Pretreatment of NPMSCs with Appropriate Concentration of H₂O₂ Enhances its Ability to Treat IVDD

Yuyao Zhang (✉ zhangyuyao@tmmu.edu.cn)

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital <https://orcid.org/0000-0002-6584-0778>

Zhilei Hu

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Yuhan Qi

China Academy of Chinese Medical Sciences

Haiyin Li

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Xian Chang

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Xiaoxin Gao

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Chenhao Liu

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Yueyang Li

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Jinhui Lou

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Yu Zhai

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital

Changqing Li

Third Military Medical University Second Affiliated Hospital: Xinqiao Hospital <https://orcid.org/0000-0002-0029-2336>

Research Article

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Abstract

Background: Nucleus pulposus mesenchymal stem cells (NPMSCs) transplantation is a promising treatment for intervertebral disc degeneration (IVDD). However, the transplanted NPMSCs exhibited weak cell proliferation, high cell apoptosis, and low ability to resist the harsh microenvironment of the degenerated intervertebral disc. There is an urgent need in exploring feasible methods to enhance the therapeutic efficacy of NPMSCs transplantation.

Objective: To identify the optimal concentration for NPMSCs pretreatment with hydrogen peroxide (H_2O_2) and explore the therapeutic efficacy of NPMSCs transplantation using H_2O_2 pretreatment in IVDD.

Methods: Rat NPMSCs were pretreated with different concentrations of H_2O_2 . The proliferation, ROS level, and apoptosis of NPMSCs were detected by CCK-8 test, EdU staining, and flow cytometry *in vitro*. The underlying signaling pathways were explored utilizing western blot. A rat needle-puncture stimulated IVDD model was established. X-ray, histological staining, and multi-mode small animal live imaging system were performed to evaluate the therapeutic effect of H_2O_2 -pretreated NPMSCs *in vivo*.

Results: 75 μM H_2O_2 pretreated NPMSCs demonstrated the strongest elevated cell proliferation by inhibiting the Hippo pathway. Meanwhile, 75 μM H_2O_2 pretreated NPMSCs exhibited significantly enhanced anti-oxidative stress ability, which is related to downregulated Brd4, Keap1, and upregulated Nrf2. 75 μM H_2O_2 pretreated NPMSCs also exhibited distinctly declined apoptosis. *In vivo* experiments results verified that 75 μM H_2O_2 pretreated NPMSCs-transplanted rats exhibited enhanced disc height index (DHI%) and better histological morphology, which means 75 μM H_2O_2 pretreated NPMSCs can better adapt to the environment of degenerative intervertebral discs and promote the repair of IVDD.

Conclusions: Pretreatment with 75 μM H_2O_2 was the optimal concentration to improve the ability of proliferation, anti-oxidative stress, and anti-apoptosis of transplanted NPMSCs, which is expected to provide a new feasible method to improve the stem cell therapy efficacy of IVDD.

Introduction

IVDD is an important cause of low back pain (LBP) and lower extremity dysfunction, which imposes a heavy economic burden on society[1, 2]. The intervertebral disc is an important connection structure between the vertebral bodies that consisted of the nucleus pulposus, the annulus fibrosus, and the upper and lower cartilage endplate, which can maintain the amplitude of spinal motion and withstand mechanical pressure[3]. When the intervertebral disc undergoes gradual degeneration, the number of nucleus pulposus cells and the anabolic processes decrease, resulting in a series of clinical symptoms such as backache, numbness, and lower limb pain[4]. Although surgery is a commonly chosen method for palliating symptoms, it failed to restore the biological and mechanical functions of the intervertebral disc, and may even alter the biomechanical function of the spine, which may lead to further degeneration

of the adjacent intervertebral disc[5, 6]. Therefore, it is necessary to explore new feasible strategies to treat IVDD.

Stem cell transplantation has become a promising therapeutic method utilized to treat IVDD experimentally and clinically in recent years[7, 8]. The intervertebral disc is recognized as an immune-exempt area, and this feature makes it possible to use exogenous stem cells to treat IVDD[9, 10]. Extensive researches have shown that a variety of stem cells can directly differentiate into nucleus pulposus cells, and facilitate the function of degenerative nucleus pulposus cells through a paracrine manner when transplanted into the degenerative intervertebral disc[11–14]. Recent studies have identified NPMSCs with mesenchymal stem cell characteristics in the nucleus pulposus tissue, which possess a stronger ability to differentiate into nucleus pulposus cells and withstand the microenvironment of the intervertebral disc compared with commonly used stem cells such as bone marrow mesenchymal stem cells (BMMSCs) and adipose mesenchymal stem cells (ADMSCs)[15–17]. However, the low viability of transplanted stem cells at the transplant site has become a bottleneck for stem-cell based IVDD therapy. The intervertebral disc is the largest tissue without blood supply in the whole body, and the degenerated intervertebral disc exhibit a harsh microenvironment with low nutrition, low pH, high mechanical pressure, and high osmotic pressure, coupled with distinct oxidative stress and local inflammation. Therefore, the transplanted cells will suffer cell cycle arrest and considerable cell death, which immensely undermine the effect of stem cell transplantation and even lead to transplantation failure[18–20].

A large number of methods have been explored to improve the therapeutic effect of transplanted stem cells in IVDD treatment, including conjunction with biological scaffolds, hydrogel materials, genetic modification, and cellular pretreatment, and pretreatment of stem cells with the mimetic harsh environment (e.g., hypoxia, acid, mechanical intervention) can improve their post-engraftment survival and differentiation characteristics[21–23]. Among them, pretreating transplanted cells with H_2O_2 is convenient to obtain and simple to operate. Previous studies have shown that pretreatment with an appropriate concentration of H_2O_2 can significantly improve the proliferation, paracrine manner, anti-apoptotic ability, and therapeutic effect of MSCs in liver fibrosis, skin wound, and myocardial infarction[24–26]. However, a high concentration of H_2O_2 is cytotoxic and will cause the increase of reactive oxygen species (ROS), which may lead to irreversible oxidative damage to the cells[27, 28]. Therefore, exploring the optimal concentration of H_2O_2 pretreatment is of great significance for improving the effect of NPMSCs in the treatment of IVDD.

In this study, the optimal H_2O_2 concentration for pretreatment of NPMSCs was explored. We found that 75 μM H_2O_2 pretreated NPMSCs demonstrated the strongest elevated cell proliferation, enhanced anti-oxidative stress ability, distinctly declined apoptosis. These effects may be related to the Hippo signaling pathway, Keap1-Nrf2 signaling pathway. *in vivo* experiments verified that 75 μM H_2O_2 pretreated NPMSCs exhibited the strongest therapeutic effects. These results demonstrate that 75 μM H_2O_2 pretreatment of NPMSCs enhances its ability to treat IVDD.

Materials And Methods

Ethical statement

Animal experiments were performed on 8-week-old male Sprague Dawley (SD) rats (n=60) provided by the Animal Center of the Xinqiao Hospital. All experiments were approved by the Ethics Committee of the Army Medical University.

Cell preparation

NPMSCs were extracted from the nucleus pulposus tissue of 30 rats. Briefly, rats were properly anesthetized with 2% pentobarbital (50 mg/kg), and then the caudal vertebrae were isolated after removing the skin, ligaments, and muscle tissues under aseptic conditions. The nucleus pulposus tissue was separated from the caudal intervertebral disk and was then Digested with 0.2% type II collagenase (C2-BIOC, Sigma-Aldrich, USA) for approximately 2 h at 37 °C, followed by centrifugation at 400 g for 5 min at room temperature. The supernatant was discarded and the sediment was suspended and cultured in a standard mesenchymal stem cell growth medium (HUBMX-90011, Guangzhou, China). NPMSCs were placed in a humidified chamber in a 5% CO₂-air mixture at 37 °C. The medium was changed every 3-4 days. Cells were subcultured when they reached a confluence of 80–90%. NPMSCs from the third generations were used throughout the experiment.

Detection of cell surface markers

NPMSCs were digested with 0.25% trypsin (SH30042, HyClone, Logan, USA) and suspended in phosphate buffer solution (PBS) at 5×10^5 cells/ml. Further, 100 µl cell suspension solution was aliquoted per tube and incubated, respectively, with a solution of FITC-conjugated-CD34, CD45, CD73, CD90, and CD105 (all from Bioworld, Minnesota, USA) at room temperature for 30 min. Isotype control was used in each case. After incubation, the cells were washed with PBS for three times, and then centrifuged at 4 °C at 250 g for 5 min. The supernatant was discarded and the cells were re-suspended in 500 µl of PBS prior flow cytometry analyses (Gallios, BECKMAN, Germany).

Detection of multilineage differentiation ability

The multilineage differentiation ability of NPMSCs was detected using an osteogenic differentiation induction kit (RASMIX-90021, Cyagen Biosciences, Suzhou, China), a chondrogenic differentiation induction kit (RASMIX-90041/90042, Cyagen Biosciences, Suzhou, China) and an adipogenic induction differentiation kit (RASMIX-90031, Cyagen Biosciences, Suzhou, China). After 21 days of induction, NPMSCs were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 30 minutes. Then,

Alizarin Red staining, Oil Red O staining, and Alcian Blue staining were performed according to the instructions.

Cell Counting Kit-8 assay

The cell viability of the NPMSCs was detected using cell counting kit-8 (C0038, Beyotime, Shanghai, China). NPMSCs were seeded into 96-well plates at a density of 5×10^3 cells. NPMSCs were able to grow adherent in the plates after one night's culture. Then, H_2O_2 (323381, Sigma-Aldrich, USA) was added to the medium for 12 h at the concentration of 25, 50, 75, 100, 150, 200, 300 μM . The medium was replaced with the fresh medium. After that, 10 μl CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. A spectramax (M2, MOLECULAR DEVICE, USA) was used to detect the optical density (OD) values at a wavelength of 450 nm. Each trial was repeated 3 times.

EdU staining assay

The cell proliferation activity was detected using the EdU-555 cell proliferation detection kit (C0075S, Beyotime, Shanghai, China). The cells were inoculated into 6-well plates at a density of 2×10^5 cells per well. The cells were then treated with H_2O_2 at the concentration of 25, 50, 75, 100, 150, 200, and 300 μM for 12 h. Meanwhile, we designed four treatment methods for further exploration: untreated group, 75 μM H_2O_2 treatment for 12 h, 300 μM H_2O_2 treatment for 12 h, and 75 μM H_2O_2 treatment for 12 h and then 300 μM H_2O_2 treatment for 12 h. 2ml of 10 μM EdU reagent was added to each well and incubated for 2 h. Cells were fixed at room temperature for 15 min with 2 ml 4% paraformaldehyde. 2 ml of immunostaining strong permeability solution (P0097, Beyotime, Shanghai, China) was added to each well and incubated for 15 min. 2 ml of Click Additive Solution was added to each well and incubated for 30 min at room temperature (shield from light). The nuclei were stained with Hoechst 33358 solution (C0021, Solarbio, Beijing, China). Immunofluorescent staining images were captured by the fluorescent microscope (Olympus, Tokyo, Japan).

Western Blot

The protein of NPMSCs was extracted by radio-immunoprecipitation assay (RIPA) solution (P0013B, Beyotime, Shanghai, China) containing 2% phenylmethanesulfonyl fluoride (PMSF) (ST507, Beyotime, Shanghai, China) and 2% phosphatase inhibitor (P1082, Beyotime, Shanghai, China). The protein was then centrifuged at 4 °C and 12,000 g for 5 min to collect the supernatant. Protein concentration was detected by bicinchoninic acid (BCA) protein concentration assay kit (PC0020, Solarbio, Beijing, China). Then protein loading buffer (Beyotime Biotechnology, Shanghai, China, P0015L) was added to each sample and heated in a boiling water bath for 5 min. The protein was separated in 12% sodium salt-polyacrylamide gel, then the protein was transferred to polyvinylidene fluoride (PVDF) membranes (ISEQ00010, Merck Millipore, Darmstadt, Germany). The membranes were soaked with 5% skim milk

powder (P0216, Beyotime Biotechnology, Shanghai, China) for 1 h at room temperature and incubated with corresponding antibodies at 4 °C overnight. After washing with TBS with Tween-20 (TBST), the membranes were immersed by the corresponding horseradish peroxidase-labeled secondary antibody (diluted concentration 1:500) and incubated at room temperature for 1 h. The ECL luminescence kit (1705060, BIO-RAD, CA, USA) and gel imaging system (BIO-RAD, CA, USA) were utilized to visualize protein content. Protein bands were analyzed using Image J software (National Institute of Health, USA). All results were quantified and normalized to β -Actin. The antibodies used are as follows: cyclin D1 (ab134175), P16 (ab51243), β -Actin (ab8227), Cytochrome C (ab133504), Brd4 (ab75898), Keap1 (ab119403) and Nrf2 (ab92946) from Abcam (Cambridge, UK). cleaved-caspase 3 (9664T), p-Lats1 (9157), p-Mst1 (49332), p-YAP (13008T), and YAP (14074T) from CST (Shanghai, China). Bcl-2 (26593-1-AP) and Bax (60267-1-Ig) from Proteintech (Chicago, USA).

Detection of ROS

ROS level in cells was detected using the ROS detection kit (S0033M Beyotime, Biotechnology, Shanghai, China). 2 ml of 10 μ M DCFH-DA (diluted by serum-free medium) was added to each well, and the cells were incubated at 37 °C for 20 min (shield from light). The cells were then washed with the serum-free medium for 3 times to remove the unloaded probes. After that, the cells were collected and the production of ROS was detected by flow cytometry.

Detection of mitochondrial membrane potential

Mito-Tracker Red CMXRos (C1049B Beyotime, Biotechnology, Shanghai, China) was used to detect mitochondrial membrane potential. Briefly, NPMSCs were inoculated into 6-well plates at a density of 2×10^5 cells per well and treated with H_2O_2 at concentrations of 75 and 300 μ M for 24 h, with or without 75 μ M H_2O_2 for 12 h prior to 300 μ M H_2O_2 treatment. Then 1 ml of Mito-Tracker Red CMXRos working solution was added to each well and incubated at 37 °C for 30 min (shield from light). The nuclei were stained with the Hoechst 33358 staining solution. The staining was observed under a laser confocal microscope (LSM880, ZEISS, Germany).

Detection of apoptosis rate

The rate of apoptosis was detected using the Annexin VPE/7-AAD apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA). NPMSCs were collected and resuspended in binding buffer at a concentration of 5×10^6 cells/ml. Then 100 μ l cell suspension was transferred to the test tube and 5 μ l of Annexin VPE and 5 μ l of 7-AAD were added. After incubating at room temperature for 15 min (shield from light), 400 μ l binding buffer was added to each sample and the apoptosis rate was detected by flow cytometry.

Labeling of NPMSCs with green fluorescent protein (GFP)

Lentiviral vectors expressing GFP (GV493, GeneChem, Shanghai, China) were transfected in NPMSCs according to the manufacturer's instructions. The multiplicity of infection (MOI) was set at 20 and the transfection time was set at 18 h. 5 µg/ml puromycin (ST551, Beyotime, Shanghai, China) was used to select stably transfected NPMSCs.

Establishment of IVDD model in rats

30 healthy male SD rats aged 8 weeks were randomly selected for animal experiments. Rats were fasted for 12 h before surgery, and water was forbidden for 4 h before surgery. Then the rats were anesthetized by intraperitoneal injection of 2% pentobarbital (50 mg/kg). The tail skin of rats was sterilized with iodophor for 3 times, and percutaneous intervertebral disc puncture was performed with no. 20 needle at coccyx 6-7 (CO6-7) level[29]. The needle was vertically inserted at a depth of 5 mm and rotated through 360 degrees for 30 seconds before exiting, Surgical areas were then sterilized with iodophor again. Rats were provided with food and water 12 h after the operation to prevent intestinal paralysis.

NPMSCs transplantation in IVDD rats

4 weeks after the operation, X-ray was used to verify if the IVDD models were successfully created. After excluding 3 rats that died accidentally and 3 rats that failed to induce IVDD, the remaining 24 rats with obvious IVDD were randomly divided into 4 groups: Untreated group (6 rats) were treated without any treatment, PBS injection group (6 rats) were injected with 2 µl PBS at CO6-7, normal cells group (6 rats) were injected with labeled NPMSCs (5×10^4 cells, suspended in PBS) at CO6-7, and pretreated cells group were injected with labeled NPMSCs (5×10^4 cells, suspended in PBS) after 75 µM pretreatment for 12 h at CO6-7. PBS and cells were injected through a microsyringe (25 µl, Gaoge, Shanghai, China).

Disc height measurement

A multi-mode small animal live imaging system (Xtremell, BRUKER, Belgium) was used to obtain X-ray images of rats. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital (50 mg/kg), Then the rats were fixed on the tray and put into the apparatus. The parameters were as follows: Modality (X-Ray), Background (None), Illumination (X-Ray), Filters (X-Ray 0.4 mm, KVP 45, Excitation 0, Emission 0), Exposure time (1.2 s), FOV (15 cm), fStop (2), Bin (1×1), Focal Plane (0 mm), Focal Zone (X-Ray). Finally, Image J was used to measure the three longitudinal lines of the intervertebral disc and the two adjacent upper and lower vertebral bodies, and then calculate the DHI% according to the method recorded in previous research[9] to evaluate the degree of IVDD.

Histological analyzes of the caudal intervertebral disc

At 60 days after treatment, the caudal vertebrae of rats were collected. The samples were fixed in 4% paraformaldehyde solution for 48 h, Then, rapid bone tissue decalcification solution (BB-23619, Bestbio, Shanghai, China) was used for decalcification for 2 days. Then the samples were dehydrated with ethanol solutions of different concentrations (75%, 85%, 95% and 100%). Paraffin sections (6 μ m) were prepared and stained with Hematoxylin Eosin staining Kit (G1120, Solarbio, Beijing, China) and Modified Safranin O-Fast Green FCF Stain Kit (G1371, Solarbio, Beijing, China) to evaluate the degree of IVDD in each group. Three observers blindly observed the shape and structure of the intervertebral disc and the type and number of cells in the intervertebral disc and scored using the grading scale (Supplementary Table S1) reported in the previous research[30].

Fluorescence imaging of rats *in vivo*

A multi-mode small animal live imaging system (Xtremell, BRUKER, Belgium) was used to obtain fluorescence images of rats. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital (50 mg/kg), Then we fixed the rats on the tray and put them into the apparatus. The parameters were as follows: Modality (Fluorescence), Background (None), Illumination (Multi-wavelength), Filters (X-Ray 0 mm, KVP 45, Excitation 470, Emission 535), Exposure time (2 s), FOV (15 cm), fStop (2), Bin (2 $\times$ 2), Focal Plane (0 mm), Focal Zone (Tray).

Statistical analysis

All the experiments were repeated three times with at least triplicated samples. The results were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS 23.0 (SPSS Inc. IL, New York, NY, USA). Multiple comparisons were evaluated using one-way or two-way ANOVA followed by Bonferroni's post hoc test. The DHI% data were analyzed by two-way repeated-measures ANOVA followed by Bonferroni's post hoc test. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of NPMSCs

NPMSCs were isolated from the rat caudal intervertebral disc. After 5 days of culture, the primary cells exhibited a short spindle shape. The third generation of NPMSCs showed accelerated and spiral cell growth (Fig. 1a).

Multilineage differentiation ability of NPMSCs was performed in the third generation of NPMSCs. After induction for 21 days, Alizarin Red staining showed the formation of mineralized nodules after osteogenic differentiation induction. Alcian Blue staining was used showed distinct sulfated

proteoglycan staining after chondrogenic differentiation induction. Oil Red O staining showed lipid vacuoles formation after adipogenic differentiation induction. These results suggested that NPMSCs possess the ability of tri-lineage differentiation (Fig. 1b). Flow cytometry results exhibited that the expression of CD73, CD90, and CD105 were positive (more than 95%) and CD34, CD45 were negative (less than 5%) in NPMSCs (Fig. 1c), which was consistent with the standardized cell surface markers of mesenchymal stem cells[31].

75 μM H_2O_2 promoted the proliferation activity of NPMSCs via inhibiting the Hippo signaling pathway

CCK-8 experiment showed that 75 μM and 100 μM H_2O_2 treatments could significantly improve the proliferation of NPMSCs, whereas 300 μM H_2O_2 dramatically inhibited the proliferation ability of NPMSCs (Fig. 2a). The results of EdU staining showed that after 75 μM H_2O_2 treatment, the percentage of EdU positive cells increased significantly, while 300 μM H_2O_2 could reduce the percentage of EdU positive cells (Fig. 2b,2c). Western blot results showed that 75 μM H_2O_2 treatment elevated the expression of cyclin D1 and suppressed the expression of cyclin dependent kinase inhibitor 2A (P16). In comparison, 300 μM H_2O_2 treatment significantly inhibited the expression of cyclin D1 and significantly raised the expression of P16 (Fig. 2d, 2e). These results showed that 75 μM H_2O_2 could promote the proliferation of NPMSCs.

The Hippo pathway is a crucial signaling pathway that can regulate cell proliferation and cell growth[27]. We speculated that H_2O_2 may regulate the proliferation of NPMSCs by inhibiting the Hippo pathway. Western blotting results showed that 75 μM H_2O_2 treatment reduced the phosphorylation levels of key proteins in the Hippo pathway including phosphorylated large tumor suppressor kinase 1 (p-Mst 1), phosphorylated mercaptopyruvate sulfurtransferase-1 (p-Lats1), and phosphorylated Yes1 associated transcriptional regulator (p-YAP) (Fig. 2f, 2g). However, NPMSCs received 300 μM H_2O_2 treatment exhibited an opposite result. These results confirmed our conjecture.

75 μM H_2O_2 pretreatment enhanced the anti-oxidative stress capacity in NPMSCs

The intracellular ROS content was detected under different concentrations of H_2O_2 treatment by flow cytometry. In the pretreatment process, we found that with the elevation of H_2O_2 concentration, the intracellular ROS concentration also increased (Fig. 3a, 3b). However, NPMSCs received 75 μM H_2O_2 pretreatment for 12 h showed significantly lower ROS content when they were exposed to 300 μM H_2O_2 again (Fig. 3c, 3d).

To further verify the protective effect of 75 μM H_2O_2 pretreatment on NPMSCs, the Mito-Tracker Red CMXRos probe was used to detect the mitochondrial membrane potential. The results showed that the

fluorescence intensity of CMXRos in NPMSCs treated with 300 μM H_2O_2 was significantly reduced. But in NPMSCs received 75 μM H_2O_2 pretreatment, the fluorescence intensity was relatively more (Fig. 3e, 3f). In conclusion, these results suggest that NPMSCs pretreated with 75 μM H_2O_2 can protect mitochondria and improve the ability of cells to resist oxidative stress.

75 μM H_2O_2 pretreatment improved the resistance of NPMSCs to oxidative stress by inhibiting Brd4 and activating the Keap1-Nrf2 signaling pathway

To explore the mechanism of enhanced resistance of NPMSCs to oxidative stress by 75 μM H_2O_2 pretreatment, we detected the expression of key proteins in the Keap1-Nrf2 signaling pathway, which can regulate the expression of a variety of antioxidant and anti-inflammatory genes[32]. Western blot results showed that when NPMSCs pretreated with 75 μM H_2O_2 were exposed to 300 μM H_2O_2 , the expression level of Keap1 significantly declined and the expression level of Nrf2 dramatically rose compared to NPMSCs exposed to 300 μM H_2O_2 solely (Fig. 4a, 4b).

As an upstream regulatory molecule of the Keap1-Nrf2 signaling pathway, Brd4 can inhibit the activation of this pathway and elevate the intracellular oxidative stress[33, 34]. We found that Brd4 expression of NPMSCs pretreated with 75 μM H_2O_2 decreased significantly when they were exposed to 300 μM H_2O_2 (Fig. 4c, 4d). Therefore, we speculated that 75 μM H_2O_2 pretreatment could inhibit Brd4 expression and then activate the Keap1-Nrf2 signaling pathway in NPMSCs. Furthermore, we treated the 75 μM H_2O_2 pretreated NPMSCs with a Brd4 inhibitor JQ-1 (13030, MCE, Shanghai, China) and then exposed the cells to 300 μM H_2O_2 . Western blot results showed that the Brd4 expression of cells in JQ-1 treatment group and 75 μM H_2O_2 pretreatment group tremendously decreased. Meanwhile, the expression level of Keap1 was significantly decreased and the expression level of Nrf2 was significantly elevated (Fig. 4e, 4f), which partially verified our speculation.

75 μM H_2O_2 pretreatment enhanced the anti-apoptotic capacity in NPMSCs

We further explored the effect of H_2O_2 pretreatment on the anti-apoptotic ability of NPMSCs. The results of flow cytometry showed that when the concentration of H_2O_2 was less than 150 μM , there was no statistically significant change in the percentage of apoptosis of NPMSCs. The percentage of apoptosis increased when NPMSCs were treated with 200 μM and 300 μM H_2O_2 (Fig. 5a, 5b). However, when NPMSCs received pretreatment with 75 μM H_2O_2 for 12 h, the percentage of apoptotic cells was significantly reduced when they were subsequently exposed to 300 μM H_2O_2 (Fig. 5c, 5d). These results

indicate that pretreatment with 75 μM H_2O_2 for 12 h can significantly improve the anti-apoptotic ability of NPMSCs.

Oxidative stress can lead to mitochondrial damage and mitochondrial apoptosis[35]. Therefore, we examined the effects of different concentrations of H_2O_2 on mitochondrial apoptosis-related proteins. The results showed that 75 μM H_2O_2 treatment exhibited the highest ratio of Bcl-2 to Bax. And over 100 μM H_2O_2 concentration could lead to distinctly increased Cytochrome C. Increased cleaved-caspase 3 levels were observed when the concentrations of H_2O_2 exceeded 150 μM (Fig. 5e,5f). However, compared with 300 μM H_2O_2 treated NPMSCs, NPMSCs pretreated with 75 μM H_2O_2 demonstrated elevated Bcl-2/Bax ratio and declined cleaved-caspase 3 and suppressed Cytochrome C when they were subsequently exposed to 300 μM H_2O_2 (Fig. 5g,5h). These results suggest that pretreatment of NPMSCs with 75 μM H_2O_2 may enhance the ability of cells to resist apoptosis by stabilizing the mitochondrial.

75 μM H_2O_2 pretreatment promoted the therapeutic effects of transplanted NPMSCs in treating IVDD

To explore whether NPMSCs pretreated with appropriate concentration of H_2O_2 can better adapt to the microenvironment of the degenerated intervertebral disc and thus result in ideal therapeutic effects in treating IVDD, A rat IVDD model and GFP-labeled NPMSCs were created. GFP protein was successfully overexpressed in NPMSCs to trace the transplanted cells (Fig. 6a). Next, NPMSCs pretreated with or without 75 μM H_2O_2 were injected into the degenerative intervertebral discs, and X-rays were taken at the 1st, 10th, 30th, and 60th days after transplantation (Fig. 6b). The DHI% of rat caudal intervertebral disc was calculated according to the previously reported calculation method[10] (Fig. 6c). On day 60, we found that both groups with cell transplantation showed an increase in DHI% compared to the untreated group (DHI% = 49.00 ± 1.52) and PBS injection group (DHI% = 46.57 ± 4.00). The treatment effect of the pretreated cell group (DHI% = 90.00 ± 4.55) was better than that of the normal cell group (DHI% = 69.51 ± 3.31) (Fig. 6d).

The histological structure of the NP tissue at 60 days after transplantation was analyzed by HE staining, Safranin O-Fast Green staining, and histological scores of IVDD (Fig. 6e, f). Reduced disc contents and disordered fibrous tissue were observed in the untreated group and the PBS group. Compared to the untreated group, the histological scores of the normal cell group and the pretreated cell group were higher, while the pretreated cell group exhibited the optimal ameliorative outcome.

In vivo fluorescence images results showed that obvious fluorescence could be observed in the caudal intervertebral disc of both groups of rats transplanted with NPMSCs. The fluorescence intensity of the rats injected with NPMSCs pretreated with 75 μM H_2O_2 was higher than that of the normal cells group (Fig. 6g, 6h). These results suggest that 75 μM H_2O_2 pretreatment promoted the therapeutic effects of transplanted NPMSCs in treating IVDD.

Discussion

Although many studies have reported that stem cell transplantation is a feasible method in the treatment of IVDD, the insufficient efficiency of transplantation and low survival rate of graft limit the therapeutic potential of this method to some extent[13, 36]. A high concentration of H_2O_2 can induce a surge of intracellular ROS, which damages the organelles and results in cell cycle arrest and cell apoptosis[27]. However, pretreatment of transplanted cells with H_2O_2 at appropriate concentrations can result in beneficial effects. For example, pretreatment with H_2O_2 enhanced the anti-apoptotic ability of BMMSCs in skin wound[24, 37]. H_2O_2 pretreatment of human umbilical cord mesenchymal stem cells (UCMSCs) or human ADMSCs improved the ability of cells to resist high dose H_2O_2 induced oxidative stress[38, 39]. In this study, we explored whether pretreating NPMSCs with H_2O_2 can improve the therapeutic efficacy of transplanted NPMSCs in IVDD and identify the optimal concentration. We found that 75 μM H_2O_2 pretreated NPMSCs demonstrated the strongest elevated cell proliferation, enhanced anti-oxidative stress ability, and distinctly reduced apoptosis. *In vivo* experiments results verified that 75 μM H_2O_2 pretreated NPMSCs transplantation exhibited distinctly enhanced DHI% and better histological morphology. These results demonstrate that 75 μM H_2O_2 is an optimal concentration in facilitating the therapeutic potential of NPMSCs transplantation in IVDD treatment.

Stem cells reported to treat IVDD include induced pluripotent stem cells (iPSCs), BMMSCs, ADSCs, and NPMSCs, etc.[8, 13, 40]. NPMSCs are derived from nucleus pulposus tissue and are better able to survive in the microenvironment of the intervertebral disc and replenish the number of cells in the intervertebral disc. NPMSCs are stem cells derived from nucleus pulposus tissue which regarded as a more suitable candidate for transplantation in IVDD treatment. NPMSCs can survive better in the microenvironment of the degenerated intervertebral disc and replenish the residual nucleus pulposus cells[15, 40, 41]. Therefore, NPMSCs were selected as research objects in our study. NPMSCs were isolated from the nucleus pulposus tissue of rats. We observed that these cells could proliferate and grow adherent in a cell culture flask. Besides, these cells expressed CD34 and CD45 at low levels and CD73, CD90, and CD105 at high levels, which were consistent with the standardized cell surface markers of mesenchymal stem cells[31]. Furthermore, these cells could undergo tri-lineage differentiation. All of the above characteristics indicated that these cells fit the characteristics of mesenchymal stem cells[31].

To determine the optimal concentration of H_2O_2 for NPMSCs pretreatment, NPMSCs were cultured with different concentrations of H_2O_2 for 12 h according to the previous article reports[24]. Firstly, the cell viability and cell proliferation of NPMSCs were detected. We found that 50 μM , 75 μM , and 100 μM H_2O_2 promoted the cell viability and cell proliferation of NPMSCs, when the 200 μM H_2O_2 and 300 μM H_2O_2 inhibited the cell viability and cell proliferation of NPMSCs. Among the designated concentrations, 75 μM H_2O_2 treated NPMSCs exhibited the highest facilitating effects. Moreover, the expression of cyclin D1, reported as one of cell proliferation facilitators[42, 43], as well as P16, a well-known cell cycle repressor[44], were detected to consolidate our results. Consistent with the above observations, we found that 75 μM H_2O_2 treated NPMSCs exhibited maximized expression of cyclin D1 and lowest expression of

P16, whereas the expression of cyclin D1 was decreased and the expression of P16 was elevated to the greatest extent in 300 μM H_2O_2 treated NPMSCs. Therefore, 75 μM H_2O_2 was selected as the pretreatment concentration and 300 μM H_2O_2 was selected as the concentration to induce inhibition of cell proliferation in subsequent experiments.

The Hippo pathway plays an important role in regulating stem cell proliferation and apoptosis[45, 46]. Previous studies have shown that the activated Hippo pathway can inhibit cell proliferation and promote cell apoptosis of the intervertebral disc cells[47-50]. Moreover, the inhibition of the Hippo pathway is closely related to the H_2O_2 induced BMMSCs proliferation upregulation and apoptosis downregulation[51]. Therefore, the expression of key proteins in the Hippo pathway were detected to explore the mechanism of H_2O_2 in promoting NPMSCs proliferation. We found that 75 μM H_2O_2 treatment significantly reduced the phosphorylation of Lats1, Mst1, and YAP, indicating that the Hippo pathway was inhibited. However, when NPMSCs received 300 μM H_2O_2 pretreatment, the phosphorylation of key proteins in this pathway decreased, indicating that the 75 μM H_2O_2 pretreatment may promote NPMSCs proliferation via inhibiting the Hippo pathway to some extent.

Oxidative stress is one of the key factors leading to cell damage in the intervertebral disc. ROS generated by intracellular metabolism can cause mitochondrial homeostasis loss and induce cell death[52-54]. We found that with increasing H_2O_2 concentration, the intracellular ROS content increased gradually.

However, we found that 75 μM H_2O_2 pretreated NPMSCs exhibited a distinct suppressed ROS level and upregulated mitochondrial membrane potential when they were supplemented with subsequent 300 μM H_2O_2 . These outcomes indicate that NPMSCs can activate their own antioxidant system after pretreatment and thus reducing the content of ROS in cells when they encounter detrimental 300 μM H_2O_2 . The Keap1-Nrf2 signaling pathway is the most important antioxidant system in cells. Activated Nrf2 can promote the expression of antioxidant factors such as NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase (SOD), etc.[35, 55, 56]. Previous studies have shown that activating the Keap1-Nrf2 signaling pathway could protect intervertebral disc cells from the oxidative stress injury[57, 58]. Therefore, we then explored whether the declined ROS and raised mitochondrial membrane potential were associated with the activation of the Keap1-Nrf2 signaling pathway. Our results found that that 75 μM pretreated H_2O_2 exhibited enhanced expression of Nrf2, and reduced expression of Keap1 when they were supplemented with subsequent 300 μM H_2O_2 . Interestingly, when NPMSCs were treated with 75 μM of H_2O_2 , Brd4, an upstream regulator of the Keap1-Nrf2 signaling pathway, was also decreased. Therefore, we hypothesized that the 75 μM H_2O_2 pretreatment might also regulate Brd4. Subsequently, we treated NPMSCs with the Brd4 inhibitor JQ-1. JQ-1 treated NPMSCs and 75 μM H_2O_2 pretreated NPMSCs exhibited similar change trends in the expression level of Brd4, Keap1, and Nrf2, which partially confirmed our hypothesis.

Meanwhile, the apoptotic rate of NPMSCs under different H_2O_2 concentrations was detected. We found that 75 μM H_2O_2 treated NPMSCs exhibited the lowest cell apoptotic rate, while 300 μM H_2O_2 treated

NPMSCs exhibited the highest cell apoptotic rate. Moreover, 75 μM H_2O_2 pretreated NPMSCs exhibited a distinct suppressed cell apoptotic rate when they were supplemented with subsequent 300 μM H_2O_2 . The ratio of anti-apoptotic protein Bcl-2 to pro-apoptotic protein Bax is an important indicator of the degree of apoptosis mediated by the mitochondrial pathway[59]. The level of Cytochrome C is the gold standard for mitochondrial damage[60]. Therefore, the expression levels of Bcl-2, Bax, apoptotic marker cleaved-caspase 3, and Cytochrome C were detected. We found that the 75 μM H_2O_2 treated NPMSCs demonstrated the highest ratio of Bcl-2 to Bax, and the lowest leaved-caspase 3 and Cytochrome C levels. The NPMSCs received 300 μM H_2O_2 demonstrated the lowest ratio of Bcl-2 to Bax, and the highest leaved-caspase 3 and Cytochrome C levels. However, NPMSCs supplemented with 300 μM H_2O_2 exhibited an elevated ratio of Bcl-2 to Bax, reduced leaved-caspase 3 and Cytochrome C levels if they were pretreated with 75 μM H_2O_2 , which is consistent with the results above. These beneficial effects may also partly be attributed to the effects of H_2O_2 on the Hippo pathway and Keap1-Nrf2 pathway. Further research will illuminate the underlying mechanisms that H_2O_2 pretreatment regulates NPMSCs apoptosis. The signaling pathway involved in the H_2O_2 in regulating cell proliferation, oxidative stress, and cell apoptosis were summarized in Figure 7.

Based on the above *in vitro* study, we determined that 75 μM H_2O_2 pretreatment may maximize the proliferation and anti-apoptotic ability of NPMSCs. Therefore, we further explored whether 75 μM H_2O_2 pretreatment NPMSCs could improve the therapeutic efficacy of NPMSCs transplantation in a rat IVDD model. As expected, compared to rats treated with normal cells alone, rats injected with 75 μM H_2O_2 pretreated NPMSCs showed increased transplanted cells survival, facilitated extracellular matrix deposition, and a distinct improvement in DHI% during a 60-day observation. Therefore, these results confirmed that NPMSCs pretreated with 75 μM H_2O_2 is an optimal concentration for NPMSCs pretreatment before transplantation. Notably, the optimal concentration of H_2O_2 pretreatment was slightly higher than that of previous studies[61, 62], This discrepancy may be attributed to the intrinsic feather of NPMSCs, which reside in a microenvironment of high mechanical pressure, low oxygen supplement, high lactic acid concentration. The NPMSCs may innately possess a certain resistance to H_2O_2 .

There are several limitations in our experiment. Firstly, our experiment directly selected the time of H_2O_2 pretreatment reported before, thus the relationship between the therapeutic effects of H_2O_2 and pretreatment time was not explored. Time gradient experiment will further explore the optimal invention time to maximize the facilitating effects of H_2O_2 in the future. In addition, the underlying mechanisms that H_2O_2 pretreatment regulates the Hippo signaling pathway and Keap1-Nrf2 signaling pathway were not investigated in our current experiments, further molecular-level understanding of the experimental observations need to be explored in the future. However, the present experiments have proved the beneficial effects in 75 μM H_2O_2 in NPMSCs pretreatment. Given that this method is convenient and efficient, the H_2O_2 pretreated NPMSCs transplantation may become a feasible method in treating IVDD clinically in the future.

Conclusions

In summary, we identified for the first time that pretreatment with 75 μM H_2O_2 can better promote cell proliferation, reduce oxidative stress and cell apoptosis in NPMSCs *in vitro*, which is related to the regulation of the Hippo signaling pathway and Keap1-Nrf2 pathway. NPMSCs pretreated with 75 μM H_2O_2 also exhibited the most satisfactory therapeutic effects when transplanted in the rat degenerated caudal intervertebral disc. These results demonstrate that 75 μM H_2O_2 is an optimal concentration in NPMSCs pretreatment, which may serve as a feasible method for the clinical application of NPMSCs-based therapy in treating IVDD in the future.

Abbreviations

IVDD: intervertebral disc degeneration; NPMSCs: nucleus pulposus mesenchymal stem cells; μM : $\mu\text{mol/ml}$; h: hour; min: minute; H_2O_2 : hydrogen peroxide; Brd4: bromodomain containing 4; Keap1: kelch-like ECH-associated protein 1; Nrf2: nuclear factor, erythroid derived 2, like 2; LBP: low back pain; BMMSCs: bone marrow mesenchymal stem cells; SD rats: Sprague Dawley rats; PBS: phosphate buffer solution; OD: optical density; RIPA: radio-immunoprecipitation assay; PMSF: phenylmethanesulfonyl fluoride; BCA: bicinchoninic acid; PVDF: polyvinylidene fluoride; TBST: TBS with Tween-20; ROS: reactive oxygen species; GFP: green fluorescent protein; MOI: multiplicity of infection; CO6-7: coccyx 6-7; SD: standard deviation; ANOVA: analysis of variance; UCMSCs: umbilical cord mesenchymal stem cells; ADMSCs: adipose mesenchymal stem cells; iPSCs: induced pluripotent stem cells; P16: cyclin dependent kinase inhibitor 2A; p-Lats 1: phosphorylated large tumor suppressor kinase 1; p-Mst 1: phosphorylated mercaptopyruvate sulfurtransferase-1; p-YAP: phosphorylated Yes1 associated transcriptional regulator; YAP: Yes1 associated transcriptional regulator; TAZ: tafazzin; ARE: antioxidant response elements; NQO1: NAD(P)H quinone dehydrogenase 1; SOD: superoxide dismutase.

Declarations

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Authors' contributions

ZYY and LCQ conceived and designed the experiments; ZYY and HZL performed the experiments; GXX and LCH participated in the creation of the experimental animal model. LHY and CX revised the article and conducted the experiments complementation; ZY and LYY analyzed the data; ZYY and LJH edited the figures, LCQ polished the language. ZYY, ZY, and QYH wrote this article. LCQ and ZY reviewed and edited the article.

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Availability of data and materials

All the data supporting the results were shown in the article and can be applicable from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethics approval was obtained with the informed consent of all participants. The Ethics Committee of the Army Medical University approved this research.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures

Figure 1

Identification of NPMSCs. (a) Representative image illustrating the morphology of NPMSCs. Scale bar = 200 μm . (b) Alizarin Red staining, Alcian Blue staining, and Oil Red O staining results of NPMSCs at 21 days post induction. Scale bar = 50 μm . (c) Flow cytometry examining the expression of CD34, CD45, CD73, CD90 and CD105 on the NPMSCs.

Figure 2

Effects of different H_2O_2 concentrations on NPMSCs proliferation. (a) The effect of H_2O_2 on NPMSCs proliferation detected by the CCK-8 assay. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). (b, c) Detection of proliferated NPMSCs (red) under different H_2O_2 concentrations by the EdU-555 proliferation assay kit and quantification. Nuclei were counterstained with Hoechst 33358 (blue). (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). Scale bar =200 μm . (d, e) Western blot analysis of cyclin D1 and P16 expressions in NPMSCs exposed to different concentrations of H_2O_2 for 12 h (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). (f, g) Western blot analysis of p-Lats1, p-Mst1, p-YAP, and YAP expressions in NPMSCs exposed to different concentrations of H_2O_2 for 12 h. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test).

Figure 3

Effect of different H_2O_2 concentration H_2O_2 on ROS and mitochondrial membrane potential in NPMSCs. (a, b) The intracellular ROS in NPMSCs received different H_2O_2 concentration detected by flow cytometry and quantifications. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). (c, d) The intracellular ROS in NPMSCs received 75 μM H_2O_2 pretreatment detected by flow cytometry and quantifications. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). (e, f) The mitochondrial membrane potential of NPMSCs received 75 μM H_2O_2 pretreatment detected by Mito-tracker Red CMXRos and quantifications. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test, ## P< 0.01 compared to the 300 μM H_2O_2 group). Scale bar = 50 μm .

Figure 4

Effect of different H₂O₂ concentrations on the Keap1-Nrf2 signaling pathway in NPMSCs. (a, b) Western blot analysis of Keap1 and Nrf2 expressions in NPMSCs exposed to different concentrations of H₂O₂. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test. # P< 0.05 compared to the 300 μ M H₂O₂ group). **(c, d)** Western blot analysis of Brd4 expressions in NPMSCs exposed to different concentrations of H₂O₂. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test. # P< 0.01 compared to the 300 μ M H₂O₂ group). **(e, f)** Western blot analysis estimating the effect of 75 μ M H₂O₂ pretreatment on Keap1, Nrf2, and Brd4 expression and quantification when NPMSCs were subsequently exposed to 300 μ M H₂O₂ (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test. ## P< 0.01 compared to the 300 μ M H₂O₂ group).

Figure 5

Effect of different H₂O₂ concentrations on the anti-apoptotic capacity of NPMSCs. (a, b) Effect of different H₂O₂ on the apoptosis ratio detected by flow cytometry and quantification. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). **(c, d)** Effect of 75 μ M H₂O₂ pretreatment on the apoptosis ratio detected by flow cytometry and quantification when NPMSCs were subsequently exposed to 300 μ M H₂O₂ (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test. ## P< 0.01 compared to the 300 μ M H₂O₂ group). **(e, f)** Western blot analysis estimating the effect of different H₂O₂ on Bcl-2, Bax, cleaved-caspase 3, and Cytochrome C expression and quantification. (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test). **(g, h)** Western blot analysis estimating the effect of 75 μ M H₂O₂ pretreatment on Bcl-2, Bax, cleaved-caspase 3, and Cytochrome C expression and quantification when NPMSCs were subsequently exposed to 300 μ M H₂O₂ (n=3, *P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test. # P<0.05, ## P< 0.01 compared to the 300 μ M H₂O₂ group).

Figure 6

75 μ M H₂O₂ pretreatment facilitated the therapeutic effects of transplanted NPMSCs in treating IVDD. (a) GFP protein was overexpressed in NPMSCs utilizing lentiviral vectors to trace the transplanted cells. Scale bar = 200 μ m. **(b)** Representative X-rays images were taken on the 0, 1st, 10th, 30th, and 60th days after injection. **(c)** Methods of calculating DHI%. **(d)** Quantification of DHI% in rats on designated days after treatment. (n=6, two-way repeated-measures ANOVA followed by Bonferroni's post hoc test. **P<0.01, ## P<0.01, compared to the normal cells group). **(e)** Representative HE staining and Safranin O-Fast Green staining illustrating the histological structure of the NP tissue at 60 days after treatment. Scale bar = 500 μ m. **(f)** Histological score of the intervertebral disc in each group (n=3, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test, # P<0.05, compared to the normal cells group). **(g, h)** 60

days after treatment, the fluorescence intensity of GFP-labeled NPMSCs in intervertebral discs was detected by the multi-mode small animal live imaging system (n=3, **P<0.01, one-way ANOVA followed by Bonferroni's post hoc test, ## P<0.01, compared to the normal cells group).

Figure 7

Signaling pathway involved in the H₂O₂ in regulating cell proliferation, oxidative stress, and cell apoptosis in NPMSCs. Optimal concentration of H₂O₂: 75 μM; The lethal concentration of H₂O₂: 300 μM. Up arrow: upregulation. Down arrow: downregulation.

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