H2S treatment improves protective effect of MSC-derived exosomes upon renal ischemia/reperfusion fibrosis by suppressing inflammatory signaling of TGF-β and NF-κB as well as ROS generation

Type
Research paper

Keywords
inflammation, exosomes, fibrosis, reperfusion, ROS, MSC, H2S, renal ischemia

Abstract

Introduction
H2S has been reported to participate in renal I/R fibrosis-associated signaling pathways. In this study, the authors hypothesized that the preconditioning with H2S could boost the effect of exosomes in the therapy of renal I/R fibrosis.

Material and methods
Real-time PCR and Western blot was performed to analyze the gene and protein expression of Nrf2, NF-κB, TGF-β, α-SMA, and Col2 (Collagen Type II) in distinct conditions. H&E and MASSON staining were carried out to examine the kidney injury and fibrosis in I/R rats.

Results
H2S-preconditioned EXOs remarkably reinforced the therapeutic role of EXOs on attenuating the kidney injury in I/R rats. EXOs treatment significantly restored the activated gene and protein expression of NF-κB, TGF-β, α-SMA, and Col2 in I/R rats and cellular models, while H2S preconditioning remarkably strengthened the efficiency of EXOs. Besides, H2S preconditioning remarkably strengthened the efficiency of EXOs in restoring the activated expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rats and cellular models and maintaining the altered activities of SOD, MDA, H2O2, GST and GPx.

Conclusions
This study utilized I/R rats to demonstrate that the administration of H2S-preconditioned exosomes showed more significant effect in inflammation and ROS generation than the unconditioned exosomes. To be specific, exosomes, especially H2S-preconditioned exosomes, could not only reduce the expression of NF-κB and the downstream inflammatory responses, but also promote the expression of Nrf2 and regulate ROS generation, leading to potential therapeutic effect on renal I/R fibrosis.
H₂S treatment improves protective effect of mesenchymal stem cells (MSCs)-derived exosomes upon renal ischemia/reperfusion fibrosis by suppressing inflammatory signaling of TGF-β and NF-κB as well as radical oxygen species (ROS) generation

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Abstract

Background: H₂S has been reported to regulate signaling pathways responsible for development of renal ischemia/reperfusion (I/R) associated fibrosis. In this study, we hypothesized that the preconditioning with H₂S may boost the protective effect of MSCs-derived exosomes on renal I/R fibrosis. Methods: Real-time PCR and Western blot was performed to evaluate mRNA and protein expression of Nrf2, NF-κB, TGF-β, α-SMA, and Col2 (Collagen Type II). H&E and MASSON staining were carried out to examine the kidney injury and fibrosis in rats. Results: H₂S-preconditioned EXOs substantially promoted protective effect of EXOs on the development of
kidney injury as well as associated fibrosis in I/R rats. EXOs treatment significantly restored the
activated gene and protein expression of NF-κB, TGF-β, α-SMA, and Col2 in I/R rats and cellular
model of H/R, while H2S preconditioning remarkably strengthened such effect of EXOs. Besides,
H2S preconditioning remarkably strengthened the efficiency of EXOs in restoring the activated
expression of IL-1α, IL-6, IL-12 and TNF-α as well as altered activities of SOD, MDA, H2O2, GST
and GPx in I/R rats and cellular model of H/R. Conclusion: This study utilized I/R rats to
demonstrate that the effect of H2S-preconditioned exosomes in suppressing inflammation and
ROS generation was better than that of unconditioned exosomes. To be specific, exosomes,
especially H2S- preconditioned exosomes, could not only reduce the expression of NF-κB and the
downstream inflammatory responses, but also promote the expression of Nrf2 and inhibit ROS
generation, which explained the molecular mechanism underlying the protective effect of H2S-
preconditioned exosomes on renal I/R associated fibrosis.

Running title: H2S improves protective effect of MSC-derived exosomes on I/R fibrosis

Key words: H2S, MSC, exosomes, renal ischemia, reperfusion, fibrosis, inflammation, ROS

Abbreviation

MSCs: mesenchymal stem cells
EXOs: exosomes
I/R: ischemia/reperfusion
H/R: hypoxia/reoxygenation
ROS: radical oxygen species
H2S: hydrogen sulfide

Introduction

Fibrosis is a pathophysiological condition developed following renal ischemia-reperfusion (I/R)
injury, and it is very common medical condition worldwide [1]. I/R produces microenvironment
in kidney tissues which lacks oxygen and necessary nutrients, leading to an increase in oxidative
stress and production of ROS. These conditions cause cell apoptosis, necrosis and injury in the interstitial cells of kidney [2, 3]. The immune response generated within renal interstitium activates mesangial cell proliferation and extracellular matrix production causing chronic renal injury and fibrosis. As a result, blood capillaries in the interstitium layer get smaller and produce an aggressive reaction like anoxia, and further worsening the kidney injury [4-6]. The I/R condition impacts the process of oxidative phosphorylation, causing increased levels of reactive oxygen species. Therefore, antioxidants have been shown to attenuate progression of I/R related kidney injury [7].

Previous studies have explored the beneficial effects of mesenchymal stem cells (MSCs) in various pathophysiological conditions, such as heart disease, stroke, and autoimmune disease [8, 9]. Considering the ability of the MSCs to differentiate into other tissues, it was believed that the injured kidney tissues can be regenerated [10, 11]. Microvesicles, also called exosomes (30–100 nm), are usually extracted from various body fluids and supernatant layers of cell culture [12-14]. Exosomes contain complex cocktail of components ranging from different types of proteins, genetic material such as RNA, lipids and enzymes. Exosomes function as effective method of cell-cell communication [14, 15].

Protective effects of in situ synthesized gaseous molecules, also known as gasotransmitters, against the tissue IRI have been reported recently. The list of recently discovered gasotransmitters include nitric oxide (NO), carbon monoxide (CO) and latest member is hydrogen sulfide (H2S) [16, 17]. The protection offered by H2S against renal injury has been shown in various models, including brain, intestine, liver lung and heart. The proposed mechanism of action of protective effect of H2S involves antiapoptotic, antioxidant and anti-inflammatory effects [18, 19]. The effects of exogenous H2S treatment on acute recovery period needed to reduce inflammation in I/R rat models was investigated in current study [20].

A complex signaling pathway is responsible for anti-inflammatory effects of H2S. Majority of inflammatory responses involved NF-kB as a intracellular signaling mediator [21]. It was also shown that, upon H2S treatment, the treated cells switched into reversible hypometabolic and hibernation-like state. The hypothesized hypometabolism induced by H2S treatment function by
lowering the mitochondrial activity via binding reversibly to cytochrome c oxidase. During hypometabolism, animals are protected from hypoxia and their organs from I/R due to lack of oxygen demand. Administration of H₂S not only scavenge reactive species, like ROS or reactive nitrogen species (RNS), but also produce glutathione, a natural antioxidant. Overall, H₂S act as a protective agent in animal models with renal injury [22].

Hypoxia-preconditioned MSCs have been proved to be an effective cell therapy method to prevent renal fibrosis and inflammation [23]. And H₂S has been reported to participate in renal I/R fibrosis-associated signaling pathways in recent studies [22, 24, 25]. In this study, we hypothesized that the preconditioning with H₂S could boost the effect of exosomes in the therapy of renal I/R fibrosis. By establishing an I/R rat model, we aimed to investigate the potential therapeutic effect of H₂S-preconditioned exosomes and underlying mechanisms upon renal I/R fibrosis.

Materials and Methods

Animal and treatment

Male SD rats with age ranging between six and eight weeks were maintained under room temperature. Bone marrow was collected from the rats at six weeks, whereas I/R model were generated using eight weeks old rats. The animal studies were performed according to an IACUC approved protocol. Renal ischemia reperfusion injury model was set up by carefully harnessing the unilateral renal artery. Before the surgery, the rats were given an intraperitoneal injection of mixture of anesthetics. The left kidneys of the rats were exposed following the procedure of laparotomy. Using a vascular clamp, the renal pedicle was clamped for 1 h. Later, the reperfusion procedure was carried out. Animals were divided in four groups with 12 animals in each group, i.e., 1.) control group (group of animals without any treatments), 2.) I/R group (group of animals were subjected to the surgery described before to make IRI model), 3.) EXOs group (group of animals were treated with 100 ug of exosomes without H₂S treatment) and 4.) EXOs with H₂S group (group of animals were treated with the H₂S pretreated 100 ug of exosomes). Institutional animal ethics committee has approved the protocols of this study.

Preparation of MSCs
Following a procedure described in previous publication [26], marrow from bones was collected from the rats. The bone marrow cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS (Sigma-Aldrich). The cells were grown in four phases and used as rat MSCs for transplantation. According to instructions from previous publication [27], NaHS (Sigma-Aldrich Chemical, St. Louis, MO, USA) was prepared at a concentration of 10 μmol/ml, and then the MSCs were preconditioned with 200 μmol/L NaHS for 30 min.

**Measurement of carbon monoxide (CO) production in kidney**

Kidney samples were homogenized and centrifuged to collect the protein content from the supernatant. And the production of CO in kidney samples was measured by gas chromatography-mass spectroscopy following instructions by previous publications [28].

**Measurement of nitric oxide (NO) production in kidney**

The NO production was determined in urine using a colorimetry assay kit (Cayman Chemical; Ann Arbor, CA, USA) following the instructions by the manufacturer.

**Real-time PCR**

To explore the effect of H₂S treated exosomes, the assays were carried out to analyze the expression of Nrf2, NF-κB, TGF-β, α-SMA, and Col2 in I/R rat models and HK2 cells. To carry out the measurements, the intact RNA was initially obtained from each sample by utilizing an RNAiso Plus RNA extraction reagent (Thermo Fisher, MA) using the provided protocol. Then, the isolated RNA from each sample was reverse transcribed into cDNA templates by making use of a Taqman Advanced cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA) along with a QuantiTect Reverse Transcription Kit (Qiagen, MD) following the specific assay procedure received along with the kit. Then, quantitative real time PCR was performed by making use of a Fast Start Universal SYBR Green Master Mix assay kit (Roche, Basel, Switzerland) following the specific assay procedure received along with the kit. The real time PCR reaction was done on an ABI Prism 7900HT real time PCR machine (Applied Biosystems, Foster City, CA) using U6 as well as GAPDH as the internal control for the normalization of measured relative expression of target genes, i.e., Nrf2, NF-κB, TGF-β, α-SMA, and Col2.
Western blot analysis

The total protein content was extracted from each sample by making use of a radioimmunoprecipitation assay kit (Abcam, CA) following the specific assay procedure received along with the kit. Then, 50 μg of each protein sample were resolved by using SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked by utilizing 5% skimmed milk at ambient temperature for 1 h before they were incubated for 24 h at 4 °C with corresponding primary antibodies of Nrf2, NF-κB, TGF-β, α-SMA, Col2 (Abcam, Cambridge, MA) followed by incubation with suitable HRP-conjugated secondary antibodies for 1 h at room temperature in accordance with the specific instructions provided by the antibody manufacturer. After color development by utilizing an enhanced chemiluminescence (ECL) assay kit (Amersham Pharmacia, Piscataway, NJ) following the specific assay procedure received along with the kit, the relative protein expression of COMT in each sample was determined by means of a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA) in accordance with the instructions provided by the machine manufacturer.

H&E staining

The tissue samples were fixated with formalin and embedded with paraffin to stain with H&E stain. The sections were observed with help of fluorescence microscope.

MASSON staining

The tissue samples were fixated with formalin, deparaffinized, and rehydrated with 100% alcohol, 95% alcohol and 75% alcohol. The tissue samples were re-fixed in Bouin's solution for 60 min at 56 °C and rinsed with running tap water. Later, stained in Weigert's iron hematoxylin for few minutes and again washed with water. Immediately after this, Biebrich scarlet-acid fuchsin solution was applied on the tissue samples for ten minutes. Rinsed with water and re-stained with phosphomolybdic-phosphotungstic acid solution for ten minutes.

ELISA
ELISA assays were performed to measure IL-1α, IL-6, IL-12 and TNF-α levels using commercial kits (Abcam, Cambridge, MA). Samples at 1/10 dilution were tested for IL-1α, IL-6, IL-12 and TNF-α according to the manufacturer's instructions.

**Cell culture**

The human renal proximal tubular epithelial cell line human kidney-2 (HK-2) was obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were preserved in RPMI-1640 medium supplied with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), and put in incubator at 37°C with 5% CO₂. HK-2 cells divided in four groups, i.e., 1.) control group (HK-2 cells without any treatments), 2.) H/R group (HK-2 cells were put under hypoxic condition, i.e., 37 °C, 1% O₂, 94% N₂ and 5% CO₂ for 12 h in glucose- and serum free medium to induce hypoxic injury. Later, the cells were placed under normal condition (5% CO₂) for reoxygenation for 12 and 24 hours., 3.) EXOs group (HK2 cells were treated with exosomes without H₂S treatment) and 4.) EXOs with H₂S group (HK-2 cells were treated with the H₂S pretreated exosomes). As per the manufacturer's protocols, the HK-2 cells were transfected using Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA).

**Enzyme assays**

The HK-2 cells were lysed to analyze the levels of MDA and SOD activity. The corresponding assay kits were used to this end following the protocols provided by the manufacturer. The HK-2 cells were cultured in DMEM medium plus fetal bovine serum. To detect amount of hydrogen peroxide present in the kidney tissues, the kidney extracted from the animals was perfused and homogenized. Later, the reagent, containing 10U/mL horseradish peroxidase, was added to the homogenized tissues according to the provider’s assay protocol. And then, the reading from the fluorescence instrument were analyzed. To detect the enzyme activity in the extracted kidney samples, adduct of GSH and CDNB was detected at 340 nm. To measure the SOD and GPx activity, an indirect method of detecting reduction of NBT by an enzyme xanthine-xanthine oxidase was used as described previously [16].

**Statistical analysis**
All outcomes were expressed as mean ± standard deviations. The statistical relevance of inter-group contrasts was performed by student t-tests. The statistical analyses were performed making use of Prism 7.0 software (GraphPad, La Jolla, CA). P < 0.05 was considered to be statistical significance.

**Results**

**H₂S-preconditioned EXOs effectively restored the kidney function impairment in I/R rat models.**

MSCs were pretreated with H₂S followed by EXOs collection. Electron microscope analysis indicated no obvious difference between EXOs collected from H₂S preconditioned MSCs and untreated MSCs (Fig.1A). Western blot was performed to analyze the surface markers CD63, CD69 and CD81 on EXOs collected from H₂S preconditioned MSCs and untreated MSCs, no significant difference was found (Fig.1B). I/R rat model was established and subjected to EXOs treatment. H&E staining analysis indicated that the kidney injury was remarkably elevated in I/R rat models. EXOs treatment notably decreased the kidney injury, moreover, EXOs from H₂S preconditioned MSCs showed a reinforced efficiency in attenuating the kidney injury in I/R rat models (Fig.1C). Furthermore, MASSON staining was performed to evaluate the kidney fibrosis in I/R rats under distinct conditions. H₂S-preconditioned EXOs apparently maintained the kidney fibrosis in I/R rats (Fig.1D). Besides, the increased kidney functional parameters SCr (Fig.1E) and BUN (Fig.1F) were also effectively restored by H₂S-preconditioned EXOs. Moreover, it was shown that H₂S preconditioning reinforced the efficiency of EXOs in suppressing NO production in I/R rats (Fig.1G), while the CO production was not influenced by these treatments (Fig.1H).

**H₂S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, α-SMA, and Col2 protein in I/R rats.**

Western blot was carried out to analyze the expression of Nrf2, NF-κB, TGF-β, α-SMA, and Col2 in I/R rats under distinct conditions (Fig.2A). The expression of Nrf2 was notably elevated in I/R rats when compared with the control. EXOs treatment further increased the Nrf2 expression and H₂S-preconditioned EXOs showed a stronger efficiency in enhancing the Nrf2 expression in I/R rats (Fig.2B). Even though the expression of NF-κB, TGF-β, α-SMA, and Col2 in I/R rats was remarkably increased, EXOs treatment effectively attenuate the up-regulation of NF-κB, TGF-β,
α-SMA and Col2 in I/R rats. And H₂S-preconditioning further decreased the elevated expression of NF-κB (Fig.2C), TGF-β (Fig.2D), α-SMA (Fig.2E), and Col2 (Fig.2F) in I/R rats. Moreover, we also performed quantitative real-time PCR upon the gene expression of Nrf2 (Fig.2G), NF-κB (Fig.2H), TGF-β (Fig.2I), α-SMA (Fig.2J), and Col2 (Fig.2K) in the rat models, and similar results were obtained.

**H₂S-preconditioned EXOs effectively restored the expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rats.**

ELISA was performed to analyze the distinct expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rat models under differential treatments. The expression of IL-1α, IL-6, IL-12 and TNF-α was remarkably activated in the kidney of I/R rats when compared with the control. EXOs treatment effectively attenuated the up-regulation of IL-1α, IL-6, IL-12 and TNF-α. Moreover, H₂S-preconditioning further decreased the elevated expression of IL-1α (Fig.3A), IL-6 (Fig.3B), IL-12 (Fig.3C) and TNF-α (Fig.3D) in the kidney of I/R rats.

**H₂S-preconditioned EXOs effectively restored the enzymatic activities of SOD, MDA, H₂O₂, GST and GPx in I/R rats.**

Enzymatic activities of SOD, MDA, H₂O₂, GST and GPx were evaluated in I/R rats under distinct conditions. The enzymatic activities of SOD, GST and GPx were notably suppressed in I/R rats when compared with the control. EXOs treatment effectively restored the suppressed activities of SOD, GST and GPx, and H₂S-preconditioning showed a strengthened efficiency on restoring the enzymatic activities of SOD (Fig.4A), GST (Fig.4D) and GPx (Fig.4E). However, the enzymatic activities of MDA and H₂O₂ were notably activated in I/R rats when compared with the control. EXOs treatment effectively decreased the elevated activities of MDA and H₂O₂, and H₂S-preconditioning showed a strengthened efficiency on restoring the enzymatic activities of MDA (Fig.4B) and H₂O₂ (Fig.4C).

**H₂S-preconditioned EXOs effectively restored the expression of α-SMA, Col2 protein and the enzymatic activities of SOD, MDA, H₂O₂, GST and GPx in HK-2 H/R models.**
HK-2 cell H/R (hypoxia/reoxygenation) model was established as described followed by EXOs treatment. Western blot was performed to analyze the expression of Nrf2, α-SMA and Col2 (Fig.5A) in HK-2 H/R models under distinct conditions. The expression of Nrf2 was notably elevated in HK-2 H/R models when compared with the control. EXOs treatment further increased the Nrf2 expression and H$_2$S-preconditioned EXOs showed a stronger efficiency in enhancing the Nrf2 expression in HK-2 H/R models (Fig.5B). Even though the expression of α-SMA and Col2 in HK-2 H/R models was remarkably increased, EXOs treatment effectively attenuated the up-regulation of α-SMA, and Col2 in HK-2 H/R models. Moreover, H$_2$S-preconditioning further decreased the elevated expression of α-SMA (Fig.5C) and Col2 (Fig.5D) in HK-2 H/R models. Moreover, we also performed quantitative real-time PCR upon the gene expression of Nrf2 (Fig.5E), α-SMA (Fig.5F), and Col2 (Fig.5G) in the rat models, and similar results were obtained. The enzymatic activities of SOD, GST and GPx were notably suppressed in HK-2 H/R models when compared with the control. EXOs treatment effectively restored the suppressed activities of SOD, GST and GPx, and H$_2$S-preconditioning showed a strengthened efficiency on restoring the enzymatic activities of SOD (Fig.5H), GST (Fig.5K) and GPx (Fig.5L). However, the enzymatic activities of MDA and H$_2$O$_2$ were notably activated in HK-2 H/R models when compared with the control. EXOs treatment effectively decreased the elevated activities of MDA and H$_2$O$_2$, and H$_2$S-preconditioning showed a strengthened efficiency on restoring the enzymatic activities of MDA (Fig.5I) and H$_2$O$_2$ (Fig.5J).

H$_2$S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, IL-1α, IL-6, IL-12 and TNF-α in THP-1 H/R models.

THP-1 cell H/R (hypoxia/reoxygenation) model was established as described followed by EXOs treatment. Western blot was performed to analyze the expression of NF-κB and TGF-β (Fig.6A). The expression of NF-κB and TGF-β in THP-1 H/R models was remarkably increased, EXOs treatment effectively attenuate the up-regulation of NF-κB and TGF-β in THP-1 H/R models. Moreover, H$_2$S-preconditioning further decreased the elevated expression of NF-κB (Fig.6B) and TGF-β (Fig.6C) in THP-1 H/R models. And quantitative real-time PCR was performed to investigate the expression of NF-κB mRNA (Fig.6D) and TGF-β mRNA (Fig.6E) in the cell models, showing similar results as the protein expressions. ELISA was performed to analyze the distinct expression
of IL-1α, IL-6, IL-12 and TNF-α in THP-1 H/R models under differential treatments. The expression of IL-1α, IL-6, IL-12 and TNF-α was remarkably activated in THP-1 H/R models when compared with the control. EXOs treatment effectively attenuate the up-regulation of IL-1α, IL-6, IL-12 and TNF-α. Moreover, H₂S-preconditioning further decreased the elevated expression of IL-1α (Fig.6F), IL-6 (Fig.6G), IL-12 (Fig.6H) and TNF-α (Fig.6I) in THP-1 H/R models.

Discussion

Many surgical procedures in kidney inevitably lead to renal ischemia-reperfusion injury (IRI) [26]. End state of kidney injury caused by IRI is diagnosed based on renal fibrosis which involve glomerulosclerosis and tubulointerstitial fibrosis [29]. This type of progression to renal failure is mostly irreversible and is the main reason for higher mortality rate of this disease [30]. A complex mixture of factors are responsible for progressive renal fibrosis which include growth factors, reactive oxygen species, cytokines and metabolic waste [31]. In this study, administration of H₂S-preconditioned exosomes successfully restored the renal function in I/R rat models. Additionally, the authors also performed Western blot analysis to correlate expression of Nrf2, NF-κB, TGF-β, α-SMA, and Col2 to treatment of H₂S pretreated or untreated exosomes in I/R rat and cellular models.

In past few decades, application of stem cell therapy has become popular as an adjuvant strategy to treat I/R diseases [32]. However, the exact mechanism of action remains elusive. Another study showed that administration of human adipose (hAD) derived mesenchymal stem cells in the I/R mice provided protections against acute and chronic renal injury. The therapy decreased penetration of inflammatory cells as well as enhanced activity of IL-10, which suggested that the therapy altered microenvironment of the kidney cells and the result of the study revealed that hAD-MSC therapy reduced chronic renal injury and fibrosis. Other pathophysiological conditions, such as unilateral urethral obstruction and vitamin B9, induced kidney fibrosis also improved followed by the hAD-MSC therapy [33, 34].

Many researchers have shown that H₂S treatment has protective effect against several pathophysiological processes as well as various types of renal ischemia/reperfusion injury [35, 36]. Previous study also proved that H₂S treatment protected against cold IRI for longer duration,
as well as against warm IRI for shorter period [20, 37]. Another study revealed that H2S treatment provided both short-term and long-term renal protection against warm IRI. It has been suggested that the regulation of iNOS activation-induced NO release is closely related to the mechanisms of H2S-related kidney protection [38, 39]. And apart from NO, other gasotransmitters such as CO can interact with NO and H2S [40]. For example, H2S were significantly minimized in the presence of CO inhibitor [41]. And previous studies have shown that H2S donor could suppress the overproduction of NO, while simultaneously inhibiting the level of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, which indicated the potential positive effect on inflammation resulting from the crosstalk between H2S and NO [42]. Moreover, H2S therapy was found to successfully restored NO concentration and eNOS functions in individuals with IR injury [43], and NOS isoforms can reduce kidney damage [44]. Moreover, it was also reported that the T allele of the eNOS gene G894T polymorphisms is associated with hypertension in women [45].

It has been shown that levels of Nrf2 protein in the renal tissue after fasting are not involved in protection against oxidative stress caused by IRI [46]. The study also suggested that mitochondrial pathways are also involved in protective effects of fasting in IRI [47]. The results showed that fasting alleviates dysregulation in oxygen consumption, action potential in mitochondrial membrane, structure of mitochondria and balance of protein levels between mitochondria and nuclear. Also, fasting also helped to lower the risk of fibrosis in the injured kidney. In this study, we performed ELISA to measure the expression level of IL-1α, IL-6, IL-12 and TNF-α in I/R rat and cellular models under specific conditions. Moreover, the H2S-pretreated exosomes successfully improved the expression levels of IL-1α, IL-6, IL-12 and TNF-α. Meanwhile, we also evaluated expression of NF-κB and H2S preconditioned EXO restored elevated expression of NF-κB caused by IRI. Several studies have shown that NF-κB is an important transcription factor involved in inflammatory regulations [48]. The NF-κB is a key player in different types of cellular damage and inflammation caused by chemical hypoxia, doxorubicin and LPS [49, 50].

Injury to cells and organs caused during reperfusion are mainly due to ROS production [51]. According to the previous findings H2S changes the conformation Keap1 therefore releasing the bound Nrf2, which subsequently protects the protein from proteasomal degradation [52, 53]. The Nrf2 accumulated in the cytoplasm translocated into the nucleus where it was
phosphorylated to promote transcription of antioxidant genes [54, 55]. In this study, we evaluated the enzymatic activities of SOD, MDA, H2O2, GST and GPx in I/R rat and cellular models under distinct conditions. H2S-preconditioned EXOs effectively restored the enzymatic activities of SOD, MDA, H2O2, GST and GPx. The results of this study shed a light on the clinical use of MSCs in the treatment and prevention of development of fibrosis following renal ischemia reperfusion injury. Furthermore, pre-conditioning of MSCs with H2S will further promote the therapeutic effect of MSCs. In addition, the level of H2S could be a biomarker for the prognosis and risk of fibrosis following renal reperfusion injury.

**Conclusion**

In conclusion, this study utilized I/R rats to demonstrate that the effect of H2S-preconditioned exosomes in suppressing inflammation and ROS generation was better than that of unconditioned exosomes. To be specific, exosomes, especially H2S- preconditioned exosomes, could not only reduce the expression of NF-κB and the downstream inflammatory responses, but also promote the expression of Nrf2 and inhibit ROS generation, which explained the molecular mechanism underlying the protective effect of H2S-preconditioned exosomes on renal I/R associated fibrosis.

**Conflict of interest**

None

**Availability of data and material**

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

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Not applicable
Authors' contributions

YPW, RXW, ZJL and LYH planned the study, YPW, XYW, XQ, TM, ZYJ, and LYH collected the literature and data. LPW and LYH composed the manuscript, and all the other co-authors approved the final manuscript.

Figure legends

Fig.1

H2S-preconditioned EXOs effectively restored the kidney function impairment in I/R rat models.

A: Microscope examination of EXOs isolated from MSCs and H2S-preconditioned MSCs.

B: Western blot analysis of CD63, CD69 and CD81 in EXOs isolated from MSCs and H2S-preconditioned MSCs.

C: H&E staining indicated that H2S preconditioning reinforced the efficiency of EXOs in restoring kidney injury in I/R rats.

D: MASSON staining indicated that H2S preconditioning reinforced the efficiency of EXOs in restoring kidney fibrosis in I/R rats.

E: H2S preconditioning reinforced the efficiency of EXOs in restoring the SCr in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

F: H2S preconditioning reinforced the efficiency of EXOs in restoring the BUN in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

G: H2S preconditioning reinforced the efficiency of EXOs in suppressing NO production in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

H: The CO production was comparable between all rat animal groups.

Fig.2

H2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats.
A: Western blot analysis of Nrf2, NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats under distinct conditions.

B: H2S-preconditioned EXOs further increased the protein expression of Nrf2 in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

C: H2S-preconditioned EXOs effectively restored the protein expression of NF-κB protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

D: H2S-preconditioned EXOs effectively restored the protein expression of TGF-β protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

E: H2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

F: H2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

G: Both EXOs and H2S-preconditioned EXOs significantly increased the gene expression of Nrf2 in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group).

H: H2S-preconditioned EXOs effectively restored the gene expression of NF-κB protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

I: H2S-preconditioned EXOs effectively restored the expression of TGF-β protein in I/R rats (* P value < 0.05 vs. SHAM group; # P value < 0.05 vs. EXOs group).

J: H2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

K: Both EXOs and H2S-preconditioned EXOs suppressed the gene expression of Col2 protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group).
H2S-preconditioned EXOs effectively restored the expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

A: H2S-preconditioned EXOs effectively restored the expression of IL-1α in I/R rats.

B: H2S-preconditioned EXOs effectively restored the expression of IL-6 in I/R rats.

C: H2S-preconditioned EXOs effectively restored the expression of IL-12 in I/R rats.

D: H2S-preconditioned EXOs effectively restored the expression of TNF-α in I/R rats.

H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD, MDA, H2O2, GST and GPx in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

A: H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD in I/R rats.

B: H2S-preconditioned EXOs effectively maintained the enzymatic activities of MDA in I/R rats.

C: H2S-preconditioned EXOs effectively maintained the enzymatic activities of H2O2 in I/R rats.

D: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GST in I/R rats.

E: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GPx in I/R rats.

H2S-preconditioned EXOs effectively restored the expression of α-SMA, Col2 protein and the enzymatic activities of SOD, MDA, H2O2, GST and GPx in HK-2 H/R models. 

A: Western blot analysis of Nrf2, α-SMA, Col2 protein in HK-2 H/R models under distinct conditions.
B: H2S-preconditioned EXOs further increased the protein expression of Nrf2 in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

C: H2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

D: H2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

E: H2S-preconditioned EXOs further increased the gene expression of Nrf2 in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

F: H2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

G: H2S-preconditioned EXOs effectively restored the gene expression of Col2 protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

H: H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

I: H2S-preconditioned EXOs effectively maintained the enzymatic activities of MDA in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
J: H2S-preconditioned EXOs effectively maintained the enzymatic activities of H2O2 in HK-2 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

K: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GST in HK-2 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

L: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GPx in HK-2 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

Fig. 6

H2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, IL-1α, IL-6, IL-12
and TNF-α in THP-1 H/R models.

A: Western blot analysis of NF-κB, TGF-β protein in THP-1 H/R models under distinct conditions.

B: H2S-preconditioned EXOs effectively restored the expression of NF-κB protein in THP-1 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

C: H2S-preconditioned EXOs effectively restored the expression of TGF-β protein in THP-1 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

D: H2S-preconditioned EXOs effectively restored the expression of NF-κB mRNA in THP-1 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).

E: H2S-preconditioned EXOs effectively restored the expression of TGF-β mRNA in THP-1 H/R
models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs.
EXOs group).
F: H2S-preconditioned EXOs effectively restored the expression of IL-1α in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

G: H2S-preconditioned EXOs effectively restored the expression of IL-6 in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

H: H2S-preconditioned EXOs effectively restored the expression of IL-12 in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

I: H2S-preconditioned EXOs effectively restored the expression of TNF-α in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

References


Corsello T, Komaravelli N, Casola A: Role of Hydrogen Sulfide in NRF2- and Sirtuin-Dependent Maintenance of Cellular Redox Balance. Antioxidants (Basel) 2018;7:
H2S-preconditioned EXOs effectively restored the kidney function impairment in I/R rat models.

A: Microscope examination of EXOs isolated from MSCs and H2S-preconditioned MSCs.

B: Western blot analysis of CD63, CD69 and CD81 in EXOs isolated from MSCs and H2S-preconditioned MSCs.

C: H&E staining indicated that H2S preconditioning reinforced the efficiency of EXOs in restoring kidney injury in I/R rats.

D: MASSON staining indicated that H2S preconditioning reinforced the efficiency of EXOs in restoring kidney injury in I/R rats.
in restoring kidney fibrosis in I/R rats.
E: H2S preconditioning reinforced the efficiency of EXOs in restoring the SCr in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).
F: H2S preconditioning reinforced the efficiency of EXOs in restoring the BUN in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).
G: H2S preconditioning reinforced the efficiency of EXOs in suppressing NO production in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).
H: The CO production was comparable between all rat animal groups.
H2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats.

A: Western blot analysis of Nrf2, NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats under distinct conditions.

B: H2S-preconditioned EXOs further increased the protein expression of Nrf2 in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

C: H2S-preconditioned EXOs effectively restored the protein expression of NF-κB protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

D: H2S-preconditioned EXOs effectively restored the protein expression of TGF-β protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

E: H2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).
F: H2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

G: Both EXOs and H2S-preconditioned EXOs significantly increased the gene expression of Nrf2 in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group).

H: H2S-preconditioned EXOs effectively restored the gene expression of NF-κB protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

I: H2S-preconditioned EXOs effectively restored the expression of TGF-β protein in I/R rats (* P value < 0.05 vs. SHAM group; # P value < 0.05 vs. EXOs group).

J: H2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

K: Both EXOs and H2S-preconditioned EXOs suppressed the gene expression of Col2 protein in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group).
H2S-preconditioned EXOs effectively restored the expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

A: H2S-preconditioned EXOs effectively restored the expression of IL-1α in I/R rats.
B: H2S-preconditioned EXOs effectively restored the expression of IL-6 in I/R rats.
C: H2S-preconditioned EXOs effectively restored the expression of IL-12 in I/R rats.
D: H2S-preconditioned EXOs effectively restored the expression of TNF-α in I/R rats.
H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD, MDA, H2O2, GST and GPx in I/R rats (* P value < 0.05 vs. SHAM group; ** P value < 0.05 vs. I/R group; # P value < 0.05 vs. EXOs group).

A: H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD in I/R rats.
B: H2S-preconditioned EXOs effectively maintained the enzymatic activities of MDA in I/R rats.
C: H2S-preconditioned EXOs effectively maintained the enzymatic activities of H2O2 in I/R rats.
D: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GST in I/R rats.
E: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GPx in I/R rats.
H2S-preconditioned EXOs effectively restored the expression of α-SMA, Col2 protein and the enzymatic activities of SOD, MDA, H2O2, GST and GPx in HK-2 H/R models.

A: Western blot analysis of Nrf2, α-SMA, Col2 protein in HK-2 H/R models under distinct conditions.

B: H2S-preconditioned EXOs further increased the protein expression of Nrf2 in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

C: H2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

D: H2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

E: H2S-preconditioned EXOs further increased the gene expression of Nrf2 in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

F: H2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

G: H2S-preconditioned EXOs effectively restored the gene expression of Col2 protein in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; #
H: H2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
I: H2S-preconditioned EXOs effectively maintained the enzymatic activities of MDA in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
J: H2S-preconditioned EXOs effectively maintained the enzymatic activities of H2O2 in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
K: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GST in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
L: H2S-preconditioned EXOs effectively maintained the enzymatic activities of GPx in HK-2 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
Fig. 6
H2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, IL-1α, IL-6, IL-12 and TNF-α in THP-1 H/R models.
A: Western blot analysis of NF-κB, TGF-β protein in THP-1 H/R models under distinct conditions.
B: H2S-preconditioned EXOs effectively restored the expression of NF-κB protein in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
C: H2S-preconditioned EXOs effectively restored the expression of TGF-β protein in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).
D: H2S-preconditioned EXOs effectively restored the expression of NF-κB mRNA in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P
value < 0.05 vs. EXOs group).

E: H2S-preconditioned EXOs effectively restored the expression of TGF-β mRNA in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

F: H2S-preconditioned EXOs effectively restored the expression of IL-1α in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

G: H2S-preconditioned EXOs effectively restored the expression of IL-6 in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

H: H2S-preconditioned EXOs effectively restored the expression of IL-12 in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).

I: H2S-preconditioned EXOs effectively restored the expression of TNF-α in THP-1 H/R models (* P value < 0.05 vs. CONTROL group; ** P value < 0.05 vs. H/R group; # P value < 0.05 vs. EXOs group).