H_2S treatment improves the protective effect of mesenchymal stem cell-derived exosomes on renal ischemia/reperfusion fibrosis by suppressing inflammatory signaling of TGF-β and NF-κB as well as radical oxygen species generation

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Abstract

Introduction: Hydrogen sulfide (H_2S) has been reported to regulate signaling pathways responsible for development of renal ischemia/reperfusion (I/R) associated fibrosis. In this study, we hypothesized that preconditioning with H_2S may boost the protective effect of mesenchymal stem cells- (MSC) -derived exosomes (EXOs) on renal I/R fibrosis.

Material and methods: Real-time polymerase chain reaction (PCR) and Western blot were performed to evaluate mRNA and protein expression of Nrf2, NF-κB, TGF-β, α-SMA, and collagen type II (Col2). H & E and Masson staining were carried out to examine the kidney injury and fibrosis in rats.

Results: H_2S-preconditioned EXOs substantially promoted the protective effect of EXOs on the development of kidney injury as well as associated fibrosis in I/R rats.EXO treatment significantly restored the activated gene and protein expression of NF-κB, TGF-β, α-SMA, and Col2 in I/R rats and the cellular model of hypoxia/reoxygenation (H/R), while H_2S preconditioning remarkably strengthened this effect of EXOs. Additionally, H_2S 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 superoxide dismutase (SOD), malondialdehyde (MDA), H_2O_2, glutathione S-transferase (GST) and glutathione peroxidase (GPx) in I/R rats and the cellular model of H/R.

Conclusions: This study utilized I/R rats to demonstrate that the effect of H_2S-preconditioned exosomes in suppressing inflammation and radical oxygen species (ROS) generation was better than that of unconditioned exosomes. To be specific, exosomes, especially H_2S-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 H_2S-preconditioned exosomes on renal I/R associated fibrosis.

Key words: H_2S, MSC, exosomes, renal ischemia, reperfusion, fibrosis, inflammation, ROS.

Introduction

Fibrosis is a pathophysiological condition that develops following renal ischemia-reperfusion (I/R) injury, and it is a very common medical condition worldwide [1]. I/R produces a microenvironment in kidney tissues which lacks oxygen and necessary nutrients, leading to an increase in oxidative stress and production of radical oxygen species (ROS). These conditions cause cell apoptosis, necrosis and injury in the interstitial cells of the kidney [2, 3]. The immune response generated within the 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 become smaller and produce an aggressive reaction like anoxia, 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 (EXOs) (30–100 nm), are usually extracted from various body fluids and supernatant layers of cell culture [12–14]. Exosomes contain a complex cocktail of components ranging from different types of proteins, genetic material such as RNA, lipids and enzymes. Exosomes function as an effective method of cell-cell communication [14, 15].

Protective effects of in situ synthesized gaseous molecules, also known as gasotransmitters, against tissue ischemia-reperfusion injury (IRI) have been reported recently. The list of recently discovered gasotransmitters includes nitric oxide (NO), carbon monoxide (CO), and the latest member is hydrogen sulfide (H_2S) [16, 17]. The protection offered by H_2S against renal injury has been shown in various models, including brain, intestine, liver lung and heart. The proposed mechanism of action of the protective effect of H_2S involves antiapoptotic, antioxidant and anti-inflammatory effects [18, 19]. The effects of exogenous H_2S treatment on the acute recovery period needed to reduce inflammation in I/R rat models were investigated in the current study [20].

A complex signaling pathway is responsible for anti-inflammatory effects of H_2S. The majority of inflammatory responses involved NF-kB as an intracellular signaling mediator [21]. It was also shown that, upon H_2S treatment, the treated cells switched into a reversible hypometabolic and hibernation-like state. The hypothesized hypometabolism induced by H_2S treatment functions 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 a lack of oxygen demand. Administration of H_2S not only scavenges reactive species, such as ROS or reactive nitrogen species (RNS), but also produces glutathione, a natural antioxidant. Overall, H_2S acts 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]. Also H_2S 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 preconditioning with H_2S could boost the effect of EXOs in the therapy of renal I/R fibrosis. By establishing an I/R rat model, we aimed to investigate the potential therapeutic effect of H_2S-preconditioned EXOs and underlying mechanisms on renal I/R fibrosis.

Material and methods

Animal and treatment

Male SD rats with age ranging between six and eight weeks were maintained at room temperature. Bone marrow was collected from the rats at six weeks, whereas the I/R model was generated using eight-week-old rats. The animal studies were performed according to an IACUC approved protocol. The 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 a 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 into four groups with 12 animals in each group, i.e., 1) the control group (a group of animals without any treatments), 2) the I/R group (animals were subjected to the surgery described before to make the IRI model), 3) the EXOs group (animals were treated with 100 µg of exosomes without H_2S treatment) and 4) EXOs with H_2S group (animals were treated with H_2S and pretreated 100 µg of EXOs). The institutional animal ethics committee has approved the protocols of this study.

Preparation of mesenchymal stem cells

Following a procedure described in a 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 a 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 production in kidney

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

Measurement of nitric oxide production in kidney

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

Real-time PCR

To explore the effect of H_2S 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 HK-2 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 using 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 with the kit. Then, quantitative real-time PCR was performed using a Fast Start Universal SYBR Green Master Mix assay kit (Roche, Basel, Switzerland) following the specific assay procedure received 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 radio immunoprecipitation assay kit (Abcam, CA) following the specific assay procedure received with the kit. Then, 50 µg of each protein sample was resolved using SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked using 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 using an enhanced chemiluminescence (ECL) assay kit (Amersham Pharmacia, Piscataway, NJ) following the specific assay procedure received with the kit, the relative protein expression of catechol-O-methyltransferase (COMT) in each sample was determined using 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 using a 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, they were stained in Weigert's iron hematoxylin for a few minutes and again washed with water. Immediately after this, Biebrich scarlet-acid fuchsin solution was applied on the tissue samples for ten minutes. Then they were rinsed with water and re-stained with phosphomolybdic-phosphotungstic acid solution for ten minutes.

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) 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 an incubator at 37°C with 5% CO_2. HK-2

cells were divided into four groups, i.e., 1) control group (HK-2 cells without any treatments); 2) hypoxia/reoxygenation (H/R) group (HK-2 cells were put in hypoxic conditions, i.e., 37°C, 1% O_2, 94% N_2 and 5% CO_2 for 12 h in glucose- and serum-free medium to induce hypoxic injury. Later, the cells were placed in normal condition (5% CO_2) for reoxygenation for 12 and 24 hours; 3) EXOs group (HK-2 cells were treated with exosomes without H_2S treatment); and 4) EXOs with H_2S group (HK-2 cells were treated with the H_2S 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 malondialdehyde (MDA) and superoxide dismutase (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 determine the amount of hydrogen peroxide present in the kidney tissues, the kidney extracted from the animals was perfused and homogenized. Later, the reagent, containing 10 U/mL horseradish peroxidase, was added to the homogenized tissues according to the provider's assay protocol. Then, the reading from the fluorescence instrument was analyzed. To detect the enzyme activity in the extracted kidney samples, the adduct of GSH and CDNB was detected at 340 nm. To measure the SOD and glutathione peroxidase (GPx) activity, an indirect method of detecting reduction of NBT by the enzyme xanthine-xanthine oxidase was used as described previously [16].

Statistical analysis

All outcomes were expressed as mean ± standard deviations. The statistical significance of inter-group differences was evaluated by Student's *t*-test. The statistical analyses were performed using Prism 7.0 software (GraphPad, La Jolla, CA). Value of $p < 0.05$ was considered to represent statistical significance.

Results

H_2S-preconditioned EXOs effectively restored the kidney function impairment in I/R rat models

MSCs were pretreated with H_2S followed by EXO collection. Electron microscope analysis indicated no obvious difference between EXOs collected from H_2S preconditioned MSCs and untreated MSCs (Figure 1 A). Western blot was performed to analyze the surface markers CD63, CD69 and CD81 on EXOs collected from H_2S preconditioned MSCs and untreated MSCs; no significant difference was found (Figure 1 B). An I/R rat model was established and subjected to EXO 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 H_2S preconditioned MSCs showed a reinforced efficiency in attenuating the kidney injury in I/R rat models (Figure 1 C). Furthermore, Masson staining was performed to evaluate the kidney fibrosis in I/R rats under distinct conditions. H_2S-preconditioned EXOs apparently maintained the kidney fibrosis in I/R rats (Figure 1 D). Additionally, the increased kidney functional parameters SCr (Figure 1 E) and BUN (Figure 1 F) were also effectively restored by H_2S-preconditioned EXOs. Moreover, it was found that H_2S preconditioning reinforced the efficiency of EXOs in suppressing NO production in I/R rats (Figure 1 G), while the CO production was not influenced by these treatments (Figure 1 H).

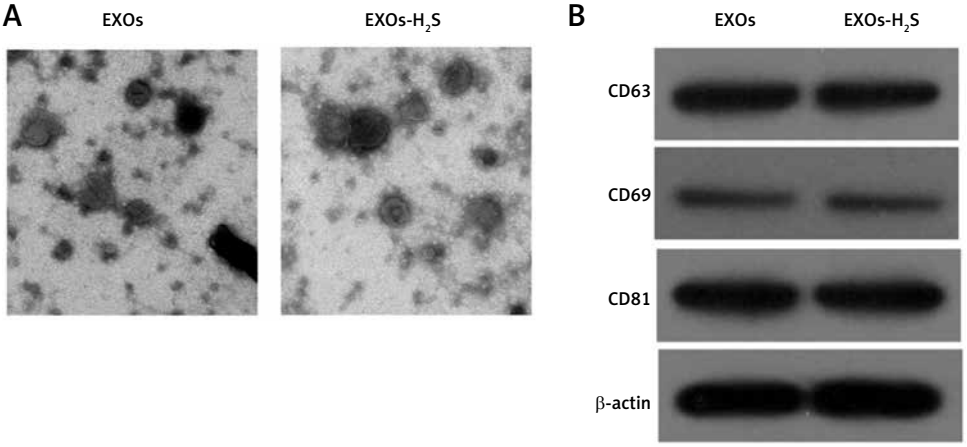

Figure 1. H_2S-preconditioned EXOs effectively restored the kidney function impairment in I/R rat models. **A** – Microscope examination of EXOs isolated from MSCs and H_2S-preconditioned MSCs. **B** – Western blot analysis of CD63, CD69 and CD81 in EXOs isolated from MSCs and H_2S-preconditioned MSCs

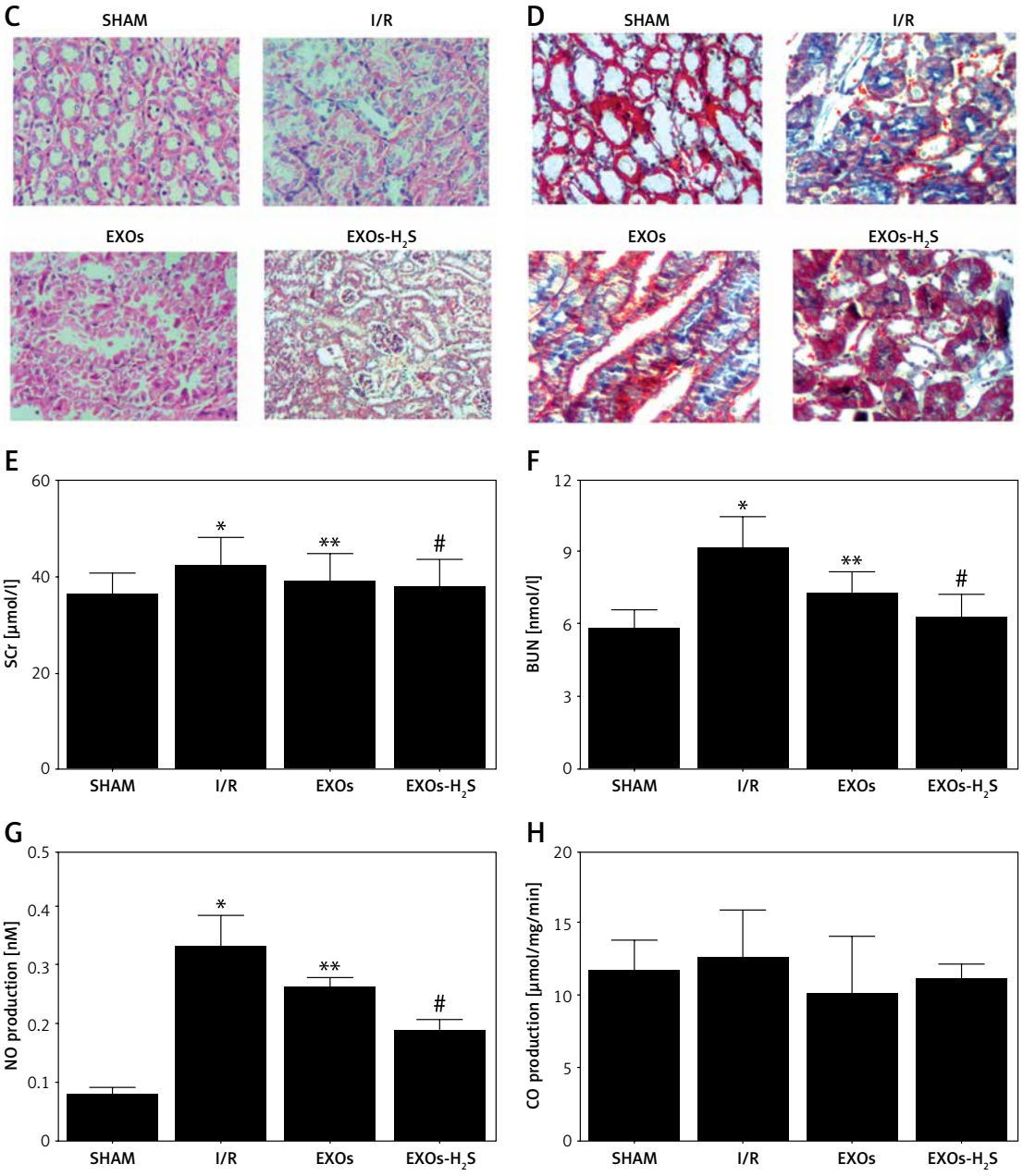

Figure 1. Cont. **C** – H & E staining indicated that H_2S preconditioning reinforced the efficiency of EXOs in restoring kidney injury in I/R rats. **D** – Masson staining indicated that H_2S preconditioning reinforced the efficiency of EXOs in restoring kidney fibrosis in I/R rats. **E** – H_2S preconditioning reinforced the efficiency of EXOs in restoring the SCr in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **F** – H_2S preconditioning reinforced the efficiency of EXOs in restoring the BUN in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **G** – H_2S preconditioning reinforced the efficiency of EXOs in suppressing NO production in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **H** – The CO production was comparable between all rat animal groups

H_2S-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 (Figure 2 A). The expression of Nrf2 was notably elevated in I/R rats compared with the control. EXO treatment further increased the Nrf2 expression and H_2S-preconditioned EXOs showed higher efficiency in enhancing the Nrf2 expression in I/R rats (Figure 2 B). Even though the expression of NF-κB, TGF-β, α-SMA, and Col2 in I/R rats was remarkably increased, EXO treatment effectively attenuated the up-regulation of NF-κB, TGF-β, α-SMA and Col2 in I/R rats. H_2S preconditioning further decreased the elevated expression of NF-κB (Figure 2 C),

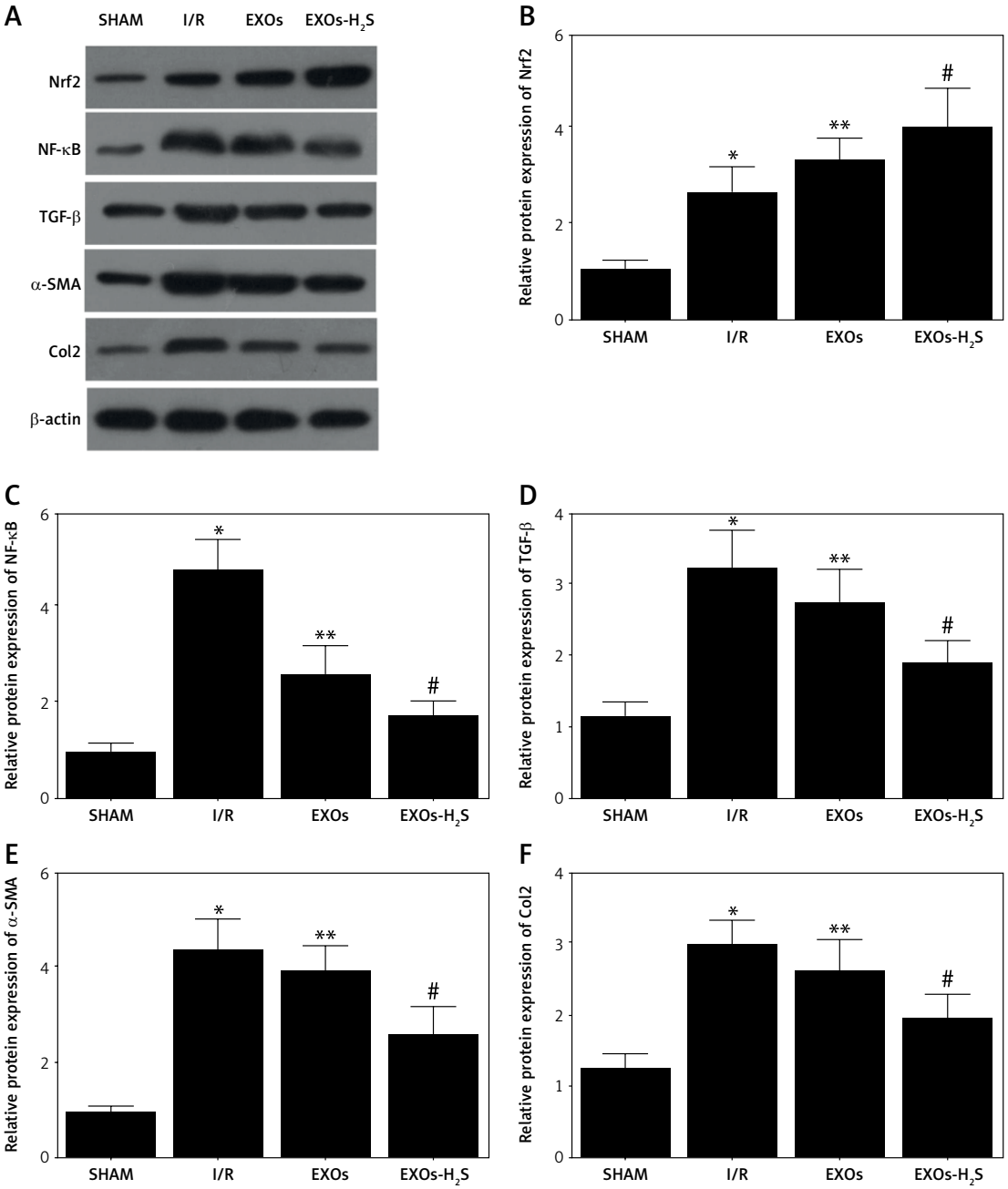

Figure 2. H_2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats. **A** – Western blot analysis of Nrf2, NF-κB, TGF-β, α-SMA, Col2 protein in I/R rats under distinct conditions. **B** – H_2S-preconditioned EXOs further increased the protein expression of Nrf2 in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **C** – H_2S-preconditioned EXOs effectively restored the protein expression of NF-κB protein in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **D** – H_2S-preconditioned EXOs effectively restored the protein expression of TGF-β protein in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **E** – H_2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **F** – H_2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group)

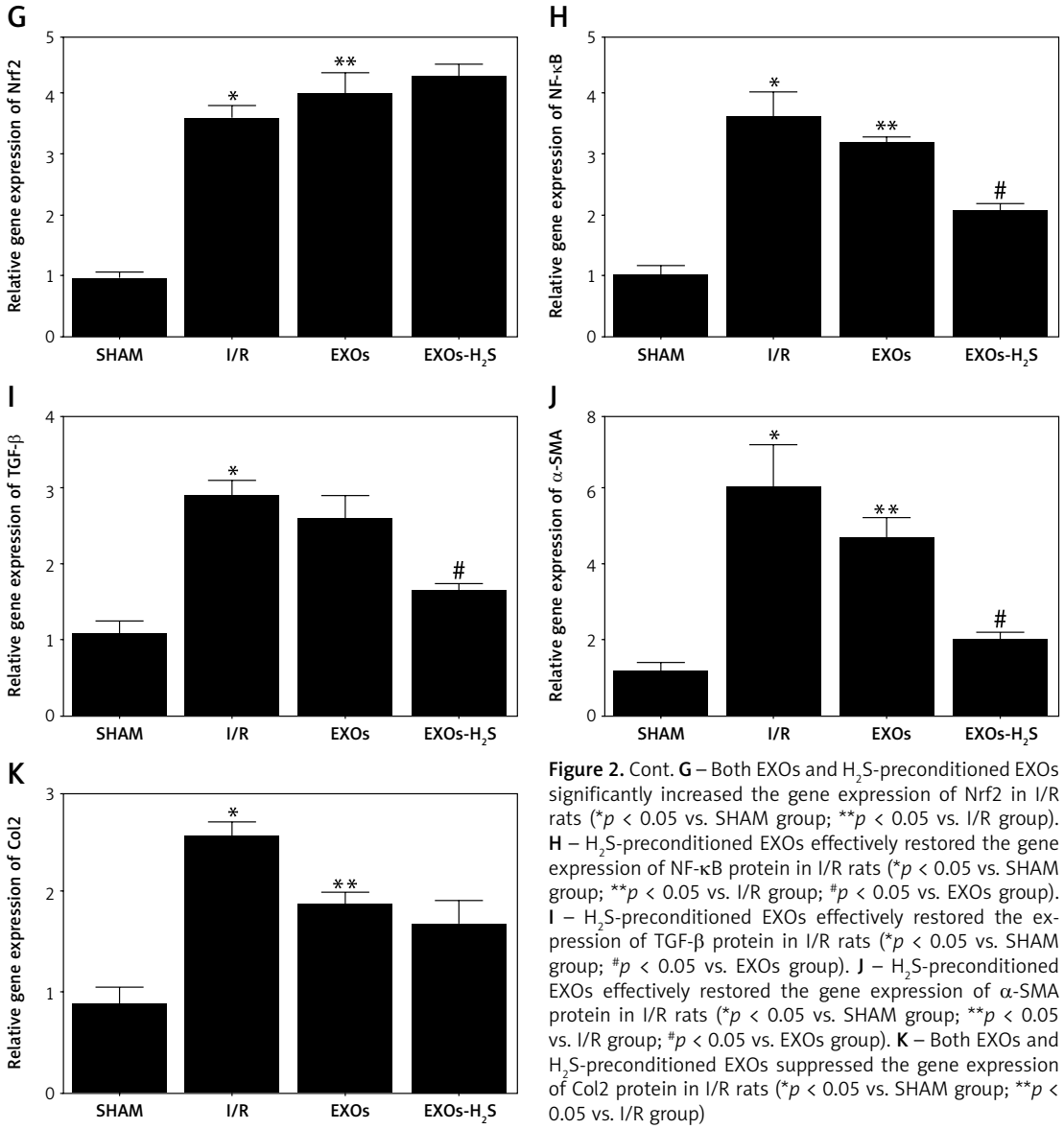

Figure 2. Cont. **G** – Both EXOs and H_2S-preconditioned EXOs significantly increased the gene expression of Nrf2 in I/R rats (*p < 0.05 vs. SHAM group; **p < 0.05 vs. I/R group). **H** – H_2S-preconditioned EXOs effectively restored the gene expression of NF-κB protein in I/R rats (*p < 0.05 vs. SHAM group; **p < 0.05 vs. I/R group; #p < 0.05 vs. EXOs group). **I** – H_2S-preconditioned EXOs effectively restored the expression of TGF-β protein in I/R rats (*p < 0.05 vs. SHAM group; #p < 0.05 vs. EXOs group). **J** – H_2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in I/R rats (*p < 0.05 vs. SHAM group; **p < 0.05 vs. I/R group; #p < 0.05 vs. EXOs group). **K** – Both EXOs and H_2S-preconditioned EXOs suppressed the gene expression of Col2 protein in I/R rats (*p < 0.05 vs. SHAM group; **p < 0.05 vs. I/R group)

TGF-β (Figure 2 D), α-SMA (Figure 2 E), and Col2 (Figure 2 F) in I/R rats. Moreover, we also performed quantitative real-time PCR upon the gene expression of Nrf2 (Figure 2 G), NF-κB (Figure 2 H), TGF-β (Figure 2 I), α-SMA (Figure 2 J), and Col2 (Figure 2 K) in the rat models, and similar results were obtained.

H_2S-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 compared with the control. EXO treatment effectively attenuated the up-regulation of IL-1α, IL-6, IL-12 and TNF-α. Moreover, H_2S-preconditioning further decreased the elevated expression of IL-1α (Figure 3 A), IL-6 (Figure 3 B), IL-12 (Figure 3 C) and TNF-α (Figure 3 D) in the kidney of I/R rats.

H_2S-preconditioned EXOs effectively restored the enzymatic activities of SOD, MDA, H_2O_2, GST and GPx in I/R rats

Enzymatic activities of SOD, MDA, H_2O_2, glutathione S-transferase (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 compared with the control. EXO treatment effectively restored the suppressed activities of SOD, GST and GPx, and H_2S preconditioning showed increased efficiency in restoring the enzymatic activities of SOD (Figure 4 A), GST (Figure 4 D) and GPx (Figure 4 E). However, the en-

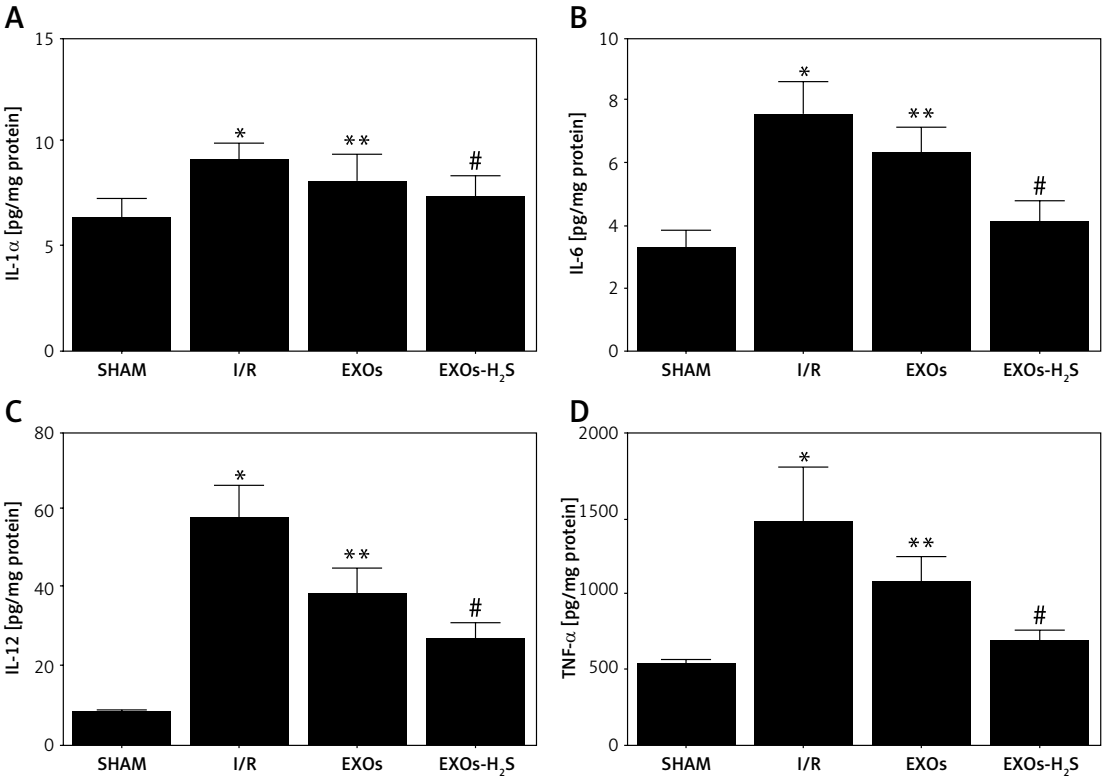

Figure 3. H_2S-preconditioned EXOs effectively restored the expression of IL-1α, IL-6, IL-12 and TNF-α in I/R rats (*$p < 0.05$ vs. SHAM group; **$p < 0.05$ vs. I/R group; #$p < 0.05$ vs. EXOs group). **A** – H_2S-preconditioned EXOs effectively restored the expression of IL-1α in I/R rats. **B** – H_2S-preconditioned EXOs effectively restored the expression of IL-6 in I/R rats. **C** – H_2S-preconditioned EXOs effectively restored the expression of IL-12 in I/R rats. **D** – v-preconditioned EXOs effectively restored the expression of TNF-α in I/R rats

zymatic activities of MDA and H_2O_2 were notably activated in I/R rats compared with the control. EXO treatment effectively decreased the elevated activities of MDA and H_2O_2, and H_2S preconditioning showed increased efficiency in restoring the enzymatic activities of MDA (Figure 4 B) and H_2O_2 (Figure 4 C).

H_2S-preconditioned EXOs effectively restored the expression of α-SMA, Col2 protein and the enzymatic activities of SOD, MDA, H_2O_2, GST and GPx in HK-2 H/R models

A HK-2 cell H/R model was established as described followed by EXO treatment. Western blot was performed to analyze the expression of Nrf2, α-SMA and Col2 (Figure 5 A) in HK-2 H/R models under distinct conditions. The expression of Nrf2 was notably elevated in HK-2 H/R models compared with the control. EXO treatment further increased the Nrf2 expression and H_2S-preconditioned EXOs showed higher efficiency in enhancing the Nrf2 expression in HK-2 H/R models (Figure 5 B). Even though the expression of α-SMA and Col2 in HK-2 H/R models was remarkably increased, EXO treatment effectively attenuated the up-regulation of α-SMA, and Col2 in HK-2 H/R models. Moreover, H_2S preconditioning further decreased the elevated expression of α-SMA (Figure 5 C) and Col2 (Figure 5 D) in HK-2 H/R models. Moreover, we also performed quantitative real-time PCR upon the gene expression of Nrf2 (Figure 5 E), α-SMA (Figure 5 F), and Col2 (Figure 5 G) 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 compared with the control. EXOs treatment effectively restored the suppressed activities of SOD, GST and GPx, and H_2S preconditioning showed higher efficiency in restoring the enzymatic activities of SOD (Figure 5 H), GST (Figure 5 K) and GPx (Figure 5 L). However, the enzymatic activities of MDA and H_2O_2 were notably activated in HK-2 H/R models compared with the control. EXO treatment effectively decreased the elevated activities of MDA and H_2O_2, and H_2S preconditioning showed increasing efficiency in restoring the enzymatic activities of MDA (Figure 5 I) and H_2O_2 (Figure 5 J).

H_2S-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 THP-1 cell H/R model was established as described followed by EXO treatment. Western blot

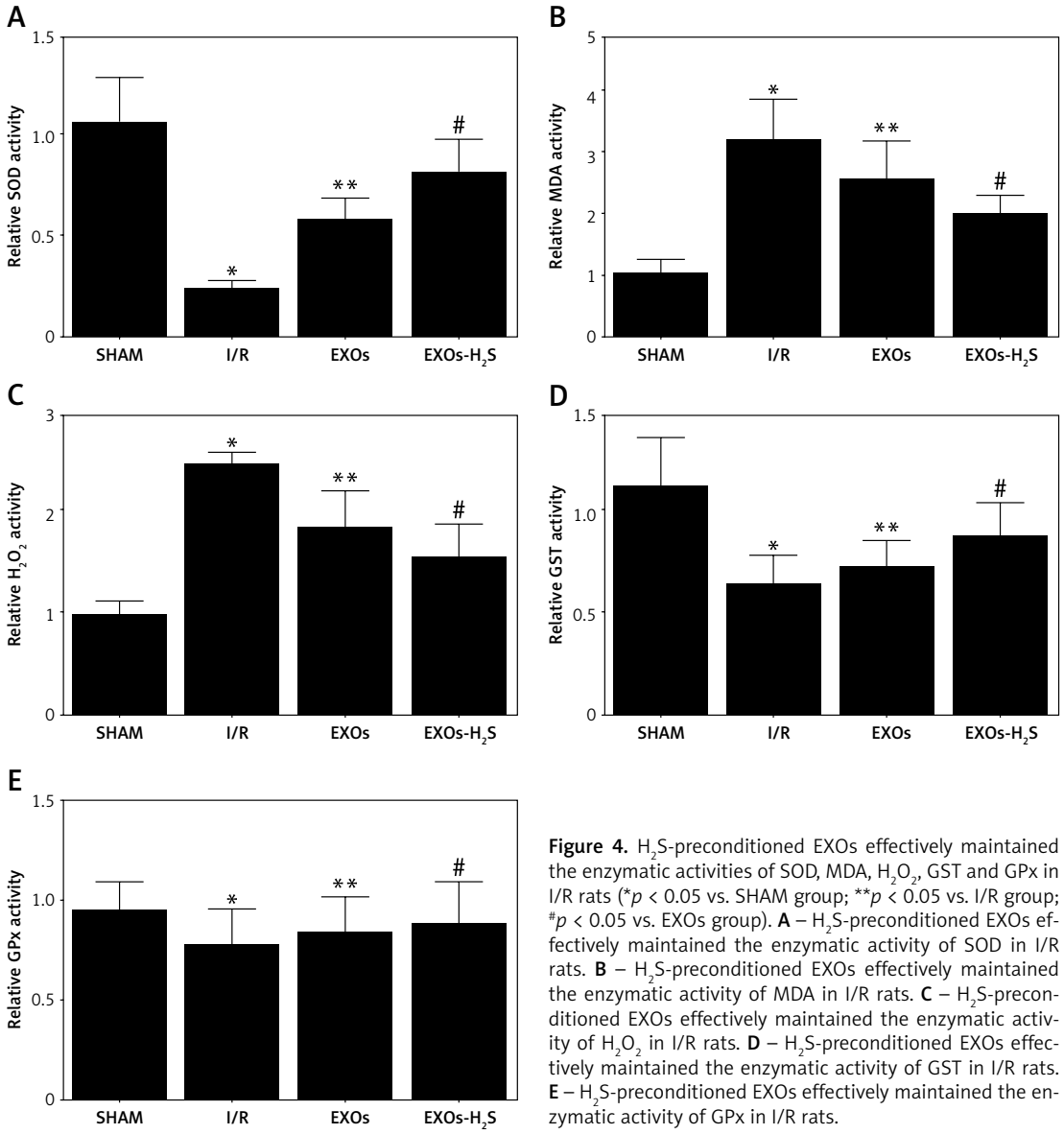

Figure 4. H_2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD, MDA, H_2O_2, GST and GPx in I/R rats (*p < 0.05 vs. SHAM group; **p < 0.05 vs. I/R group; #p < 0.05 vs. EXOs group). **A** – H_2S-preconditioned EXOs effectively maintained the enzymatic activity of SOD in I/R rats. **B** – H_2S-preconditioned EXOs effectively maintained the enzymatic activity of MDA in I/R rats. **C** – H_2S-preconditioned EXOs effectively maintained the enzymatic activity of H_2O_2 in I/R rats. **D** – H_2S-preconditioned EXOs effectively maintained the enzymatic activity of GST in I/R rats. **E** – H_2S-preconditioned EXOs effectively maintained the enzymatic activity of GPx in I/R rats.

was performed to analyze the expression of NF-κB and TGF-β (Figure 6 A). The expression of NF-κB and TGF-β in THP-1 H/R models was remarkably increased, and EXO treatment effectively attenuated the up-regulation of NF-κB and TGF-β in THP-1 H/R models. Moreover, H_2S preconditioning further decreased the elevated expression of NF-κB (Figure 6 B) and TGF-β (Figure 6 C) in THP-1 H/R models. Also quantitative real-time PCR was performed to investigate the expression of NF-κB mRNA (Figure 6 D) and TGF-β mRNA (Figure 6 E) in the cell models, showing similar results as the protein expression. 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 compared with the control. EXO treatment effectively attenuated the up-regulation of IL-1α, IL-6, IL-12 and TNF-α. Moreover, H_2S preconditioning further decreased the elevated expression of IL-1α (Figure 6 F), IL-6 (Figure 6 G), IL-12 (Figure 6 H) and TNF-α (Figure 6 I) in THP-1 H/R models.

Discussion

Many surgical procedures in the kidney inevitably lead to renal IRI [26]. The end state of kidney injury caused by IRI is diagnosed based on renal fibrosis which involves glomerulosclerosis and tubulointerstitial fibrosis [29]. This type of progression to renal failure is mostly irreversible and is the main reason for the higher mortality rate of this disease [30]. A complex mixture of factors is responsible for progressive renal fibrosis, which include growth factors, reactive oxygen species, cytokines and metabolic waste [31]. In this study, administration of H_2S-preconditioned exosomes

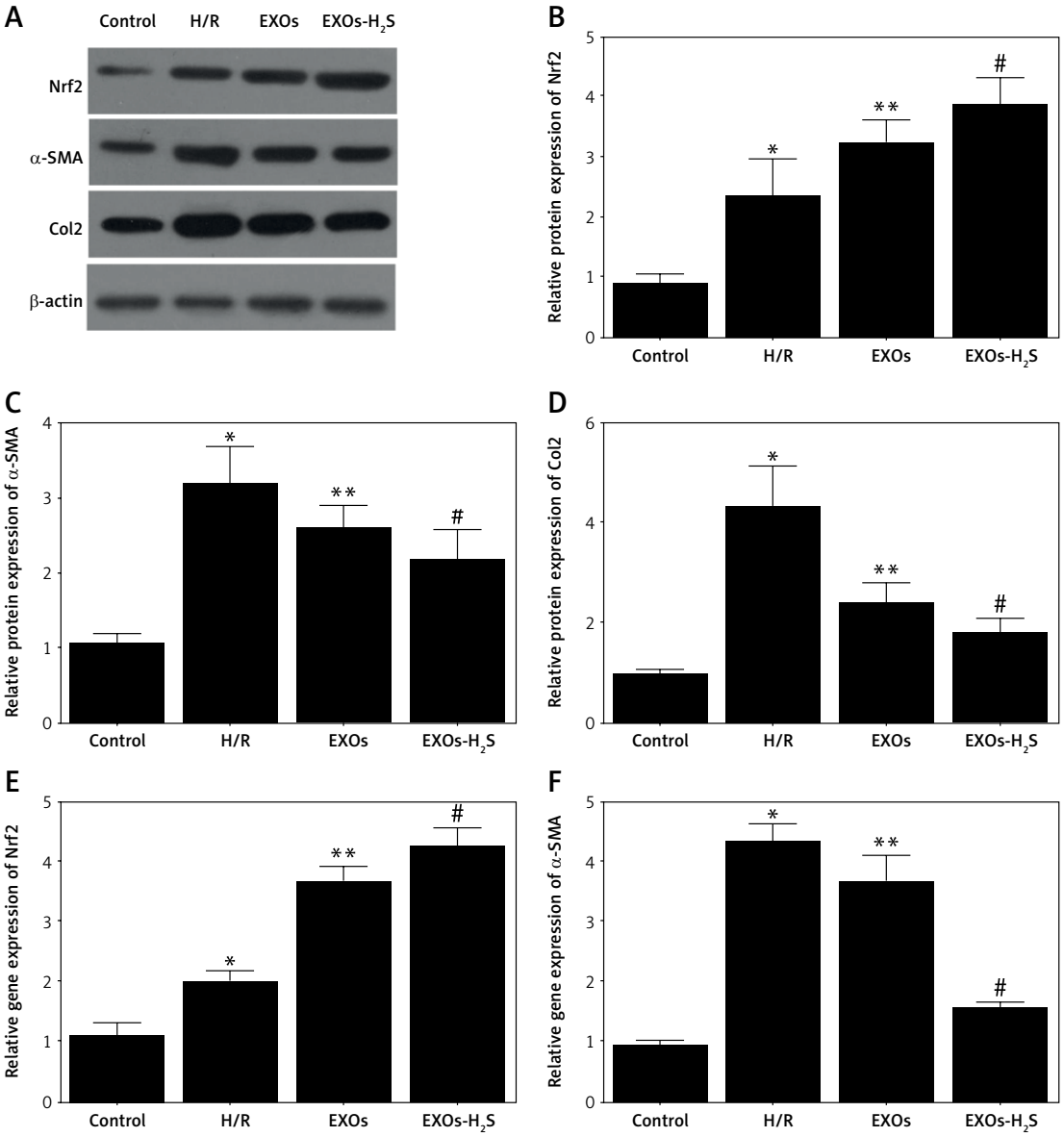

Figure 5. H_2S-preconditioned EXOs effectively restored the expression of α-SMA, Col2 protein and the enzymatic activities of SOD, MDA, H2O2, GST and GPx in HK-2 H/R models. **A** – Western blot analysis of Nrf2, α-SMA, Col2 protein in HK-2 H/R models under distinct conditions. **B** – H_2S-preconditioned EXOs further increased the protein expression of Nrf2 in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **C** – H_2S-preconditioned EXOs effectively restored the protein expression of α-SMA protein in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **D** – H_2S-preconditioned EXOs effectively restored the protein expression of Col2 protein in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **E** – H_2S-preconditioned EXOs further increased the gene expression of Nrf2 in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **F** – H_2S-preconditioned EXOs effectively restored the gene expression of α-SMA protein in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group)

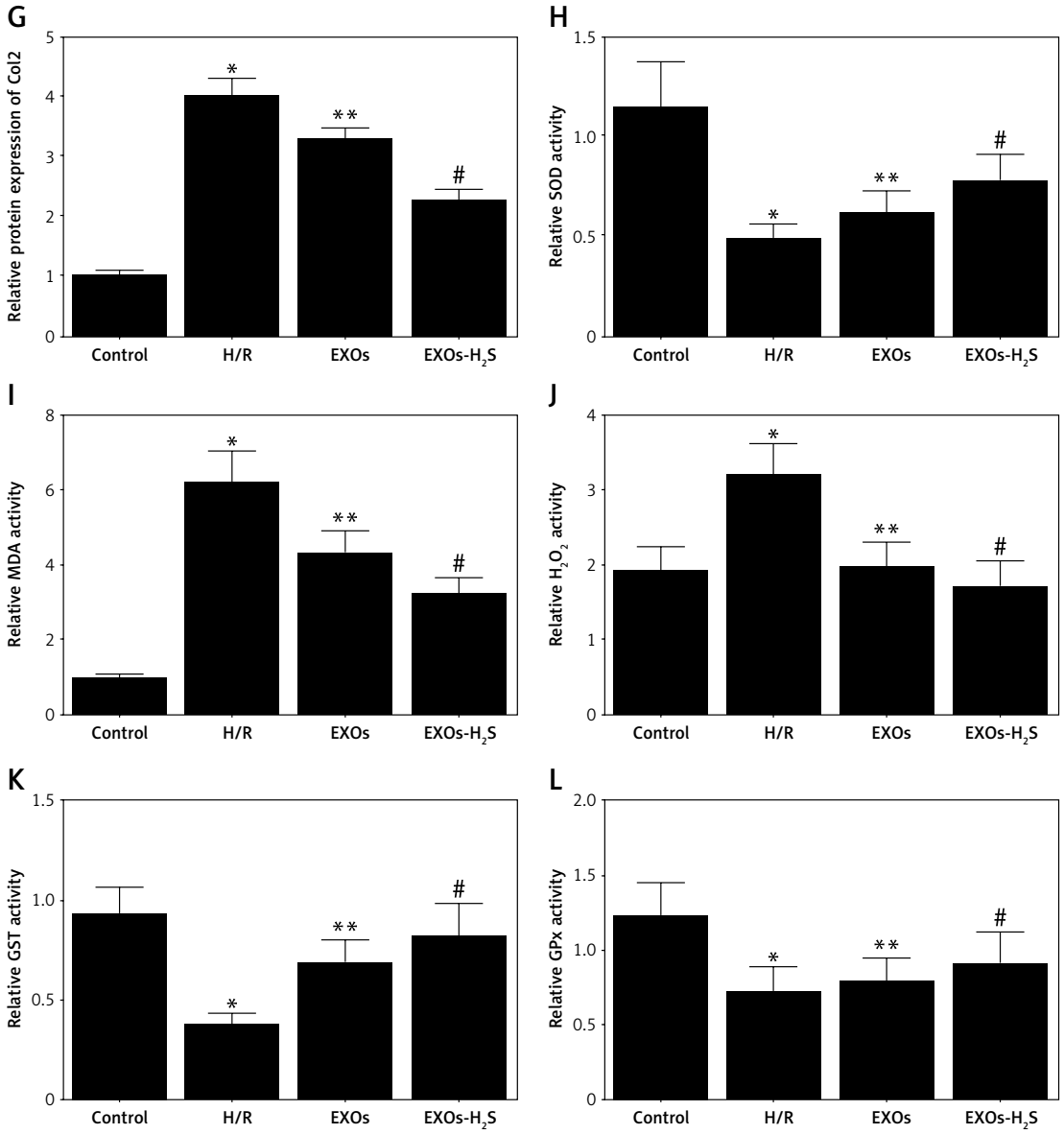

Figure 5. Cont. **G** – H_2S-preconditioned EXOs effectively restored the gene expression of Col2 protein in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **H** – H_2S-preconditioned EXOs effectively maintained the enzymatic activities of SOD in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **I** – H_2S-preconditioned EXOs effectively maintained the enzymatic activities of MDA in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **J** – H_2S-preconditioned EXOs effectively maintained the enzymatic activities of H_2O_2 in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **K** – H_2S-preconditioned EXOs effectively maintained the enzymatic activities of GST in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **L** – H_2S-preconditioned EXOs effectively maintained the enzymatic activities of GPx in HK-2 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group)

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 with treatment of H_2S pretreated or untreated exosomes in I/R rat and cellular models.

In the 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 I/R mice provided protection against acute and chronic renal injury. The therapy decreased penetration of inflammatory cells as well as enhancing activity of IL-10, which suggested that the therapy altered the microenvironment of the kidney cells and the result of the study revealed that hAD-MSC

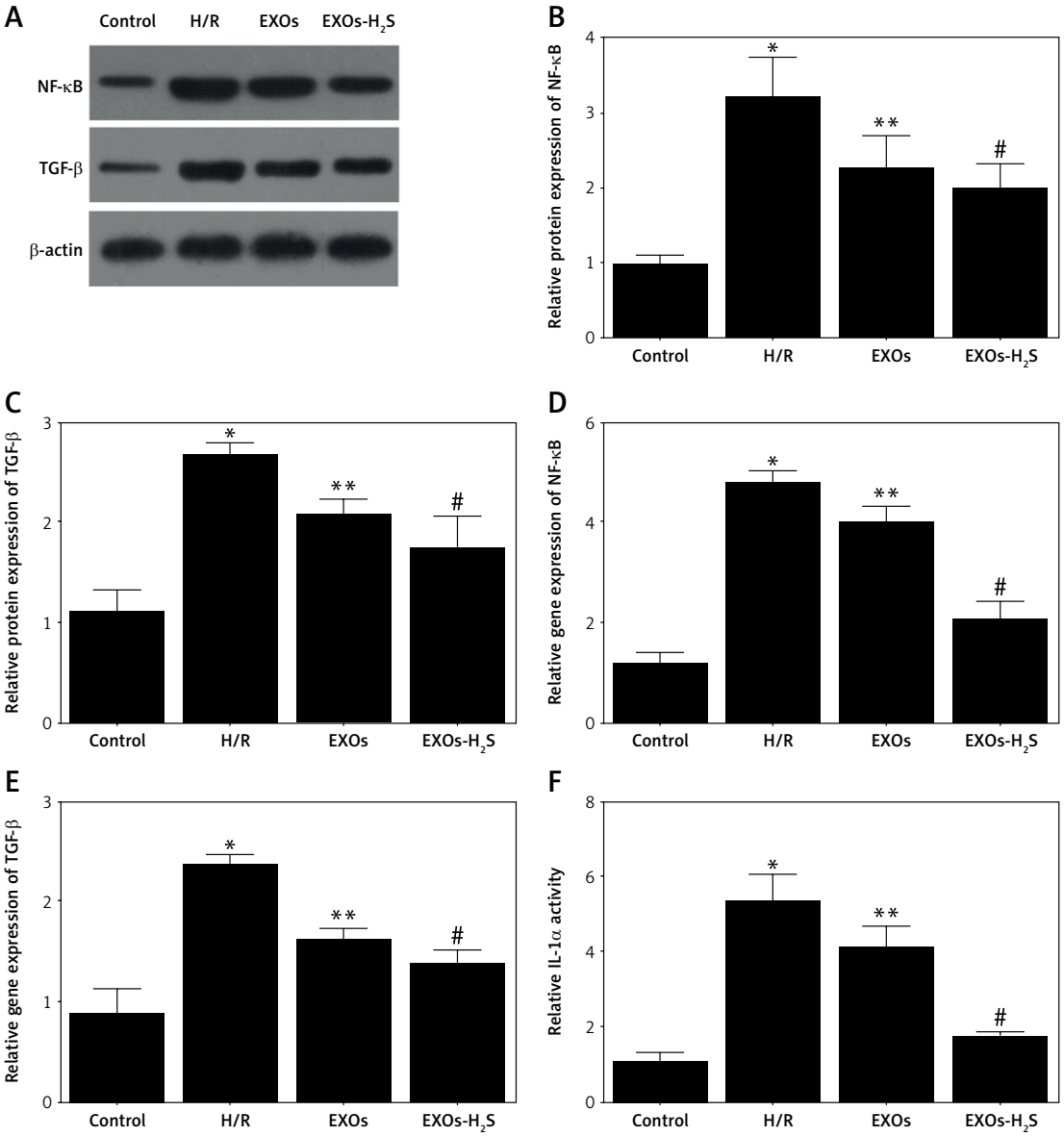

Figure 6. H_2S-preconditioned EXOs effectively restored the expression of NF-κB, TGF-β, IL-1α, IL-6, IL-12 and TNF-α in THP-1 H/R models. **A** – Western blot analysis of NF-κB, TGF-β protein in THP-1 H/R models under distinct conditions. **B** – H_2S-preconditioned EXOs effectively restored the expression of NF-κB protein in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **C** – H_2S-preconditioned EXOs effectively restored the expression of TGF-β protein in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **D** – H_2S-preconditioned EXOs effectively restored the expression of NF-κB mRNA in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **E** – H_2S-preconditioned EXOs effectively restored the expression of TGF-β mRNA in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **F** – H_2S-preconditioned EXOs effectively restored the expression of IL-1α in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group)

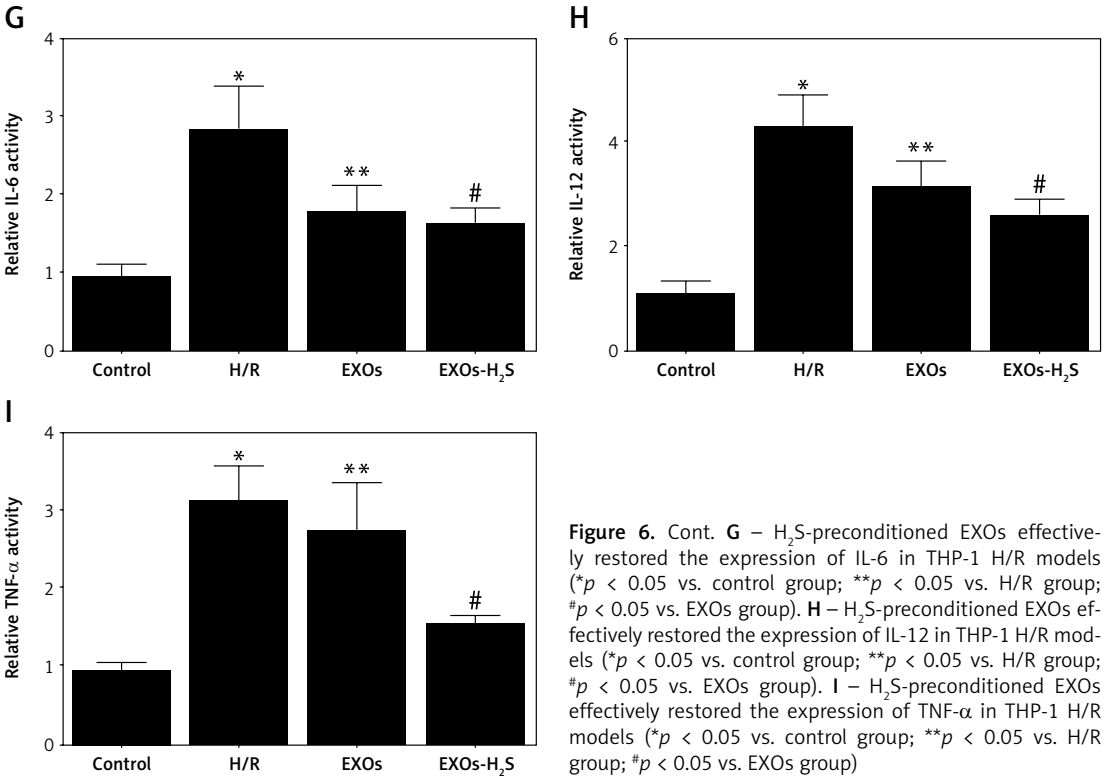

Figure 6. Cont. **G** – H_2S-preconditioned EXOs effectively restored the expression of IL-6 in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **H** – H_2S-preconditioned EXOs effectively restored the expression of IL-12 in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group). **I** – H_2S-preconditioned EXOs effectively restored the expression of TNF-α in THP-1 H/R models (*p < 0.05 vs. control group; **p < 0.05 vs. H/R group; #p < 0.05 vs. EXOs group)

therapy reduced chronic renal injury and fibrosis. Other pathophysiological conditions, such as unilateral urethral obstruction and vitamin B9 induced kidney fibrosis, also improved following hAD-MSC therapy [33, 34].

Many researchers have shown that H_2S treatment has protective effects against several pathophysiological processes as well as various types of renal ischemia/reperfusion injury [35, 36]. Previous research also proved that H_2S treatment protected against cold IRI for a longer duration, as well as against warm IRI for a shorter period [20, 37]. Another study revealed that H_2S 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 H_2S-related kidney protection [38, 39]. Apart from NO, other gasotransmitter such as CO can interact with NO and H_2S [40]. For example, H_2S was significantly minimized in the presence of CO inhibitor [41]. Previous studies have shown that a H_2S 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 H_2S and NO [42]. Moreover, H_2S therapy was found to successfully restore 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 polymorphism 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 the nucleus. Also, fasting also helped to lower the risk of fibrosis in the injured kidney. In this study, we performed ELISA to measure the expression levels of IL-1α, IL-6, IL-12 and TNF-α in I/R rat and cellular models under specific conditions. Moreover, the H_2S-pretreated exosomes successfully improved the expression levels of IL-1α, IL-6, IL-12 and TNF-α. Meanwhile, we also evaluated expression of NF-kB and H_2S-preconditioned EXO restored elevated expression of NF-kB caused by IRI. Several studies have shown that NF-κB is an important transcription factor involved in inflammatory regulation [48]. The NF-kB 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 H_2S changes the conformation of 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, H_2O_2, GST and GPx in I/R rat and cellular models under distinct conditions. H_2S-preconditioned EXOs effectively restored the enzymatic activities of SOD, MDA, H_2O_2, GST and GPx. The results of this study shed 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 H_2S will further promote the therapeutic effect of MSCs. In addition, the level of H_2S could be a biomarker for the prognosis and risk of fibrosis following renal reperfusion injury.

In conclusion, this study utilized I/R rats to demonstrate that the effect of H_2S-preconditioned exosomes in suppressing inflammation and ROS generation was better than that of unconditioned exosomes. To be specific, exosomes, especially H_2S-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 H_2S-preconditioned exosomes on renal I/R associated fibrosis.

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Conflict of interest

The authors declare no conflict of interest.

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