BM-MSCs protect against liver ischemia/reperfusion injury via HO-1 mediated autophagy

XUN WANG[1*], SHUAI WANG[2*], YUAN ZHOU[1], HALMURAT OBULKASIM[1], ZHI-HENG ZHANG[1], BO DAI[2], WEI ZHU[3] and XIAO-LEI SHI[1]

[1]Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School; [2]Department of Hepatobiliary Surgery, Drum Tower Clinical Medical College of Nanjing Medical University; [3]Department of Anesthesiology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, P.R. China

Received June 1, 2017; Accepted February 23, 2018

DOI: 10.3892/mmr.2018.9207

Abstract. Ischemia/reperfusion (I/R) injury is considered to be a contributing factor in liver injury following major hepatic resection or liver transplantation. Bone marrow mesenchymal stem cells (BM-MSCs) have the potential to protect against liver I/R injury; however, the precise mechanisms have not been completely elucidated. Autophagy serves an important role in protecting against various injuries, including I/R injury. The present study aimed to determine the role of autophagy and its potential regulatory mechanism in BM-MSC-mediated protection against liver I/R injury in rats. The results demonstrated that BM-MSCs mitigated I/R injury and enhanced autophagy *in vivo*. In addition, inhibition of autophagy by 3-methyladenine reversed the positive effects of BM-MSCs. Furthermore, heme oxygenase-1 (HO-1) expression was promoted by BM-MSCs. Using zinc protoporphyrin IX to inhibit HO-1 demonstrated that HO-1 was important for the promotion of autophagy. In conclusion, the present study revealed that BM-MSCs protected against liver I/R injury via the promotion of HO-1-mediated autophagy.

Correspondence to: Dr Wei Zhu, Department of Anesthesiology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: chzhuwei118@qq.com

Dr Xiao-Lei Shi, Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: njsxl2000@163.com

*Contributed equally

Key words: ischemia/reperfusion injury, mesenchymal stem cells, autophagy, heme oxygenase-1

Introduction

Ischemia/reperfusion (I/R) is a process that may damage tissues or organs that depend on aerobic metabolism (1). I/R is widely considered to be a contributing factor in liver injury; it may lead to hepatic failure following major hepatic resection, as portal vein occlusion is a necessary step. In addition, I/R injury is attributed to approximately 10% of acute graft dysfunction cases post-liver transplantation (2,3). Thus, mitigating the adverse effects of I/R injury is an important clinical issue. Researchers have developed myriad therapeutic strategies as potential solutions to prevent hepatic I/R injury, including surgical interventions, pharmacotherapy or preconditioning with medication (4-6). In addition, the benefits of mesenchymal stem cells (MSCs) in liver I/R injury have received considerable attention (7).

As demonstrated previously, MSCs exhibited promising efficacy in reducing I/R injury in different organs, including the brain, myocardium, kidney, small bowel and liver (8-12). Bone marrow MSCs (BM-MSCs) are a type of MSCs derived from bone marrow. Studies have revealed that BM-MSCs have protective effects against liver I/R injury; they are able to attenuate liver injury by promoting liver regeneration or paracrine actions, including cytokines, growth factors or chemokines. However, the precise mechanisms through which BM-MSCs confer protection against I/R injury have not been completely elucidated. Recently, autophagy has been reported to be a potential mechanism underlying the protective effects of BM-MSCs against I/R injury (13).

Autophagy, including macroautophagy, microautophagy and chaperone-mediated autophagy, is regarded as a rudimentary cellular response to injury; it removes macromolecules and organelles via lysosomes, restricts cell death, enables cells to withstand diverse insults and prevents irreversible organ damage (14). In addition, a growing body of evidence has indicated that autophagy has positive effects in liver diseases (15,16). In liver I/R injury, the promotion of autophagy may ameliorate liver damage (17). Furthermore, BM-MSCs are able to alleviate damage in CCl_4-injured livers in addition to I/R-induced lung injury by autophagy (13,18). To the best of our knowledge, autophagy has not yet been

evaluated in BM-MSC-mediated protection against liver I/R injury.

Heme oxygenase-1 (HO-1) is a stress-inducible enzyme that has anti-inflammatory, anti-apoptotic, pro-survival and antioxidant potential. HO-1 has been confirmed to serve a role in protecting against liver I/R injury by regulating oxidative stress or inflammation (19-21). Furthermore, recent studies have demonstrated that HO-1 attenuates liver I/R injury through autophagy (22). However, it remains unclear as to whether HO-1 mediated autophagy is a mechanism through which BM-MSCs protect against liver I/R injury.

Based on these findings, it was hypothesized that BM-MSCs may protect against liver I/R injury by promoting autophagy. In addition, the present study aimed to investigate the association between autophagy and HO-1 in experimental I/R injury *in vivo*.

Materials and methods

Isolation, culture, identification, and differentiation of BM-MSCs. BM-MSCs were obtained from the femurs and tibias of 4-week-old Wistar rats by flushing the bone marrow cavity with complete culture medium as previously described (23). The extract was centrifuged, resuspended and cultured in a 75-cm^2 culture flask containing Dulbecco's modified Eagle's medium with low glucose (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin/streptomycin at 37˚C and 5% CO_2. One-half of the medium was replaced following 24 h of culture, and the entire culture medium every 2-3 days. Cells were subcultured when the adherent cells reached 70-80% confluence.

Cells were identified as BM-MSCs by their morphology, adherence and surface markers. BM-MSCs of 3-4 passages were identified by antibodies against cluster of differentiation (CD)29-phycoerythrin (PE)-A, CD34-PE-A, CD44-PE-A, CD45-fluorescein isothiocyanate (FITC)-A, and CD90-PE-A (eBioscience; Thero Fisher Scientific, Inc.). Briefly, resuspended cells were incubated with CD29-PE-A (1:2; cat. no. 12-0291-82), CD34-PE-A (1:40; cat. no. MA1-10205), CD44-PE-A (1:40; cat. no. MA5-16908), CD45-FITC-A (1:20; cat. no. 11-0461-82) and CD90-PE-A (1:40; cat. no. MA1-80650) at room temperature. After 20 min, the cells were washed with three times PBS and a flow cytometer was used (FACS Aria II; BD Biosciences, San Jose, CA, USA). Software was used for data analysis (FlowJo software 7.6; FlowJo LLC, Ashland, OR, USA).

Cells were additionally identified by their multilineage differentiation potential, as previously reported (24). For adipogenic differentiation, adipogenic induction medium (Cyagen Biosciences, Co., Ltd., Suzhou, China) was added to the BM-MSCs at 37˚C for 3 days, followed by maintenance medium (Cyagen Biosciences, Co., Ltd.) at 37˚C for 1 day. Following three cycles, the cells were cultured in maintenance medium for a further 7 days. The cells were subsequently stained with Oil Red O (Cyagen Biosciences, Co., Ltd.) at room temperature for 30 min. The sections were washed and examined using a light microscope (magnification, x40, Olympus Corporation, Tokyo, Japan). For osteogenic differentiation, the BM-MSCs were cultured with rat BM-MSC osteogenic differentiation medium (Cyagen Biosciences, Co., Ltd.) when subconfluent at 37˚C. Subsequent to 3 weeks of differentiation, the calcium depositions were stained with Alizarin red (Cyagen Biosciences, Co., Ltd.) at room temperature for 3-5 min. The sections were washed and examined using a light microscope (magnification, x40, Olympus Corporation).

Animals and treatment. A total of 30 healthy 4-6-week-old male Wistar rats weighing ~200 g were purchased from the Laboratory Animal Center of The Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China), and housed under specific pathogen-free conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School, under the National Institutes of Health (Bethesda, MD, USA) Guide for the Care and Use of Laboratory Animals. All experiments were conducted under isoflurane anesthesia, and all efforts were made to minimize suffering.

The rats were randomly divided into five groups as follows: The sham group, the I/R group, the I/R+MSCs group, the 3-methyladenine (3-MA) group and the zinc protoporphyrin IX (ZnPP) group. A hepatic warm I/R model was used as previously described (25). The rats were anesthetized with isoflurane followed by laparotomy. A sterile microvascular clamp was placed around all structures in the portal triad for the left and median liver lobes to interrupt the blood supply in all groups except the sham group, which underwent the same procedure without vascular occlusion. Reperfusion was initiated via removal of the clamp after 1 h. In addition, $1x10^6$ MSCs suspended in 0.5 ml PBS were injected via the penis dorsal vein 30 min prior to hepatic warm I/R (26) in all the groups, except the sham group and the I/R group. In the 3-MA group, the autophagy inhibitor 3-MA was administered (30 mg/kg; intraperitoneal; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) 0.5 h prior to ischemia. The rats in the ZnPP group were treated twice with ZnPP (10 mg/kg; intraperitoneal; Sigma-Aldrich; Merck KGaA) at 16 and 3 h prior to ischemia to inhibit HO-1 *in vivo*. All the rats were sacrificed following 6 h of reperfusion, and blood samples and liver tissues were obtained. Blood samples were stored at 4˚C overnight. A portion of the liver tissues were fixed in 10% formalin solution at room temperature for ≥24 h, and then prepared for HE staining and immunohistochemistry (5 μm) as described below. The remaining liver tissues were stored at -80˚C for further use.

In order to detect the implantation of BM-MSCs in the liver following I/R injury, the cells were stained with lipophilic membrane dyes (DiO; Invitrogen; Thermo Fisher Scientific, Inc.) prior to intravenous administration of BM-MSCs. The BM-MSCs were suspended at a concentration of 10^6 cells/ml and incubated with 10 mM DiO for 20 min at 37˚C. Frozen sections were fixed at 4˚C for 10 min in acetone and air-dried. The nuclei were stained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.). The sections were washed and examined using a Leica TCS SP8 confocal laser-scanning microscope (magnification, x200; Leica Microsystems GmbH, Wetzlar, Germany).

Blood biochemistry. Blood samples was harvested via the postcava and centrifuged at 3,000 x g at 4˚C for 10 min. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic analyzer (Fujifilm Global, Tokyo, Japan), as previously described (27).

Histological analysis and immunohistochemistry. Following fixation of the liver tissues in 10% buffered formalin at room temperature for at least 24 h, paraffin embedding was performed using a standard protocol. Paraffin sections (5 μm) were stained with hematoxylin at room temperature for 3-5 min and stained with eosin at room temperature for 2 min, then examined under a light microscope (magnification, x100) for histological evidence of liver injury. Immunohistochemical staining for HO-1 was performed on the paraffin sections which were blocked with blocking serum (OriGene Technologies, Inc., Beijing, China) at room temperature for 10 min, incubated with primary antibodies against HO-1 (1:200; cat. no. ab13243, Abcam, Cambridge, UK) overnight at 4˚C and developed using a horseradish peroxidase-conjugated secondary antibody (PV-6000, undiluted, OriGene Technologies, Inc., Beijing, China) at 37˚C for 30 min and a 3,3-diaminobenzidine substrate kit (ZLI-9018, undiluted; OriGene Technologies, Inc.) at room temperature for 5-20 min, according to standard methods in routine pathology. The sections were washed and examined using a light microscope (x100; Leica Microsystems GmbH, Wetzlar, Germany).

Gel electrophoresis and western blotting. Western blotting was conducted as previously described (22). Equal amounts (40 μg) of the proteins were separated on 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4˚C with primary antibodies against HO-1 and microtubule-associated proteins 1A/1B light chain 3B (LC3B; 1:2,000; cat. no. ab192890; Abcam). GAPDH (1:1,000; cat. no. ab9484; Abcam; incubated at 4˚C overnight) expression served as a loading control. The membrane was treated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:8,000; KGAA37; Nanjing KeyGen Biotech Co,. Ltd., Nanjing, China) or horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:8,000; KGAA35; Nanjing KeyGen Biotech Co,. Ltd.) at room temperature for 2 h. Blots were developed using enhanced chemiluminescence (ECL) western blotting substrate (EMD Millipore, Billerica, MA, USA) and visualized on a Tanon 5200 Multi Image Station (Tanon Science & Technology Co., Ltd., Shanghai, China). The results were quantified by Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) and data are expressed as the mean ± standard error of the mean; results were repeated in triplicate. Differences between the groups were evaluated for significance using one-way analysis of variance combined with Bonferroni's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture, identification and detection of BM-MSCs. At passage 3 or passage 4, the cells from the rat bone marrow existed as a monolayer of typical fibroblastic, spindle-shaped and plastic-adherent cells (Fig. 1A). As presented in Fig. 1B, identification of BM-MSCs was performed using flow cytometry. These cells exhibited little or no expression of hematopoietic markers (CD34 and CD45) and positive expression of stromal markers (CD29, CD44 and CD90). In addition, the BM-MSCs had the potential to differentiate into osteoblastic or adipogenic lineages (Fig. 1C). Based on these results, the cells from rat bone marrow were confirmed to be BM-MSCs. The DiO-labeled BM-MSCs were used to clarify whether BM-MSCs migrated to the liver following I/R. BM-MSCs were observed in the I/R+MSCs group, although not in the sham group or the I/R group (Fig. 1D).

Pretreatment with BM-MSCs ameliorates liver injury following I/R. In order to determine whether BM-MSCs attenuate hepatic I/R injury, the rats were pretreated with BM-MSCs prior to ischemia. Serious liver damage was observed following 6 h of reperfusion, as previously reported (25). The rats were sacrificed 6 h post-insult, and liver tissues and blood were collected for further research. Compared with the rats in the I/R group, which had the highest ALT and AST levels, a significant improvement was noted in the I/R+MSCs group (Fig. 2A and B). In addition, the liver samples from the I/R group exhibited marked abnormalities, including severe hepatocellular necrosis and cytoplasmic vacuolization, following 6 h of reperfusion, which were improved in the I/R+MSCs group (Fig. 2C).

Autophagy is promoted by pretreatment with BM-MSCs. Autophagy is part of the adaptive stress response to infection or lipopolysaccharide (LPS) exposure to support cell survival. In order to clarify the effects of pretreatment with BM-MSCs on autophagy, the expression of the autophagy associated marker LC3B in the rat liver was examined in the sham group, the I/R group and the I/R+MSCs group by western blotting. Upon the induction of autophagy, LC3B is a terminal autophagic protein used as a classic marker to monitor alterations in autophagy (28). As presented in Fig. 3, the expression of the autophagic signaling factor LC3B was markedly increased in the I/R+MSCs group compared with the other study groups. These findings confirmed that autophagy increased following pretreatment with BM-MSCs.

BM-MSCs protect against liver I/R injury via the induction of autophagy. To evaluate whether autophagy contributes to BM-MSC-mediated protection against I/R injury, the rats were pretreated with 3-MA prior to ischemia, which is widely regarded as an inhibitor of autophagy. The expression of autophagy was measured using western blotting to determine the presence of LC3B. Compared with the I/R+MSCs group, treatment with 3-MA resulted in a decrease in LC3B expression in response to liver I/R injury (Fig. 3). Notably, compared with the I/R+MSCs group, the serum levels of AST and ALT and the histological abnormalities were reversed in the 3-MA group (Fig. 2). Based on the results following pretreatment with

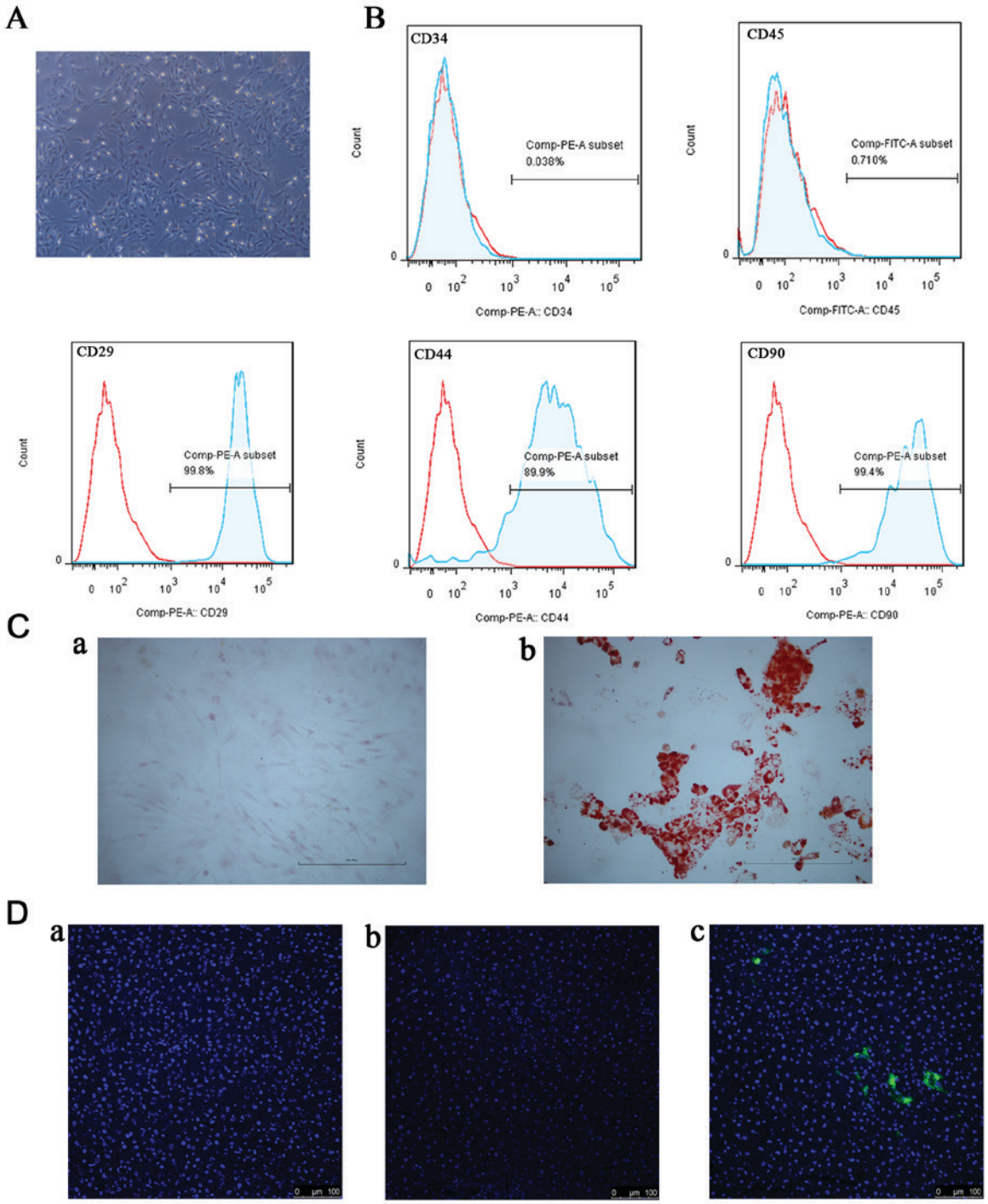

Figure 1. Identification and detection of BM-MSCs. (A) Morphological appearance of primary BM-MSCs expanded in culture. At passage 3 or passage 4, the cells exhibited typical fibroblastic, spindle-shaped morphology (magnification, x200). (B) Analysis of the surface markers of BM-MSCs by flow cytometry demonstrated that they did not express CD34 or CD45, although they positively expressed CD29, CD44 and CD90. (C) Multilineage differentiation of BM-MSCs stained with (a) Alizarin red and (b) Oil Red O. Scale bar, 100 μm. (D) Detection of DiO-labeled BM-MSCs in the liver samples following reperfusion: (a) The sham group; (b) the I/R group; (c) the I/R+MSCs group. Scale bar, 100 μm. BM-MSCs, bone marrow mesenchymal stem cells; CD, cluster of differentiation; I/R, ischemia/reperfusion.

3-MA, it was concluded that autophagy served a notable role in BM-MSC-mediated protection against cell death following I/R injury.

BM-MSC-promoted autophagy is dependent on HO-1. The present study subsequently aimed to analyze how BM-MSCs promote autophagy following I/R injury. A previous study reported that upregulation of HO-1 was able to mitigate I/R injury (29). Furthermore, HO-1 has been demonstrated to be associated with autophagic activity (22,30). Consistent with these findings, alterations in HO-1 expression were observed in the case of pretreatment with BM-MSCs in response to I/R injury. HO-1 expression was increased in the rat livers subjected to I/R injury, while in the I/R+MSCs group, HO-1 expression was even more significantly increased (Fig. 4). Since BM-MSCs increased HO-1 expression and autophagic

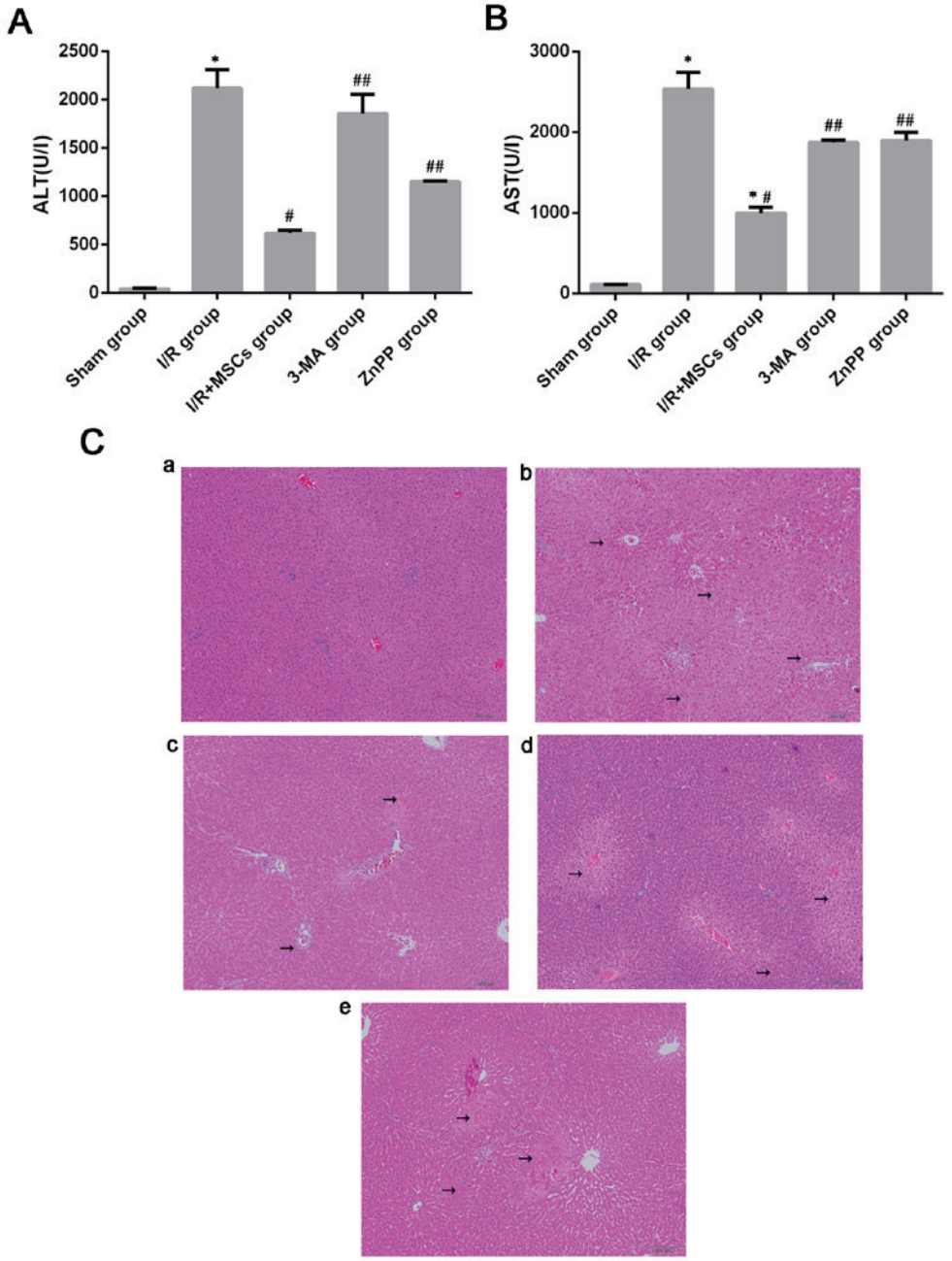

Figure 2. Pretreatment with BM-MSCs attenuates I/R-induced liver injury. The (A) ALT and (B) AST levels obtained following 6 h of reperfusion in the respective groups are presented. The data are presented as the mean ± standard error of the mean. *P<0.05 vs. sham group; #P<0.05 vs. I/R group; ##P<0.05 vs. I/R+MSCs group. (C) Representative hematoxylin-eosin stained images of liver sections (magnification, x100): (a) The sham group; (b) the I/R group; (c) the I/R+MSCs group; (d) the 3-MA group; (e) the ZnPP group. The arrows denote hepatocellular necrosis and cytoplasmic vacuolization. ALT, alanine aminotransferase; AST, aspartate aminotransferase; I/R, ischemia/reperfusion; BM-MSCs, bone marrow mesenchymal stem cells; 3-MA, 3-methyladenine; ZnPP, zinc protoporphyrin IX.

activity, it was preliminarily suggested that HO-1 may serve a role in the promotion of autophagy via pretreatment with BM-MSCs. Therefore, in order to identify the association between HO-1 and autophagy, HO-1 was inhibited by ZnPP. ZnPP significantly decreased the expression of LC3B (Fig. 5). Furthermore, inhibition of HO-1 by ZnPP significantly inhibited the protective effect provided by BM-MSCs following I/R insult. The serum levels of AST and ALT in the ZnPP-treated rats were increased compared with those in the I/R+MSCs group (Fig. 2). The I/R injury-associated histopathological alterations in the livers of rats were more severe in the ZnPP group compared with the I/R+MSCs group (Fig. 2C). Taken together, these data demonstrate that HO-1, increased by pretreatment with BM-MSCs, promoted autophagy, which protected against I/R injury *in vivo*.

Discussion

MSCs have been widely studied as anti-apoptotic and pro-survival cells, and they have been demonstrated to successfully mitigate liver injury caused by D-galactosamine/LPS or toxins, in addition to organ I/R injury (31-34). However, little

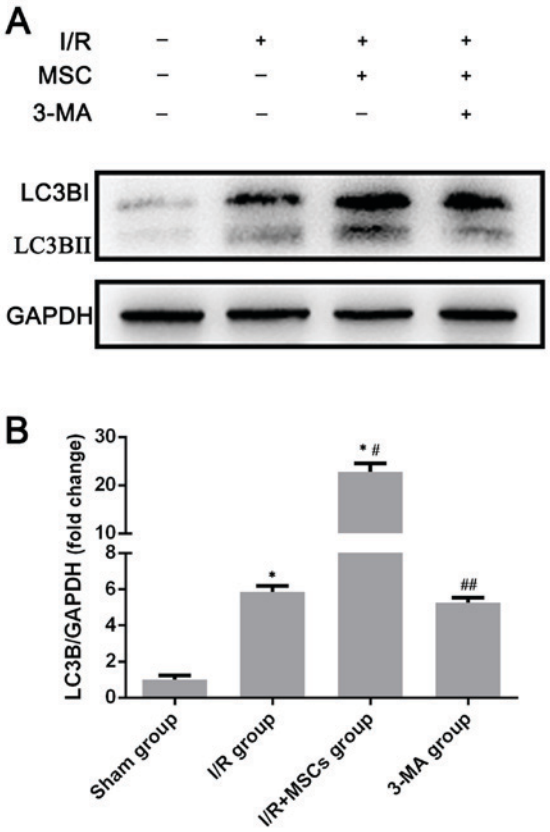

Figure 3. BM-MSCs afford protection against liver I/R injury via autophagy. The rats were pretreated with BM-MSCs 0.5 h prior to ischemia. The rats were additionally pretreated with 3-MA to ascertain the role of autophagy in BM-MSC-mediated protection against I/R injury. (A) The expression levels of the autophagic proteins LC3B I and II in the liver samples were examined by western blotting. (B) Relative density analysis of the protein bands. The data are presented as the mean ± standard error of the mean. *P<0.05 vs. sham group; #P<0.05 vs. I/R group; ##P<0.05 vs. I/R+MSCs group. LC3B, microtubule-associated proteins 1A/1B light chain 3B; BM-MSCs, bone marrow mesenchymal stem cells; 3-MA, 3-methyladenine; I/R, ischemia/reperfusion.

is known about the role of autophagy in BM-MSC-mediated protection and its detailed regulatory mechanism in liver I/R injury is unclear. MSCs have recently been demonstrated to ameliorate liver fibrosis by inducing autophagy (35), which is regarded as a protective mechanism against I/R injury in the liver or other organs (22,36,37). Therefore, the present study focused on evaluating whether BM-MSCs protected against liver I/R injury by increasing the expression level of HO-1. BM-MSCs are one of the most studied stem cells; they have previously been identified by their morphology, surface markers or multilineage differentiation potential (38,39). Therefore, BM-MSCs were identified by the same method in the present study. The results demonstrated that the cells we obtained were BM-MSCs. BM-MSCs hold great potential in protecting organs from I/R injury. Sheashaa *et al* (9) reported that adipose-derived MSCs were able to significantly attenuate injury caused by I/R. Similarly, Lu *et al* (40) reported that BM-MSCs significantly reversed lung injury following I/R by alleviating inflammation induced by I/R injury. The beneficial effect of BM-MSCs was further confirmed by Fu *et al* (25), who documented that the hepatoprotection provided by BM-MSCs following I/R injury was dependent on the inhibition of hepatocellular apoptosis and the stimulation of N-acetyltransferase 8 regeneration *in vivo* or *in vitro*. Consistent with these observations, the present study confirmed that BM-MSCs had beneficial effects against liver I/R injury *in vivo* by observing levels of ALT/AST and alterations in histomorphology that were consistent with certain published papers (41,42). Previous studies revealed that MSCs attenuated I/R injury in solid organs through various complex mechanisms, including an anti-inflammatory reaction (12,40), angiogenesis (43), anti-oxidative stress (44,45), and immunomodulation (46). The present study focused on whether BM-MSCs mitigated liver I/R injury through autophagy.

Autophagy is regarded as a cellular self-digestion process in response to a wide range of deleterious stimuli, through which cytoplasmic materials or organelles integrate into lysosomes for further degradation (47). Previous studies have reported that, autophagy serves a crucial role in the development, differentiation, survival and homeostasis of cells (48-51). During organ I/R injury, autophagy may help cells respond to injury. Liu *et al* (52) reported that autophagy is a critical homeostatic mechanism in maintaining renal tubular cell integrity during renal I/R. Zhang *et al* (37) suggested that autophagy evoked by I/R is involved in the process of neuroprotection via mitophagy-associated mitochondrial clearance and the inhibition of downstream apoptosis. Autophagy was additionally suggested to be a pro-survival mechanism in the field of liver I/R injury (53). Zhao *et al* (54) observed that increasing autophagy by inhibiting calpain2 was able to decrease the sensitivity of fatty liver to I/R injury. Wang *et al* (55) revealed that the restoration of autophagy inhibited the activation of mitochondrial permeability transition and attenuated damage in aged livers with I/R injury. However, liver injury is aggravated following the suppression of I/R-induced autophagy by chloroquine, which demonstrated the benefit of autophagy for I/R injury of livers (56). These previous studies strongly support the view that autophagy orchestrates a cytoprotective mechanism in I/R injury. It has been demonstrated that MSCs are associated with autophagy. Park *et al* (35) indicated that the mechanism of BM-MSCs in the resolution of CCl_4-induced liver fibrosis was partially due to the accumulation of autophagy-associated proteins. Shin *et al* (57) reported that BM-MSCs were able to exert a neuroprotective effect in Alzheimer's disease based on enhanced autophagy, which resulted in the clearance of amyloid-β. Zhou and You (58) proved that BM-MSCs alleviated LPS-induced acute lung injury by reducing the levels of microRNA 142a-5p and increasing Beclin1-mediated autophagy in pulmonary endothelial cells.

The present study demonstrated that pretreatment with BM-MSCs provided hepatoprotection against liver I/R injury accompanied by the increased expression of autophagic signaling molecules, including LC3B. To further determine the role of autophagy in the protection mediated by BM-MSCs, the rats were pretreated with BM-MSCs and concomitant 3-MA, a specific autophagy inhibitor, prior to I/R injury. The results demonstrated that pretreatment with 3-MA abrogated the increased in autophagy mediated by BM-MSCs *in vivo*. This abrogation translated into increased serum levels of AST and ALT, severe histological abnormalities. From these investigations, it was confirmed that BM-MSCs afforded hepatoprotection against liver I/R injury, partially by promoting

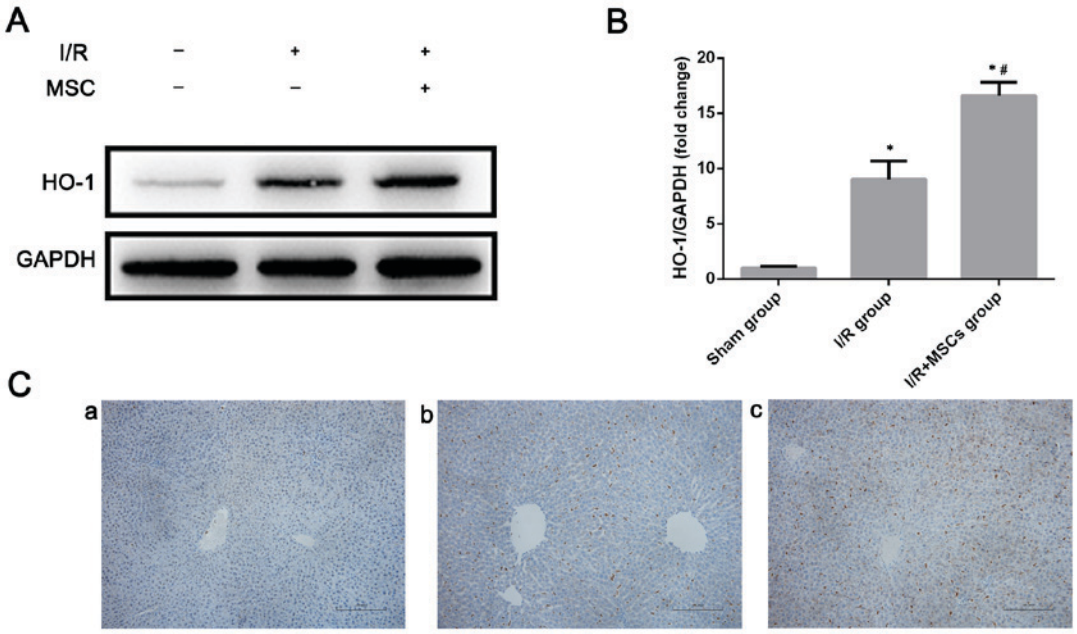

Figure 4. Pretreatment with BM-MSCs increases the expression of HO-1. (A) The expression levels of HO-1 in the liver samples were examined by western blotting. (B) Relative density analysis of HO-1/GAPDH. The data are presented as the mean ± standard error of the mean. *P<0.05 vs. sham group; #P<0.05 vs. I/R group. (C) Immunohistochemistry analysis of the expression of HO-1 in (a) the sham group, (b) the I/R group and (c) the I/R+MSCs group. Scale bar, 100 μm. HO-1, heme oxygenase-1; BM-MSCs, bone marrow mesenchymal stem cells; I/R, ischemia reperfusion.

autophagy. In the present study, LC3B was regarded as an indicator of autophagy, as reported previously (28), which was a limitation of the present study. In addition, there is a discrepancy between the present results and the findings reported in several previous investigations, which demonstrated that MSCs repaired I/R injury via anti-autophagic mechanisms (59). Since autophagy is highly variable during liver I/R, a potential explanation for this contradiction is that it is likely that autophagy serves different roles at various time points during reperfusion. Future research is required to study the alterations in autophagic activity influenced by MSCs at different time points following reperfusion.

The regulatory mechanisms of BM-MSCs on autophagy during liver I/R injury remained unclear. HO-1 is an indispensable protein in various organs, including the liver, and is involved in restoring cellular homeostasis in response to multiple insults (30). Previous studies have demonstrated that autophagy is elevated by HO-1. Carchman *et al* (30) revealed that autophagy orchestrated protection against liver injury due to sepsis, which was dependent on HO-1. In addition, HO-1-mediated autophagy was the mechanism by which baicalein and remote ischemic preconditioning prevented hepatocellular injury due to I/R (5,22). Yun *et al* (60) confirmed that the HO-1 system was a possible regulator of autophagy in liver I/R injury. Conversely, Wang *et al* (61) used a HO-1 inhibitor to demonstrate that HO-1 was a key inducer of autophagy in response to liver I/R injury, thereby preventing aggravation of liver damage. In the present study, the groups pretreated with BM-MSCs exhibited increased HO-1 expression and autophagic activity. Thus, it appears that MSC-promoted autophagy occurred via HO-1. In order to elaborate on the role of HO-1 in MSC-promoted autophagy, HO-1 induced by BM-MSCs was inhibited by ZnPP prior to ischemia. The results of the present study demonstrated that inhibition of HO-1 by ZnPP

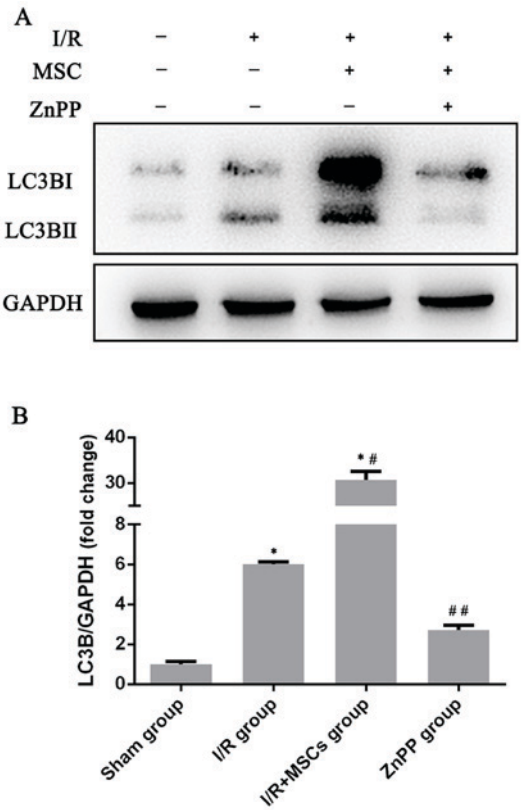

Figure 5. HO-1 regulates BM-MSCs-mediated increased autophagy. The rats were pretreated with ZnPP prior to ischemia. (A) The association between HO-1 and autophagy was examined by western blot analysis following pretreatment with ZnPP. (B) The relative density analysis of the protein bands above are presented in the histogram. The data are presented as the mean ± standard error of the mean. *P<0.05 vs. sham group; #P<0.05 vs. I/R group; ##P<0.05 vs. I/R+MSCs group. HO-1, heme oxygenase-1; BM-MSCs, bone marrow mesenchymal stem cells; I/R, ischemia reperfusion; ZnPP, zinc protoporphyrin IX; LC3B, microtubule-associated proteins 1A/1B light chain 3B.

prevented autophagy due to pretreatment with BM-MSCs; it additionally prevented the protective effect of BM-MSCs on I/R injury *in vivo*.

From the results of previous studies and the present observations, it was concluded that HO-1 was the regulator through which the BM-MSCs increased autophagic activity in liver I/R injury. However, the way in which treatment with BM-MSCs increased in the expression of HO-1 in the liver remained unresolved. As previously reported, HO-1 may be increased in the liver by the phosphatidylinositol 3-kinase/RAC-α serine/threonine-protein kinase/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (62), the kelch-like ECH-associated protein 1/Nrf2/thioredoxin 1/hypoxia inducible factor-1α pathway (20), or the cyclic AMP-dependent transcription factor ATF3-mediated Nrf2 pathway (63). Further study is required to determine the mechanism of HO-1 expression increased by BM-MSCs. In addition, HO-1 is an oxidation/antioxidation regulator. A previous study revealed that BM-MSCs protect against liver I/R injury by suppressing oxidative stress (7). Therefore, the present study did not discuss whether BM-MSCs were able to attenuate I/R injury via HO-1-mediated antioxidant action, which may merit further research. Furthermore, the present study emphasized the influence of BM-MSCs on the whole liver tissue as before (18). The present study aimed to supplement the current protection mechanism of MSCs against liver I/R injury; the origin of HO-1 or autophagy also requires further investigation.

In conclusion, the present findings provide evidence that the protective effects of BM-MSCs may be associated with the promotion of autophagy by increasing the levels of HO-1 *in vivo*.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81670566) and the Natural Science Foundation of Jiangsu Province, China (grant no. BK20131084).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW and SW conceived and designed the study, conducted the experiments, acquired data, analyzed and interpreted the data, and wrote the manuscript. YZ, HO, ZHZ, and BD conceived and designed the study, collected data, and wrote the manuscript. WZ and XLS conceived and designed the study, acquired financial support and study materials, wrote and gave final approval of the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School, under the National Institutes of Health (Bethesda, MD, USA) Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. de Groot H and Rauen U: Ischemia-reperfusion injury: Processes in pathogenetic networks: A review. Transplant Proc 39: 481-484, 2007.
2. Ke B, Lipshutz GS and Kupiec-Weglinski JW: Gene therapy in liver ischemia and reperfusion injury. Curr Pharm Des 12: 2969-2975, 2006.
3. Kim YI: Ischemia-reperfusion injury of the human liver during hepatic resection. J Hepatobiliary Pancreat Surg 10: 195-199, 2003.
4. Selzner N, Rudiger H, Graf R and Clavien PA: Protective strategies against ischemic injury of the liver. Gastroenterology 125: 917-936, 2003.
5. Liu A, Huang L, Guo E, Li R, Yang J, Li A, Yang Y, Liu S, Hu J, Jiang X, *et al*: Baicalein pretreatment reduces liver ischemia/reperfusion injury via induction of autophagy in rats. Sci Rep 6: 25042, 2016.
6. Bahde R and Spiegel HU: Hepatic ischaemia-reperfusion injury from bench to bedside. Br J Surg 97: 1461-1475, 2010.
7. Sun CK, Chang CL, Lin YC, Kao YH, Chang LT, Yen CH, Shao PL, Chen CH, Leu S and Yip HK: Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats. Crit Care Med 40: 1279-1290, 2012.
8. Zhang H, Xiang M, Meng D, Sun N and Chen S: Inhibition of myocardial ischemia/reperfusion injury by exosomes secreted from mesenchymal stem cells. Stem Cells Int 2016: 4328362, 2016.
9. Sheashaa H, Lotfy A, Elhusseini F, Aziz AA, Baiomy A, Awad S, Alsayed A, El-Gilany AH, Saad MA, Mahmoud K, *et al*: Protective effect of adipose-derived mesenchymal stem cells against acute kidney injury induced by ischemia-reperfusion in Sprague-Dawley rats. Exp Ther Med 11: 1573-1580, 2016.
10. Chang CL, Sung PH, Sun CK, Chen CH, Chiang HJ, Huang TH, Chen YL, Zhen YY, Chai HT, Chung SY, *et al*: Protective effect of melatonin-supported adipose-derived mesenchymal stem cells against small bowel ischemia-reperfusion injury in rat. J Pineal Res 59: 206-220, 2015.
11. Lin YC, Ko TL, Shih YH, Lin MY, Fu TW, Hsiao HS, Hsu JY and Fu YS: Human umbilical mesenchymal stem cells promote recovery after ischemic stroke. Stroke 42: 2045-2053, 2011.
12. Semedo P, Palasio CG, Oliveira CD, Feitoza CQ, Gonçalves GM, Cenedeze MA, Wang PM, Teixeira VP, Reis MA, Pacheco-Silva A and Câmara NO: Early modulation of inflammation by mesenchymal stem cell after acute kidney injury. Int Immunopharmacol 9: 677-682, 2009.
13. Li J, Zhou J, Zhang D, Song Y, She J and Bai C: Bone marrow-derived mesenchymal stem cells enhance autophagy via PI3K/AKT signalling to reduce the severity of ischaemia/reperfusion-induced lung injury. J Cell Mol Med 19: 2341-2351, 2015.
14. Rautou PE, Mansouri A, Lebrec D, Durand F, Valla D and Moreau R: Autophagy in liver diseases. J Hepatol 53: 1123-1134, 2010.
15. Denaes T, Lodder J, Chobert MN, Ruiz I, Pawlotsky JM, Lotersztajn S and Teixeira-Clerc F: The cannabinoid receptor 2 protects against alcoholic liver disease via a macrophage autophagy-dependent pathway. Sci Rep 6: 28806, 2016.

16. Zhu S, Wu Y, Ye X, Ma L, Qi J, Yu D, Wei Y, Lin G, Ren G and Li D: FGF21 ameliorates nonalcoholic fatty liver disease by inducing autophagy. Mol Cell Biochem 420: 107-119, 2016.
17. Wang D, Ma Y, Li Z, Kang K, Sun X, Pan S, Wang J, Pan H, Liu L, Liang D and Jiang H: The role of AKT1 and autophagy in the protective effect of hydrogen sulphide against hepatic ischemia/reperfusion injury in mice. Autophagy 8: 954-962, 2012.
18. Jung J, Choi JH, Lee Y, Park JW, Oh IH, Hwang SG, Kim KS and Kim GJ: Human placenta-derived mesenchymal stem cells promote hepatic regeneration in CCl4-injured rat liver model via increased autophagic mechanism. Stem Cells 31: 1584-1596, 2013.
19. Yun N, Eum HA and Lee SM: Protective role of heme oxygenase-1 against liver damage caused by hepatic ischemia and reperfusion in rats. Antioxid Redox Signal 13: 1503-1512, 2010.
20. Ke B, Shen XD, Zhang Y, Ji H, Gao F, Yue S, Kamo N, Zhai Y, Yamamoto M, Busuttil RW, *et al*: KEAP1-NRF2 complex in ischemia-induced hepatocellular damage of mouse liver transplants. J Hepatol 59: 1200-1207, 2013.
21. Ke B, Shen XD, Ji H, Kamo N, Gao F, Freitas MC, Busuttil RW and Kupiec-Weglinski JW: HO-1-STAT3 axis in mouse liver ischemia/reperfusion injury: Regulation of TLR4 innate responses through PI3K/PTEN signaling. J Hepatol 56: 359-366, 2012.
22. Wang Y, Shen J, Xiong X, Xu Y, Zhang H, Huang C, Tian Y, Jiao C, Wang X and Li X: Remote ischemic preconditioning protects against liver ischemia-reperfusion injury via heme oxygenase-1-induced autophagy. PLoS One 9: e98834, 2014.
23. Xia X, Chen W, Ma T, Xu G, Liu H, Liang C, Bai X, Zhang Y, He Y and Liang T: Mesenchymal stem cells administered after liver transplantation prevent acute graft-versus-host disease in rats. Liver Transpl 18: 696-706, 2012.
24. Zhang X, Li J, Nie J, Jiang K, Zhen Z, Wang J and Shen L: Differentiation character of adult mesenchymal stem cells and transfection of MSCs with lentiviral vectors. J Huazhong Univ Sci Technolog Med Sci 30: 687-693, 2010.
25. Fu J, Zhang H, Zhuang Y, Liu H, Shi Q, Li D and Ju X: The role of N-acetyltransferase 8 in mesenchymal stem cell-based therapy for liver ischemia/reperfusion injury in rats. PLoS One 9: e103355, 2014.
26. Saidi RF, Rajeshkumar B, Shariftabrizi A, Bogdanov AA, Zheng S, Dresser K and Walter O: Human adipose-derived mesenchymal stem cells attenuate liver ischemia-reperfusion injury and promote liver regeneration. Surgery 156: 1225-1231, 2014.
27. Wang S, Shi XL, Feng M, Wang X, Zhang ZH, Zhao X, Han B, Ma HC, Dai B and Ding YT: Puerarin protects against CCl4-induced liver fibrosis in mice: Possible role of PARP-1 inhibition. Int Immunopharmacol 38: 238-245, 2016.
28. Huang YH, Yang YL, Tiao MM, Kuo HC, Huang LT and Chuang JH: Hepcidin protects against lipopolysaccharide-induced liver injury in a mouse model of obstructive jaundice. Peptides 35: 212-217, 2012.
29. Schmidt R, Tritschler E, Hoetzel A, Loop T, Humar M, Halverscheid L, Geiger KK and Pannen BH: Heme oxygenase-1 induction by the clinically used anesthetic isoflurane protects rat livers from ischemia/reperfusion injury. Ann Surg 245: 931-942, 2007.
30. Carchman EH, Rao J, Loughran PA, Rosengart MR and Zuckerbraun BS: Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. Hepatology 53: 2053-2062, 2011.
31. Zhang Y, Cai W, Huang Q, Gu Y, Shi Y, Huang J, Zhao F, Liu Q, Wei X, Jin M, *et al*: Mesenchymal stem cells alleviate bacteria-induced liver injury in mice by inducing regulatory dendritic cells. Hepatology 59: 671-682, 2014.
32. Lee KC, Lin HC, Huang YH and Hung SC: Allo-transplantation of mesenchymal stem cells attenuates hepatic injury through IL1Ra dependent macrophage switch in a mouse model of liver disease. J Hepatol 63: 1405-1412, 2015.
33. Sun L, Fan X, Zhang L, Shi G, Aili M, Lu X, Jiang T and Zhang Y: Bone mesenchymal stem cell transplantation via four routes for the treatment of acute liver failure in rats. Int J Mol Med 34: 987-996, 2014.
34. van Poll D, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW and Yarmush ML: Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. Hepatology 47: 1634-1643, 2008.
35. Park M, Kim YH, Woo SY, Lee HJ, Yu Y, Kim HS, Park YS, Jo I, Park JW, Jung SC, *et al*: Tonsil-derived mesenchymal stem cells ameliorate CCl4-induced liver fibrosis in mice via autophagy activation. Sci Rep 5: 8616, 2015.
36. Li X, Zeng Z, Li Q, Xu Q, Xie J, Hao H, Luo G, Liao W, Bin J, Huang X and Liao Y: Inhibition of microRNA-497 ameliorates anoxia/reoxygenation injury in cardiomyocytes by suppressing cell apoptosis and enhancing autophagy. Oncotarget 6: 18829-18844, 2015.
37. Zhang X, Yan H, Yuan Y, Gao J, Shen Z, Cheng Y, Shen Y, Wang RR, Wang X, Hu WW, *et al*: Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. Autophagy 9: 1321-1333, 2013.
38. Zou Z, Cai Y, Chen Y, Chen S, Liu L, Shen Z, Zhang S, Xu L and Chen Y: Bone marrow-derived mesenchymal stem cells attenuate acute liver injury and regulate the expression of fibrinogen-like-protein 1 and signal transducer and activator of transcription 3. Mol Med Rep 12: 2089-2097, 2015.
39. Zhao X, Shi X, Zhang Z, Ma H, Yuan X and Ding Y: Combined treatment with MSC transplantation and neutrophil depletion ameliorates D-GalN/LPS-induced acute liver failure in rats. Clin Res Hepatol Gastroenterol 40: 730-738, 2016.
40. Lu W, Si YI, Ding J, Chen X, Zhang X, Dong Z and Fu W: Mesenchymal stem cells attenuate acute ischemia-reperfusion injury in a rat model. Exp Ther Med 10: 2131-2137, 2015.
41. Liu A, Fang H, Wei W, Dirsch O and Dahmen U: Ischemic preconditioning protects against liver ischemia/reperfusion injury via heme oxygenase-1-mediated autophagy. Crit Care Med 42: e762-e771, 2014.
42. Li SP, He JD, Wang Z, Yu Y, Fu SY, Zhang HM, Zhang JJ and Shen ZY: miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury via decreasing the Atg12-Atg5 conjugate. World J Gastroenterol 22: 4501-4514, 2016.
43. Jiang YB, Zhang XL, Tang YL, Ma GS, Shen CX, Wei Q, Zhu Q, Yao YY and Liu NF: Effects of heme oxygenase-1 gene modulated mesenchymal stem cells on vasculogenesis in ischemic swine hearts. Chin Med J (Engl) 124: 401-407, 2011.
44. Nong K, Wang W, Niu X, Hu B, Ma C, Bai Y, Wu B, Wang Y and Ai K: Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. Cytotherapy 18: 1548-1559, 2016.
45. Chen YT, Sun CK, Lin YC, Chang LT, Chen YL, Tsai TH, Chung SY, Chua S, Kao YH, Yen CH, *et al*: Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. J Transl Med 9: 51, 2011.
46. Zhang B, Adesanya TM, Zhang L, Xie N, Chen Z, Fu M, Zhang J, Zhang J, Tan T, Kilic A, *et al*: Delivery of placenta-derived mesenchymal stem cells ameliorates ischemia induced limb injury by immunomodulation. Cell Physiol Biochem 34: 1998-2006, 2014.
47. Gracia-Sancho J, Guixé-Muntet S, Hide D and Bosch J: Modulation of autophagy for the treatment of liver diseases. Expert Opin Investig Drugs 23: 965-977, 2014.
48. Awan MU and Deng Y: Role of autophagy and its significance in cellular homeostasis. Appl Microbiol Biotechnol 98: 5319-5328, 2014.
49. Hale AN, Ledbetter DJ, Gawriluk TR and Rucker EB III: Autophagy: Regulation and role in development. Autophagy 9: 951-972, 2013.
50. Mizushima N and Levine B: Autophagy in mammalian development and differentiation. Nat Cell Biol 12: 823-830, 2010.
51. Liu Q, Guan JZ, Sun Y, Le Z, Zhang P, Yu D and Liu Y: Insulin-like growth factor 1 receptor-mediated cell survival in hypoxia depends on the promotion of autophagy via suppression of the PI3K/Akt/mTOR signaling pathway. Mol Med Rep 15: 2136-2142, 2017.
52. Liu S, Hartleben B, Kretz O, Wiech T, Igarashi P, Mizushima N, Walz G and Huber TB: Autophagy plays a critical role in kidney tubule maintenance, aging and ischemia-reperfusion injury. Autophagy 8: 826-837, 2012.
53. Cursio R, Colosetti P and Gugenheim J: Autophagy and liver ischemia-reperfusion injury. Biomed Res Int 2015: 417590, 2015.
54. Zhao Q, Guo Z, Deng W, Fu S, Zhang C, Chen M, Ju W, Wang D and He X: Calpain 2-mediated autophagy defect increases susceptibility of fatty livers to ischemia-reperfusion injury. Cell Death Dis 7: e2186, 2016.
55. Wang JH, Ahn IS, Fischer TD, Byeon JI, Dunn WA Jr, Behrns KE, Leeuwenburgh C and Kim JS: Autophagy suppresses age-dependent ischemia and reperfusion injury in livers of mice. Gastroenterology 141: 2188-2199.e6, 2011.

56. Fang H, Liu A, Dahmen U and Dirsch O: Dual role of chloroquine in liver ischemia reperfusion injury: Reduction of liver damage in early phase, but aggravation in late phase. Cell Death Dis 4: e694, 2013.
57. Shin JY, Park HJ, Kim HN, Oh SH, Bae JS, Ha HJ and Lee PH: Mesenchymal stem cells enhance autophagy and increase β-amyloid clearance in Alzheimer disease models. Autophagy 10: 32-44, 2014.
58. Zhou Z and You Z: Mesenchymal stem cells alleviate LPS-induced acute lung injury in mice by MiR-142a-5p-Controlled pulmonary endothelial cell autophagy. Cell Physiol Biochem 38: 258-266, 2016.
59. Yin F, Meng C, Lu R, Li L, Zhang Y, Chen H, Qin Y and Guo L: Bone marrow mesenchymal stem cells repair spinal cord ischemia/reperfusion injury by promoting axonal growth and anti-autophagy. Neural Regen Res 9: 1665-1671, 2014.
60. Yun N, Cho HI and Lee SM: Impaired autophagy contributes to hepatocellular damage during ischemia/reperfusion: Heme oxygenase-1 as a possible regulator. Free Radic Biol Med 68: 168-177, 2014.
61. Wang Y, Xiong X, Guo H, Wu M, Li X, Hu Y, Xie G, Shen J and Tian Q: ZnPP reduces autophagy and induces apoptosis, thus aggravating liver ischemia/reperfusion injury in vitro. Int J Mol Med 34: 1555-1564, 2014.
62. Park J, Kang JW and Lee SM: Activation of the cholinergic anti-inflammatory pathway by nicotine attenuates hepatic ischemia/reperfusion injury via heme oxygenase-1 induction. Eur J Pharmacol 707: 61-70, 2013.
63. Rao J, Qian X, Li G, Pan X, Zhang C, Zhang F, Zhai Y, Wang X and Lu L: ATF3-mediated NRF2/HO-1 signaling regulates TLR4 innate immune responses in mouse liver ischemia/reperfusion injury. Am J Transplant 15: 76-87, 2015.