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Hydrogen sulfide regulates autophagy in nucleus pulposus cells under hypoxia

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Abstract

Objective: Hydrogen sulfide (H_2S) has been found to act as an important gasotransmitter to regulate cell activities. This study aimed to investigate the effect of H_2S on autophagy of nucleus pulposus (NP) cells under hypoxia and possible mechanism.

Materials and Methods: NP cells were isolated from rat caudal discs. Cobalt chloride was used to mimic hypoxia, sodium hydrosulfide was used to emulate exogenous H_2S and 3-methyladenine was used to block cell autophagy. Cell viability was assessed by phase contrast microscope and Cell Counting Kit-8 method. Moreover, expression of key autophagic proteins was analyzed via western blotting, and transmission electron microscopy was performed to detect autophagosomes.

Results: Hypoxia markedly impaired NP cell proliferation compared with control. Whereas H_2S provided pro-proliferation and pro-autophagy effects on hypoxic NP cells. However, these beneficial impact of H_2S on hypoxic NP cells were reversed by autophagy inhibitor.

Conclusions: Our results showed that H_2S played a cytoprotective role in NP cells exposed to hypoxia in an autophagy-dependent manner.

KEYWORDS
autophagy, hydrogen sulfide, nucleus pulposus

1 | INTRODUCTION

Intervertebral disc degeneration (IVDD) is the major contributor to a variety of spinal disorders but its underlying mechanism has not yet been fully clarified.[1] IVDD is typically characterized by progressive decline in disc nutrient supply and alterations in extracellular matrix (ECM) composition, which weakens the mechanical strength of tissue and alters the microenvironment.[2] The gelatinous core of disc, nucleus pulposus (NP), produces ECM and plays a critical role in maintaining the integrity of intervertebral discs. Growing evidence suggests that aberrant NP cell activities are key to IVDD

pathogenesis.[3,4] NP cells physiologically locate in hypoxic environment and autophagy activity is essential to the survival of NP cells under hypoxia.[5]

After nitric oxide and carbon monoxide, hydrogen sulfide (H_2S), a kind of colorless gas with unpleasant smells, became the third gaseous signal molecule in mammals. The effects of H_2S on the cell biological activities have been investigated in various cell scenarios.[6] Our previous work has shown that H_2S may have beneficial effects on survival of NP cells by attenuating hypoxia-induced apoptosis.[7] However, the effect of H_2S on autophagy activity remains debated because it may be dose-dependent, time-dependent, and cell-type-dependent,[8] and

to date there are no studies assessing the effect of H_2S on hypoxic NP cells. This study aimed to illustrate the role of H_2S on NP cell survival under hypoxia and its underlying mechanism.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture

Four-week-old male Wistar rats (225-250 g) were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (100 mg/kg). The gelatinous NP tissue was isolated under a microscope, washed with phosphate buffered saline (PBS, Keygen, China), and digested with trypsin (2.5 g/L) at 37°C for 10 to 15 minutes. Subsequently, 10% fetal bovine serum (FBS, Gibco, USA) medium was used to terminate digestion. Centrifugation was performed to extract the precipitate,

and 0.1% type II collagenase (Sigma, USA) was added for shaking digestion at 37°C for 6 hours. After filtration and centrifugation, Dulbecco's Modified Eagle Medium/Ham's F-12 medium containing 20% FBS and 1% cyan streptomycin mixture (Keygen, China) was added to prepare cell suspension. The cell suspension was inoculated into a 35-mm Petri dish at 1×10^5 cells/mL, and then cultured in an incubator with saturated humidity and 5% CO_2 at 37°C. Cells at the third passage were used in the following experiments. The study was approved by animal ethics committee of Peking University First Hospital.

2.2 | Concentration determination

Due to dose-dependent effect of the treatments (cobalt chloride [$CoCl_2$], NaHS, and 3-methyladenine [3-MA]), optimal concentration
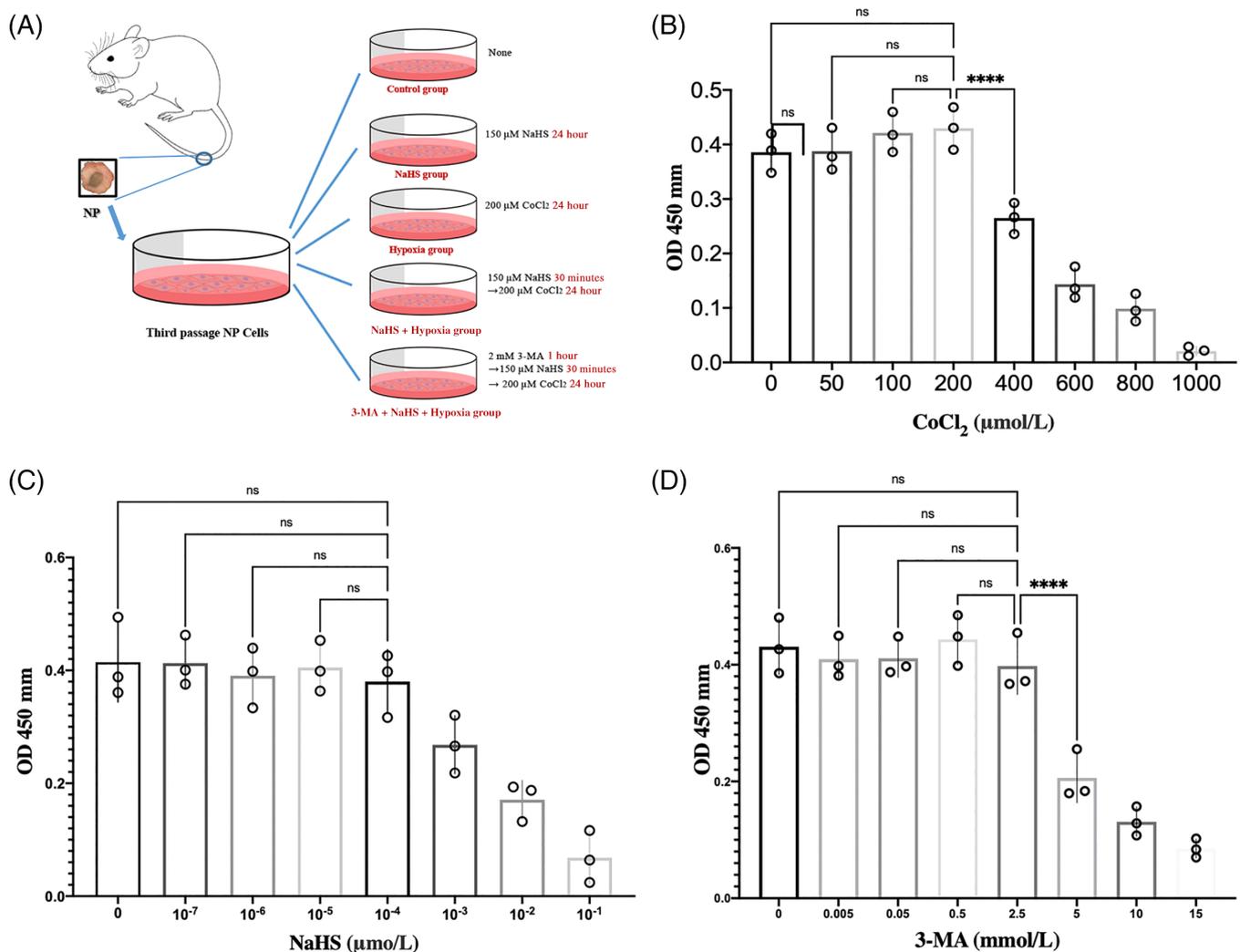

FIGURE 1 Cell grouping and determination of optimal concentration. (A) Isolation of rat nucleus pulposus cells from caudal disc and cell groupings according to different treatments of the third-passage NP cells; (B-D) The optimal concentration of different treatments was determined after 2 hours of different treatments by CCK-8 assay, n = 3 replicates per group. ****$P < .0001$ by one-way ANOVA test followed by Tukey's post hoc. 3-MA, 3-methyladenine; $CoCl_2$, cobalt chloride; NaSH, sodium hydrosulfide; NP, nucleus pulposus; OD, optical density. Appendix S2 provided the numeric values of all the bar graphs

of treatments was determined by gradient concentration by utilizing Cell Count Kit-8 (CCK-8) assay. After different treatments, third-passage NP cells (1×10^5/mL) were seeded in triplicate into 96-well plates and allowed to attach for 2 hours. After PBS washing, 100 μL medium and 10 μL CCK-8 working solution (Keygen, China) were applied into each well at 37°C for 2 hours. The optical density (OD) value of each well was detected at 450 nm via Microplate Reader (Bio-Rad, Hercules, California). Each assay was biologically replicated three times.

2.3 | Cell subgrouping and viability assay

When the confluence of third-passage rat NP cells was close to 60% to 70%, the NP cells were inoculated into five new petri dishes (1×10^5 cells/mL) as different groups: Group A (control), Group B (NaHS), Group C (Hypoxia), Group D (NaHS + hypoxia), and Group E (3-MA + NaHS + hypoxia) (Figure 1). Phase contrast microscope was used to observe cell viability of different groups daily in the 4 days following different treatments.

CCK-8 was also used to assess cell viability of different groups. The protocol was similar to that of concentration determination method, and the only difference was that cells seeded into 96-well plates attached for 24 hours before adding the working solution. CCK-8 assay was biologically replicated three times.

2.4 | Transmission electron microscopy

NP cells were fixed with 1% glutaraldehyde for 2 hours and 1% osmic acid for 20 minutes at 4°C. The cells were then dehydrated by acetone gradient, embedded with epoxy embedding medium (Sigma-Aldrich, USA). Ultrathin sections were cut, stained with 4% uranium acetate and lead citrate and observed under a transmission electron microscope (TEM) (Jeol JEM-1230, Japan).

2.5 | Western blotting

NP cells were lysed in RIPA buffer (Keygentec, China), and total protein concentration was measured using the BCA protein assay kit (Keygentec, China). Same amount of total protein (40-80 μg) from cell lysate was loaded to each well. Protein extract was electrophoresed through 5% to 12% Bis-Tris gel, then transferred to nitrocellulose membrane (NC membrane, Applygen, China). Membranes were washed in Tris-Buffered Saline + Tween 20 (TBST) and then blocked in 5% skimmed milk (Sigma, USA) for 1 hour at room temperature to prevent non-specific protein binding. The membrane was then incubated with primary antibodies, including rabbit anti-rat LC3B (1:1000, L7543, Sigma-Aldrich, USA), rabbit anti-rat Beclin-1 (1:1000, #3495, Cell Signaling Technology, USA), rabbit anti-rat Bcl-2 (1:1000, #3498, Cell Signaling Technology, USA), rabbit anti-rat Col2A1 (1:1000,
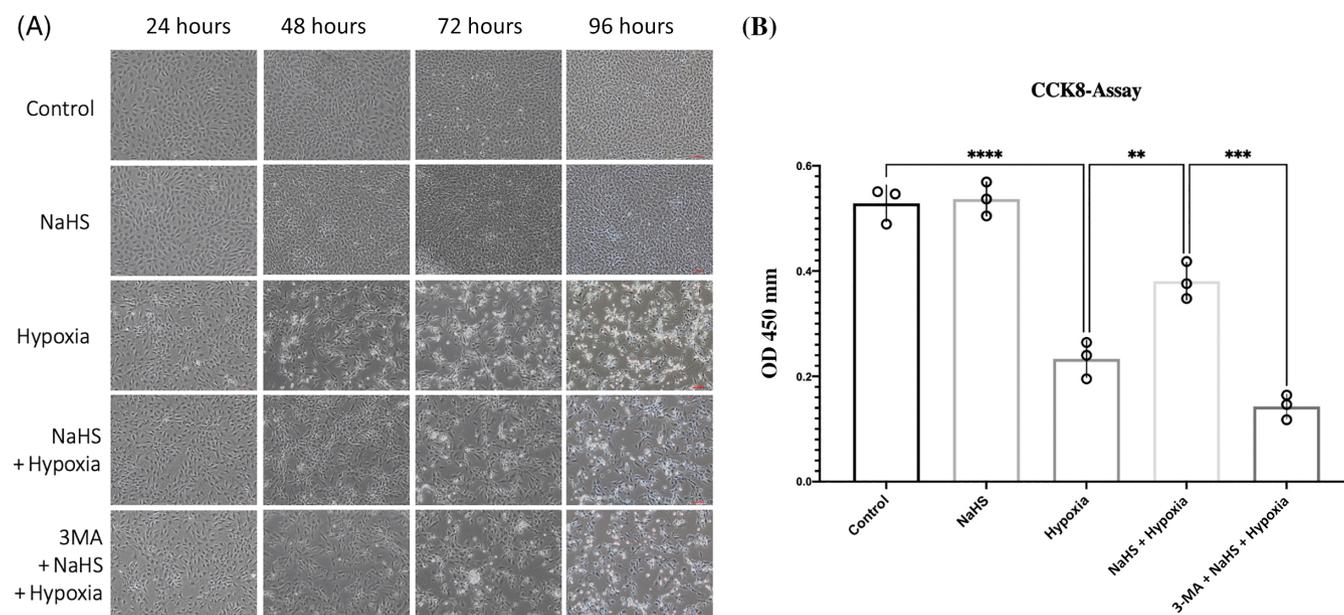

FIGURE 2 Effect of hypoxia, NaHS, and autophagy inhibitor on viability, proliferation and apoptosis of nucleus pulposus cells. (A) Cell proliferation of 4 days in different groups observed under phase-contrast microscope, necrosis was defined as collapse of the plasma membrane and lysis of the cells[33]; (B) CCK-8 assay of NP cells was counted after 24 hours of different treatments. *P < .05 vs the control group, n = 3 replicates per group. **P < .01 by one-way ANOVA test followed by Tukey's post hoc; ***P < .001 by one-way ANOVA test followed by Tukey's post hoc; ****P < .0001 by one-way ANOVA test followed by Tukey's post hoc. 3-MA, 3-methyladenine; CoCl$_2$, cobalt chloride; NaSH, sodium hydrosulfide. Appendix S2 provided the numeric values of the bar graph

ab34712, abcam, UK), rabbit anti-rat β-actin (1:1000, ab8226, abcam, UK), and rabbit anti-rat GAPDH (1:500, sc-47724, Santa Cruz, USA), at 4°C overnight with gentle agitation. Western blots were visualized with HRP-conjugated goat anti-rabbit secondary antibody (1:8000, sc-2004, Santa Cruz, California) for 1 hour, and then through ECL chemiluminescent reagents (Millipore, USA) in a luminescent image analyzer GBOX CHEMI XT4 gel image analysis system (Syngene, Synoptics Group, USA). The results were analyzed using ImageJ software (Rawak Software Inc., Stuttgart, Germany). Western blotting assay was biologically replicated three times.

2.6 | Statistical analysis

All results are presented as means ± SD of three independent experiments. Differences between groups were assessed by the one-way ANOVA with Tukey post hoc analysis. A value of $P < .05$ was considered statistically significant. Statistical results were processed and plotted by GraphPad Prism 9 (GraphPad Software Inc., San Diego, California).

3 | RESULTS

3.1 | Optimal concentration of treatments determined by CCK-8 assay

The final concentration of 200 μmol/L of $CoCl_2$ was used as hypoxia inducing concentration, 150 μmol/L of NaHS was used to emulate exogenous hydrogen sulfide and 2 mmol/L of 3-MA was applied to block autophagy, similar to results of previously studies.[9-11] The above-mentioned treatments did not significantly alter the phenotype
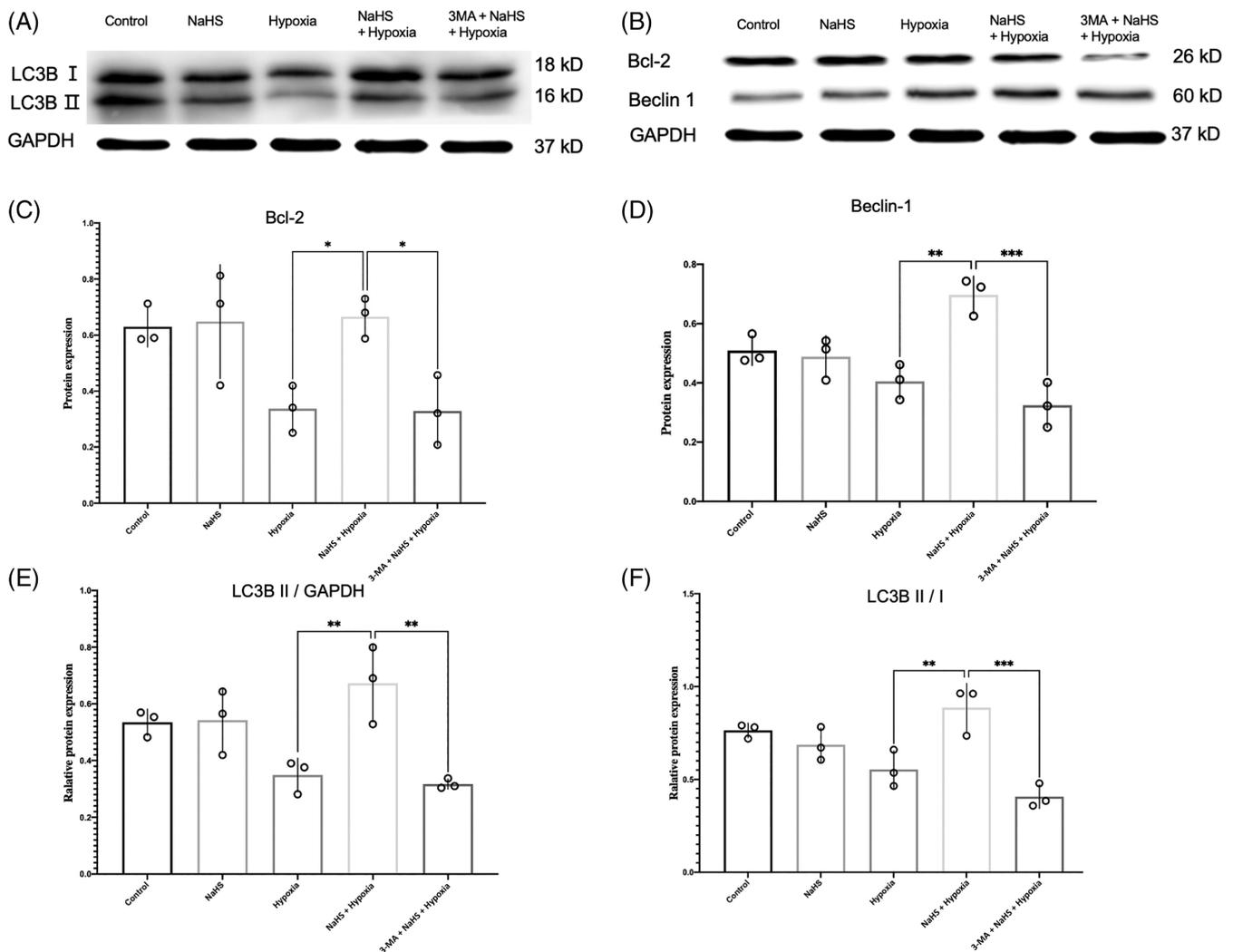

FIGURE 3 Expression of key autophagy markers in nucleus pulposus cells. Western blot analysis and density measurements. The expression level of (C) Bcl-2 (n = 3 replicates per group), (D) Beclin-1 (n = 3 replicates per group.), (E) ratio of LC3B II/GAPDH (n = 3 replicates per group.) and (F) ratio of LC3B II/LC3B I (n = 3 replicates per group) in hypoxia group was insignificantly lower than control group and significantly lower than NaHS + hypoxia group. The ratio was lowest in 3-MA + NaHS + hypoxia group. *$P < .05$ by one-way ANOVA test followed by Tukey's post hoc; **$P < .01$ by one-way ANOVA test followed by Tukey's post hoc; ***$P < .001$ by one-way ANOVA test followed by Tukey's post hoc. 3-MA, 3-methyladenine; $CoCl_2$, cobalt chloride; NaHS, sodium hydrosulfide. Appendix S2 provided the numeric values of all the bar graphs

of NP cells as all groups expressed similar Col2A1 detected by western blotting (see Appendix S1). Determination of optimal concentration of treatments by gradient concentration was shown in Figure 1.

3.2 | NaHS rescued NP cell proliferation under hypoxia condition

Under phase contrast microscope, NaHS did not pose significant effect on cell proliferation under normoxia, whereas decreased cell population and increased cell death were observed in hypoxia group during the 4 days. However, NaHS enhanced cell proliferation and reduced cell death of NP cells under hypoxia (Figure 2A). These differences were further validated by CCK-8 assay at 24 hours of different treatments. Hypoxia group manifested significantly lower OD value compared with control group (0.233 ± 0.035 vs 0.529 ± 0.034, $P < 0.0001$) and NaHS + hypoxia group (0.233 ± 0.035 vs 0.381 ± 0.035, $P < 0.01$), respectively (Figure 2B).

3.3 | Autophagic inhibitor reversed the protective effects of NaHS under hypoxia

When NP cells were preincubated with 3-MA before NaHS + hypoxic culture, decreased cell population and increased cell necrosis were noticed under microscope during observation compared with NaHS + hypoxia group. Similarly, CCK-8 assay showed that the OD value of 3-MA + NaHS + hypoxia group was significantly lower than that of the NaHS + hypoxia group (0.143 ± 0.024 vs 0.381 ± 0.035, $P < 0.0001$) (Figure 2B).
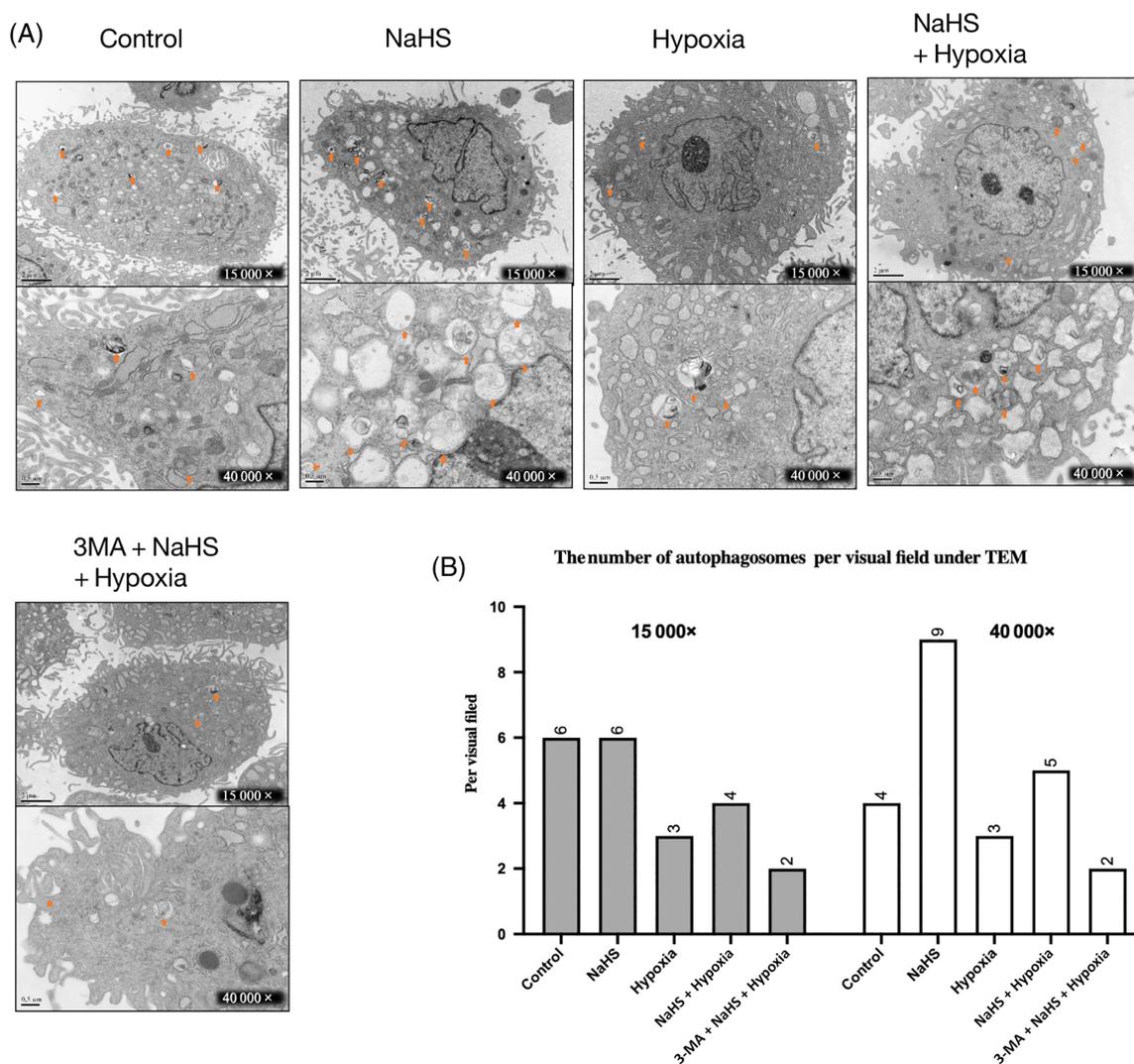

FIGURE 4 Autophagosome detection under TEM. (A) The number of autophagosomes (double-membrane sequestering vesicles, marked by arrows) of nucleus pulposus cells in hypoxia group was attenuated compared with that of control group. The number of autophagosomes in NP cells of NaHS + hypoxia group was higher than in hypoxia group. The number of autophagosomes of NP cells in 3-MA + NaHS + hypoxia group was the lowest among all groups (Scale bar: 2 μm in A1, B1, C1, D1, E1 and 0.5 μm in A2, B2, C2, D2, E2); (B) Bar chart of semi-quantification of autophagosomes of different groups of cells of random visual fields under TEM. 3-MA, 3-methyladenine; CoCl$_2$, cobalt chloride; NaSH, sodium hydrosulfide; TEM, transmission electron microscope
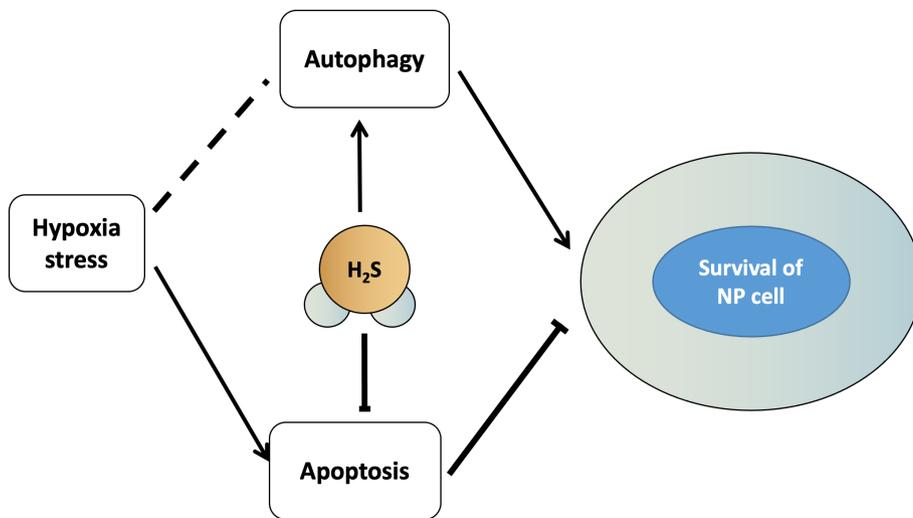
FIGURE 5 Schematic diagram of the cytoprotective effect of H_2S on nucleus pulposus cells under hypoxia. H_2S upregulates autophagy and downregulate apoptosis, resulting in beneficial effects on NP cells under hypoxia. The dotted line represents uncertain mechanism. H_2S, hydrogen sulfide; NP, nucleus pulposus

3.4 | NaHS retrieved autophagy under hypoxia

The analysis of western blotting results showed that the expression of Bcl-2, Beclin-1, and the ratio of LC3B II/GAPDH and LC3B II/LC3B I in hypoxia group were lower than in the control group, but the differences were not statistically significant. The expression of autophagy-related markers significantly increased by the added preincubation of NaHS in hypoxic culture ($P < 0.05$). However, the positive effects on autophagy of NaHS was reclaimed by the pretreatment of 3-MA, as shown in Figure 3. On TEM, the number of autophagosomes of NP cells in hypoxia group slightly decreased compared with that of control group, whereas increased compared with that of NaHS + hypoxia group. However, the number of autophagosomes of NP cells in 3-MA + NaHS + hypoxia group was the lowest among all groups (Figure 4).

4 | DISCUSSION

IVDD is the leading cause of multiple spinal disorders, yet there are currently no effective therapies for IVDD for its unknown pathogenesis. The process of IVDD, accompanied by the inhibition of cell viability, is correlated with the decline in nutrients and O_2 supply.[12] Our previous study showed that H_2S attenuated hypoxia-induced apoptosis in rat NP cells.[7] The results of current study further demonstrated that H_2S improved cell proliferation under hypoxia by upregulating autophagy.

Autophagy is a fundamental biological process in which cytoplasmic constituents are encapsulated within double-membraned autophagosomes for degradation in response to various stimuli such as malnutrition or hypoxia, while basal autophagy is sustained in NP cells for maintaining cellular homeostasis due to avascular physiological feature in intervertebral discs.[13] During autophagy process, LC3-I in the cytoplasm can be transformed into LC3-II on the mature autophagosome membranes via the addition of a phosphatidylethanolamine, and LC3 conversion (LC3-I to LC3-II) is often used as an indicator for maturation of autophagosomes.[14] Meanwhile, Beclin-1

and Bcl-2 together form a complex to regulate post-translational modification and protein-protein interactions to promote autophagosome maturation.[15] The effects of hypoxia and autophagy of NP cells is still controversial. The study of Chen et al showed that autophagy of NP cells decreased under hypoxia, while Choi et al and Kim et al found that autophagy was elevated by hypoxic culture conditions.[5,16,17] In the present study, the expression of autophagy-related markers (ie, Beclin-1, Bcl-2, and LC3-II/ LC3-I conversion) in hypoxia group were all lower than in the control group, but the differences were not significantly different.

H_2S has been regarded for a long time as a metabolic waste.[18,19] It was not until quite recently did researchers start to realize that H_2S is an important endogenous gas signaling molecule, which plays important roles in a wide range of physiological and pathological conditions in various types of cells.[8] In this study, H_2S was generated by NaHS, a dependable source to boost H_2S concentrations.[20] Administration of H_2S-releasing molecules was found to alleviate progression and symptoms of musculoskeletal degeneration such as osteoarthritis and IVDD in vitro and in vivo.[21-24] Our previous work has shown that the key H_2S producing enzymes, CBS and CSE, were negatively correlated with of the grade of intervertebral disc protrusion in human, whereas another study found that the expression of CBS and CSE was negatively correlated with the grade of disc degeneration in human.[7,23] These results implied that the progression of IVDD might be connected to the decrease of endogenous H_2S.[7,23] Additionally, previous studies have elucidated the anti-apoptosis function of H_2S on chondrocytes and NP cells, two physiologically similar cells, via multiple signaling pathways including GRP78/mTOR signaling pathway, PI3K/Akt signaling pathway, NF-κB signaling pathway, MAPK/ERK signaling pathway, and Fas/CytC-dependent pathways.[7,23,25,26]

In the current study, H_2S significantly elevated expression of autophagic proteins and increased accumulation of autophagosomes under hypoxia. However, the impact of H_2S on cell survival under hypoxia was reversed by autophagy inhibitor, indicating the cytoprotective effect of H_2S relies largely on the upregulation of autophagy. Recent studies have shown that the dual role of H_2S on

autophagy, which may be due to the concentration, time frame, and reaction time of H_2S as well as the pathological conditions.[8,27,28] Studies have indicated that a number of signaling pathways are involved in the pro-autophagy effect of H_2S, such as LKB1/STRAD/MO25 and miR-30c signaling pathways, while SR-A, PI3K/SGK1/GSK3β, Nrf2-ROS-AMPK, and JNK1 signaling pathways are involved in anti-autophagy effects of H_2S. Additionally, PI3K/Akt/mTOR and AMPK/mTOR signaling pathways possess dual effect on autophagy.[8] Here 3-MA, a PI3K selective inhibitor, reversed the pro-autophagic effects of H_2S, we therefore presume H_2S promotes autophagy in a PI3K-dependent manner. The relationship among H_2S, autophagy, apoptosis, and cell survival, according to our works, was illustrated in Figure 5.

There are certain limitations in this study. Firstly, due to technical limitation, hypoxia was mimicked by $CoCl_2$ instead of in low oxygen chamber. $CoCl_2$ increases hypoxia-inducible factor (HIF)-1α/2α in a dose-dependent manner, similar to that observed in low oxygen.[29] However, $CoCl_2$ only mimicked part of the hypoxia response and altered the expression of many genes not regulated by hypoxia.[30] The insignificant effect of hypoxia on autophagy might also be due to the incomplete hypoxia-mimicking effect of $CoCl_2$. Besides, previous studies used the $CoCl_2$ at the concentration of 100, 150, 200, and 400 μM for mimicking hypoxia for NP cells, the current study determined the optimal concentration as 200 μM through gradient concentration, and the toxic effect of $CoCl_2$ might occur above 200 μM.[7,29,31,32] Then, we did not rule out that the increase in autophagy may be due to the appearance of autophagic flux or the inhibition of the fusion of autophagosome and lysosome. Additionally, the exact signaling pathways of H_2S on autophagy was not explored here. It would be interesting to perform further experiments to verify our findings through signaling verification.

Taken together, this study provides novel evidence that H_2S may play a role in the pathogenesis of IVDD by promoting autophagy of NP cells. We found pro-autophagy impact of H_2S on NP cells under hypoxia. Our study may help to better understand the role of H_2S in pathogenesis of IVDD as well as provide insights in developing novel strategies for treating degenerative disc diseases.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Lei Yue: Conceived and designed the project, performed the experiments, analyzed data, interpreted the results of experiments, prepared the figures, drafted the original manuscript, contributed to this study equally. **Yongkai Hu:** Conceived and designed the project, performed the experiments, contributed to this study equally. **Haoyong Fu:** Performed the experiments. **Longtao Qi:** Interpreted the results of experiments. **Haolin Sun:** Edited and revised the manuscript. All authors read and approved the final manuscript.

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