

# SOD2 overexpression in bone marrow-derived mesenchymal stem cells ameliorates hepatic ischemia/reperfusion injury

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**Abstract.** Hepatic ischemia/reperfusion injury (HIRI) is a complex pathophysiological process that may develop after liver transplantation and resection surgery, as well as in uncontrolled clinical conditions. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are potential targets for liver diseases. Thus, the present study aimed to investigate the effects of superoxide dismutase 2 (SOD2) overexpression in BM-MSCs on HIRI by constructing a HIRI rat model. The adenoviral vector containing SOD2 and the corresponding control vector were designed and constructed, and SOD2-overexpressing BM-MSCs were injected into the tail vein of the rats. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, as well as pathological changes and the remnant liver regeneration rate were determined. The activities of SOD and glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) content were measured. Reactive oxygen species (ROS) were determined with 2',7'-dichlorofluorescein diacetate and measured via fluorescence microscopy. Cell apoptosis was assessed using TUNEL staining. Moreover, the expression levels of Bax, Bcl-2 and caspase-3 were detected via western blotting. SOD2-overexpressing BM-MSCs significantly reduced the elevation of serum AST and ALT levels. Furthermore, SOD2-overexpressing BM-MSCs enhanced SOD and GSH-Px activities, and suppressed the production of MDA and ROS. Histopathological findings revealed that SOD2-overexpressing BM-MSCs decreased the number of TUNEL-positive cells in the liver. It was also found that SOD2-overexpressing BM-MSCs promoted Bcl-2 expression, but inhibited Bax and caspase-3 expression in HIRI. Collectively, these findings suggest that SOD2-overexpressing BM-MSCs may provide

therapeutic support in HIRI by inhibiting oxidative stress and hepatocyte apoptosis.

## Introduction

Ischemia/reperfusion (I/R) injury refers to the destruction of the normal structure and physiological function of the tissues and organs after the interruption and restoration of the blood supply (1). I/R injury often occurs when tissues and organs are traumatized, such as after thrombosis, frostbite burns, organ transplantation and surgery (2). Hepatic I/R injury (HIRI) is also a common complication after liver tissue injury and includes two stages of ischemia injury and reperfusion injury, and is associated with calcium overload, oxidative stress, cell apoptosis and metabolic acidosis (3,4). However, there are currently no ideal treatments or prevention interventions that can effectively avoid HIRI.

Accumulating evidence has suggested that stem cells secrete a variety of cytokines, improve the local micro-environment, regulate the process of cell apoptosis and exert anti-inflammatory and anti-apoptotic effects, as well as promote the endogenous repair of tissues in the injured area (5-7). With the development of stem cells in the field of disease treatment, bone marrow-derived mesenchymal stem cells (BM-MSCs) have been attracting increased attention due to their wide-ranging sources, weak immunogenicity and multidirectional differentiation across germ layers (8,9). After continuous subculturing and cryopreservation, BM-MSCs maintain their multidirectional differentiation ability, as well as their normal karyotype and telomerase activity, and can be induced to differentiate into hepatocytes, neuronal cells, cartilage cells and adult cells under specific conditions *in vitro* (10,11). Moreover, BM-MSCs can attenuate injury of the brain, heart, kidney, liver and other organs, which activates strong chemical signals from damaged tissues, via differentiating into a variety of cells, secreting cytokines and chemokines, and through cell fusion (12).

A number of studies have reported that MSCs can alleviate liver histological damage, promote hepatocyte proliferation and accelerate liver tissue recovery (13,14). Accumulating evidence has indicated that BM-MSC-derived hepatocyte-like exosomes reduce HIRI by enhancing autophagy (13). In a rat HIRI model, MSCs improve PTEN induced kinase 1-dependent mitochondrial-mediated apoptosis of hepatocytes

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via AMP-activated protein kinase  $\alpha$  subunit activation (15). Other studies have further confirmed that allogeneic BM-MSCs can reduce HIRI by inhibiting oxidative stress and apoptosis (16). These findings indicate that BM-MSCs serve an important role in HIRI. Therefore, BM-MSCs may have potential therapeutic prospects for the treatment of HIRI.

It is worth noting that BM-MSCs can also be used as vectors to deliver protective genes by overexpressing the transfected genes in damaged parts. This not only improves the therapeutic effect, but also promotes local tissue repair (17). Superoxide dismutase 2 (SOD2) gene transfection of BM-MSCs inhibits inflammation, and it has been found that the transfected gene can be stably expressed in the body and improve glucose tolerance (18). Furthermore, upregulation of SOD2 can promote the proliferation of human umbilical cord blood-derived MSCs, and improve the oxidative stress and apoptosis of bone tissue (19). These findings suggest that BM-MSCs can be used as cell therapy, and that they are also optimal gene carriers.

Based on the aforementioned research findings, the present study aimed to establish an animal model of HIRI that closely simulated hepatic transplantation injury to investigate the protective role of SOD2-overexpressing BM-MSCs and the underlying molecular mechanisms during HIRI.

## Materials and methods

**Animals.** All animal procedures were conducted in accordance with the International Guidelines for the Care and Use of Laboratory Animals and local ethics committee approval (20), and were also approved by the Laboratory Animal Center, The Second Xiangya Hospital of Central South University (Changsha, China). A total of 80 male Wistar rats (weight, 200–220 g; age, 8–10 weeks) were used for this study. All rats were kept in a temperature and humidity-controlled environment with a 12-h light/dark cycle at 22–25°C, with 50–65% humidity and free access to food and water.

**Isolation of BM-MSCs.** BM-MSCs were obtained using density centrifugation as previously described (18). Briefly, MSCs were flushed from the femurs and tibias of male 4-week-old Wistar rats, and were cultured with MEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (cat. no. F8687; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 mg/l streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Non-adherent hematopoietic cells were removed from the adherent BM-MSCs. The culture medium was changed every 3 days. The cells were subcultured when they reached 70–80% confluence. Third-passage BM-MSCs were used in all the experiments.

**Characterization of MSCs.** When the cultured cells reached 90% confluence, adherent cells were trypsinized and passaged. Cells underwent five passages before use in subsequent experiments. BM-MSCs were analyzed via antibody staining using CD90 (cat. no. SAB4700719; Sigma-Aldrich; Merck KGaA), CD105 (cat. no. MABT117; Sigma-Aldrich; Merck KGaA), CD34 (cat. no. RAB1334; Sigma-Aldrich; Merck KGaA) and CD45 (cat. no. APREST79682; Sigma-Aldrich; Merck KGaA).

MSC marker antibody staining was performed using a FACSCanto II flow cytometer (BD Biosciences).

**Adenovirus production and transduction.** Empty adenovirus vectors (Ad.null) and vectors encoding SOD2 (Ad.SOD2) were purchased from Shanghai Liangtai Biotechnology Company, and transductions were performed as previously described (21). To yield the adenovirus, the linearized construct DNA was transfected into 293 cells (American Type Culture Collection) using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 24 h, the cells were supplied with fresh medium, and the incubation was continued for an additional 5 days. The virus was released from the cells by freezing and thawing for three consecutive cycles. After the third freeze-thaw cycle, the cells were briefly centrifuged at 4,000 x g to pellet the debris at 4°C for 10 min, and the lysate was collected in sterile centrifuge tubes and stored at -20°C for subsequent use. For adenovirus transduction, the MSCs were plated into 6-well plates, and the next day, the adenovirus was added into 2 ml serum-free DMEM at a MOI of 10. The plate was centrifuged at 220 x g for 90 min at 37°C. Then, the cells were incubated in a 5% CO<sub>2</sub> incubator at 37°C for an additional 4 h. Next, the medium was removed, and fresh complete growth medium (cat. no. 12558011; Gibco; Thermo Fisher Scientific, Inc.) was added. The cells were incubated for another 24 h at 37°C prior to analysis.

**Animal model of HIRI.** Rats were divided into the following groups (n=8 per group): Sham group, rats were subjected to laparotomy only; I/R group, rats were induced with I/R and received PBS; +MSCs, rats were induced with I/R and then transplanted with MSCs; and +SOD2-MSCs, rats were induced with I/R and then transplanted with SOD2-overexpressing MSCs. HIRI was induced as previously described (17). All surgical procedures were performed under anesthesia with pentobarbital sodium (60 mg/kg). A midline laparotomy was performed to expose the portal circulation, and a microaneurysm clamp was placed on the hepatic artery and portal vein to block the blood supply. This method resulted in 70% of segmental liver ischemia and prevented mesenteric veins. After 60 min, the clamp was removed, and 1x10<sup>6</sup> PKH26-labeled MSCs were immediately resuspended in 200  $\mu$ l PBS with a 30-gauge needle. Sham-operated rats only underwent laparotomy. After the operation, a 4/0 silk suture was used. After the animals were fully awake, they were provided with free access to food and water. During the entire procedure, the core body temperature of each rat was continuously monitored and maintained at 37.0 $\pm$ 0.4°C with a heating lamp. All animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) until the animals lost consciousness, and then sacrificed by exsanguination.

**Assessment of liver functions.** To assess the severity of HIRI, the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined. Rats were anesthetized with pentobarbital sodium (60 mg/kg), and 2 ml blood was collected from the inferior vena cava with a 20-gauge needle, which was then placed in a microtainer tube with serum separator (Eppendorf), and centrifuged at 4°C at 3,000 x g

for 12 min. AST (cat. no. C010-2-1; Nanjing Jiancheng Bioengineering Institute) and ALT (cat. no. C009-2-1; Nanjing Jiancheng Bioengineering Institute) levels were measured using commercial kits.

**Hematoxylin and eosin (H&E) staining.** Liver tissues were collected, fixed in 4% paraformaldehyde for 24 h at room temperature and dehydrated until transparent. The tissues were embedded in paraffin, and cut into 5- $\mu$ m sections. Sections were stained with H&E at room temperature and observed in three random areas under a light microscope (Zeiss GmbH) connected to a digital camera.

The severity of hepatic injury was evaluated in accordance with the modified Suzuki classification (22). Scores for the corresponding indicators of liver severity were determined as follows: None, 0; minimal, 1; moderate, 2; and severe, 3. For each rat, three liver sections were examined and three randomly selected high-power fields (magnification, x200) were analyzed in each section. The mean score for each animal was then determined by a summation of all scores.

**Determination of SOD, glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) in the liver.** Liver tissues were collected and homogenized in ice-cold 0.9% saline. Following centrifugation at 3,000 x g for 10 min at 4°C, the supernatant was collected and the activities of SOD (cat. no. 19160; Sigma-Aldrich; Merck KGaA), GSH-Px (cat. no. CS0260; Sigma-Aldrich; Merck KGaA) and MDA (cat. no. MAK085; Sigma-Aldrich; Merck KGaA) content were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute).

**Measurement of ROS.** The total ROS level was measured using a ROS assay kit (cat. no. MAK143; Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. Briefly, intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2V, 7V-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) using a fluorospectrophotometer (Thermo Fisher Scientific, Inc.) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

**Detection of apoptotic cells in liver tissues.** A TUNEL kit was used to detect the apoptotic cells. Liver sections (thickness, 5  $\mu$ m) were excised and fixed with 4% paraformaldehyde in PBS at room temperature for 24 h. Fixed tissues were embedded in paraffin and stained using a TUNEL kit (cat. no. 11684795910; Roche Diagnostics), according to the manufacturer's protocol, and six sections were analyzed for each rat. The numbers of apoptotic cells and total hepatic cells in each section were counted in three randomly selected fields (magnification, x400). The apoptosis index (AI) was expressed as the mean percentage of apoptotic cells within the total number of hepatic cells for each animal.

**Western blotting.** A total of 100 mg liver tissue was homogenized with lysate (cat. no. P0013; Beyotime Institute of Biotechnology), and the supernatant was centrifuged at 4,000 x g at 4°C for 10 min. Protein quantification was performed using a BCA protein assay (Abcam) and protein samples (20  $\mu$ g) were

collected and subjected to SDS-PAGE (10% separation gel and 6% concentration gel). Proteins in the gel were subsequently transferred to PVDF membranes and blocked with 5% skimmed milk in TBS-0.1% Tween-20 at 37°C for 1 h. The membranes were then incubated with the following primary antibodies: Rabbit anti-Bcl-2 (1:1,000; cat. no. SAB4500003; Sigma-Aldrich; Merck KGaA), rabbit anti-Bax (1:1,000; cat. no. SAB4502546; Sigma-Aldrich; Merck KGaA) and rabbit anti-caspase-3 (1:1,000; cat. no. C8487; Sigma-Aldrich; Merck KGaA) in blocking solution at 4°C overnight. Membranes were then washed and incubated for 5 min at room temperature with HRP-conjugated anti-mouse (1:5,000; cat. no. AP160P; Sigma-Aldrich; Merck KGaA) or anti-rabbit IgG secondary antibodies (1:2,000; cat. no. 31402; Invitrogen; Thermo Fisher Scientific, Inc.). The bound secondary antibodies were analyzed with an Odyssey Infrared Imaging system (LI-COR Biosciences), and proteins were normalized to  $\beta$ -actin (1:5,000; cat. no. SAB3500350; Sigma-Aldrich; Merck KGaA). Densitometric analysis was performed using ImageJ version 2 software (National Institutes of Health).

**Reverse transcription-quantitative (RT-q)PCR.** Total RNA was extracted using a RNeasy Mini kit (Qiagen, Inc.) from the MSCs after adenovirus transduction and purified with 75% ethanol. RNA concentration was determined by spectrophotometry. The purified total RNA (200 ng/sample) was reverse transcribed into cDNA using a transcription kit (cat. no. RR037A; Takara Bio, Inc.). qPCR reactions were performed in triplicate using a SYBR<sup>®</sup> Green Master Mix (Bio-Rad Laboratories, Inc.) and run on a LightCycler 480 system (Roche Diagnostics GmbH). The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec; followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 31 sec. The following primers were used in the current study: SOD2 forward, 5'-ACGTACTAGACGCGCAATT-3' and reverse, 5'-ACTTGTTAGAGTTGCGGTGG-3'; and GAPDH forward, 5'-CAGTACTTCCCCAGCAA-3' and reverse, 5'-CACGACTCATACAGCACCT-3'. The relative gene expression was quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (23).

**Statistical analysis.** SPSS 21.0 (IBM Corp.) was used for statistical analyses. Data are presented as the mean  $\pm$  SD of experiments repeated in triplicate. For multiple comparisons, data were analyzed using one-way ANOVA followed by a Tukey's post hoc test. Analysis between two groups was performed using an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression level of SOD2 in BM-MSCs with adenovirus vector transduction.** To verify whether the collected cells were BM-MSCs, MSC-specific cell surface markers were detected via staining. As shown in Fig. 1A-D, BM-MSCs were positive for CD90 and CD105, which are specific MSC surface markers (24). Additionally, these cells were negative for CD34 and CD45, which are non-MSC markers. The findings suggested that these BM-MSCs were typical MSCs, and were used for subsequent experiments.

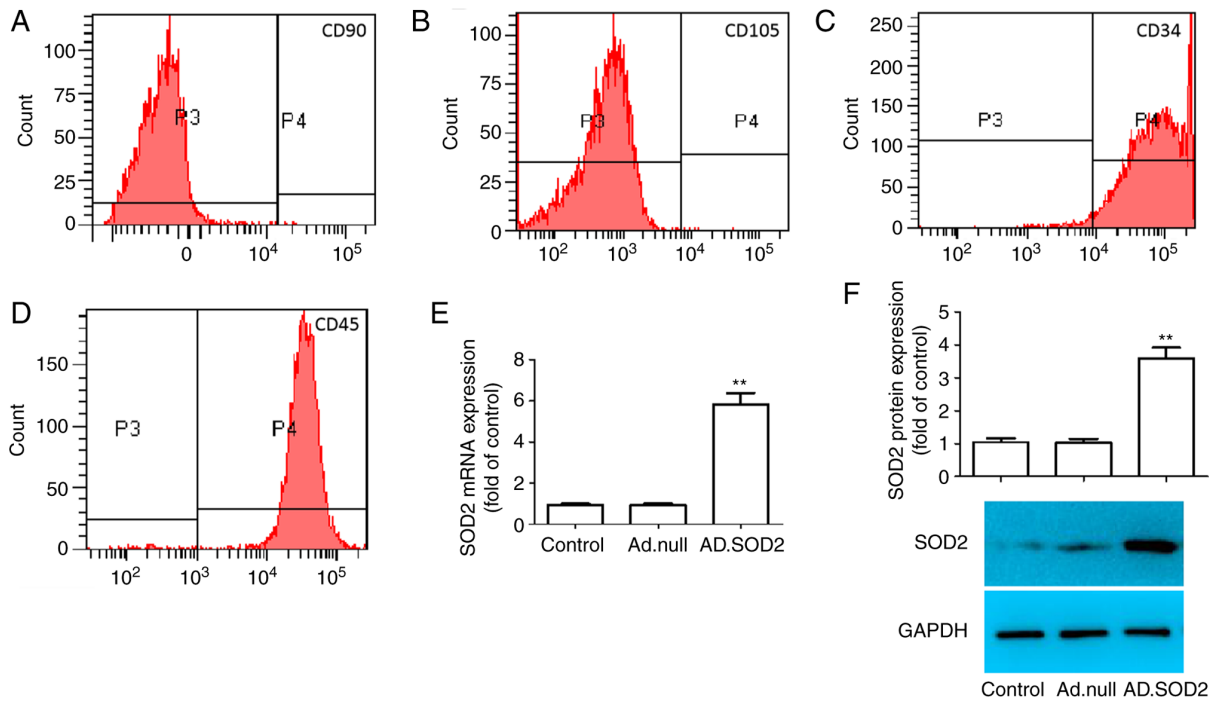


Figure 1. Flow cytometric identification of third-passage MSCs. Histograms show isotype controls (light red area) and cell surface marker expression (red line). The percentage of the expression level of the indicated markers was defined as the M1 region. Flow cytometric analysis identified that BM-MSCs were positive for (A) CD90 and (B) CD105, and negative for (C) CD34 and (D) CD45. Histograms show isotype controls (light red area) and cell surface marker expression (red line). The percentage of the expression level of the indicated markers was defined as the M1 region. (E) SOD2 mRNA expression was analyzed via reverse transcription-quantitative PCR. (F) SOD2 protein expression was determined using western blot analysis. Data are presented as the mean  $\pm$  SD from three independent experiments. \*\* $P < 0.01$  vs. Control. BM-MSCs, bone marrow-derived mesenchymal stem cells; SOD2, superoxide dismutase 2; Ad, adenovirus.

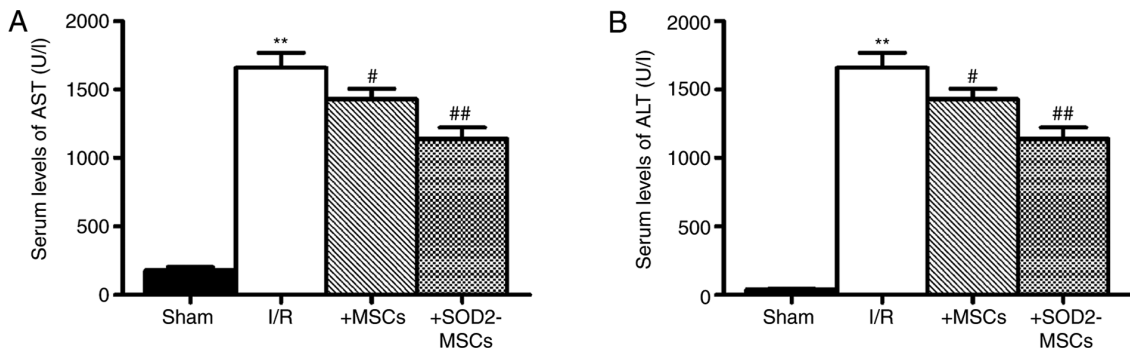


Figure 2. Effects of SOD2-overexpressing MSC transplantation on AST and ALT levels in the serum of rats with hepatic I/R injury. (A) AST and (B) ALT levels in the serum. Blood samples were collected after I/R and cell transplantation for ALT and AST analysis. Sham group, rats were subjected to laparotomy only; I/R group, rats were induced with I/R and received PBS; +MSCs, rats were induced with I/R and then transplanted with MSCs; and +SOD2-MSCs, rats were induced with I/R and then transplanted with SOD2-overexpressing MSCs.  $n = 8$  rats in each experimental group. Data are expressed as the mean  $\pm$  SD. \*\* $P < 0.01$  vs. sham; # $P < 0.05$ , ## $P < 0.01$  vs. I/R. I/R, ischemia/reperfusion; SOD2, superoxide dismutase 2; MSCs, mesenchymal stem cells; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

To verify SOD2 overexpression in BM-MSCs, Ad.SOD2 was transduced into BM-MSCs. After infection for 24 h, the mRNA and protein expression levels of SOD2 were assessed. The results demonstrated that SOD2 mRNA and protein expression levels were significantly increased (Fig. 1E and F) in Ad.SOD2-transduced BM-MSCs compared with mock group or Ad.null-transduced BM-MSCs (\*\* $P < 0.01$ ).

*Effects of BM-MSCs overexpressing SOD2 on AST and ALT levels after HIRI.* Elevated AST and ALT levels are important signs of severe liver injury (25). To determine the degree

of I/R-induced hepatic injury in the liver tissues of the rat receiving BM-MSCs or SOD2-overexpressing BM-MSCs, ALT and AST levels were assessed after I/R and cell transplantation. Compared with the sham group, markers of liver damage, including ALT and AST, were significantly increased in I/R model rats (\*\* $P < 0.01$ ; Fig. 2A and B). However, there were significantly decreased levels of ALT and AST in the I/R + MSCs and I/R + SOD2-MSCs groups compared with the I/R group (# $P < 0.05$ ). These findings suggested that the transplantation of SOD2-overexpressing BM-MSCs improved HIRI.



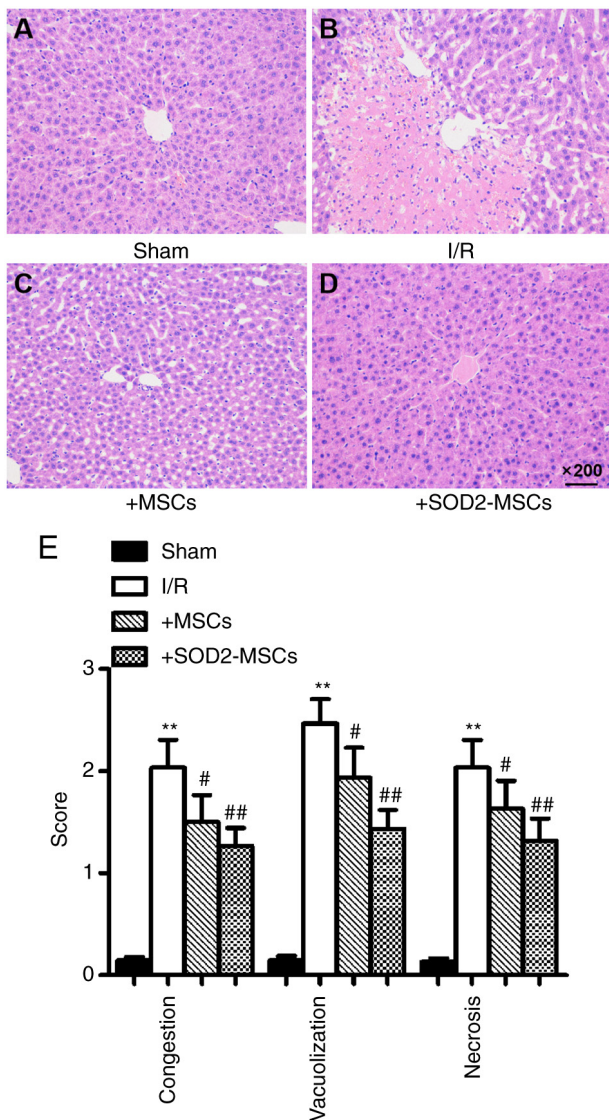


Figure 3. Histopathological analysis of liver tissues in rats. Liver tissue sections were stained with hematoxylin and eosin and scored according to the Suzuki Score System. Represented micrographs of liver tissue staining. Representative micrographs of livers from the (A) sham, (B) I/R, (C) +MSCs and (D) +SOD2-MSCs groups. (E) Suzuki scores from each group. Sham group, rats were subjected to laparotomy only; I/R group, rats were induced with I/R and received PBS; +MSCs, rats were induced with I/R and then transplanted with MSCs; and +SOD2-MSCs, rats were induced with I/R and then transplanted with SOD2-overexpressing MSCs. n=8 rats in each experimental group. Data are expressed as the mean  $\pm$  SD. \*\*P<0.01 vs. sham; #P<0.05, ##P<0.01 vs. I/R. I/R, ischemia/reperfusion; SOD2, superoxide dismutase 2; MSCs, mesenchymal stem cells.

**BM-MSCs overexpressing SOD2 improve the histopathological changes of HIRI.** To further confirm the role of SOD2 overexpression on HIRI in rats, the histopathology of livers harvested after I/R induction and cell transplantation were examined. As shown in Fig. 3A-D, the pathological findings identified sinusoidal congestion, cytoplasmic vacuolization and necrosis in the I/R group, which are indicative of severe damage. Moreover, the results of the Suzuki scores indicated worse histopathology in the I/R group compared with the sham group (\*\*P<0.01), whereas the scores indicated improved histopathology in the I/R + MSCs and I/R + SOD2-MSCs groups compared with the I/R group (#P<0.05; Fig. 3E).

**BM-MSCs overexpressing SOD2 attenuate the oxidative stress response in I/R injury.** The oxidative stress response is considered to be an important contributor to HIRI (26). Therefore, SOD, GSH-Px and MDA levels were assessed after HIRI induction. It was found that SOD and GSH-Px levels were markedly decreased, while the MDA level was notably increased in I/R rats compared with the sham group (\*P<0.05; Fig. 4A-C). These findings indicated that elevated oxidative stress was an important contributor in liver injury. Moreover, in the I/R + MSCs and I/R + SOD2-MSCs groups, increased SOD and GSH-Px activities were observed, while MDA levels were decreased compared with the I/R group (#P<0.05; Fig. 4A-C). In line with the results of the biochemical index, the production of reactive oxygen species (ROS) was detected in the liver tissues. The results demonstrated that an increased number of apoptotic cells was observed in the hepatic I/R group compared with the sham group, whereas the number of apoptotic cells was notably decreased in the I/R + MSCs and I/R + SOD2-MSCs groups compared with the I/R group (Fig. 4D-G). These findings suggested that transplanted SOD2-overexpressing BM-MSCs attenuated the oxidative stress in HIRI.

**BM-MSCs overexpressing SOD2 inhibit cell apoptosis after HIRI.** To investigate the effect of SOD2-overexpressing BM-MSCs against HIRI-related apoptosis, TUNEL staining was firstly utilized to examine the number of apoptotic cells and western blotting was used to detect the expression levels of Bcl-2, Bax and caspase-3. It was identified that hepatic I/R caused a markedly higher AI, suggesting that I/R resulted in hepatocyte apoptosis (Fig. 5A and B). Furthermore, the findings indicated a significant downregulation in Bcl-2 expression, and an upregulation in Bax and caspase-3 expression in the I/R group compared with the sham group (\*P<0.05; Fig. 5E-H). It was also found that these effects were partially reversed in the I/R + MSCs and I/R + SOD2-MSCs groups compared with the I/R group (#P<0.05; Fig. 5C-H). Collectively, these results suggested that SOD2-overexpressing BM-MSCs exerted an inhibitory effect on cell apoptosis in HIRI.

## Discussion

HIRI is a widespread clinical concern, which is common across multiple pathological conditions and liver surgical procedures, such as hemorrhagic shock, liver resection and liver transplantation (27). Hepatic I/R can lead to hepatic sinusoidal endothelial cell damage, Kupffer cell and leukocyte activation, leukocyte and platelet adhesion and microcirculation disorders, which directly affect the prognosis of the disease, the success rate of surgery and the survival rate of patients (28). The main mechanisms of HIRI are associated with intracellular calcium overload and oxygen free radicals (29). Thus, it is important for investigators to develop for novel strategies for hepatic I/R treatment.

BM-MSCs have a strong self-replication ability and multi-differentiation potential, which are based on their pluripotency and the secretion of beneficial molecules; therefore, these cells are widely used in basic and clinical trials (6,13). It has been reported that BM-MSCs possess potent migratory and differentiation abilities under both physiological and pathological conditions, and may be cultured *in vitro* under specific

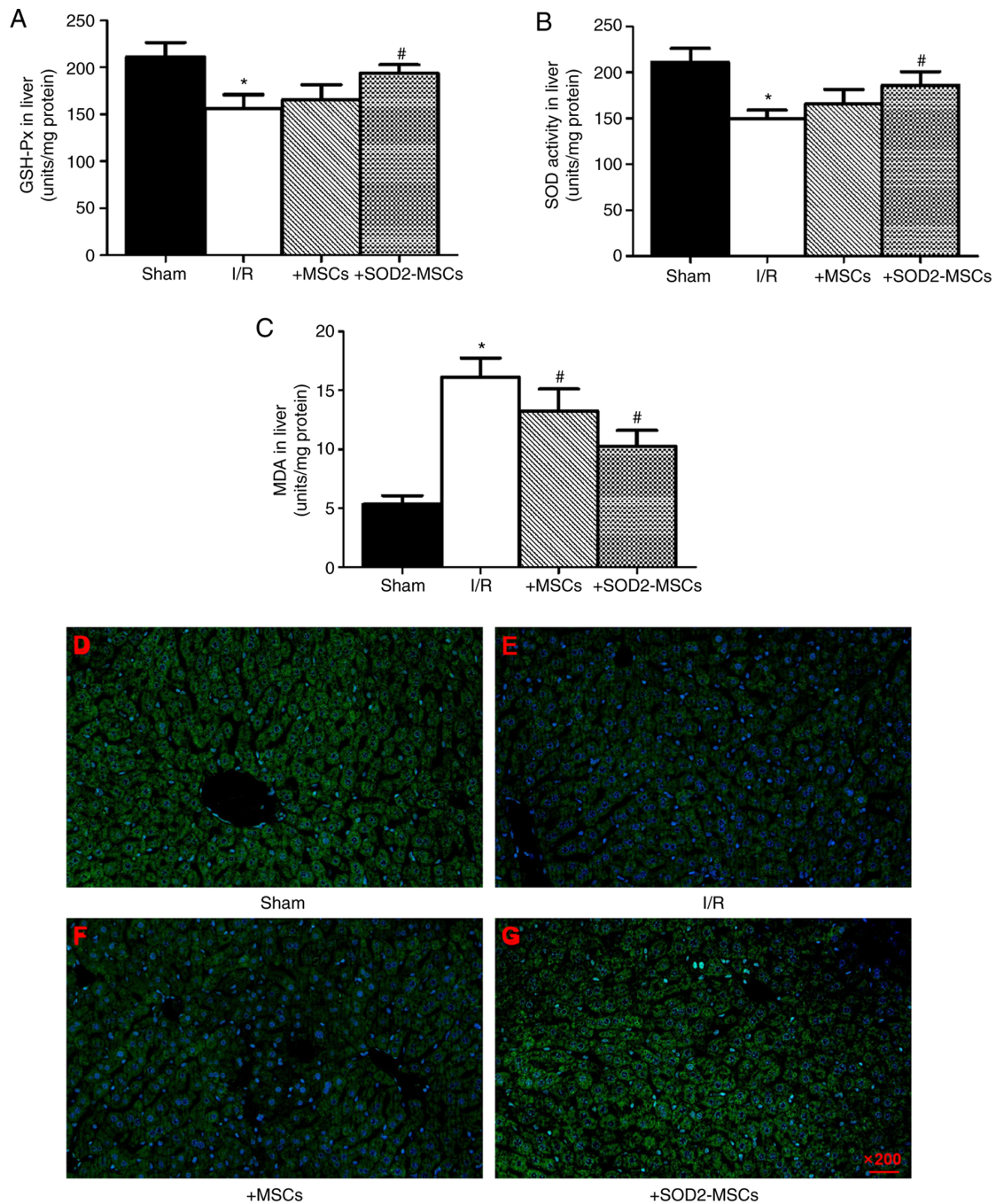


Figure 4. SOD2-overexpressing MSCs attenuate the oxidative stress response in hepatic I/R injury. (A) GSH-Px and (B) SOD activity; (C) MDA content. (D-G) ROS levels. Sham group, rats were subjected to laparotomy only; I/R group, rats were induced with I/R and received PBS; +MSCs, rats were induced with I/R and then transplanted with MSCs; and +SOD2-MSCs, rats were induced with I/R and then transplanted with SOD2-overexpressing MSCs. n=8 rats in each experimental group. Data are expressed as the mean  $\pm$  SD. \*P<0.05 vs. sham; #P<0.05 vs. I/R. I/R, ischemia/reperfusion; SOD, superoxide dismutase; MSCs, mesenchymal stem cells; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

conditions to differentiate into cells derived from multiple germ layers, such as hepatocyte-like and adult cells (30,31).

Accumulating evidence has indicated that nerve cells, adipocytes, osteoblasts and stem cell transplantation therapy are conducive to tissue damage repair and functional recovery (32-34). HIRI is a common pathological event during liver surgery, and is widely accepted as a common and inevitable complication during liver resection and liver transplantation, particularly in patients with end-stage liver disease (35). Moreover, the commonly used treatment methods

for this include liver resection and liver transplantation, and thus, a method to reduce the I/R injury of the liver during surgery will be of great significance to the clinical treatment of liver diseases (36). Previous studies have confirmed that BM-MSC transplantation exerts positive effects in treatment and has a protective function against liver tissue damage, and may be a potential intervention in the field of medicine and biology (37,38). It has also been revealed that the overexpression of cytokines and growth factors targeting certain diseases may be effective strategies for dysfunction-targeted therapy (39).



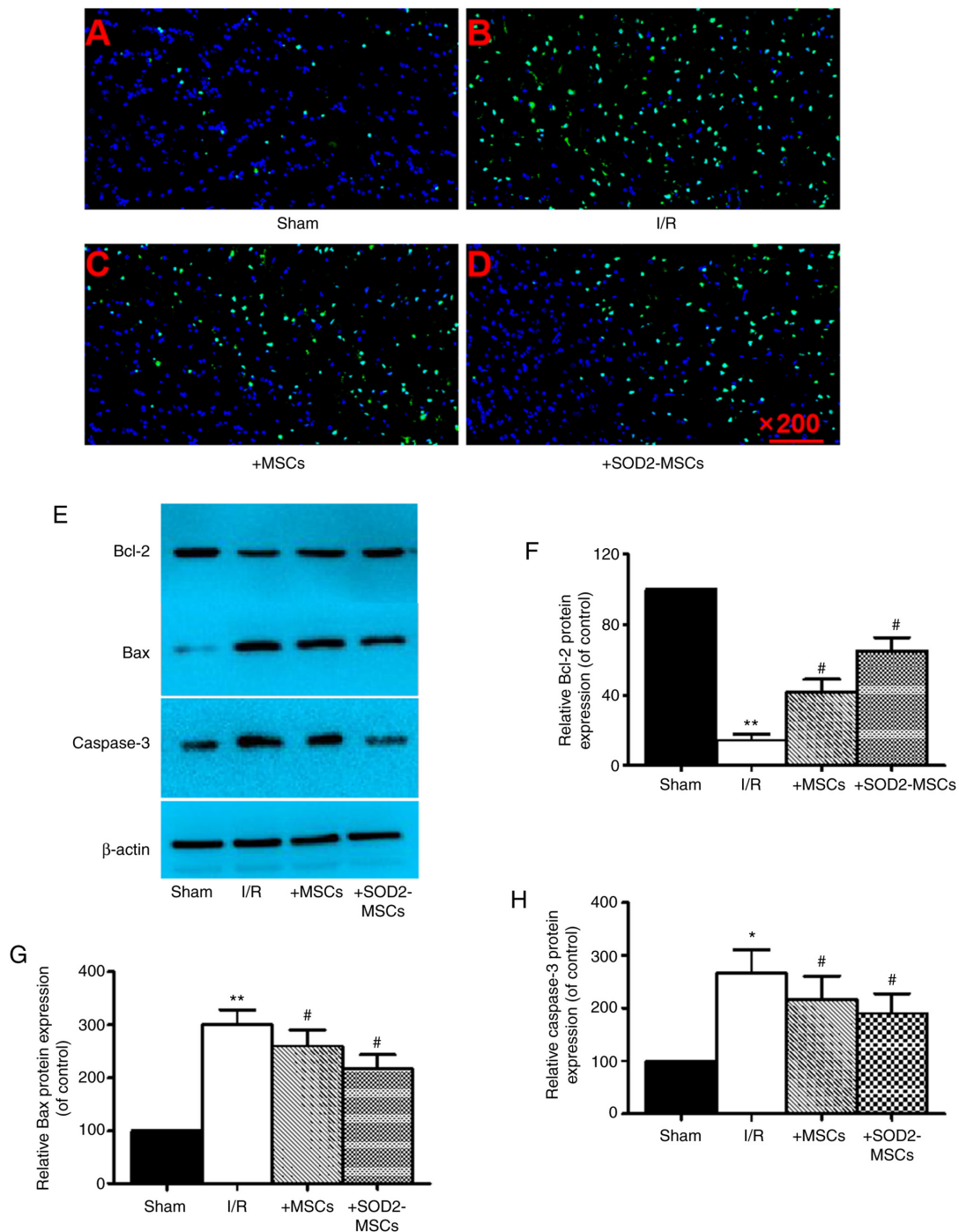


Figure 5. SOD2-overexpressing MSCs inhibit hepatocyte apoptosis. (A-D) TUNEL staining results. (E) Representative expression levels of Bcl-2, Bax and caspase-3 were analyzed via western blotting. (F-H) The relative expression levels of these proteins in relation to  $\beta$ -actin in each sample are presented. Sham group, rats were subjected to laparotomy only; I/R group, rats were induced with I/R and received PBS; +MSCs, rats were induced with I/R and then transplanted with MSCs; and +SOD2-MSCs, rats were induced with I/R and then transplanted with SOD2-overexpressing MSCs.  $n=8$  rats in each experimental group. Data are expressed as the mean  $\pm$  SD. \* $P<0.05$ , \*\* $P<0.01$  vs. sham; # $P<0.05$  vs. I/R. I/R, ischemia/reperfusion; SOD2, superoxide dismutase 2; MSCs, mesenchymal stem cells.

Therefore, the method of gene transfer allows BM-MSCs to express additional genes that are beneficial to treatment, and can enhance the efficacy of BM-MSC transplantation.

Intravenous administration of BM-MSCs has become a widely accepted treatment for decreasing intracellular calcium overload and oxygen free radicals, as well as for hepatoprotection following HIRI (13). The present study used the method of adenovirus transfection to overexpress the SOD2 gene in

MSCs, and RT-qPCR and western blotting demonstrated that SOD2 expression was upregulated in MSCs after adenovirus transfection, suggesting that SOD2 overexpression was successfully established. Moreover, SOD2 overexpression decreased oxidative stress and increased the expression levels of apoptosis-related genes.

Oxidative stress caused by hepatic I/R is the main mechanism contributing to the deterioration of liver function

induced by I/R (40). SODs are endogenous antioxidants that catalyze the conversion of  $O_2^-$  to  $H_2O_2$ , and helps maintain the redox balance by diffusing the superoxide (41). SOD2, a member of the SOD family, is a mitochondrial antioxidant enzyme that scavenges superoxide radicals (42). The continuous expression of SOD2 is regulated by the cell oxygen concentration, and once hepatic hypoxic-ischemic injury occurs, SOD2 is activated (43). By regulating its downstream target genes, SOD2 participates in regulating anaerobic metabolism, cytokine production and regulating cell apoptosis (44). Therefore, SOD2 overexpression may be a promising novel intervention for the treatment of hepatic ischemia and hypoxic injury.

An increasing number of basic and clinical experiments have reported that SOD2 may serve a protective role in hepatocytes during HIRI (45). Reduction of SOD2 activity has been shown to decrease liver function induced by transient ischemia (44,46). Moreover, overexpression of SOD2 can provide direct hepatic-protection via increasing levels of serum transaminases (including AST and ALT) and reversing pathological changes (47,48). In animal models of HIRI, significant increases in liver SOD2 mRNA and protein expression levels have been observed (47,48). These findings support the idea that SOD2-overexpressing BM-MSC transplantation may mitigate HIRI. In the present study, it was demonstrated that HIRI caused increases in the levels of AST and ALT, and SOD2-overexpressing BM-MSC transplantation obviously decreased the abnormal increases of these indicators, which in line with the reversal of pathological changes in HIRI.

In the current study, it was observed that SOD2-overexpressing BM-MSCs exerted the protective effect of SOD2 against oxidative stress in rats subjected to hepatic I/R. Furthermore, SOD2 overexpression in BM-MSCs exerted protective effects in promoting liver function recovery and decreasing oxidative stress in a rat model of hepatic I/R. These effects were mediated via the amelioration of oxidative stress and apoptosis inhibition after BM-MSC transplantation. SODs are endogenous antioxidants, and their activities are associated with SOD2 levels. The present study demonstrated that SOD and GSH-Px activities were significantly increased, and MDA content was decreased after SOD2-overexpressing BM-MSC transplantation.

The concentration of lipid peroxides in the plasma during HIRI is closely associated with serum transaminase activity, and is parallel to the severity of liver morphological damage (49). The present findings indicated that I/R caused obvious necrosis and congestion in the injured liver lobules, accompanied by the presence of a large number of red blood cells in the venules and inflammatory cell infiltration. However, after SOD2-overexpressing BM-MSC transplantation, the liver tissue structure was partially restored to normal, although changes, such as dilated central veins, were still visible (50). These results suggested that MSC treatment regenerated damaged liver tissues subjected to I/R.

SOD2 exerts a hepatoprotective effect in HIRI, and several studies have reported that the increase in sirtuin (Sirt)3 activity promotes the deacetylation of SOD2, which can decrease oxidative stress by eliminating ROS (46,51). Sirt3 can stabilize hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), and HIF-1 $\alpha$  can

activate the Sirt3 gene promoter, which leads to increased Sirt3 mRNA transcript synthesis and the inactivation of cyclophilin D, thereby reducing the opening of the mitochondrial permeability transition pore channel. In turn, the extent of liver damage can be reduced (52). The present results demonstrated that SOD2 overexpression enhanced the therapeutic effect of BM-MSCs in HIRI, improved liver function and effectively reduced the degree of tissue injury. Compared with the BM-MSCs group and control group, liver tissue damage in the SOD2-MSCs group was significantly reduced. These results indicated that SOD2 overexpression could exert a positive synergistic effect with BM-MSCs in the treatment of liver injury.

However, the limitations of the current study have yet to be considered. Despite progress in revealing the ameliorative potentials of SOD2 overexpression in MSCs, the complete regulatory molecular targets of SOD2 overexpression require additional research. Moreover, the application of SOD2 overexpression in MSCs for the treatment of other liver I/R-related injuries, including liver transplantation, requires more rigorous and scientific verification, as well as subsequent clinical trials.

In conclusion, the present study demonstrated that BM-MSCs could be a potential new method for cell therapy, particularly in liver injury. At present, the mortality rate of liver injury is increasing worldwide annually, making the development of novel strategies for liver injury treatment highly important. The present study identified that transplantation of SOD2-overexpressing BM-MSCs may have the potential to ameliorate HIRI via the inhibition of oxidative stress and apoptosis.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Authors' contributions

QL, WZ and EX designed and performed experiments, and analyzed, interpreted and presented the results for group discussions. QL, WZ and EX confirm the authenticity of all the raw data. EX made substantial contributions to the conception of the study. All authors read and approved the final manuscript.



## Ethics approval and consent to participate

The protocol of the present study was approved by the Laboratory Animal Center, The Second Xiangya Hospital of Central South University (Changsha, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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