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Original Article

Effects of transplantation of bone marrow mesenchymal stem cells on hepatic injury and metabolism in rats with acute liver failure

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Abstract: This study was conducted to investigate the effect of transplantation of bone marrow mesenchymal stem cells (BMSCs) on liver injury repair and metabolism in rats with acute hepatic failure for better clinical treatment of the disease. D-galactosamine (D-gal) and lipopolysaccharide (LPS) were used to construct rat models of acute liver failure (ALF). HE staining was used to examine the pathological changes of liver, liver function, and hepatic injury. Western blot was used to assay the expression of acute liver injury marker-betaine homocysteine S-methyltransferase (BHMT). BMSCs were isolated using the Percoll method and cultured in vitro for transplantation. SD rats were randomly divided into control, model and BMSC transplantation groups. For BMSC transplantation, the models were transplanted BMSC (10^7 cells/kg) at the third passage by tail vein injection while the rats in the model group received intraperitoneal injection of normal saline. Apoptosis of liver cells was determined using the TUNEL method and analyzed for the correlation with BMSC transplantation using Pearson correlation at different time points. Blood and liver tissue samples were analyzed for the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and lactate dehydrogenase (LDH), liver function, total protein (TP), cholesterol (CHO), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), and liver metabolites. The expression of IL-18 and its activating enzyme caspase-1 was using qRT-PCR. Based on tissue examination and BHMT level, it was confirmed that the ALF animal modeling was successful following D-Gal and LPS administration with a success rate of about 86.2%. 48 h after BMSC transplantation, the numbers of apoptosis-positive cells in control, model, and BMSC transplantation groups were 25.2±5.4, 58.6±8.4 and 32.1±2.2, respectively. Compared with the control group, the number was significantly upregulated ($P < 0.05$) in the models, while transplantation of BMSC reduced the number. Compared with the control group, serum levels of ALT, AST, TBIL, LDH, TG and LDL were significantly ($P < 0.05$) up-regulated in the model group, while TP level was reduced ($P < 0.05$). The mRNA level of IL-18 and Caspase-1 were significantly higher ($P < 0.01$) in the model than in the control group. Again, 48 h after BMSC transplantation, IL-18 and Caspase-1 expression were reduced to a certain extent. D-Gal combined with LPS is easy and effective to construct ALF rat model. BMSC transplantation can reduce apoptosis in liver tissue, restore metabolism and blood biochemical indicators. It also down-regulates the expression of IL-18 and caspase-1 at mRNA level.

Keywords: Stem cells, MSCs, liver failure, liver injury

Introduction

Acute liver failure (ALF) is a serious liver damage caused by a variety of factors, resulting in severe obstacles, disorders or decompensation in liver functions such as synthesis, detoxification, excretion and biotransformation. The clinical syndrome also includes blood coagulation, jaundice, hepatic encephalopathy and ascites [3]. ALF occurs quickly and has high mortality. No effective treatment methods are clinically available. Therefore, it is very important to study the response of the disease to various intervention options for better treatment of the disease.

Bone marrow mesenchymal stem cell (BMSC) is an important member of stem cell family. It is non-hematopoietic mainly coming from early mesoderm and ectoderm, and has strong proliferation ability and the potential of multi-directional differentiation. BMSC is an important tool

for stem cell tissue engineering and gene therapy, and has become an important means for the treatment of various tissue damaging diseases [4, 5]. Studies have confirmed that BMSC transplantation to injured tissues can assist tissue regeneration and repair and is also attempted for the treatment of ALF [6, 7]. The pathogenesis of ALF is shown to associate with various immune and inflammatory response in the host [8]. BMSC intervention is being due to its low immunogenicity and relatively low cost and has now become an important option for the treatment of liver failure after liver transplantation [9, 10]. BMSCs are easy to prepare, biologically stable and safe, and psychologically acceptable to the patients. Therefore, BMSCs are well adopted clinically. However, the mechanisms underlying the protective effect of BMSC transplantation on liver injury repair is still largely unclear. There is also a gap in study data to support its clinical application. IL-18 is a lymphocyte with a variety of biological effects. It plays an important role in immune regulation, such as the activation of T cells to produce cytokines. The study showed that it positively modulated the repair of hepatic injury [11].

To better understand the protection of BMSC on liver injury and effect of liver function, we constructed the animal model of ALF using D-galactosamine (D-gal) and lipopolysaccharide (LPS); transplanted them with BMSCs and assayed cell apoptosis and a number of blood biochemical indicators. The results will provide insights on the mechanisms underlying the protective effect of BMSC transplantation on liver injury repair.

Materials and methods

ALF modeling

Male SPF SD rats, weighing 250±50 g, aged three weeks, were purchased from Silaike Experimental Animal Technology (Shanghai, China), hosted in standard animal cages (5 per cage) in well vented room with natural sunlight at 18-25°C and had free access to food and drinking during the experiment. The animal experimental protocols were approved by the ethic committee of Guangxi TCM University.

After adaptive feeding for 7 d, the rats were intraperitoneally injected with D-Gal (SIGMA, USA) at a dose of 500 mg/kg and 0.005% LPS (SIGMA, USA) at 20 μg/kg as previously described [12, 13]. The control group was given equal volume of normal saline.

Isolation and culture of BMSC

Isolation and culture of BMSC were conducted as described [14]. Briefly, four week-old SD rats were sacrificed by cervical dislocation, the femur and tibia and cut open at the middle, washed with 10 mL of low glucose DMEM (GIBCO, USA) containing 100 U/ml heparin. The obtained marrow cell suspension was purified by Percoll gradient centrifugation according to the supplier instructions (GE Healthcare, USA). Mononuclear cells were collected, washed 2 times with serum free medium and resuspended in BMSC culture medium (low glucose DMEM with 15% fetal bovine serum (FBS, GIBCO, USA), 100 U/ml penicillin and streptomycin). The cells were inoculated into culture flasks and cultured at 37°C in 5% CO_2. The cell surface markers CD11b, CD44 and CD29 were detected using flow cytometry (FACSCanto, BD, USA) after staining with FITC-CD11b, FITC-CD44, FITC-CD29 multicolor antibodies (BD, USA). 48 hours later, the culture medium was changed to remove the non-adherent cells. When the cells grew to 80% confluence, they were digested with 0.25% trypsin and subcultured (EDTA) digested cells were cultured.

BMSC transplantation

Sixty rats were randomly divided into control group, model group, BMSC transplantation group, where BMSCs (10^7 cells/kg) at the third passage were injected through tail vein. The rats in model group were injected with normal saline.

Determination of biochemical indicator and apoptosis

Serums from blood samples collected 48 h after BMSC transplantation from the tail veins were analyzed d for aspartate aminotransferase (AST), alanine aminotransferase [1], total bilirubin (TBIL), lactate dehydrogenase (LDH), total protein (TP), cholesterol (CHO), triglyceride [2], low density lipoprotein (LDL), high density lipoprotein (HDL) on an automatic analyzer (Beckman, USA). Apoptosis was assay using a TUNEL test kit according to the supplier's instruction (Vazyma, USA), positive cells were

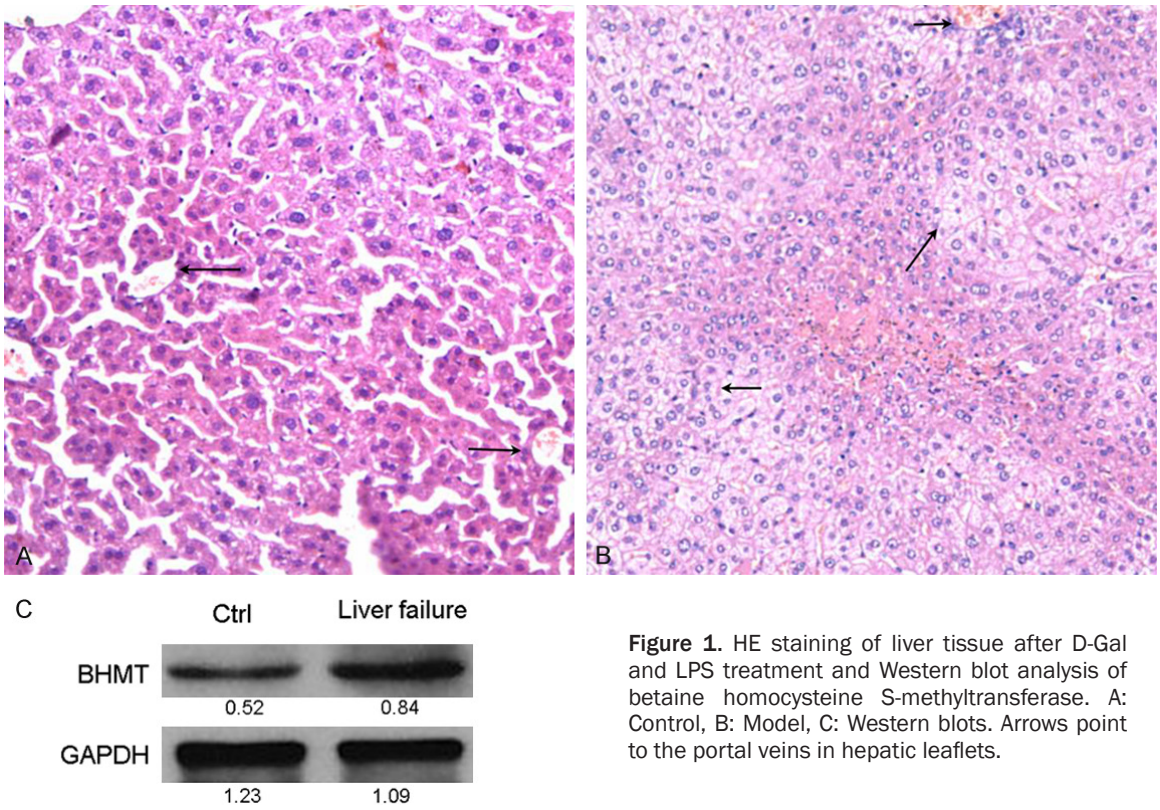

Figure 1. HE staining of liver tissue after D-Gal and LPS treatment and Western blot analysis of betaine homocysteine S-methyltransferase. A: Control, B: Model, C: Western blots. Arrows point to the portal veins in hepatic leaflets.

counted using Plus Image-Pro 6 software in 3 fields, each for 100 nuclei. The percentage of number apoptotic cells over total number of cells observed was calculated as apoptotic index (AI).

Western blot

Total protein as extracted from liver tissue and quantified using BCA Protein Quantitation Kit (Vazyme Biotech, USA). The proteins were fractioned on SDS-PAGE gel and transferred to PVDF membranes (Millipore, USA). The membranes were stained with primary antibody against BHMT (Santa Cruz Biotechnology, USA) for 2 h at room temperature, washed, stained with horseradish peroxidase (HRP)-labeled secondary antibody (Origene, Beijing), detected with a ECL kit (Millipore, USA), and quantified with the Quantity One (v4.62) software.

Real-time PCR

Total RNA was extracted by using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quantity and quality were using Nanodrop 2000 (Applied, USA). Reverse transcription was performed with 200 ng of RNA in a total volume of 10 μl using the High Capacity cDNA Transcriptase Reverse kit (Applied Biosystems by Life Technologies, Carlsbad, California, USA) according to manufacturer's recommendations. A total of 2.5 μl of the resulting cDNA was subjected to pre-amplification using the TaqMan Pre-Amp Master Mix (Applied Biosystems) in a total volume of 20 μl. Non-fluorescent probes were used at 1X. Pre-amplification cycling conditions were 10 min at 95°C followed by 14 cycles, each one consisting of 33 s at 95°C and 4 min at 60°C. RT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes (Applied Biosystems), using primers for IL-18 (Forward, GTGAACCCCAGACCAGACTG; reverse, CCTGGAACACGTTTCTGAAAGA), caspase-1 (Forward, ACAAGGCACGGGACCTATG; reverse, TCCCAGTCAGTCCTGGAAATG) and GAPDH (Forward, AATGGATTTGGACGCATTGGT; reverse, TTTGCACTGGTACGTGTTGAT) as internal control. The PCR was carried out in a total volume of 20 μl containing 1.5 μl of diluted and pre-amplified cDNA, 10 μl of TaqMan Gene Expression Master Mix and 1 μl of each fluorescence TaqMan probe. The cycling conditions were 50°C for 2 min, 95°C

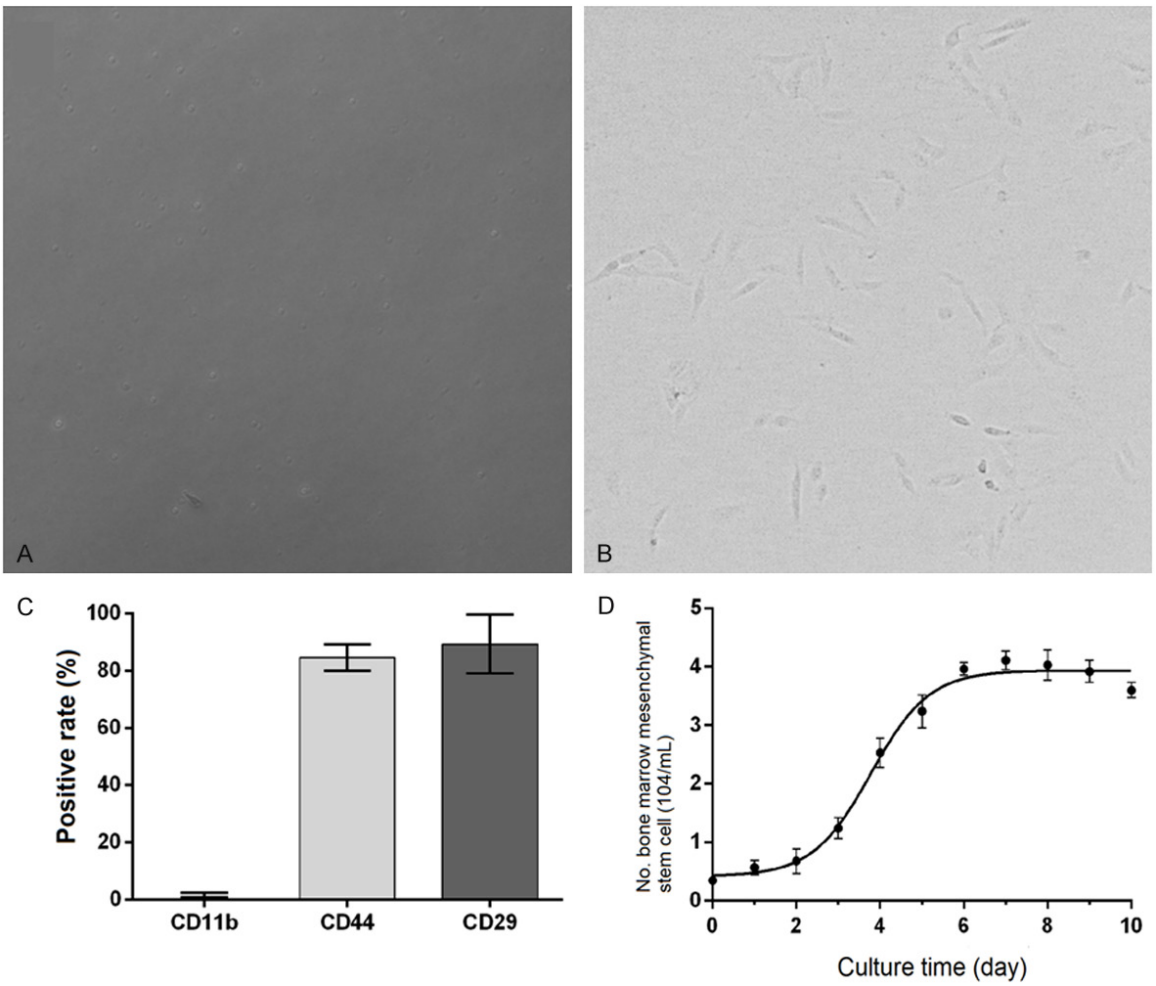

Figure 2. Isolation, culture and characterization of mesenchymal stem cells from rat bone marrow. A: Primary bone marrow mesenchymal stem cells; B: Bone marrow mesenchymal stem cells at the third passage; C: Possible rate of CD11b, CD44 and CD29, and D: Growth curve.

for 10 min followed by 39 cycles, each one consisting of 33 s at 95°C and 13 s at 60°C. Samples were run in triplicate and the mean value was calculated for each case.

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using comparative Ct method and obtaining the fold change value (2-ΔΔCt) according to previously described protocol [2].

Statistical analysis

Statistical analysis was conducted using statistical software Graphpad prism 6.0 and data were expressed as means (x) ± standard derivation [15]. All experiments were repeated at least three times and performed in triplicate. Means were compared using the student's t-test or two-way ANOVA with the corresponding post-test. A p-value ≤ 0.05 or was ≤ 0.01 considered statistically significant or highly significant.

Results

Establishment and identification of animal models

HE staining showed that 48 h after modeling with D-Gal and LPS, the livers had normal morphology with clearly visible lobules and arteries in the control group (**Figure 1A**) while the models had inflammatory invasions (**Figure 1B**, arrows), where the lobules looked unclear and tissue boundaries were fuzzy, suggesting the modeling was successful.

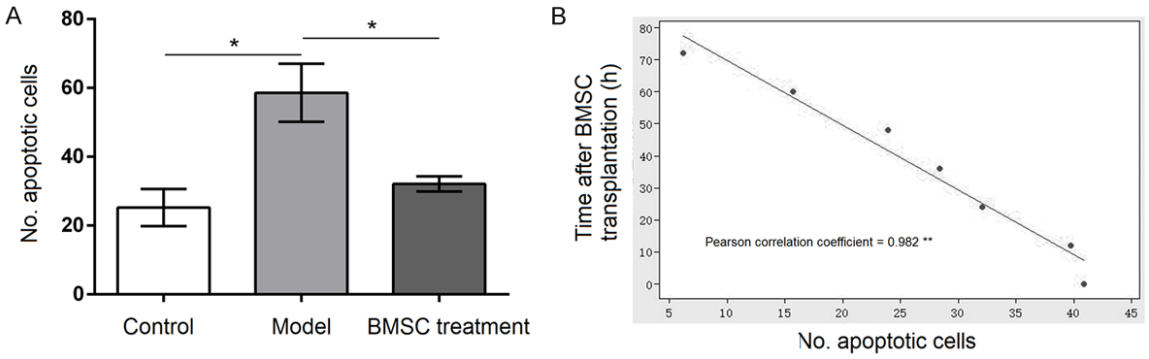

Figure 3. Apoptosis of liver cells after BMSC transplantation. A: Number of apoptotic cells determined using TUNEL assays; B: Pearson correlation analysis between the apoptotic cells and time after BMSC transplantation.

Table 1. Serum levels of biochemical indicators in vein bloods in rats after BMSC transplantation

Indicator	Control	Model	BMSC transplantation
ALT (U/L)	75.1±0.2	86.2±0.3*	80.2±0.2
AST (U/L)	177.9±2.5	251.7±2.7**	223.9±2.4$^{\Delta}$
TBIL (μM)	0.65±0.02	3.05±0.02**	2.85±0.02$^{\Delta\Delta}$
LDH (U/L)	1330.7±14.3	1628.5±11.7**	1410.5±14.9$^{\Delta}$
TP (g/L)	82.1±2.3	62.5±1.6*	65.7±1.4
CHO (mM)	2.08±0.05	2.16±0.05	1.97±0.05$^{\Delta}$
TG (mM)	0.94±0.01	1.26±0.01*	1.13±0.01$^{\Delta}$
LDL (mM)	0.21±0.06	0.89±0.05**	0.73±0.35
HDL (mM)	2.16±0.10	2.23±0.07	2.05±0.14

*P < 0.05, **P < 0.01 vs control group; $^{\Delta}P$ < 0.05, P < 0.01 vs model group.

The expression of BHMT was further examined using Western blot. The results showed that its expression was significantly up-regulated (**Figure 1C**, P < 0.01).

Isolation, culture and characterization of BMSC

The freshly isolated primary BMSCs were singular and round with strong light refraction and clear boundary (**Figure 2A**). After 12 h culture, some cells began to adhere to the wall of culture vessels and showed varied morphology (**Figure 2B**). The cells were subcultured 48 later to remove non-adherent cells and checked for CD expression. The results showed that the positive rates for CD11b, CD44 and CD29 were 1.6%, 84.7% and 89.4%, respectively, suggesting that the purity of BMSC is higher (**Figure 2C**). Growth curve determination showed that the cells were at a rapid growth phase after they were inoculated at a density of 0.5 × 10^4 cells/ml and 6 d later, the growth was peaked and plateaued off (**Figure 2D**).

Protection of BMSC on acute liver injury

TUNEL assays showed that the numbers of apoptotic cells were 25.2±5.4, 58.6±8.4, 32.1±2.2 in control, model and BMSC transplantation groups, respectively, 48 h after BMSC transplantation. Analysis showed that the number was significantly higher in model and in control rats (P < 0.05), and lower in transplantation than in model group (P < 0.05) (**Figure 3A**). Pearson correlation analysis showed that the number of apoptotic cells was negatively related to the time after BMSC transplantation (**Figure 3C**).

We then analyzed the biochemical indicators in vein bloods such as ALT, AST, TBIL, LDH, TP, CHO, TG, LDL and HDL. The results showed that after the ALF modeling, the rats had significantly higher serum levels of ALT, AST, TBIL, LDH, TG, LDL and significantly lower level of TP (P < 0.05) as compared with the control. After BMSC transplantation, most of these indicators were reduced as compared to the model (**Table 1**), although they were still mostly higher than these in control.

Effect of BMSC transplantation on IL-18 and Caspase-1 expression

We further examined the IL-18 and caspase-1 expression in liver tissue using RT-PCR method. Results showed that at the mRNA level, IL-18 and Caspase-1 were significantly up-regulated in in model than in control (P < 0.01) (**Figure**

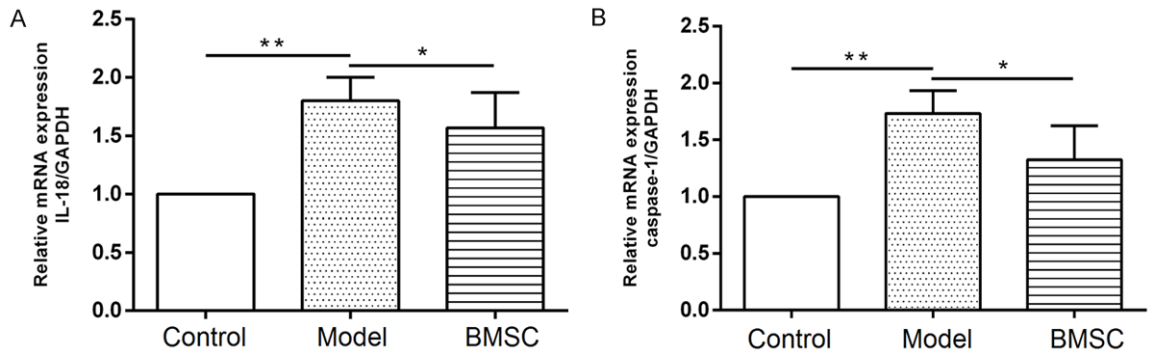

Figure 4. mRNA level of IL-18 and Caspase-1 after BMSC transplantation in rats. A: IL-18; B: Caspase-1.

4A), while 48 h after BMSC transplantation the two gene was significantly down-regulated as compared with model ($P < 0.05$, **Figure 4A**, **4B**).

Discussion

In 1968, Friedenstein et al. found a non hematopoietic stem cell in the bone marrow, which was later named BMSC [16] in 1984. BMSC has been shown to be able to differentiate into many different type cells such as osteoblasts, fibroblasts, adipocytes and endothelial cells, and are therefore of great potential in the treatment of some difficult diseases. Lagesse et al. showed that BMSC can differentiate into liver cells not only in normal liver environments, but also in pathological conditions [17]. At present, BMSC transplantation has been clinically used for the treatment of ALF [18, 19]. However, it is still not well understood regarding the mechanism of BMSC in protecting and repairing liver injury.

D-gal is an amino acid sugar. Treatment with D-gal results in the depletion of uridine (UTP) and inhibition of transcription in liver and is a well adopted method for ALF modeling. D-gal induced degranulation of mast cells to release histamine, resulting in increased intestinal absorption of LPS, cascade inflammation reaction, and eventually necrosis of liver cells [20, 21]. In this study, we observed typical morphological characteristics after the modeling and found BHMT, an important and sensitive marker for liver injury [22, 23], was significantly upregulated. All these findings clearly indicate that our modeling was successful.

In this study, we obtained BMSCs of high purity based on expression of CD11b, CD44 and CD29 through gradual removal of non-adherent cells. At the third generation, the purity of BMSC was high enough for transplantation. When the model was transplanted with the BMSCs, reduced apoptosis was observed in comparison with saline-injected models, suggesting that BMSC is functional in reducing liver injury. Also Pearson correlation analysis showed the reduction is dependent on the time after BMSC injection, suggesting that the BMSCs in the rats is likely responsible for reducing apoptosis in the liver during the initial 72 h. Since liver is important for metabolism, we also analyzed a number of biochemical indicators after BMSC injection. The results are consistent with HE staining and TUNEL assays that after ALF modeling, the liver functions were significantly deteriorated, and BMSC transplantation improved these functions to certain extend. Since these assays were conducted relatively shortly after the transplantation, it is likely that there may be more prominent improvement over the time.

IL-18 is a newly discovered inflammatory cytokine. It is similar to IL-1 in term of structure activation receptor and signal transduction. IL-18 is shown to have various biological activities, including immunomodulation, and stimulation of T cell proliferation and secretion of IFN-γ to trigger a cascade of immunologic responses. Previous studies have indicated that IL-18 and IL-18-induced cytokines are closely related to the liver damage caused by excessive immune response during the process of liver injury [24]. Therefore, we investigated the effect of BMSC on the expression of IL-18 and its activating enzyme Caspase-1 in hepatocytes. The results showed that both IL-18 and Caspase-1 were significantly up-regulated after the modeling, suggesting that during ALF, Caspase-1 may activate IL-18 to exert stronger induction of

apoptosis. However, how Caspase-1 is induced and up-regulated in the damaged tissue is still unclear. At the same time, the expression of the two genes was found down-regulated after BMSC transplantation. Based on HE staining and TUNEL assay, our findings suggest that Caspase-1 is related to cell apoptosis, and it may activate IL-18 to amplify the apoptosis and result in liver failure.

Acknowledgements

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Disclosure of conflict of interest

None.

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