibiting H_2O_2 -induced oxidative stress in NP cells

To investigate the protective effect of puerarin on H_2O_2 -induced oxidative stress, the serum levels of MDA, SOD, CAT and GSH were analyzed in this study. H_2O_2 observably induced the content of MDA and GSH (Figure 4A, 4D), and suppressed the levels of SOD and CAT in NP cells (Figure 4B, 4D) compared with those of control group. As shown in Figure 5, these indexes were observably reversed by treatment with puerarin (10, 20 and 50 μ M) compared with those of H_2O_2 -induced-no puerarin group (Figure 4).

Protective effect of puerarin on inflammatory in NP cells

To examine the protective effect of puerarin on H_2O_2 -induced inflammatory responses, the levels of NF- κ B, TNF- α , IL-1 β and IL-6 were measured in present study. H_2O_2 markedly activated the levels of NF- κ B, TNF- α , IL-1 β and IL-6 in NP cells compared with those of control group (Figure 5). However, the levels of NF- κ B, TNF- α , IL-1 β and IL-6 were markedly suppressed by treatment with puerarin (10, 20 and 50 μ M)

compared with those of H_2O_2 -induced-no puerarin group (Figure 5).

Protective effect of puerarin on cleaved caspase-3 in NP cells

To further analyze the protective effect of puerarin on cell apoptosis, caspase-3 activity was inspected in our study. We found that H_2O_2 significantly increased the activity of cleaved caspase-3 in NP cells as compared to the control group (Figure 6). Nevertheless, the increase of cleaved caspase-3 activity was markedly reduced by treatment with puerarin (10 20 and 50 μ M) compared with those of H_2O_2 -induced-no puerarin group (Figure 6).

Protective effect of puerarin on Bax in NP cells

To further explore the protective effect of puerarin on cell apoptosis, the protein expression of Bax were measured using western blot analysis. Western blot showed that H_2O_2 significantly induced the protein expression of Bax in NP cells as compared to the control group (Figure 7). However, puerarin pretreatment significantly reduced the protein expression of Bax compared with that of H_2O_2 -induced-no puerarin group (Figure 7).

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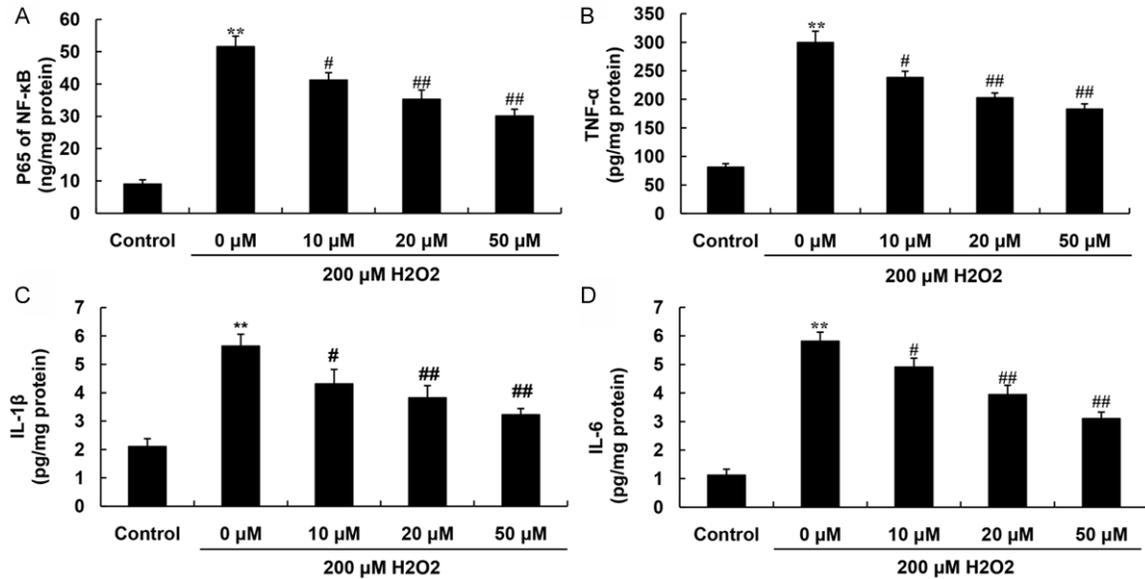


Figure 5. Effects of puerarin on the activities of antioxidant enzymes (A) NF-κB (B) TNF-α, (C) IL-1β and (D) IL-6 following H₂O₂-induced oxidative damage. (n=4, mean ± standard deviation). **P<0.01, compared with the control; #P<0.05, compared with 0 μM puerarin group; ##P<0.01, compared with 0 μM puerarin group. Control, control group; 0 μM, no puerarin-treated group; 20 μM, puerarin (20 μM)-treated group and 50 μM, puerarin (50 μM)-treated group. 200 μM H₂O₂ and bars, pre-treated with 200 μM H₂O₂. NF-κB, Nuclear factor kappa B, TNF-α, tumor necrosis factor-α, IL-1β, interleukin-1β, IL-6, interleukin-6.

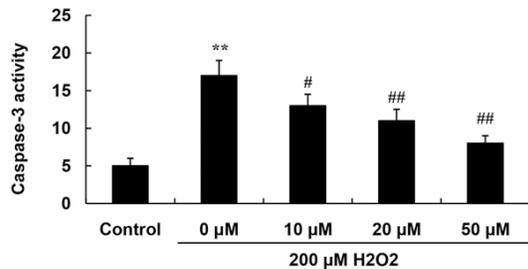


Figure 6. The effects of puerarin on the activity of caspase-3 following H₂O₂-induced oxidative damage. (n=3, mean ± standard deviation). **P<0.01, compared with the control; #P<0.05, compared with 0 μM puerarin group; ##P<0.01, compared with 0 μM puerarin group. Control, control group; 0 μM, no puerarin-treated group; 20 μM, puerarin (20 μM)-treated group and 50 μM, puerarin (50 μM)-treated group. 200 μM H₂O₂ and bars, pre-treated with 200 μM H₂O₂.

Discussion

Discogenic lower back pain, a clinical frequently-occurring orthopedic disease with various clinical manifestations, is a common problem that affects people's life and work [6]. Of all the low back pain fundamental causes, intervertebral disc degeneration is among the most common. However, there are no effective interven-

tion measures available to relieve pain symptoms based on clinical interventions of discogenic lower back pain. It is believed that the existence of nerve fibers transmit pain in the intervertebral disc is the precondition of intervertebral disc source of low back pain, but there are few studies on the mechanisms of intervertebral disc nerves grow [7, 8].

In this study, the growth of NP cells exposed in H₂O₂ was significantly inhibited and its cellular apoptosis increased. As seen in **Figure 3**, incubation with H₂O₂ causes a significant damage on NP cells in which many apoptotic cells increase obviously. After co-treatment with puerarin, fewer apoptotic cells were detected in a dose-dependent manner, which indicated that puerarin significantly inhibited the apoptosis of H₂O₂-induced NP cells. Inconsistent with the results from some reports [9], we found for the first time that treatment with puerarin increased the cell growth and suppressed cell apoptosis of H₂O₂-induced NP cells, and our results keep consistent with the previous report [10]. To investigate the effects of puerarin on the apoptosis of H₂O₂-induced NP cells, flow cytometry was used to detect the apoptotic rate of NP cells treated by puerarin, we found that puerarin

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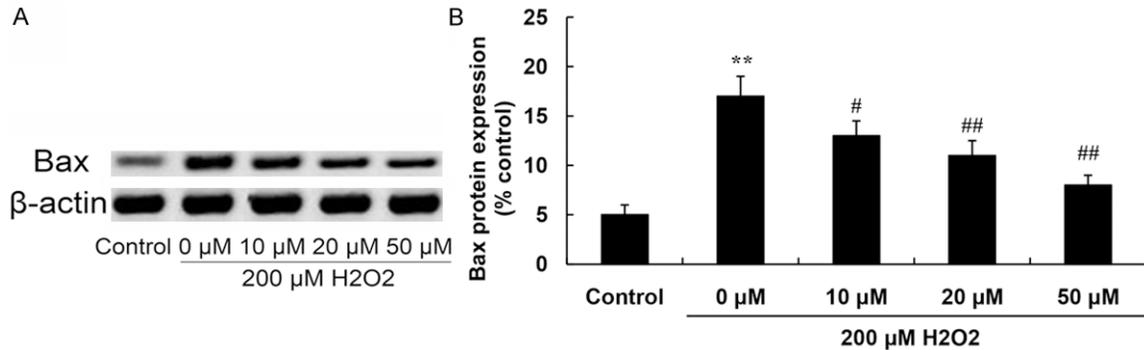


Figure 7. Effects of puerarin on Bax protein expression following H_2O_2 -induced oxidative damage. (n=5, mean \pm standard deviation). A. Representative western blot showing the protein expression levels of Bax. Bax, 21 kDa; β -actin: 43 kDa. B. Quantitative analysis of the protein level of Bax from different groups. The data were normalized to the loading control β -actin. ** $P < 0.01$, compared with the control; # $P < 0.05$, compared with 0 μM puerarin group; ### $P < 0.01$, compared with 0 μM puerarin group. Control, control group; 0 μM , no puerarin-treated group; 20 μM , puerarin (20 μM)-treated group and 50 μM , puerarin (50 μM)-treated group. 200 μM H_2O_2 and bars, pre-treated with 200 μM H_2O_2 .

rin significantly reduced H_2O_2 -induced apoptosis and fewer dead cells could be seen (Figures 2B and 4). Many research confirmed that puerarin suppressed cell apoptosis on H_2O_2 -induced injury [11, 12]. Its underlying mechanism may be that puerarin prevents apoptosis via inhibition of JNK pathway and activation of PI3K/Akt signaling pathway in NP cells, dependent on the mitochondrial apoptotic pathway [13]. Meanwhile the apoptotic pathway of degenerative disc NP cells is predominantly mediated through mitochondrial pathway [14].

Oxidative stress induced by free radical damage is one of the basic causes of cell apoptosis, antioxidant is therefore an effective option to protect cells from procedural apoptosis [15], and primary enzymatic defenses are important aspects of cellular antioxidant defense systems, which consist of MDA, SOD, catalase and glutathione peroxidase. H_2O_2 is one of reactive oxygen species closely related to oxidative stress reaction, its main product has a strong oxidizing and free to enter cells, probably causing cellular damage in many cell culture models [16]. Many diseases are involved in oxidative stress injury, such as intervertebral disc degeneration (IDD) [17]. The cell culture model can cause cellular damage in NP cells, and its potential mechanism may be that H_2O_2 -induced oxidative stress disrupts MMP and results in mitochondrial dysfunction [18-20]. In our experiment, H_2O_2 observably induced the content of MDA and GSH, and inhibited the levels of SOD and CAT, which indicate that MDA, GSH, SOD

and CAT-dependent oxidative response was reacting, moreover, after co-treatment with puerarin we found that puerarin markedly up-regulated SOD and CAT expression, and MDA and GSH were significantly down-regulated. This implies that puerarin is capable of reducing oxidative activities through regulating cytokines of MDA, GSH, SOD and CAT-dependent pathway. Our results keep consistent with some related reports [21, 22], but inconsistent with some other reports [23, 24]. Previous reports shows that puerarin could up-regulate apoptosis-related genes, including Bcl-2 and SOD, and down-regulated the NF- κ B and TNFR1/FADD/caspase signal pathways in streptozotocin (STZ)-induced diabetic rats [25, 26]. Furthermore, puerarin could attenuate neuronal degeneration in 6-OHDA-lesioned rats through blocking of oxidative stress [27]. In addition, a study indicated that puerarin protects H_2O_2 -induced pancreatic islets damage through suppressing oxidative stress [9]. According to the literature and our study, treatment with puerarin prevents H_2O_2 -induced oxidative stress in NP cells.

Through the animal experiment and clinical observation, especially, pulp check tissue inflammatory reaction, some international scholars found that cervical spondylosis, lumbar intervertebral disc protrusion in individuals with intervertebral disc degeneration, the nucleus pulposus prominent lesion are caused by degeneration of intervertebral disc [28]. Intervertebral disc tissue show pathological chang-

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es including edema, fibrin deposition, obvious polymorphism nuclear cells, lymphocytes and plasma cells invasion; Excited expression of chemical substance include factor such as monocyte chemotactic protein-1, macrophage inflammatory protein-1, fiber ring are found around the blood vessels, the disc without original vascular also had growing blood vessels surrounding a large number of inflammatory cells [29]. Inflammatory mediators receptor through the positive feedback loop between fiber ring outer damage and inflammatory cells, the penetration of continuous blood vessels development promoted inflammation development, and caused plate source neck/lumbar pain, which could seriously cause spinal cord and peripheral nerve fiber source of immune, chemical lesions, producing demyelinating degeneration, necrosis, axon damaged cells, and Schwann cells [30]. To investigate the underlying mechanisms of inflammatory response H_2O_2 -induced in NP cells, cytokines of NF- κ B, TNF- α , IL-1 β and IL-6 were detected. We found that H_2O_2 could obviously up-regulate NF- κ B, TNF- α , IL-1 β and IL-6 expression and production in NP cells, which indicated that NF- κ B, TNF- α , IL-1 β and IL-6 dependent inflammatory response was evoked. Meanwhile, after treatment with puerarin, we found that NF- κ B, TNF- α , IL-1 β and IL-6 expression and production were markedly suppressed by puerarin in a dose-dependent manner. The evidence strongly suggested that puerarin inhibit inflammatory response through a NF- κ B, TNF- α , IL-1 β and IL-6-dependent pathway in NP cells, which is in consistent with related reports [31, 32]. Furthermore, research reported that puerarin inhibited the production of interleukin IL-1 β , IL-6, TNF- α and regulated the transcriptional level through suppression of NF- κ B and MAPK signal pathways [33, 34]. These results showed that puerarin protects H_2O_2 -induced injury through decreasing the level of NF- κ B, TNF- α , IL-1 β and IL-6, which was connected with anti-inflammatory effects of puerarin.

To study the potential mechanisms underlying H_2O_2 -mediated cell antiapoptosis in NP cells, we also evaluated cleaved caspase-3 activity and Bax protein expression. Studies have shown that the caspase family (Caspases) is the starter and executor of mammalian cells apoptosis, among them, Caspase-3 is Caspases cascade "waterfall" downstream key apoptotic protease [35]. Caspase-3 is a pro-apoptotic

protein that plays a role as an executioner of apoptosis via both death receptor pathway and mitochondrial pathway. In our experiment, data showed that the expression of cleaved caspase-3 was significantly increased in the H_2O_2 -treatment group, but after co-treatment with puerarin, we found that cleaved caspase-3 expression was markedly reduced by puerarin in a concentration-dependent manner, and this result keep consistent with the literature [10, 13, 36, 37]. Its potential mechanisms may be that puerarin suppresses caspase-3 activation though both death receptor pathway and mitochondrial pathway. In addition, caspase activity could be promoted by Bax. Bax is by interfering with the release of cytochrome C and blocking the upstream caspase protease activation that inhibits the cell apoptosis. Bax protein as components of mitochondrial membrane ion channels, directly induces release of cytochrome C from mitochondrial membrane, and further activates the Caspas-3, causing apoptosis [35]. Our results demonstrated that incubation of NP cells with H_2O_2 evidently increases Bax protein expression; on the contrary, after treatment with puerarin, we found that Bax expression was significantly suppressed. This implies that puerarin could inhibit cell apoptosis through down-regulating Bax expression, similar to the related reports [10, 11, 37, 38]. Research suggested that puerarin could decrease Bax expression though the PI3K/Akt signaling pathway [11]. Moreover, puerarin suppresses Bax expression, protects against beta-amyloid-induced cell injury in PC12 cells [39], and it protects Alzheimer's disease neuronal hybrids through decreasing Bax/Bcl-2 ratio and inhibiting caspase-3 activation via inhibition of the JNK pathway and activation of the PI3K/Akt signaling pathway [40]. These results hinted that puerarin may act as an anti-apoptotic activity, and protect NP cells against H_2O_2 -induced injury.

In summary, our findings in this study suggest that the protective effect of puerarin promotes the cell growth and inhibits cell apoptosis in H_2O_2 -induced NP cells compared with the control group. These results clearly demonstrate the antioxidant, anti-inflammatory and anti-apoptotic properties of puerarin in NP cells which were previously induced by H_2O_2 . In conclusion, these results indicate that puerarin is able to reverse H_2O_2 -induced damage through antioxidant, anti-inflammatory and anti-apop-

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otic treatment, which may provide an experimental basis for treatment of low back pain.

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Disclosure of conflict of interest

None.

Address correspondence to: Min Yang, Department of Orthopedics and Traumatology, Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, PR China. E-mail: pkuyang@hotmail.com

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