Hindawi
International Journal of Polymer Science
Volume 2019, Article ID 8925807, 7 pages
https://doi.org/10.1155/2019/8925807

Research Article

Protective Effect of Polygonatum sibiricum Polysaccharides on Apoptosis, Inflammation, and Oxidative Stress in Nucleus Pulposus Cells of Rats with the Degeneration of the Intervertebral Disc

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Received 26 April 2019; Revised 7 June 2019; Accepted 24 June 2019; Published 5 September 2019

Guest Editor: Jianxun Ding

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Objective. Polygonatum sibiricum polysaccharide (PSP) has antioxidant activity, immune enhancement, and other biological properties. However, the effect of PSP on intervertebral disc degeneration has not been reported. In this study, we mainly investigated the effect of PSP on the apoptosis, inflammation, and oxidative stress of nucleus pulposus cells (NPCs) during the process of intervertebral disc degeneration. *Methods*. A rat NPC model induced by H_2O_2 was constructed. The CCK8 method was used to measure the effects of PSP on the apoptosis of rat NPCs induced by H_2O_2. The effects on the activity of SOD and content of MDA were also determined. The rat model of intervertebral disc degeneration was treated with PSP for 1 month, and the mRNA expression levels of IL-1β, COX2, iNOS, Col2α1, Col10α1, and MMP3 were measured by qPCR in the tissue of intervertebral disc. NPCs from the degenerated intervertebral discs were separated, and the cell viability was measured by the CCK8 method. The contents of SOD and MDA in NPCs were determined as well. *Results*. PSP significantly reduced the apoptosis of NPCs induced by H_2O_2, significantly increased the SOD content, and decreased the content of MDA in H_2O_2-induced NPCs. The expression level of IL-1β, COX2, and iNOS in the rat model with intervertebral disc degeneration was significantly downregulated after 1 month of PSP treatment. PSP treatment increased the expression of Col2α1 type and significantly decreased the expression of Col10α1 type collagen and MMP3 in rats with disc degeneration. PSP treatment significantly reduced NPC apoptosis and increased its SOD content and reduced MDA content, which is consistent with the results from cell-level experiments. *Conclusion*. PSP can effectively reduce the apoptosis, inflammation, and oxidative stress of H_2O_2-induced NPCs in rats with intervertebral disc degeneration and mitigate the progression of intervertebral disc degeneration, which has the potential to be developed as new drugs for the treatment of intervertebral disc degeneration.

1. Introduction

Polygonatum kingianum Coll.et Hemsl is widely and abundantly distributed in China. Polygonatum sibiricum is the dry rhizome of Polygonatum kingianum Coll.et Hemsl which is a well-known traditional Chinese herbal medicine and functional food [1, 2]. The major extract from Polygonatum sibiricum is Polygonatum sibiricum polysaccharides (PSP) mainly consisting of mannose, galactose, glucose, and xylose, which is also rich in various bioactive components such as alkaloids, steroidal saponins, flavonoids, and lignin [3, 4]. In recent years, PSP has been reported to have many pharmacological applications and biological activities, such as antioxidant, antiaging, antiosteoporosis, antidiabetes, anticancer, and antifatigue, as well as immune enhancement, neuroprotection [5, 6].

Low back pain is a common disease that places a huge burden on public health and the economy. There are lots of

reason for causing the low back pain, of which disc degeneration is one of the most important pathological changes; nevertheless, the molecular mechanism of the pathogenesis in intervertebral disc degeneration remains unclear [7]. There is increasing evidence that the role of nucleus pulposus cells (NPCs) in producing extracellular matrix components and secreting cytokines is critical to maintaining disc integrity. Importantly, apoptosis of NPCs, inflammation, and oxidative stress is involved in the development of intervertebral disc degeneration [8]. However, whether PSP with a variety of bioactivity can effectively treat intervertebral disc degeneration has not been reported yet.

2. Methods

2.1. PSP Extraction and HPLC Detection. The hot water leaching method was used to extract the PSP. The Polygonatum sibiricum pieces (Golden Leaf Pharmaceutical Co. Ltd., China) were mixed with water at the volume ratio of 1 : 8, which was then boiled for 2 h and repeated three times, and the aqueous extract was precipitated by ethanol (80%) (Sinopharm Group, China) to remove the bioactive protein substance. PSP was obtained after precipitation and evaporation of ethanol [4]. The PSP was then subjected to acid hydrolysis; specifically, PSP (0.25 g) was mixed with 0.1 L of 2 mol/L TFA (Sinopharm Group, China) solution and dried in DHG-9070A oven (Shamghai Bluepard Instruments Co. Ltd) at 100°C, and 0.1 L methanol (Sinopharm Group, China) was added afterward to remove the TFA. After 3 times of repeated acid hydrolysis, the product was subjected to PMP (1-phenyl-3-methyl-5-pyrazolone) derivatization to obtain an HPLC test solution. In detail, the acid hydrolyzate was mixed with 0.1 L of 0.3 mol/L NaOH (Sinopharm Group, China), and 0.12 L of 0.5 mol/L methanol solution was added after that, the mixer was then incubated at 70°C in a water bath for 30 min. After that, 0.1 L of 0.3 mol/L HCl (Sinopharm Group, China) was added and an equal volume of chloroform was used for extraction. The aqueous solution was taken for drying, and the extracted product is dissolved in 0.2 L of methanol to obtain an HPLC test solution. HPLC detection was then carried out using a mixed monosaccharide solution as a control; mannose, glucose, galactose, xylose, glucuronic acid, rhamnose, galacturonic acid, arabinose, and trehalose were all purchased from Sinopharm Group, China.

2.2. CCK8 Assay. NPCs were seeded at a density of 5000 cells per well in 96-well plates. The cells were washed with phosphate-buffered saline (PBS) for 3 times after H_2O_2 or PSP treatment. The CCK8 test was performed according to the kit manual, and the absorbance at 450 nm was measured with a spectrophotometer to calculate the cell survival rate.

2.3. Apoptosis Detected with Flow Cytometry. The cells were collected and fixed using absolute ethanol precooled at -20°C. The fixed cells were slowly and fully resuspended with RNase A solution (Beijing Solabao Technology Co. Ltd., China) to digest the RNA in the cells. 400 μL of a Propidium Iodide solution (PI, 50 μg/mL) was added to each cell sample to stain the nuclei for 10 min in the dark. Flow cytometry detection was completed within 24 hours after dyeing was completed. The red fluorescence was detected at an excitation wavelength of 488 nm through the BD flow cytometer channel FL2, and the light scattering was detected at the same time.

2.4. Detection of SOD and MDA. The amount of SOD reflecting the content of reducing substances in the cells was quantitatively measured by the formazan dye, which indicates the state of the antioxidant substances in the cells. The formazan dye is a substance that can be removed by SOD. In this study, we used the SOD test kit (Dojindo, Japan) to quantify the amount of formazan dye in turn to study SOD activity. According to the instructions, the OD value of the test sample and the standard at 450 nm were measured, and the SOD activity was calculated based on the formazan dye content.

The level of malondialdehyde (MDA) reflecting the level of oxidation in the cells was determined by the thiobarbituric acid (TBA) method. The OD value of the test sample and the standard at 530 nm were measured according to the instruction, and the content of MDA in the sample was calculated accordingly.

2.5. qPCR. NPCs were collected and total RNA was extracted with the RNeasy Protect Mini kit (74104, QIAGEN, Germany). The absorbance at 260 nm and 280 nm of the total RNA was determined by a spectrophotometer. And the RNA was reversely transcribed into cDNA if the OD_{260}/OD_{280} is between 1.6 and 2.0. The qPCR assay was performed according to the protocol, and the reaction was conducted in the ABI PRISM 7700 Sequence Detection System with the ABI PRISM 7700 Sequence Detection Software 1.9.1. GAPDH was used as an internal reference gene. The relative expression level of each gene of interest was represented by $2^{-\Delta\Delta Ct}$.

2.6. NPC Separation. All experiments in this study were approved by the Animal Experimental Ethics Committee. NPCs were isolated from the intervertebral discs of 180 g-200 g SD rats. The cells were first treated with PBS containing 10% penicillin at 37°C for 10 minutes and then cultured at 37°C for 18 h in DMEM-F12 containing 10% fetal bovine serum, 0.05 mg/ml ascorbic acid, 1% penicillin, and 0.2% collagenase. NPCs were collected afterward and continued to be cultured in a DMEM-F12 medium, and the medium was changed every 3 days.

2.7. Animal Model. Male SD rats were randomly divided into the following 3 groups (6 in each group): control group, model group, and PSP treatment group. Rats in the control group did not receive any treatment and were kept in an ordinary cage. The forelimbs of the rats in the model the PSP treatment group were excised from the shoulder joint. Specifically, after shaving the front limbs of the rats, the upper limb skin was disinfected with iodophor. The skin was cut at the proximal end of the forearm, fascia and muscles were then removed, the vascular nerve bundle under the deltoid muscle were ligatured, and the humerus was clamped at the distal

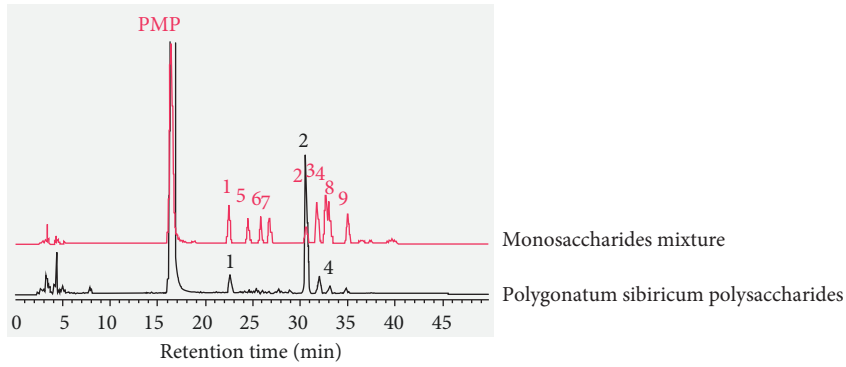


Figure 1: Chromatography of Polygonatum sibiricum polysaccharide (PSP) derivatization. 1: mannose; 2: glucose; 3: galactose; 4: xylose; 5: glucuronic acid; 6: rhamnose; 7: galacturonic acid; 8: arabinose; 9: fucose.

end of the ligation. After muscles, blood vessels, nerves, and skin were cut, the gentamicin hydrochloride was distilled into the incision; the muscles, fascia, and skin were then sutured layer by layer. The postoperative rat was forced to maintain an upright posture for seven months in a specially designed cage. Rats in the PSP treatment group was given PSP (600 mg/(kg∗d)) treatment 6 months after their operation for 30 days. The rats were then sacrificed, and the lumbar spine was analyzed.

2.8. Data Analysis. Data were analyzed using SPSS 21.0. The results for each group are expressed as mean ± standard deviation ($\bar{x} \pm s$). For the comparison between two groups, independent sample t-tests were used when the variance was uniform and the Satterthwaite approximate t-test was used when the variance was not uniform. $P < 0.05$ indicates that the difference was statistically significant.

3. Results

3.1. HPLC Detection of PSP Extract. As shown in Figure 1, compared with the control curve of the monosaccharide mixture, a variety of monosaccharides were well separated in PSP extract. Four peaks were observed from the test curve. Compared with the control curve, the majority of monosaccharides in the test sample were mannose, glucose, galactose, and xylose and the glucose content was much higher than the other three monosaccharides.

3.2. Protective Effect of PSP on Apoptosis of Rat NPCs Induced by H_2O_2. The cytotoxicity of PSP on NPCs at different concentrations (0.625 mg/L, 1.250 mg/L, 2.5 mg/L, and 5 mg/L) was determined by CCK8 assay. The results shown in Figure 2(a) indicated that PSP from 0.625 mg/L to 5 mg/L had no significant cytotoxic effect on NPCs ($P > 0.05$). We further explored the optimal concentration of H_2O_2 for the induction of apoptosis in NPCs. The cell viability of NPCs treated with 100 μM, 200 μM, and 400 μM H_2O_2 was determined by CCK8, respectively. The result showed that the three concentration gradients of H_2O_2 inhibited the viability of NPCs to various degrees and H_2O_2 at a concentration of 200 μM reached a 55% inhibition rate ($P < 0.05$) after 6 h of treatment as shown in Figure 2(b). To investigate whether PSP has a protective effect on H_2O_2-induced apoptosis in rat NPCs, NPCs were treated with PSP at concentrations of 0.625 mg/L, 1.250 mg/L, 2.5 mg/L, and 5 mg/L separately for 48 h before the induction of apoptosis in NPCs with 200 μM H_2O_2. The control group was treated only with 200 μM H_2O_2, and finally, the cell viability of NPCs was determined using CCK8. The results are shown in Figure 2(c). Compared with the control group, the PSP group with 0.625 mg/L to 5 mg/L concentration had a significant protective effect on H_2O_2-induced apoptosis in NPCs ($P < 0.05$), and the cell viability was similar under the treatment of PSP at 1.250 mg/L, 2.5 mg/L, and 5 mg/L, respectively ($P > 0.05$). Therefore, the smallest dose of 1.250 mg/L was selected in the subsequent experiments. To further verify that the PSP had a significant protective effect on H_2O_2-induced apoptosis in NPCs, we used flow cytometry to detect the apoptosis rate of H_2O_2-induced NPCs that were treated with PSP from 0.625 mg/L to 5 mg/L. As shown in Figure 3, compared with the apoptosis rate of 30.4% in the control group, the apoptosis rate of H_2O_2-induced NPCs was significantly decreased by PSP (0.625 mg/L to 5 mg/L) to 24.6%, 16.2%, 15.4%, and 15.6% separately ($P < 0.05$).

3.3. Protective Effect of PSP on H_2O_2-Induced Oxidative Stress in Rat NPCs. The amount of SOD reflecting the content of reducing substances in the cells was quantitatively determined by the formazan dye method. The results showed that the level of SOD in rat NPCs induced by H_2O_2 was significantly lower than that in the blank group ($P < 0.05$). Whereas after pretreatment with PSP for 48 hours, the level of SOD in NPCs induced with H_2O_2 was significantly higher than that in the model group ($P < 0.05$) as indicated in Figure 4(a).

The level of malondialdehyde (MDA) reflecting the level of oxidation in the cells was determined by the thiobarbituric acid (TBA) method. As shown in Figure 4(b), the MDA level in rat NPCs induced with H_2O_2 was significantly higher than that of the blank group ($P < 0.05$). However, after pretreatment with PSP for 48 hours, the MDA level was significantly reduced compared to the model group ($P < 0.05$).

3.4. Protective Effect of PSP on Inflammation of Rats with Degeneration of the Intervertebral Disc. The rat model with intervertebral disc degeneration was constructed, and qPCR

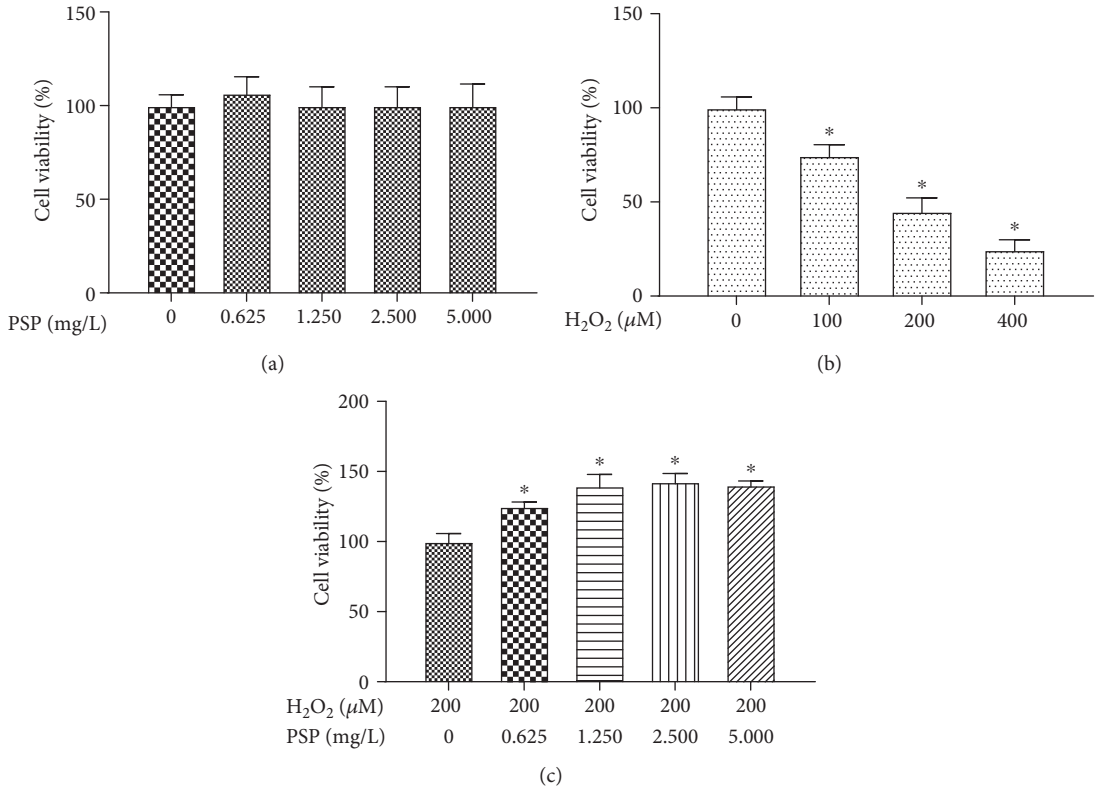


Figure 2: PSP inhibits H_2O_2-induced apoptosis in NPCs. (a) The toxicity of different concentrations of PSP on NPCs. $^*P < 0.05$, compared with the blank group. (b) The effects on cell viability by different concentrations of H_2O_2. $^*P < 0.05$, compared with 0 μM PSP groups. (c) The effects of different concentrations of PSP on the cell viability affected by 200 μM H_2O_2. $^*P < 0.05$, compared with the control group.

was used to measure the expression level of IL-1β, COX2, and iNOS in the cells, which indicate the inflammatory state of the intervertebral disc. The result in Figure 5 showed that compared with the blank group, the model group rats maintaining the upright posture for a long time had a heightened expression of IL-1β, COX2, and iNOS ($P < 0.05$). Subsequently, we administered PSP intragastrically to rats with the degeneration of intervertebral disc for 1 month and found that PSP could effectively alleviate the inflammation caused by long-term upright posture. Accordingly, compared with the model group, the expression level of IL-1β, COX2, and iNOS in the PSP treatment group was significantly downregulated as shown in Figure 5 ($P < 0.05$).

3.5. Effect of PSP on Collagen Synthesis and Catabolism in Rats with Degeneration of the Intervertebral Disc. The mRNA expression level of Col2α1 and Col10α1 type collagen, as well as the expression level of Col2α1 degrading enzyme MMP3, was determined via qPCR. We found that the upright posture induced the downregulation of Col2α1 and upregulation of Col10α1 as well as MMP3 ($P < 0.05$), while PSP pretreatment completely restored the decrease of Col2α1 mRNA expression and downregulated the expression of MMP3 and Col10α1, as shown in Figure 6.

3.6. Effect of PSP on the Activity of NPCs in Rats with Degeneration of the Intervertebral Disc. NPCs were separated from rats with degeneration of intervertebral discs, and cell viability was measured by CCK8 assay. The results showed that NPC activity in the model group was significantly lower than that in the blank group ($P < 0.05$) and PSP treatment effectively reversed the decrease of cell viability, as shown in Figure 7.

3.7. Effect of PSP on Oxidative Stress of NPCs in Rats with Degeneration of the Intervertebral Disc. NPCs were separated from rats with degeneration of intervertebral discs, the SOD activity and MDA content were measured according to the instruction of the kit, and the test results were then normalized. The result showed that the SOD activity in the NPCs in the model group was significantly lower than that in the blank group ($P < 0.05$) and the MDA content was significantly increased ($P < 0.05$). However, with the PSP treatment, the SOD activity was effectively increased ($P < 0.05$) and the content of MDA in the cells was decreased ($P < 0.05$), as shown in Figure 8.

4. Discussion

In recent years, significant progress has been made in the study of active polysaccharides [9, 10], among which Polygonatum sibiricum polysaccharide a health food has received extensive appreciation. Because of its rich biological activity, many studies have explored its role in osteoporosis, tumors, and other diseases [11]. Angiogenesis and inflammation in the pathological state of intervertebral disc degeneration

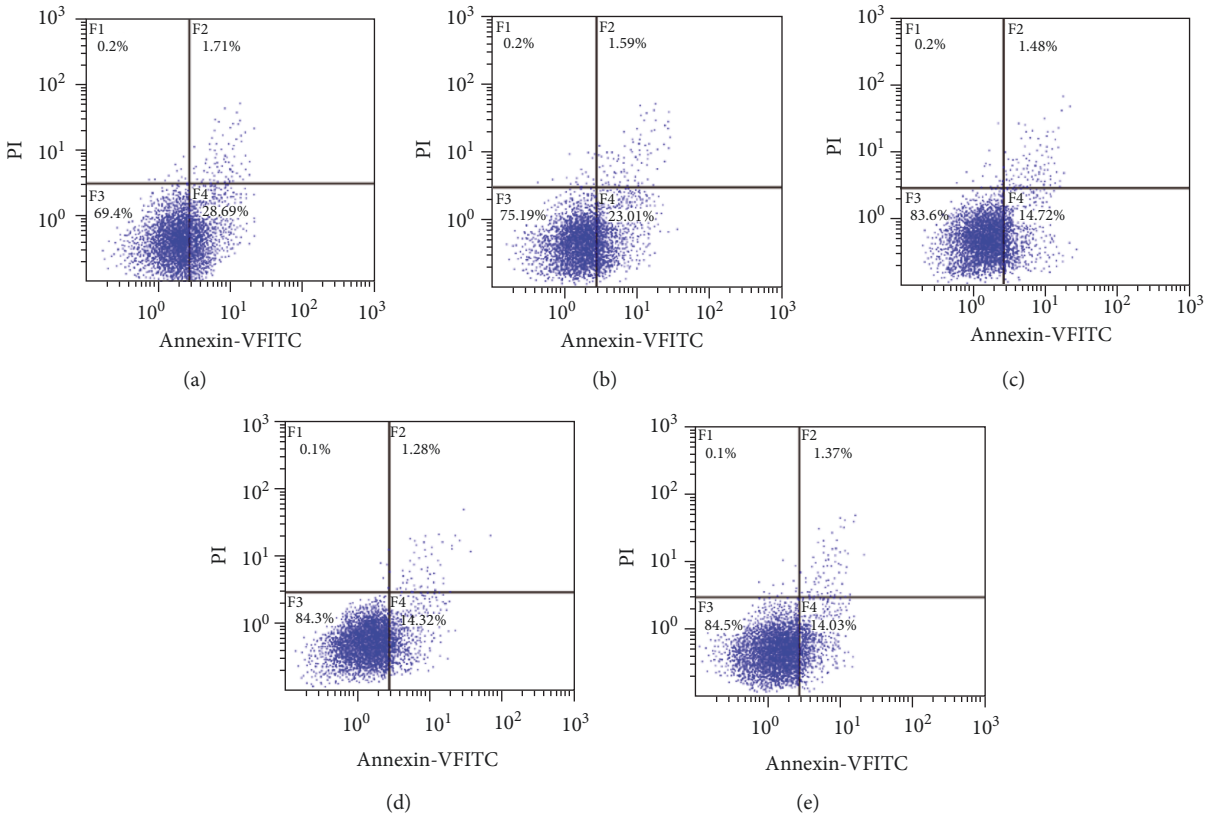


Figure 3: Flow cytometry was used to detect the effect of PSP on apoptosis induced by 200 μM H_2O_2. (a) Apoptosis rate in the control group: 30.4%. (b) Apoptosis rate (24.6%) under the treatment of 0.625 mg/L PSP. (c) Apoptosis rate (16.2%) under the treatment of 1.250 mg/L PSP. (d) Apoptosis rate (15.4%) under the treatment of 2.5 mg/L PSP. (e) Apoptosis rate (15.6%) under the treatment of 5 mg/L PSP.

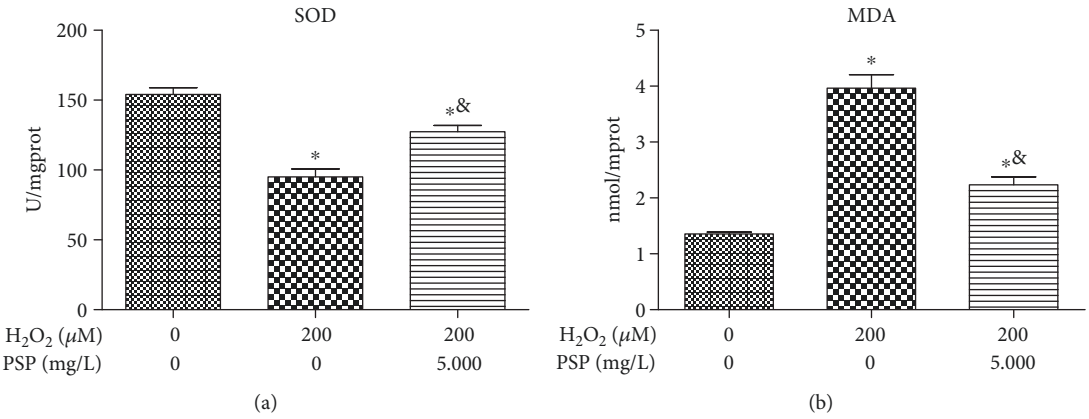


Figure 4: Protective effect of PSP on oxidative stress in rat NPCs. (a) Detection of the intracellular reducing substance SOD. (b) Detection of intracellular oxidation product MDA. $^*P < 0.05$, compared with the blank group. $^{\&}P < 0.05$, compared with the H_2O_2 induction group.

aggravate the oxidative stress of NPCs, promote the progression of IVDD, and form a vicious circle [12]. Under pathological conditions, excessive reactive oxygen species and inflammation were often generated which, in turn, could induce apoptosis of NPCs and were considered to be key targets for the treatment of IVDD [13]. We demonstrated that H_2O_2 could induce higher levels of MDA in NPCs and could increase NPC apoptosis in vitro but PSP treatment could alleviate this change. It has been reported that pretreatment of PSP significantly reduced Aβ(25-35)-induced PC12 cell death, increased Bax/bcl-2 ratio, inhibited mitochondrial dysfunction, and released cytochrome c to the cytosol. In addition, PSP significantly inhibited Aβ(25-35)-induced caspase-3 activation and enhanced phosphorylation of Akt in PC12 cells [1]. This line of evidence suggests that the protective effect of PSP may be related to the upregulation of the PI3K/Akt signaling pathway, which however needs further research and verification. Treatment of NPCs with

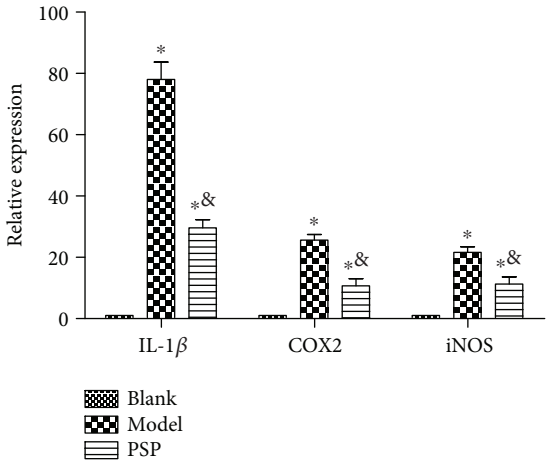


Figure 5: Protective effect of PSP on inflammation of rats with degeneration of the intervertebral disc. $^{*}P < 0.05$, compared with the blank group. $^{\&}P < 0.05$, compared with the model group.

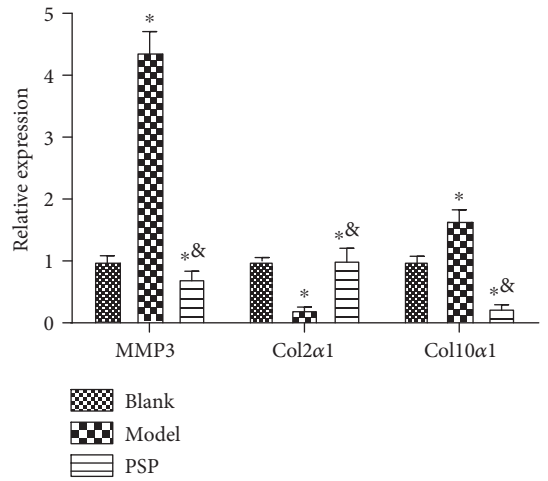


Figure 6: Effect of PSP on the expression of Col2α1, Col10α1, and MMP3 in rats with degeneration of the intervertebral disc. $^{*}P < 0.05$, compared with the blank group. $^{\&}P < 0.05$, compared with the model group.

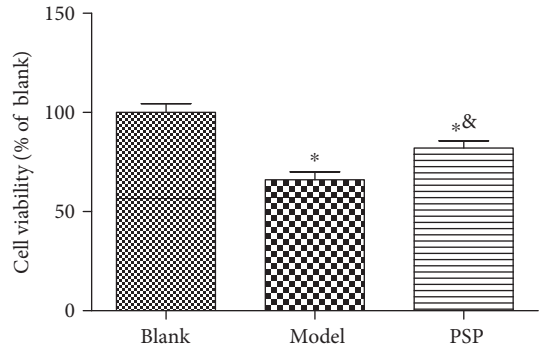


Figure 7: Effect of PSP on the activity of NPCs in rats with degeneration of the intervertebral disc.

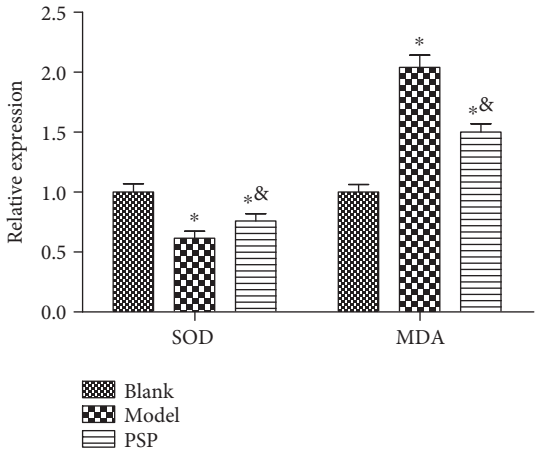


Figure 8: Effect of PSP on oxidative stress in NPCs of rats with degeneration of the intervertebral disc. $^{*}P < 0.05$, compared with the blank group. $^{\&}P < 0.05$, compared with the model group.

PSP prior to H_2O_2 treatment can alleviate oxidative stress suggesting the antioxidant activity of PSP. PSP has the function of regulating the activity of the enzyme and the ability to chelate metal. We have shown a higher SOD content in the PSP treatment group compared with the model group. By constructing a rat model with intervertebral disc degeneration, it was further showed that PSP could effectively improve oxidative stress and mitigate apoptosis of NPCs during intervertebral disc degeneration.

Many studies have also shown that disc degeneration is typically accompanied by a certain degree of inflammation, and inflammation, in turn, promotes the development of the disease [14–16]. It has also been reported that PSP could reduce myocardial inflammatory injury in type I diabetes by inhibiting inflammatory response via the inhibitory effects on the expression of TLR4, MIF, and NF-κB [11]. Our in vivo studies have shown that PSP could inhibit the expression of inflammatory mediators in the intervertebral disc and reduce the expression of Col10α1, as well as increasing the content of Col2α1 in the intervertebral disc by inhibiting the expression of Col2α1 degrading enzyme MMP3.

5. Conclusion

Our in vitro and in vivo experiments confirmed that PSP is effective for the alleviation of oxidative stress and apoptosis in NPCs and lumbar disc degeneration caused by prolonged standing posture. Results from this study showed that PSP is a potential candidate for the treatment of IVDD-related diseases. Based on these findings, we firmly believe that PSP can play an effective role in inhibiting the degeneration of the intervertebral disc and is a promising drug for the treatment and prevention of disc degeneration.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Zhaohui Zhai and Zhaoxin Li contributed equally to this work. Also, Xiaosheng Lu and Zhonglei Ji contributed equally to this work.

Acknowledgments

This work was supported by Shandong Province Medical and Health Science and Technology Development Plan (2018WS062) and Weifang Science and Technology Development Plan (2018YX026).

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