

Original Article

Bone marrow-derived mesenchymal stem cells inhibits hepatocyte apoptosis after acute liver injury

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Abstract: Objective: To investigate the protective effect of bone marrow-derived mesenchymal stem cells (BMSCs) transplantation on acute liver injury (ALI) rats. Material and Methods: BMSCs were extracted from rat bone marrow, cultured and expansion in vitro, and identified by flow cytometer. Rat model with acute liver injury was established by employing D-galactosamine and Lipopolysaccharide. Male rats were randomly divided into ALI model group and BMSCs transplantation group. Rats were sacrificed 24 h, 72 h and 120 h after BMSCs injection to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum. Proliferating cell nuclear antigen (PCNA) immunohistochemistry staining and quantitative reverse transcription polymerase chain reaction (RT-PCR) of α -fetoprotein (AFP) and glypican-3 (GPC3) were performed to analysis proliferation. Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assays were used to analyze apoptosis and mitochondria-dependent-pathway related factors Bax and Bcl-2 were examined by Western blot. Results: Compared with the ALI model group, the BMSCs transplantation group presented the lower levels of ALT, AST, decreased Bax proteins expression, and increased Bcl-2 expression. The mRNA levels of AFP and GPC3 and expression of PCNA were significantly higher in BMSCs transplantation group. Conclusions: BMSCs transplantation could significantly restore liver function. These effects were supposed to be mediated by suppressing hepatocyte apoptosis as well as promoting proliferation. Reduction of apoptosis seemed to correlate with mitochondria-dependent-pathway.

Keywords: Bone marrow-derived mesenchymal stem cells, acute liver injury, apoptosis

Introduction

Acute liver diseases can be caused by diverse etiology and may aggravate to severe clinical outcomes including hepatic encephalopathy, hepatorenal syndrome, severe infection, multiple organ failure and even death [1]. Liver transplantation is currently the most effective therapy for end-stage liver disease. However, its application is limited for shortage of available donor organs, high costs and lifelong immunosuppressive therapy [2]. In recent years, Mesenchymal Stem cells (MSCs) have emerged as a promising drug to various diseases due to their multi-potency, immune-modulation and secretome activities [3]. Many studies have demonstrated that mesenchymal stem cells (MSCs) promote repair of damaged tissue by inhibiting apoptosis [4-6].

As previously reported, derangements in apoptosis of liver cells are mechanistically important in the pathogenesis of end stage liver disease [7]. Furthermore, another research demonstrated that increased hepatocyte protection is associated with down-regulation of pro-apoptotic signaling in an acute liver injury model [8]. Therefore, therapeutic strategies to inhibit apoptosis in liver injury may have the potential to provide a powerful tool for the treatment of liver disease [9].

Although the functions of BMSCs have been widely studied, there is limited information about the anti-apoptosis function of BMSCs during acute liver injury. In this study, we aimed to explore the effectiveness of bone marrow-derived mesenchymal stem cells (BMSCs) transplantation on the prevention of acute liver

Mesenchymal stem cells reverse acute liver injury

injury in vivo and explore the potential mechanism of their protection.

Materials and methods

Isolation and culture of BMSCs from rats

Take one SPF healthy Sprague Dawley rat, male, 9 weeks old, and 170 g in weight, and sacrifice it by over-dose injection of chloral hydrate. The bilateral femur and tibia were separated under sterile conditions. Subsequently, whole bone marrow of the bilateral femur and tibia was collected and cells from bone marrow were seeded in a 25 cm² plastic bottle to separate the BMSCs using the adherence method. α -MEM (Thermo Scientific, USA) and fetal bovine serum (Gibco, USA) with the volume fraction of 10% were added. Then transferred the plastic bottle into the incubator for cultivation. After 24 hours, the non-adherent cells were removed, the solutions were changed 3 days afterwards. 0.25% trypsin (Gibco, USA) was used for digestion and regeneration once adherent cells reached confluence of 70%-80% and cells were replated at 1:2 dilution. The flow cytometer analysis was performed when BMSCs were at 3rd passage for identification. The cells from passages 3-5 were used for subsequent experiments.

Acute liver injury induction and BMSCs transplantation procedures

All experimental protocols were approved by the Animal Care Ethics Committee of Wenzhou medical University, and all rats received humane care according to the Guide for the Care and Use of Laboratory Animals. Fifty male SPF Sprague Dawley rats weighing 150 ± 20 g were purchased from Shanghai Experimental Animal Co., Ltd (Shanghai, China). All rats were randomized allocated to two groups: Acute Liver Injury (ALI) model group as control group (24 rats), and BMSCs transplantation group as therapeutic group (24 rats). 48 rats in two groups underwent acute liver injury induction with D-galactosamine (Sigma, USA) at a dose of 400 mg/kg and lipopolysaccharide (Sigma, USA) at a dose of 80 μ g/kg via intraperitoneal injection. Subsequently, 24 rats in ALI model group were received a transfusion of 0.5 ml normal saline, whereas BMSCs transplantation group were received a transfusion of 3×10^7 /kg BMSCs suspended in 0.5 ml normal saline through tail vein injection, respectively. All samples in both groups were collected at 24 h, 72

h, 120 h after transplantation with each 8 rats, respectively. At each time point, blood samples were collected through portal vein and centrifuged to obtain serum for detection of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST). And fresh liver tissues were collected for further studies such as hematoxylin and eosin (HE) staining, proliferating cell nuclear antigen (PCNA) immunohistochemistry assay, reverse transcription polymerase chain reaction (RT-PCR), and western blotting.

Serum ALT, AST determination and hematoxylin-eosin (HE) staining

The serum biochemical parameters ALT, AST which closely reflect the liver function were analyzed by the Department of Laboratory Medicine, First Affiliated Hospital of Wenzhou medical University (Wenzhou, China). The liver tissue were fixed in 4% paraformaldehyde, embedded in paraffin, and 4 μ m thick sections were cut from each paraffin block. Slices were taken for Hematoxylin-Eosin (HE) staining, PCNA staining and TUNEL assay.

PCNA immunohistochemistry

The samples were dewaxed, rehydrated and treated with 3% H₂O₂. Sections were applied 350 W microwave irradiation for 5 minutes in 0.1 M citrate buffer solution (pH 6.0) for antigen retrieval. Slides were incubated for 25 minutes with 5% bovine serum albumin and incubated overnight at 4°C with rabbit monoclonal anti-PCNA (1:16000 dilution, Cell Signaling Technology, USA). After that, Slides were incubated for 30 minutes with appropriate peroxidase-conjugated secondary antibody (PV-6001; Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). Then 3, 3'-diaminobenzidine (DAB) served as the chromogen and hematoxylin as the counterstain. Subsequently, slides were sealed with neutral gum. Samples treated with phosphate buffer served as the negative control. Sections were examined microscopically for specific staining and nuclei with brown color regardless of staining intensity were regarded as positive. PCNA positivity was calculated under 40x magnifications by dividing the number of positive cells by the total number of cells counted in 5 random visual fields and expressed as percentage for PCNA [10]. Photographs were taken with a digital image-capture system (Nikon Eclipse, Japan).

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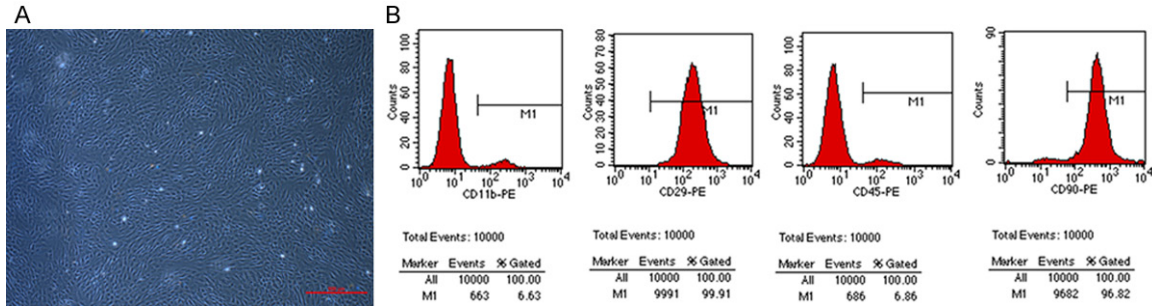


Figure 1. BMSCs prepared for the transplantation. A: BMSCs exhibited a spindle shape morphology (magnification $\times 10$). Scale bar, 50 μm . B: Determination of rat BMSCs phenotype by flow cytometer. BMSCs, bone marrow-derived mesenchymal stem cells.

Detection of apoptosis by TUNEL assay

The TUNEL (DNA fragmentation by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay kit was purchased from Roche Applied Science for detection of apoptosis. According to the manufacturer's instructions, paraffin-embedded tissue sections were dewaxed in xylene, rehydrated through graded ethanol, and pretreated with proteinase-K. Endogenous peroxidase activity was blocked by immersing in 3% H_2O_2 in methanol for 10 minutes. TUNEL reaction mixture and Converter-POD were then added. Each slice was stained by DAB, and liver cell apoptosis was observed under light microscopy. TUNEL-positive cells per field were counted in 5 random fields under $40\times$ magnifications, and positive cell percentages were averaged.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by using the RNAiso Plus reagent (Aidlab Biotechnologies Co., China). According to manufacturer's instruction, first-strand cDNA was synthesized by using Power RT kit (Biotek Corporation, China). AFP, GPC3, and internal control primers were synthesized by Invitrogen Corporation. The sequences of AFP primers were designed as follows: forward, ACCATCGAGCTCGGCTATTG; reverse, GAGACAGGAAGGTTGGGGTG. The sequences of GPC3 primers were designed as follows; forward, TGTGCTGGAACGGACAAGAG; reverse, TGGGCACAGACATGGTTCTC. Expression data were normalized to the geometric mean of housekeeping gene β -actin (forward: CACCGCG-AGTACAACCTTC and reverse: CCCATACCCACCAT-CACACC) to control the variability in expression levels. The ABI 7500 Real-Time PCR System was applied to determine Ct value of product.

Protein extraction and western blot analysis in the liver tissue

Liver tissue lysates were obtained by using lysis buffer supplemented with a protease inhibitor cocktail from Roche (Summerville, NJ, USA). Following heat denaturation at 95°C for 3 min, the samples (15 μg protein each) were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride membrane (PVDF) (Bio-Rad, USA). Skimmed milk was then employed to blocked membrane for 1 h at room temperature. The primary antibodies against Bax, Bcl-2 and GAPDH which were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) were used for incubation with the membrane overnight at 4°C , respectively. After primary antibody incubation, the membranes were took to be washed with PBS three times, and the secondary antibody was added for incubation at room temperature for 1 h, and then the film exposure was performed.

Statistical analysis

The data was analyzed by SPSS19.0 statistical software and expressed as mean \pm SD. Multi-group comparisons were performed using one-way ANOVA multiple comparisons among means. Student's t test was used for comparisons of two groups. $P < 0.05$ denoted a statistically significant difference.

Results

Identification of BMSCs and transplantation of BMSCs assist to reverse liver function

After 15 days from isolation of cells from bone marrow, adherent cells exhibited spindle-shape

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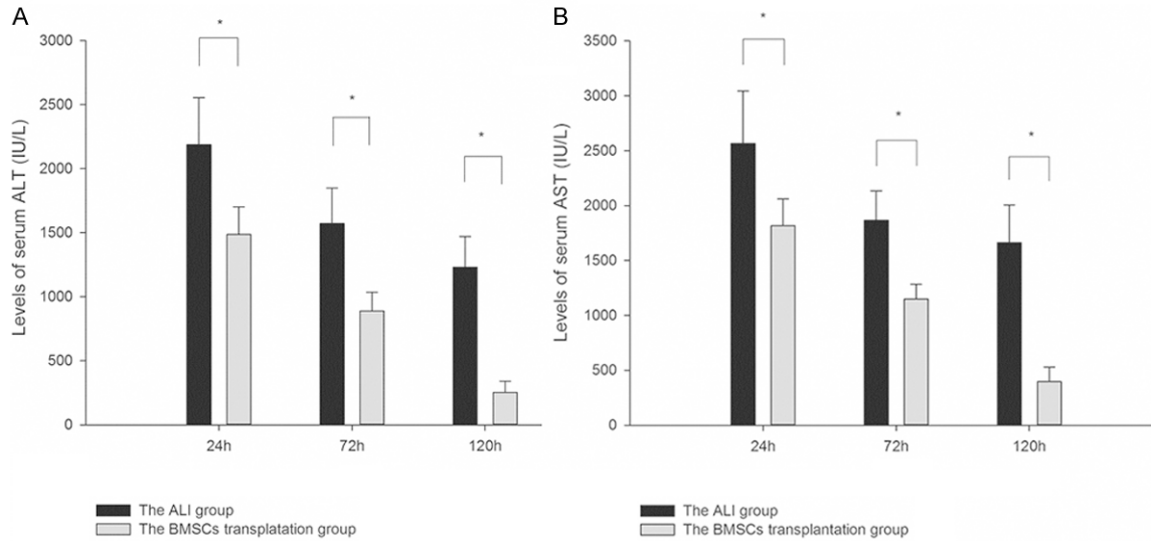


Figure 2. Results of the biochemical assays. Serum was collected from the ALI group and the BMSCs transplantation group at 24 h, 72 h, and 120 h after BMSCs transplantation. Data were expressed as $mean \pm S$, $*P < 0.05$, the ALI group versus the BMSCs transplantation group. ALI, acute liver injury; BMSCs, bone marrow-derived mesenchymal stem cells; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

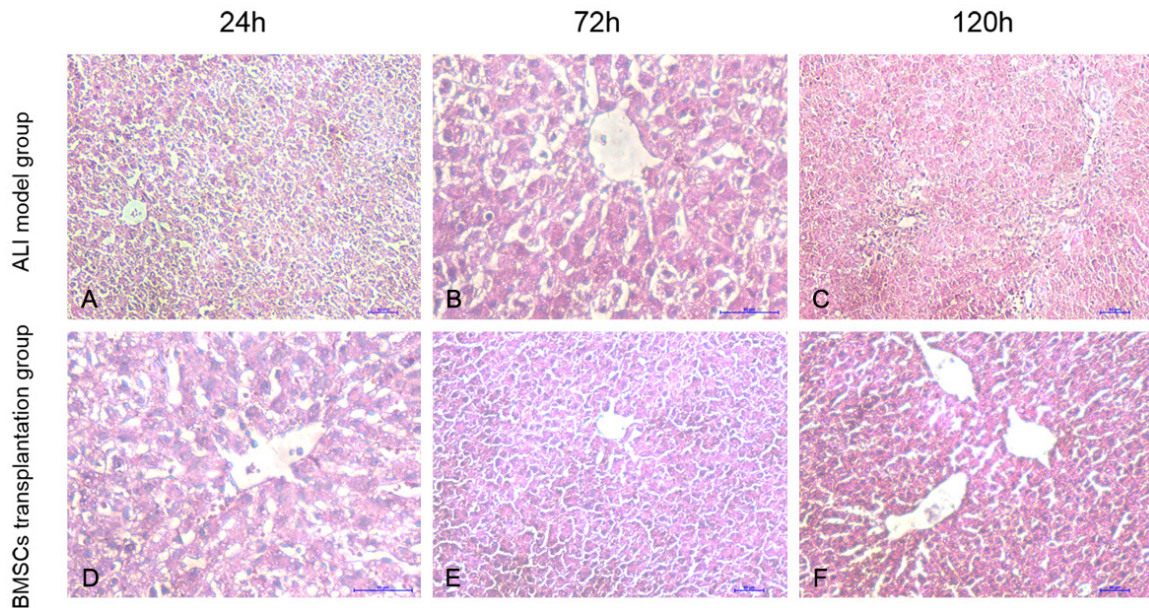


Figure 3. H&E staining. A: ALI livers, 24 h after D-gal/LPS injection, showed a typical ALI histology with hepatocyte necrosis and hemorrhage. A majority of hepatocyte were present had a swollen cytoplasm vacuolization and a moderate increase in inflammatory cells ($\times 100$). B and C: 72 h and 120 h later, the liver injury is ameliorated ($\times 200$, $\times 100$). D-F: The BMSCs transplantation significantly ameliorated D-gal/LPS-induced liver injury at 24 h ($\times 200$), 72 h ($\times 100$), and 120 h ($\times 100$). Scale bar, 50 μ m. H&E, hematoxylin and eosin; ALI, acute liver injury; D-gal, D-galactosamine; LPS, lipopolysaccharide; BMSCs, bone marrow-derived mesenchymal stem cells.

morphology as showed in **Figure 1A**. The BMSCs of passages 3 generation were obtained for flow cytometer analysis. The analysis results (**Figure 1B**) showed that the BMSCs from pas-

sages 3 were positive for CD29 and CD90 but negative for CD11b and CD45, indicating that the cells used for transplantation exhibited classic MSCs phenotype [11]. The effect of

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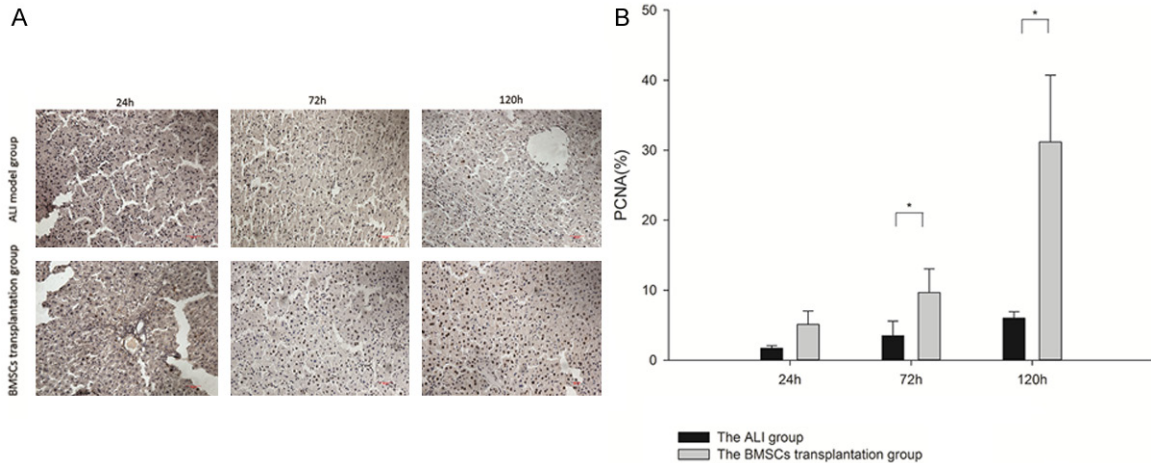


Figure 4. PCNA immunohistochemistry. A: PCNA immunohistochemistry; B: Histogram of immunohistochemistry. The PCNA positive rates were $1.69 \pm 0.38\%$, $3.50 \pm 2.07\%$, $6.01 \pm 0.93\%$, $5.12 \pm 1.9\%$, $9.65 \pm 3.38\%$, and $31.1 \pm 9.54\%$, respectively, in ALI model 24 h, 72 h, 120 h, BMSCs transplantation 24 h, 72 h, 120 h. $*P < 0.05$. Scale bar, 50 μm . PCNA, proliferating cell nuclear antigen; ALI: acute liver injury; BMSCs: bone marrow-derived mesenchymal stem cells.

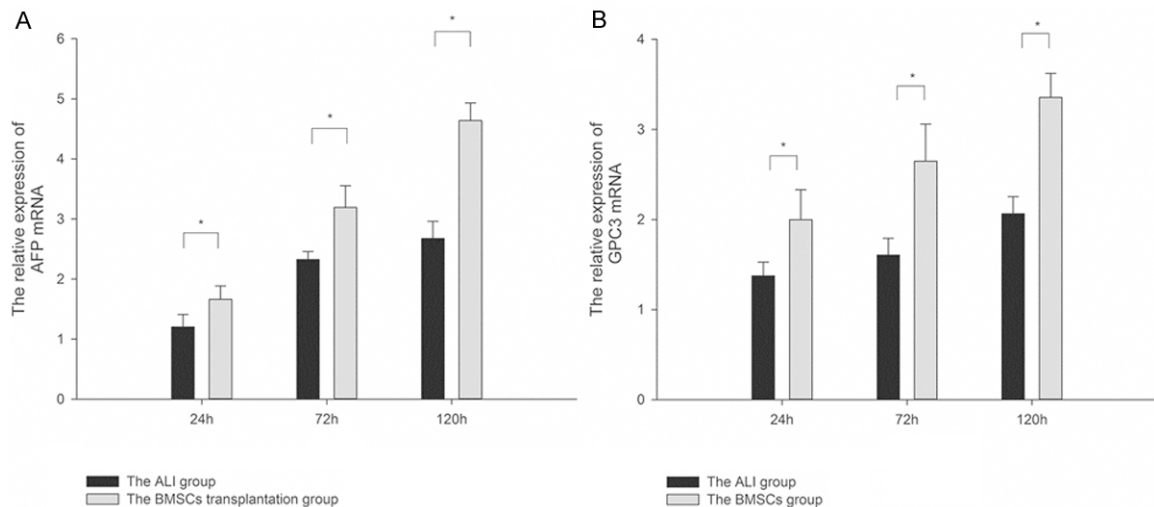


Figure 5. RT-PCR detection of the mRNA expression in liver tissues. The ABI 7500 Real-Time PCR system was used to determine Ct value of product. The relative amount of $2^{-\Delta\Delta\text{Ct}}$ method was used to compare expression of gene AFP and GPC3 in rat liver tissues. A: AFP mRNA expression in liver tissue. B: GPC3 mRNA expression in liver tissue. $*P < 0.05$. RT-PCR: reverse transcription-polymerase chain reaction; ABI: Applied Biosystems; AFP: α -fetoprotein; GPC3: glypican-3.

BMSCs was demonstrated by the data from the serum markers of liver injury. Compared to normal range, both the serum levels of ALT, AST in ALI model group and BMSCs transplantation group significantly increased after D-gal/LPS injection indicating a successful establishment of acute liver injury model. In addition, the serum makers from the BMSCs transplantation group significantly decreased compared with those in the ALI model group, especially at 72

h, and 120 h after transplantation (**Figure 2**, $P < 0.05$), suggesting a role of BMSCs in the improvement of liver function.

Histopathological examination of liver tissue

As showed in **Figure 3**, in the ALI model group, significant anomalies of liver cells and degeneration of structure were observed in D-gal/LPS induced rats, such as vacuolization of cyto-

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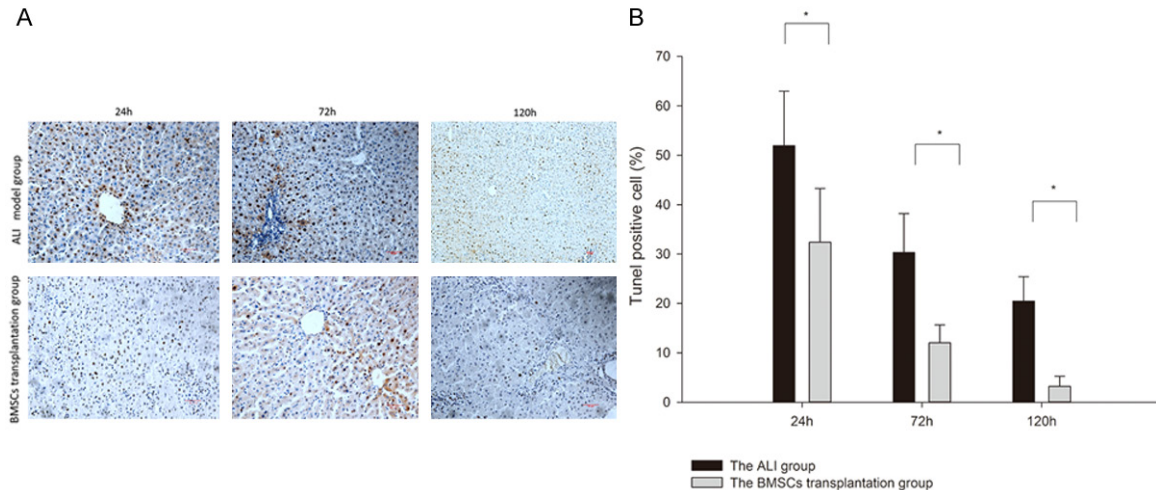


Figure 6. Effects of BMSCs on apoptosis of hepatocytes in D-Gal/LPS-induced acute hepatic injury in rats. Apoptosis of hepatocytes was determined by TUNEL assay. A: The apoptotic cells showed a dark-brown nucleus. B: The TUNEL positive cell rates were $51.92 \pm 11.02\%$, $30.29 \pm 7.89\%$, $20.48 \pm 4.93\%$, $32.39 \pm 10.89\%$, $12.04 \pm 3.62\%$, and $3.22 \pm 2.02\%$, respectively, in ALI model 24 h, 72 h, 120 h, BMSCs transplantation 24 h, 72 h, 120 h. * $P < 0.05$. Scale bar, 50 μm . TUNEL: terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; ALI: acute liver injury; BMSCs: bone marrow-derived mesenchymal stem cells.

plasm, hemorrhage and infiltration of inflammatory cell in portal area, but were alleviated in the BMSCs-treated group at 72 h and 120 h. At 120 h, the animal showed a relatively normal liver structure with minor cytoplasmic vacuolization and periportal inflammatory cell infiltration (**Figure 3F**). Overall, these results suggested the treatment with BMSCs could effectively protect the liver from D-gal/LPS-induced acute liver damage, supporting the observed biochemical effects of BMSCs.

BMSCs transplantation promotes liver cells proliferation

To clarify the effect of BMSCs on the proliferation of liver cells, the PCNA immunohistochemistry was performed (**Figure 4A**). As showed in **Figure 4B**, the PCNA positive rates were $1.69 \pm 0.38\%$, $3.50 \pm 2.07\%$, $6.01 \pm 0.93\%$, $5.12 \pm 1.9\%$, $9.65 \pm 3.38\%$, and $31.1 \pm 9.54\%$, respectively, in ALI model 24 h, 72 h, 120 h, BMSCs transplantation 24 h, 72 h, 120 h. At 72 h, 120 h time points, the PCNA positive rate of BMSCs transplantation group was significantly higher than ALI model group ($P < 0.05$), indicating an evident regeneration after the treatment of BMSCs. Consistent with PCNA immunohistochemistry findings, the RT-PCR results indicated that AFP and GPC3 mRNA expressions were significantly increased in BMSCs transplantation group compared with ALI model group at 24 h, 72 h and 120 h after transplantation

(**Figure 5**). Moreover, AFP and GPC3 mRNA expressions were gradually increased as the liver function improved in both groups. Taken together, these data demonstrated that infusion of BMSCs promote liver proliferation after an acute liver injury.

BMSCs transplantation alleviate apoptosis in the liver cells

To investigate whether BMSCs transplantation reduced the ALI-related apoptosis, the TUNEL assay was performed to examine the levels of apoptosis in each group. In sections from ALI model group rats, many large, apoptotic hepatocyte nuclei were observed, whereas only few were present after BMSCs treatment (**Figure 6A**). Number of positively stained apoptotic cells with round shape and brown nucleus were counted. In ALI model group, the apoptotic cell rates at 24 h, 72 h, and 120 h were $51.92 \pm 11.02\%$, $30.29 \pm 7.89\%$, $20.48 \pm 4.93\%$, however, after infusion of BMSCs, the rates fell to $32.39 \pm 10.89\%$, $12.04 \pm 3.62\%$, and $3.22 \pm 2.02\%$, respectively (**Figure 6B**). Thus, these present data demonstrated the capability of BMSCs to alleviate apoptosis of liver cells.

Expression of Bcl-2 and Bax protein

To further investigate the effect to apoptosis of liver cells by BMSCs transplantation, western blotting was performed to detect the expres-

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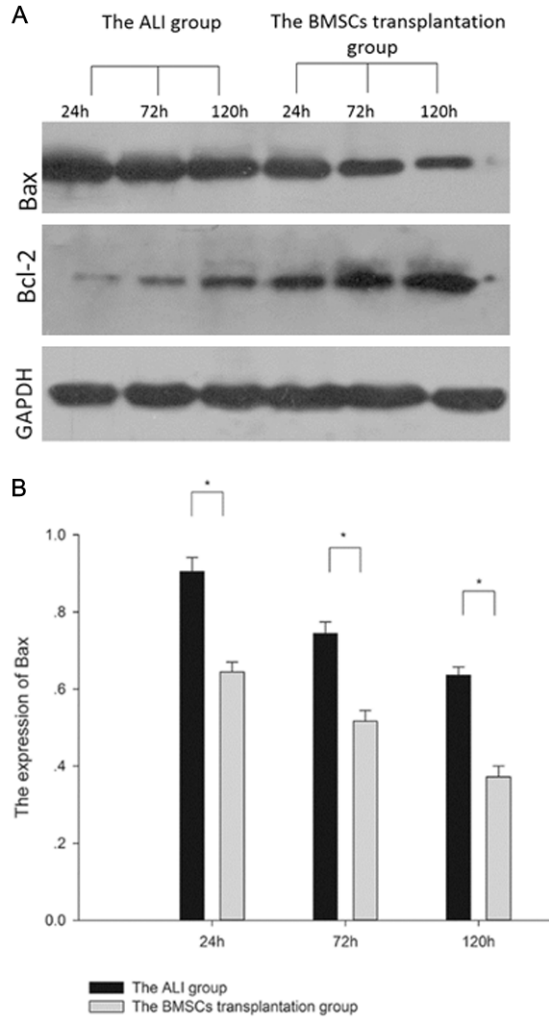


Figure 7. Levels of Bax and Bcl-2 in liver tissues were determined by Western blot analysis. A: The bands of Bax, Bcl-2 and GAPDH. B: Western blots were scanned by densitometry and data presented as relative intensity units. Data were expressed as mean \pm SD. n = 8. * $P < 0.05$. Bax, Bcl-2 associated x protein; Bcl-2, B cell lymphoma/leukemia-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

sion of Bcl-2 and Bax protein in liver tissue. As illustrated in **Figure 7**, compared with the ALI model group, the group treated with BMSCs resulted in an evident decline of Bax and significant increase of Bcl-2. The results revealed that BMSCs transplantation induced the up-regulation of Bcl-2 expression and down-regulation of Bax expression in a time-dependent manner as liver function improved. Hence, these findings further confirmed that BMSCs do play a role in alleviating liver cells apoptosis and then ameliorating acute liver injury.

Discussion

In last decade, MSCs had gained a wave of enthusiasm in clinical applications for their properties such as regeneration, immunomodulation, anti-inflammation and trophic effects [12]. The results of MSCs treatment in the severe disease, such as graft versus-host dis-

ease (GvHD) [13], osteogenesis imperfecta [14], ischaemic cardiomyopathy [15], Crohn's disease [16], are encouraging. However, the underlying mechanism of the therapy has yet to be clearly elucidated. It has been preliminarily reported that BMSCs were capable of transdifferentiating into hepatocytes and bile duct cells in the repair process after severe liver injury [17-19]. Intriguingly, Parekkadan and Xagorari et al. both performed infusion of MSC-derived conditioning medium to liver injury animals and demonstrated that these improvements could be more attributed to the secretion of soluble factors by MSCs, rather than transdifferentiation into hepatocytes [20, 21]. Anyway, BMSCs therapy seems to be a promising strategy in acute liver injury treatment.

In the present study, we established an acute liver injury rat model by induction of D-galactosamine and lipopolysaccharide, which is gen-

erally considered to mimic clinical liver dysfunction and useful for evaluating the efficiency of treatment [22-24]. The rats exposed to D-gal/LPS presented significant increase of ALT and AST, commonly used markers of liver cytolysis, whereas, these increase were attenuated by the transplantation of BMSCs. In addition to the decrease of serum makers, the histopathological results showed that BMSCs administration significantly alleviate the cytoplasmic vacuolization and infiltration of inflammatory cells. Taking these results together, we reported that BMSCs are of certain of hepatoprotective effect on this model of liver damage.

However, a successful liver cell therapy requires a better understanding of the underlying mechanisms protecting against liver injury and of their implication in cell transplantation. Preliminary trials reported that the positive effect most likely do not involve repopulation of liver parenchyma with bone marrow-derived cells themselves but might result from the production of soluble growth factors or cytokines by the infused cells. By releasing these molecules, BMSCs might stimulate pro-proliferation or anti-apoptosis functions to favor the recovery of liver injury [21]. In our study, we suspended BMSCs into saline and then transfer it into model rats to explore if BMSCs themselves could reverse acute liver injury by influencing hepatocyte proliferation or apoptosis.

To determine the effect of BMSCs on liver cells proliferation, we performed PCNA immunohistochemistry. We found the PCNA positive rates were significantly higher in BMSCs group, indicating high regeneration rates. However, whether the promotion of regeneration was produced by directly effects of BMSCs or the subsequent effects of providing pro-proliferation factors or reducing anti-proliferation factors was unknown. Therefore, further study was needed to explore underlying mechanism of BMSCs pro-proliferation function. In order to further study the effect of pro-proliferation function of BMSCs, the expression of GPC3 and AFP mRNA was detected. As a maker for hepatic progenitor cells, GPC3 is reported to be high expression during embryogenesis and organogenesis [25]. Mostly, after an induction of acute liver injury by D-gal/LPS, hepatic progenitor/oval cells proliferate when the regenerative capacities of hepatocytes are compromised [26]. Moreover, it has been suggested that overex-

pression of GPC3 inhibit liver regeneration and hepatocyte proliferation [27], indicating GPC3 may play an important negative feedback regulating role in the mechanism of liver regeneration. In this study, we showed the levels of GPC3 mRNA expression gradually increased as time extended after BMSCs transplantation and liver function improved. Compared with ALI model group, the GPC3 mRNA expression is much higher in BMSCs transplantation group at three time points, indicating that BMSCs might enhance liver regeneration. AFP, which was a marker of hepatocyte proliferation as well, was commonly used to evaluate the level of liver proliferation [28]. In concordance with the results of GPC3 mRNA, AFP mRNA expression significantly increased following liver function restored after BMSCs transplantation. The available data above support the notion that BMSCs transplantation leads to the pro-proliferation of hepatocyte in liver injury, although the underlying mechanism remains to be elucidated.

Traditionally, hepatocyte apoptosis is considered as a characteristic feature of acute liver injury, and increasing evidence indicates hepatocyte apoptosis plays a dominant role in pathogenesis of fulminant hepatic failure as well [29]. To restore the function of the damaged recipient liver, BMSCs not only enhanced the ability of liver regeneration, but also inhibit apoptosis of hepatocytes. In our study, TUNEL assay, a widely used technique designed to detect and quantify apoptotic cell death at tissues via labeling of DNA strand breaks by Terminal deoxynucleotidyl transferase, was performed and expression levels of Bcl-2 family of proteins which determines the commitment of cells to apoptosis were observed. The results revealed that hepatocytes underwent obviously apoptosis after D-gal/LPS induction but BMSCs transplantation intervention decreased the extent of apoptosis. However, the underlying mechanism that how BMSCs regulate the apoptotic process is uncertain. As the intrinsic pathway of apoptotic signal, mitochondrion-dependent pathway was reported previously to be regulated by Bcl-2 family members [28]. In our study, the expression levels of the Bax and Bcl-2 were significantly altered. Based on these results, we demonstrated that BMSCs might reduce hepatocyte apoptosis via mitochondrion-dependent pathway. Given evidence that reactive oxygen species (ROS) was involved in

LPS/D-Gal-induced liver damage and the mitochondrial pathway can be triggered by ROS, we further speculate that BMSCs might affect mitochondrial pathway by reducing ROS [30, 31]. Thus, it would be of interest to verify this hypothesis in a future study by investigating mitochondria-dependent-pathways (e.g., cytochrome c or caspase-9) in the protective effect of BMSCs on liver injury.

Li et al. had reported the effectiveness of BMSCs in the treatment of fulminant hepatic failure using human BMSCs and pig fulminant hepatic failure (FHF) model [32]. Nevertheless, our study was different from theirs by the reasons that we further confirmed the protection of BMSCs transplantation on liver injury rat model and predicted the potential underlying mechanism. BMSCs were injected via the tail vein as previously reported. BMSCs treatment significantly inhibits liver cells apoptosis and promotes liver cells proliferation, and ultimately improved the liver function of liver injury rats. By up-regulation of Bax and down-regulation of Bcl-2, we give a hypothesis that BMSCs may reduce cells apoptosis through mitochondria-dependent-pathways. Further studies are needed to clarify the exact mechanism and to improve the effectiveness of BMSCs transplantation.

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Disclosure of conflict of interest

None.

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References

- [1] Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet* 2010; 376: 190-201.
- [2] Stravitz RT, Kramer DJ. Management of acute liver failure. *Nat Rev Gastroenterol Hepatol* 2009; 6: 542-553.

- [3] Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008; 8: 726-736.
- [4] Poynter JA, Herrmann JL, Manukyan MC, Wang Y, Abarbanell AM, Weil BR, Brewster BD, Meldrum DR. Intracoronary mesenchymal stem cells promote postischemic myocardial functional recovery, decrease inflammation, and reduce apoptosis via a signal transducer and activator of transcription 3 mechanism. *J Am Coll Surg* 2011; 213: 253-260.
- [5] Zhang H, Fang J, Wu Y, Mai Y, Lai W, Su H. Mesenchymal stem cells protect against neonatal rat hyperoxic lung injury. *Expert Opin Biol Ther* 2013; 13: 817-829.
- [6] Yuan S, Jiang T, Sun L, Zheng R, Ahat N, Zhang Y. The role of bone marrow mesenchymal stem cells in the treatment of acute liver failure. *Biomed Res Int* 2013; 2013: 251846.
- [7] Malhi H, Gores GJ. Cellular and molecular mechanisms of liver injury. *Gastroenterology* 2008; 134: 1641-1654.
- [8] Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; 182: 1545-1556.
- [9] Guicciardi ME, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 2005; 54: 1024-1033.
- [10] Zeng H, Yuan Z, Zhu H, Li L, Shi H, Wang Z, Fan Y. Expression of hPNAS-4 radiosensitizes Lewis lung cancer. *Int J Radiat Oncol Biol Phys* 2012; 84: e533-540.
- [11] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8: 315-317.
- [12] de Girolamo L, Lucarelli E, Alessandri G, Avanzini MA, Bernardo ME, Biagi E, Brini AT, D'Amico G, Fagioli F, Ferrero I, Locatelli F, Maccario R, Marazzi M, Parolini O, Pessina A, Torre ML, Italian Mesenchymal Stem Cell Group. Mesenchymal stem/stromal cells: a new "cells as drugs" paradigm. Efficacy and critical aspects in cell therapy. *Curr Pharm Des* 2013; 19: 2459-2473.
- [13] Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363: 1439-1441.
- [14] Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard

Mesenchymal stem cells reverse acute liver injury

- P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999; 5: 309-313.
- [15] Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, Pattany PM, Zambrano JP, Hu Q, McNiece I, Heldman AW, Hare JM. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci U S A* 2009; 106: 14022-14027.
- [16] Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidler HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010; 59: 1662-1669.
- [17] Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, Rae F, Forrester L, Turner ML, Hayes PC, Harrison DJ, Bickmore WA, Plevris JN. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology* 2003; 124: 1891-1900.
- [18] Kisseleva T, Gigante E, Brenner DA. Recent advances in liver stem cell therapy. *Curr Opin Gastroenterol* 2010; 26: 395-402.
- [19] Sordi V, Piemonti L. Therapeutic plasticity of stem cells and allograft tolerance. *Cytotherapy* 2011; 13: 647-660.
- [20] Xagorari A, Siotou E, Yiangou M, Tsolaki E, Bougiouklis D, Sakkas L, Fassas A, Anagnostopoulos A. Protective effect of mesenchymal stem cell-conditioned medium on hepatic cell apoptosis after acute liver injury. *Int J Clin Exp Pathol* 2013; 6: 831-840.
- [21] van Poll D, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology* 2008; 47: 1634-1643.
- [22] Medline A, Schaffner F, Popper H. Ultrastructural features in galactosamine-induced hepatitis. *Exp Mol Pathol* 1970; 12: 201-211.
- [23] Jonker AM, Dijkhuis FW, Kroese FG, Hardonk MJ, Grond J. Immunopathology of acute galactosamine hepatitis in rats. *Hepatology* 1990; 11: 622-627.
- [24] Rahman TM, Hodgson HJ. Animal models of acute hepatic failure. *Int J Exp Pathol* 2000; 81: 145-157.
- [25] Filmus J. Glypicans in growth control and cancer. *Glycobiology* 2001; 11: 19R-23R.
- [26] Grozdanov PN, Yovchev MI, Dabeva MD. The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. *Lab Invest* 2006; 86: 1272-1284.
- [27] Liu B, Bell AW, Paranjpe S, Bowen WC, Khillan JS, Luo JH, Mars WM, Michalopoulos GK. Suppression of liver regeneration and hepatocyte proliferation in hepatocyte-targeted glypican 3 transgenic mice. *Hepatology* 2010; 52: 1060-1067.
- [28] Sell S. Is there a liver stem cell? *Cancer Res* 1990; 50: 3811-3815.
- [29] Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. *Compr Physiol* 2013; 3: 977-1010.
- [30] Jia M, Jing Y, Ai Q, Jiang R, Wan J, Lin L, Zhou D. Potential role of catalase in mice with lipopolysaccharide/D-galactosamine-induced fulminant liver injury. *Hepatol Res* 2014; 44: 1151-8.
- [31] Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; 281: 1309-1312.
- [32] Li J, Zhang L, Xin J, Jiang L, Li J, Zhang T, Jin L, Li J, Zhou P, Hao S, Cao H, Li L. Immediate intraportal transplantation of human bone marrow mesenchymal stem cells prevents death from fulminant hepatic failure in pigs. *Hepatology* 2012; 56: 1044-1052.