Hydrogen sulfide is expressed in the human and the rat cultured nucleus pulposus cells and suppresses apoptosis induced by hypoxia

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Abstract

Apoptosis plays pivotal role in the pathogenesis of degenerative disc diseases, which is the primary contributor to low back pain. Although the role of hydrogen sulfide (H\textsubscript{2}S) in cell apoptosis is well appreciated, the effects and mechanism that H\textsubscript{2}S regulates the program death of intervertebral disc cell are not yet elucidated. In this study, we utilized the nucleus pulposus (NP) from patients with lumbar disc herniation to investigate the relationship between endogenous H\textsubscript{2}S and NP cells apoptosis in human. Furthermore, we analyzed primary rat NP cells to study the effects of exogenous H\textsubscript{2}S on hypoxia induced cell apoptosis. Human NP samples were obtained from patients with lumbar disc herniation and were divided into uncontained and contained herniation groups. Using immunohistochemistry staining and sulphur-sensitive electrode, we detected the expression of cystathionine-\beta-synthase (CBS) and cystathionine \gamma-lyase (CSE), as well as the production of endogenous H\textsubscript{2}S in human NP. Tunel staining showed increased apoptosis in NP from herniated disc; and there was significant correlation between H\textsubscript{2}S generation and apoptosis in human NP. CoCl\textsubscript{2} was then used to induce hypoxia in cultured primary rat NP cells. Annexin V staining indicated that exogenous NaHS attenuated hypoxia induced apoptosis in rat NP cells. Furthermore, hypoxia significantly increased the levels of multiple apoptosis associated proteins (Fas, Cytochromes C, Caspase 9 and cleaved-Caspase-3) in cells, which were eliminated by NaHS.

Our study demonstrates the presence of endogenous H\textsubscript{2}S in human intervertebral disc; and the endogenous H\textsubscript{2}S generation rate is associated with NP apoptosis in herniated disc. In vitro study showes exogenous H\textsubscript{2}S donor attenuates hypoxia induced apoptosis in primary rat NP cells. Thus, our work provides insights that H\textsubscript{2}S may have beneficial effects in treating degenerative disc diseases.

Introduction

Intervertebral disc degeneration is frequently associated with disc herniation, low back pain and sciatica, thus leads to global burden with severe health-care and socioeconomic consequences [1, 2]. Apoptosis is a prerequisite process for the development of nucleus pulposus
(NP) cells and the maintenance of tissue homeostasis. However, excess apoptosis associated with hypoxia may lead to degenerative disc diseases [3, 4], which the underlying mechanisms remain largely unknown.

Hydrogen sulfide (H$_2$S) is recognized as a third gasotransmitter following nitric oxide (NO) and carbon monoxide (CO), and is physiologically present in a variety of mammalian tissues including cardiovascular system, digesting system, brain, etc [5]. Endogenously, H$_2$S is synthesized from the precursor L-cysteine via two pyridoxal-5'-phosphate dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Although H$_2$S was proved to be involved in multiple physiological cellular changes, including cell cycle, oxygen transduction, etc [6, 7], Unregulated H$_2$S may contribute to the development of variety of diseases such as degenerative disease [8], cancer, inflammation and ischemia-reperfusion-induced injury [9–11].

The current knowledge regarding endogenous H$_2$S in intervertebral disc is limited. Recently, Xu and colleagues showed H$_2$S plays a protective role in intervertebral disc degeneration via reducing endoplasmic reticulum stress and mitochondrial injury in NP cells [12]. In this study, we focused on assessing the relationship between endogenous H$_2$S production, NP cells apoptosis and intervertebral disc herniation. In addition, the effect of H$_2$S on hypoxia induced NP cells apoptosis was investigated in cultured primary rat NP cells.

Materials and methods

Collection of human lumbar intervertebral disc

Human lumbar intervertebral disc samples were obtained during discectomy surgery from patients suffering lumbar disc herniation (between L4-S1 levels). 23 females and 17 males with average age of 49.3±7.2 were recruited from Jan 2016 to June 2016. The intervertebral disc herniation was further classified into contained (displaced disc tissue is wholly held within intact outer annulus, n = 20) or uncontained (the outer annulus is not intact and the displaced disc tissue leaks into the vertebral canal fluid, n = 20) group based on magnetic resonance imaging results and surgical findings [3]. The intervertebral disc tissues from 5 adolescent idiopathic scoliosis patients were used as control, with average age at 17.6±3.7 and pathological changes at L1-L5 levels. Before operation, surgeon presented a detailed written informed consent to the patients. All participants must signed the consent form before initiation of this study. If patients were minors, parents or guardians must signed the consent. The study was approved by the Human Subjects Institutional Review Board at Peking University First Hospital.

Immunohistochemistry and TUNEL staining in human lumbar nucleus pulposus

NP was isolated immediately from intervertebral disc after surgery, post-fixed in 10% formalin and embedded in paraffin. Transverse sections (4um thick) were then obtained and immunohistochemistry staining was performed as previously described [13]. In brief, the sections were deparaffinized and rehydrated, endogenous peroxidase was removed using 3% hydrogen peroxide for 20 min and the non-specific antibody binding sites were blocked using 10% normal goat serum for 30 minutes at 37°C. Sections were then incubated in the mouse monoclonal anti-CBS antibody (1:100, Abova, TW) and mouse monoclonal anti-CSE antibody (1:100, Abova, TW) at 4°C overnight, followed with the polymer-HRP conjugated secondary antibody (DAKO).

Apoptosis was detected using in situ cell death detection kit (KeyGEN, CN) following the manufacture instructions. Shortly, after deparaffinization and rehydration, the sections were
treated with proteinase K (20 μg/mL) for 30 minutes then quenched in 3% hydrogen peroxide for 20 min. Sections were then incubated in terminal deoxynucleotidyl transferase for 60 min at 37°C in dark, followed with streptavidin–HRP reaction buffer for 30 min at 37°C. Color was developed using DAB substrate and hematoxylin counterstaining was applied.

Staining pictures were obtained using high-power field microscopy (Olympus, BX41, JP), quantitative image analysis was performed by 2 investigators in a blinded manner. The total number of NP cells and CBS/CSE positive cells were counted in randomly acquired 10 non-overlapping high-magnification imaging fields (400x) in each section and an average of CBS/CSE positive cells proportion was calculated.

H2S production in human lumbar nucleus pulposus

Another cohort of human NP tissue was used for H2S measurement. The endogenous H2S generation was detected using sulphur-sensitive electrode based on three steps chemical reaction formula: i) \( HS^- + H^+ \rightleftharpoons H_2S \); ii) \( 2HS^- + 2OH^- \rightleftharpoons 2S^{2-} + 2H_2O \); iii) \( H_2S + 2OH^- \rightleftharpoons S^{2-} + 2H_2O \) [14, 15]. In brief, NP tissues were homogenized in 50 mmol/L ice-cold potassium phosphate buffer (pH = 6.8). A reaction mixture was set up for H2S detection: 100 mmol/L potassium phosphate buffer (pH = 7.4), 10 mmol/L L-cysteine, 2 mmol/L pyridoxal 5-phosphate, and 10% (w/v) tissue homogenate. Cryovial test tubes (2 ml) were used as the center wells, and each contained 0.5 ml of 1% zinc acetate as a trapping solution along with a filter paper of 2–2.5 cm² to increase the air/liquid contact surface. The reaction was performed in a 25 ml Erlenmeyer flask. The flasks containing reaction mixture and the center wells were flushed with nitrogen before being sealed with a double layered parafilm. The reaction was initiated by transferring the flasks from ice to a shaking water bath at 37°C. After incubation at 37°C for 90 minutes, 0.5 ml of 50% trichloroacetic acid was added to stop the reaction. The flasks were sealed again and incubated at 37°C for 60 minutes to ensure complete trapping of the H2S released from the mixture. The contents of the center wells were transferred to test tubes containing 3.5 ml of water. Then, 0.5 ml of 20 mmol/L N,N-diethyl-p-phenylenediamine sulfate in 7.2 mol/L HCl and 0.5 ml of 30 mmol/L FeCl3 in 1.2 mol/L HCl were added. The absorption rate of the resulting solution was determined at 670 nm with a spectrophotometer. The H2S concentration was calculated against the calibration curve of the standard H2S solution.

Isolation of primary rat nucleus pulposus cells

NP cells were isolated from 4-week-old male Wistar rats (150–200 g) as previously described [1]. Rats were sacrificed with CO2, the spinal columns were removed en bloc under aseptic conditions and the lumbar intervertebral disc were collected. Using a dissecting microscope, the gel-like NP was separated from the fibrous annulus and digested with 0.1% type II collagenase (Sigma, St. Louis, MO) and 2 U/ml hyaluronidase (Sigma, St. Louis, MO) for 4 hours. The partially digested tissue was maintained as an explant in a humidified atmosphere containing 5% CO2 at 37°C in complete culture medium: Dulbecco’s Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and antibiotics. After the cultured cells reached confluence, trypsin (0.25%) /EDTA (1 mmol/L) solution (Invitrogen, USA) was applied and cells were collected and sub-cultured. The third passage of cells were collected for following analysis.

Type II collagen and aggrecan in NP cells was detected by immunohistochemistry staining. Rat NP cells were fixed with 4% paraformaldehyde on ice for 20 minutes and permeabilized for 15 minutes with PBS containing 0.5% triton-X 100, then incubated in blocking solution (10% FBS in PBS) for 45 minutes. Primary antibody of rabbit anti-Type II collagen (1:100, Zhong shan jin qiao, CN) and mouse anti-aggrecan (1:100, Usbiological, USA) were applied.
overnight at 4˚C. Cells incubated with mouse isotype IgG control were used as control. Cells were then incubated with FITC conjugated anti-mouse IgG (1:200, Zhong shan jin qiao, CN) for 1 hour followed with DAPI incubation for 20 minutes at room temperature. Staining pictures were obtained using laser scanning confocal microscope (Olympus Fluoview, Olympus Corp, JP). Ultra structure features were observed by transmission electron microscope. The study was approved by the Animal Ethics Committee of Peking University First Hospital.

**Immunofluorescence staining in rat nucleus pulposus cells**

Rat NP cells were fixed with 4% paraformaldehyde on ice for 20 minutes and permeabilized for 15 minutes with PBS containing 0.5% triton-X 100 then incubated in blocking solution (10% FBS in PBS) for 45 minutes. Primary antibody of mouse anti-CBS and mouse anti-CSE (1:100, Santa Cruz, USA) were applied overnight at 4˚C. Cells incubated with mouse isotype IgG control were used as control. After washing with PBS, cells were incubated with FITC conjugated anti-mouse IgG (1:200, Zhong shan jin qiao, CN) for 1 hour followed with DAPI incubation for 20 minutes at room temperature. Images were obtained using laser scanning confocal microscope (Olympus Fluoview, Olympus Corp, JP).

**Hypoxia in primary rat nucleus pulposus cells**

Hypoxia in rat NP cells was induced using CoCl$_2$ (150μmol/L) [16]. NaHS (150μmol/L) was used as exogenous H$_2$S donor [17, 18]. The third passage of NP cells were used for analysis and were divided into 4 groups: 1) Control; 2) NaHS; 3) Hypoxia; 4) NaHS+Hypoxia. The cells were then cultured for 72 hours after the treatments.

**Annexin V—FITC staining detection**

After treatments NP cells were incubated with 5ul of annexin V (Keygen, CN) for 15 minutes in the dark at room temperature. Cells were observed using fluorescence inverted phase contrast microscope (excitation 490nm, emission 520nm, DMI 60000, Leica, GE). Samples were also quantified with the inverted phase contrast lens of the same field to document all the cells in the image field. Each sample was evaluated by 2 investigators in blinded manner, and the percentage of annexin V positive cells in total cells was calculated.

**Western blot**

NP cells were harvested in RIPA buffer (Keygenbio, China), and total protein concentration was quantified using the BCA protein assay kit (Keygenbio, China). Protein extracts were electrophoresized in 5–12% Bis–Tris gel, then transferred into polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% fat-free milk (Sigma, USA) for 1 hours at room temperature, and incubated with primary antibodies rabbit anti-Fas (1:500, Santa Cruz, USA), rabbit anti-Cytochromes C (CytC) (1:500, Santa Cruz, USA), rabbit anti-Caspase 9 (1:600, Santa Cruz, USA), rabbit anti-cleaved Caspase 3 (1:600, Santa Cruz, USA), and rabbit anti-GAPDH (1:5000, Santa Cruz, USA) at 4˚C overnight with gentle agitation. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:8000, Santa Cruz, USA) for 1 hours at room temperature. Immunolabeling was detected using enhanced chemiluminescence reagent (Millipore, USA). Protein bands were quantified using densitometry and analyzed using image J software (version 1.42q, National Institutes of Health, US).
Statistical analysis
Data were analyzed by Statistical package for program (Version12, SPSS, Chicago, IL), one-way ANOVA followed by Bonferroni post hoc test was used for comparison between groups. Data are presented as means ± SEM, statistical significance was defined as p<0.05.

Results

CBS and CSE expression in human lumbar nucleus pulposus
To determine whether endogenous H\textsubscript{2}S has impact on the intervertebral disc herniation, we detected the CBS and CSE expression in the NP cells isolated from herniated discs. The immunohistochemistry showed CBS and CSE positive staining are significantly increased in NP isolated from patients with contained and uncontained herniation (p<0.001 vs control respectively, Fig 1). Furthermore, cells from patients with uncontained herniation showed significant higher CSE expression (0.52 ± 0.01% CSE positive cells) compared to the contained herniation group (0.31 ± 0.01%, p<0.001, Fig 1A and 1C).

H2S generation in human nucleus pulposus
After determining the relationship between CBS, CSE and intervertebral disc herniation, we further evaluated the H\textsubscript{2}S generation from NP biopsy. The H\textsubscript{2}S production rates was significantly higher in both contained (4.18 ± 0.31 mmol/g/min) and uncontained herniation group (7.25 ± 0.35 mmol/g/min) compared to control (2.02 ± 0.16 mmol/g/min, p<0.05 and p<0.001 respectively). Furthermore, NP cells isolated from patients with uncontained herniation showed significant higher H\textsubscript{2}S production rate compared to contained group (p<0.001, Fig 2).

Apoptosis in human nucleus pulposus
NP isolated from herniated disc contains increased proportion of apoptotic cells compared to control. The TUNEL positive cells were 0.14 ± 0.01% in contained herniation group and 0.22 ± 0.01% in uncontained herniation group (p = 0.07 and p<0.001 vs control respectively, Fig 3A and 3B). The proportion of apoptotic cells was significant higher in uncontained herniation group compared to the contained group (p<0.01, Fig 3A and 3B). In addition, there was significant correlation between H\textsubscript{2}S generation rate and cells apoptosis in NP samples (R\textsuperscript{2} = 0.3843, p<0.001, Fig 3C).

Expression of CBS and CSE in rat nucleus pulposus cells
The third passage of rat NP cells appeared round or multi-angular under inverted phase contrast microscope. Cells that were positive in type II collagen (Fig 4A) and aggrecan (Fig 4B) were identified as NP cells. Endoplasmic reticulums, Golgi complexes, free ribosomes and multilamellar bodies were observed under transmission electron microscope (Fig 4C). Immunofluorescence staining showed both CBS and CSE were expressed in the cytoplasm of the rat NP cells (Fig 4D).

H2S inhibits hypoxia induced apoptosis in rat nucleus pulposus cells
The Annexin V staining showed significant increased apoptosis in primary rat NP cells after CoCl\textsubscript{2} induced hypoxia (p<0.001 vs control, Fig 5A and 5B), which was prevented by simultaneous exogenous NaHS treatment (p<0.001, Fig 5A and 5B). The NaHS alone did not affect the apoptosis in rat NP cells.
To further investigate the mechanisms how H_2S affects hypoxia induced apoptosis in NP cells, we determined multiple proteins associated with Fas-mediated pathway which plays pivotal role in regulating apoptosis. Hypoxia significantly increased the protein levels of Fas, CytC, Caspase 9 and cleaved-Caspase-3 in both NaHS treated and non-treated NP cells. These increases were significantly eliminated by the administration of NaHS (Fig 5C and 5D).

**Discussion**

The intervertebral disc is the largest avascular structure in body. Within intervertebral disc, NP cells consume glucose to generate energy thus there is a small but significant consumption...
of oxygen [19]. Aging and cumulative damages on intervertebral disc lead to degenerative changes in disc, which coincide with reduction in oxygen and other nutrition supply. In this manuscript, we document the endogenous generation of \( \text{H}_2\text{S} \) in human intervertebral disc and demonstrate this endogenous \( \text{H}_2\text{S} \) generation rate is associated with NP cells apoptosis. Using primary rat NP cells, we show that the hypoxia induced apoptosis can be attenuated by exogenous \( \text{H}_2\text{S} \) donor.

The role of NO in regulating cell apoptosis in intervertebral disc and in disc degeneration have been well discussed [20, 21]. \( \text{H}_2\text{S} \) is another pivotal gasotransmitter and has similar biological effects as NO in multiple diseases scenarios. Studies have revealed the important role of \( \text{H}_2\text{S} \) in the degenerative diseases such as Alzheimer’s disease, Parkinson’s disease and retinal degeneration [22–24]. One of the proposed mechanism is that \( \text{H}_2\text{S} \) protects cells from hypoxia induced apoptosis [7].

In this study, we utilized the biopsy from patients with disc herniation and for the first time, proved the presence of \( \text{H}_2\text{S} \) in the NP tissue using sulphur-sensitive electrode, which is a well-established method to specifically detect \( \text{H}_2\text{S} \) production in tissue [14, 15]. In addition, we found two key enzymes of \( \text{H}_2\text{S} \), CBS and CSE, were also expressed in human NP tissue. Thus, both direct and indirect methods indicated the presence of \( \text{H}_2\text{S} \) in human intervertebral disc.

![Fig 2. The \( \text{H}_2\text{S} \) production in human nucleus pulposus tissue. The \( \text{H}_2\text{S} \) production in human NP biopsy were detected using sulphur-sensitive electrode. There is significant increased \( \text{H}_2\text{S} \) in both contained and uncontained herniation groups compared to control. Furthermore, NP cells isolated from patients in uncontained group show significant higher \( \text{H}_2\text{S} \) production compared to contained group. (‘ \( p<0.05; \quad ** p<0.001; n = 5 \) in control; \( n = 20 \) in contained and uncontained groups).](https://doi.org/10.1371/journal.pone.0192556.g002)
The severity of disc herniation accompanies with different hypoxic conditions [3]. We validated that the disc herniation is associated with NP cell apoptosis, and the percentage of apoptotic cells is significantly linked to the severity of herniation. Interestingly, the H2S generation rate also shows significant positive correlation with cells apoptosis in NP biopsy. These data indicated that H2S may play a pivotal role in regulating NP cells apoptosis in hypoxic environment. In addition, we also observed that the expression of CBS and CSE in NP biopsy is associated with the severity of the disc herniation. Study from Xu et al also demonstrated the protective effects of H2S against NP cells apoptosis and intervertebral disc degeneration [12]. Interestingly, the authors found advanced disc degeneration was accompanied with reduced CBS and CSE expression. We should point out that our work evaluated the relationship between CBS/CSE expression and the severity of disc herniation from patients at similar ages. However, Xu’s study compared the CBS/CSE expression in discs with different degeneration grades, while the patients were at different ages and with different disc diseases.

To further validate the potential biological function of H2S in NP cells apoptosis, we cultured primary rat NP cells and used CoCl2 to mimic hypoxic condition. To avoid aging and dedifferentiation of NP cells during monolayer culture [25, 26], we only used the third passage.
We also verified the NP cells by positive type II collagen and aggrecan staining [27, 28].

We then identified the cytoplasmic expression of CBS and CSE in the NP cells. This further validates the presence of endogenous H$_2$S in the cultured rat NP cells. Accumulating evidences showed that H$_2$S effectively modulates cell apoptosis in different systems and this function is associated with hypoxic environment [6, 7]. NaHS, a potent H$_2$S donor, can attenuate the death of myocardiocytes under hypoxia [29]. In our study, we found the CoCl$_2$ induced NP cells apoptosis was significantly eliminated by NaHS. In addition, NaHS significantly

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Fig 4. Identification of cultured rat NP cells and immunofluorescent detection of CBS and CSE in rat NP cells. Images indicate the third passage of rat NP cells are positive in type II collagen (A) and aggrecan (B). Transmission electron microscope show NP cells contain endoplasmic reticulum, Golgi complexes, free ribosomes and several multilamellar bodies (C). Immunofluorescent staining showed CBS and CSE are expressed in the cytoplasm of the rat NP cells (D) (n = 6/group; CBS: cystathionine-β-synthase; CSE: cystathionine γ-lyase).

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attenuated the increase of Fas, CytC, Caspase-9 and cleaved Caspase-3 in NP cells following hypoxia. Fas signaling plays critical role in regulating p53-dependent apoptosis during hypoxia [30]. The release of CytC from mitochondria is a pivotal initiator for caspase activation, and subsequently mediates cell death [31]. Thus, our data suggested the NaHS regulates NP cells apoptosis via Fas and CytC dependent pathways. This may also be an important mechanism that H_{2}S regulating the degenerative changes in the intervertebral discs during aging and diseases. The underlying signaling pathways of H_{2}S regulating programed cell death in intervertebral disc is a critical question and requires further investigation.

Taking together, our study demonstrated that human NP tissue generates endogenous H_{2}S, and exogenous H_{2}S donor prevents the hypoxia induced NP cell apoptosis in vitro. Understanding the mechanisms of how H_{2}S modulates the programed death of NP cells may provide insights in developing novel strategies for treating and preventing degenerative disc diseases.
Supporting information

S1 Dataset. Data of Figs 1 and 3.
(XLS)

S2 Dataset. Data of Fig 2.
(XLS)

S3 Dataset. Data of Fig 5B.
(XLS)

S4 Dataset. Data of Fig 5D.
(XLSX)

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