

Transplantation of bone mesenchymal stem cells effectively promotes intestine regeneration to eliminate endotoxemia and reduce mortality in the animal model of acute liver failure

Ting Jiang

Kunming Medical University

Geng Xia

Kunming Medical University

Bo Yang

Kunming Medical University

Hong-wei Zhang

Chinese Academy of Medical Sciences and Peking Union Medical College

Yue-shan Yin

Kunming Medical University

Cheng-wei Tang

Sicuan University

Jin-hui Yang (✉ 17787003686@163.com)

The Second Affiliated Hospital of Kunming Medical University <https://orcid.org/0000-0002-7234-0923>

Research

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Abstract

Background

Endotoxemia based on liver failure has been reported to be related to the worse clinical outcomes, but its management remains unsatisfactory. The addition of bone marrow mesenchymal stem cells (BMSCs) could promote the recovery of liver function and increase the survival with the liver failure. However, little is known about the potential of cell therapy with endotoxemia based on liver failure.

Methods

BMSCs were isolated from rats, and their morphology, differentiation potential, surface markers, and cell cycle were assayed. Thioacetamide-induced acute liver failure rats were randomized to groups with or without BMSCs. During the experiment, survival was recorded. Diamine oxidase (DAO), endotoxin, interleukin-6 (IL-6) and tumor necrosis factor- alpha (TNF- α) and tissue were analyzed by enzyme-linked immunosorbent assay (ELISA), histology, and western blot. Bromodeoxynucleoside uracil (BrdU) incorporation assay was performed to observe the migration of BMSCs. The intestinal epithelial differentiation of BMSCs was induced by co culture with small intestinal crypt in rats (IEC-6). Immunofluorescence was used to analyze the expression of intestinal endothelial markers. Western blot analysis was further performed to examine the differentiation effect when inhibiting the phosphoinositide kinase-3 (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway.

Results

BrdU-traced BMSCs targeted migrate to intestinal injury sites. Mortality was significantly decreased and intestinal damage was repaired following BMSCs transplantation. Proteomics revealed higher expression of DAO, endotoxin, IL-6 and TNF- α in the model animals, but these changes were reversed after BMSCs transplantation. In the in vitro study, the intestinal epithelial differentiation of BMSCs was exhibited following co-culture. Moreover, the blocking of PI3K/AKT/mTOR signal pathway inhibited this differentiation.

Conclusions

These evidences indicate that BMSCs eliminate endotoxemia and reduce mortality in the animal model of acute liver failure by reducing intestine damage.

Background

Acute liver failure is characterized by sudden development of liver dysfunction and rapid hepatocellular necrosis, may progress to exhibit hepatic coma even death due to various of etiologies, including drug

toxicity, hepatic ischemia, immune-mediated attack, and viral hepatitis such as hepatitis B and hepatitis C [1, 2]. Endotoxemia represents the severe stage of acute liver failure and is associated with poor quality of life, mortality, and economic burden [3]. Reasons for severe endotoxemia mainly include intestinal and hepatic factors, which also interact with each other [4]. Although liver transplantation is considered as a curative treatment, the disparity between demand and supply and its long time and heavy price limit the applicability [5, 6]. As the exhibition of endotoxemia is very urgent, hepatic coma and death are caught soon. Therefore, at the same time of saving hepatocytes, prevention and intervention of the endotoxemia are particularly important.

Endotoxin are mainly produced by overgrowth of gram-negative bacteria in damaged gut with the increased permeability. The increased damage of intestine in the liver failure is usually associated with liver dysfunction, congestion of portal vein, and bacterial infection [7]. The degradation of many harmful protein metabolites such as putrescine, histamine, spermine, and spermidine is catalyzed in the liver and obstructed when hepatocellular necrosis occurs. The high level of toxic substances is not only harmful to brain cells called hepatic coma, but also a great challenge to enterocytes. Meanwhile, the increased portal vein pressure caused by liver failure leads to intestinal congestion, which increases the accumulation of harmful substances in the intestine and affects the intestinal immune responses, cell proliferation, and apoptosis. Moreover, the bacterial infection has been demonstrated to increase the damage of intestine and causes bacteremia or even sepsis. Thus, a huge burden for intestine through internal and external negative factors not only endangers the intestine itself, but also aggravates the condition of the liver, forming malignant circle [8]. However, the effect of current treatment for endotoxemia in liver failure is not satisfactory.

Mesenchymal stem cells (MSCs) immediately attracted a lot of interests owing to the excellent ability of self-renewal and differentiation into various tissues. Furthermore, compared with embryonic (ESCs) and induced pluripotent stem cells (iPSCs), MSCs are easily isolated from bone marrow called the bone marrow mesenchymal stem cells (BMSCs) and without the limitation of ethical problems, the way of cell retention and immunological rejection [9, 10]. Recently, studies have described the applications of BMSCs for liver diseases. BMSCs could migrate to the injured liver and regenerate in the mice receiving partial hepatectomy (PH) and even improve the alleviation of liver fibrosis [11, 12] Moreover, the application of BMSCs have demonstrated the potential for the alleviation of liver injury and inflammation by inhibiting inflammatory factors' activation [13]. However, few researches focus on the role of intestine and the core is made no mention of the gut when it involves stem cells in the liver failure.

In this study, we aimed to assess whether the administration of BMSCs could have beneficial effects both in gut and liver in an animal model with acute liver failure. Moreover, we explored the possibly signaling-based mechanism underlying this therapy.

Methods And Materials

The study obeyed the laboratory animal welfare and ethic principle, and all the experimental animal procedures were carried out strictly in accordance with the protocol approved by Animal Experimental Ethical Committee of Kunming Medical University (Approval number KMMU2018020).

Isolation and culture of rat BMSCs

The BMSCs were isolated from SD rats (half male and female, 6-8 weeks old, 200±10g) which purchased from the experimental animal center of Kunming Medical University (Number of qualification: SCXK2015-0002). BMSCs were maintained in clean cell culture bottles in maintenance medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah, USA), 15% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin (Hyclone), and 100 µg/ml streptomycin (Hyclone). In order to observe the morphology and obtain more cells for following studies, cells were placed in an incubator at 37 °C and 5% CO² for 3–4 passages.

In vitro adipogenic differentiation and oil red O staining

The cells in passage 3 were cultured with adipogenic medium (Saiye Bio, Guangzhou, China) for 16 days in order to induce adipogenic differentiation. After 18 days, cells were stained with Oil Red O (Saiye Bio). In brief, cells were washed with phosphate buffer saline (PBS; Hyclone) and fixed with 4% paraformaldehyde (Saiye Bio) for 30 minutes. After washing with PBS (Hyclone) three times, cells were incubated with Oil Red O working solution for 30 minutes. Haematoxylin (Saiye Bio) was used for intensifying the staining of the nuclei. Red colour lipid droplets and purple nucleus could be checked microscopically in the final step.

Flow cytometry

Cells harvested from SD rats were treated with trypsin (Hyclone) in the third generation, washed twice with PBS (Hyclone), and incubated with anti-CD34 ([Santa Cruz Biotechnology](#), sc-7324, USA), anti-CD45 (Biolegend, 202207, San Diego, USA), anti-CD90 (Biolegend, 202526) and anti-CD29 antibodies (Abcam, ab36219, Cambridge, UK) at room temperature in dark for half an hour. A FACS flow cytometer was used for Analysis.

Cell-Cycle Analysis

For [cell-cycle analysis](#), cells were stained with propionyl iodide (PI; Biyuntian Bio, Shanghai, China) and analyzed using a flow cytometer.

An animal model of acute liver failure and cell transplantation

In this study, acute liver failure was induced by thioacetamide (TAA; Sigma-Aldrich, Bornem, Belgium) as previously described [14-16]. To get an ideal animal model, we divided the rats into groups to explore the best TAA dose and check point. The rats were randomly allocated to 4 groups (n = 25 each). Group 1 (control group) received saline, and group 2-4 (model groups) received different doses (200mg, 300mg,

400mg/Kg/day) of TAA via gavage. Then, after confirming the appropriate dose of modeling, we transplanted BMSCs to rats with liver failure, rats were divided into 3 groups: saline, TAA + saline, and TAA + saline + BMSCs (1×10^6 , intravenous injection). To collect samples, all rats were sacrificed by CO² asphyxiation.

Histology

Samples (livers and intestines) were fixed in 4% paraformaldehyde (Saiye Bio), dehydrated, and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin (H&E; Saiye Bio). Images ($\times 100$ magnification) were taken from each sample, and histopathological evaluation of the entire section was performed in a blinded manner. The injury of liver and intestine was scored using the modified histopathologic score described previously [17-19]. These criteria for histological score were shown in Table 1.

Bromodeoxynucleoside uracil (BrdU) Assay

BrdU incorporation assay was performed to observe the location of BMSCs. Cells were treated with BrdU for 60 min following the manufacturer's instructions (FITC BrdU Flow Kit, CST, Shanghai, China) before the administration.

Determination of diamine oxidase (DAO) and endotoxin

DAO activity was used a kit (Sigma). In brief, 3 mL of phosphate buffer (0.2 mol/L, pH 7.2), 0.1 mL (4 μ g) of horseradish peroxidase solution, 0.1 mL of *o*-dianisidine methanol solution, 0.5 mL of plasma and 0.1 mL of substrate solution (175 μ g of cadaverine dihydrochloride) made up the assay mixture. The DAO activity could be measured as the absorbance at 436 nm when incubated for 30 minutes at 37°C. The endotoxin of plasma was assayed by the test kit according to the manufacturer's instructions (Pierce™ Chromogenic Endotoxin Quant Kit, Thermo, Shanghai, China).

Enzyme-linked immunosorbent assay (ELISA)

The levels of interleukin-6 (IL-6) and tumor necrosis factor- alpha (TNF- α) in serum were determined according to the manufacturer's instruction of the ELISA kit (IL-6, BMS625, Thermo; TNF- α , KRC3011, Thermo). The content was calculated according to the standard curve and OD value. Data statistics were carried out for the average value of the test results.

Immunofluorescence analysis

Cells were fixed with PBS (Hyclone) containing 4% paraformaldehyde (Saiye Bio) for 30 min at room temperature, and blocked for another 30 min. The cells were then incubated with primary antibodies (Cytokeratin (CK), ab68260, Abcam; Villin, ab201989, Abcam) overnight at 4°C and further incubated with secondary antibody (FITC conjugated IgG; Merck Millipore, USA). Olympus phase-contrast fluorescent

microscope was used for capturing the images. Scoring was based on the previously proposed method [20].

Transwell co-culture system

Transwell chambers (Millipore) were used in the co-culture induction system. Briefly, the lower chamber was loaded with BMSCs (1×10^6 cells/well) and the upper insert was loaded with epithelial cells of small intestinal crypt in rats (IEC-6; 1×10^6 cells/well) which were obtained from Kunming Cell Bank of Chinese Academy of Sciences (No: KCB200720YJ). The induction system was cultured in an incubator with 5% CO₂ at 37°C for 10 days. To interfere with the phosphoinositide kinase-3 (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway, the inhibitor LY294002 (Abcam, ab120243) was added to the co-culture system.

Western blot analysis

Cells and intestines were homogenized and lysed following 30 min incubation on ice. The lysates were centrifuged at 12,000 rpm for 10 min. Proteins isolated from samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore). Blots were blocked for 1 h at room temperature with 5% nonfat dried milk. After washing in tris-buffered saline Tween-20 (TBST, Saiye Bio), the membranes were immunoblotted with the following antibodies: anti-IL-6 (Abcam, ab9770), anti-TNF- α (Abcam, ab66579), anti-AKT (Abcam, ab8805), anti-mTOR (Abcam, ab32028), anti-CK (Abcam, ab115293), anti-Villin (Abcam, ab109516), anti- β actin (Abcam, ab8227). The blots were then incubated with the corresponding horseradish peroxidase-conjugated IgG (Abcam, ab7090, ab7125). MyImage (SLB, Korea) was used to detect the immunoreactive proteins, and Labworks 4.0 software was used for quantitative analysis (UVP Inc., USA).

Statistical analysis

The GraphPad Prism version 7 program (GraphPad Software, Inc., San Diego, CA, USA) was used for graphs. Data are presented as mean \pm standard deviation. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA. A P value of < 0.05 was considered statistically significant.

Results

Identification of BMSCs from rats was revealed by their typical morphology and potency to differentiate to multilineage as well as their immunophenotypic analysis as showed later.

The bone marrow of SD rats was used for isolation of the BMSCs. These cells showed a fibroblast-like, spindle-shaped morphology at passage P0 (Fig. 1a) and gradually adherence to the bottom of culture bottle without differences between groups (Fig. 1b). The BMSCs must be differentiated to various cell

types to demonstrate their pluripotency. Then we used the adipogenic differentiation medium to check the potency. Oil Red O staining revealed droplets of fat in the adipogenic induction group of BMSCs (Fig. 1c and Fig. 1d). The cell surface antigens for the BMSCs characterization were detected by flow cytometry. Obviously, we could notice that CD90 and CD29 but were negative for CD45 and CD34 (Fig. 1g and Fig. 1h). Also, in order to ensure the follow-up experiments, we tested the cell cycle of these separated BMSCs. As shown in Fig. 1e and Fig. 1f, these cells were in a phase of proliferation to meet experimental requirements

Effects of different doses of TAA on animal model

Obvious pathological manifestations of intestine injury based on acute liver failure and appropriate survival rate were two basis this study would be performed. Then, we divided the rats into several groups to explore the effect of different doses of TAA (Fig. 2a). Both group of TAA300mg/Kg/day and group of TAA400mg/Kg/day showed typical hepatoenteropathology of acute liver failure (Fig. 2c and Fig. 2d), while group of TAA400mg/Kg/day had a higher mortality (Fig. 2b), which was difficult to guarantee the follow-up experiments. Therefore, for this study, the dosage of TAA300mg/Kg/day was adopted.

The administration of BMSCs reduced the mortality of TAA-induced acute liver failure rat and decreased the damage of intestine

The damage of intestinal mucosal barrier is critical to the progress of liver failure. This damage could induce severe endotoxemia leading to death. To determine whether the administration of BMSCs can solve this clinical problem, we divided the model rats into several groups to observe the effects of BMSCs on survival and pathology (Fig. 3a). Our data showed that the mortality with BMSCs was significantly lower as compared to those without BMSCs (Fig. 3b). The damage of intestine in the group with BMSCs administration was also significantly decreased (Fig. 3c). Moreover, we found out that BrdU-traced BMSCs targeted migrated to intestinal injury sites (Fig. 3d).

BMSCs ameliorated endotoxemia and inflammation of TAA-induced rat

To characterize the effects of BMSCs on endotoxemia in liver failure rats, DAO and endotoxin in plasma were tested with ELISA. For IL-6 and TNF- α , important inflammatory factors with wide biological effects, were assayed both in plasma and intestine. As shown in Fig. 3e, DAO and endotoxin were significantly less content than the group without BMSCs. This suggests that BMSCs ameliorated endotoxemia, possibly being the result of BMSCs migration and following intestine repair. Furthermore, the inflammatory factors in group with BMSCs, including the IL-6 and TNF- α , were quantified at a lower level compared to group without BMSCs (Fig. 3e and Fig. 3f).

Association of PI3K/AKT/mTOR signal pathway in the differentiation of BMSCs into enterocytes

The above results in this study suggested that BMSCs could targeted migrate to intestinal injury sites and repair intestine damage as the result in improvements of endotoxemia and mortality. Thus, we assumed that BMSCs might be able to differentiate into enterocytes to repair intestinal epithelial barrier. According

to previous reports, PI3K/AKT/mTOR signaling pathway is involved in cell fate and likely to be associated with proliferation and differentiation of stem cells [21, 22]. To investigate whether the PI3K/AKT/mTOR signaling pathway is associated with the differentiation of BMSCs into enterocytes, we performed the transwell co-culture system and evaluated the characteristic molecules of enterocytes by immunofluorescence staining. CK and villin, two typical enterocytes molecules, revealed an abundance in BMSCs which were cultured with IEC-6 (Fig. 4a). Statistical analysis showed that after 10-day co-culture, the amounts of CK and villin in BMSCs with IEC-6 were obviously more than those in BMSCs without IEC-6 (Fig. 4b). Furthermore, the PI3K/AKT/mTOR signaling pathway inhibition resulted in significant decreases in CK and villin compared with the control groups (Fig. 4c and Fig. 4d). These data suggested that the PI3K/AKT/mTOR signaling pathway might be involved in the differentiation of BMSCs into enterocytes.

Discussion

In this study, we exhibited the characteristics of BMSCs from SD rats and showed the fine capacity for differentiation and proliferation, and high probability of PI3K/AKT/mTOR signaling pathway in inducing enterocytes differentiation. TAA-induced intestine injury based on acute liver failure was established appropriately and ameliorated by BMSC transplantation, which was revealed by a reduction in intestine damage, mortality, endotoxemia and inflammation.

The survival of patients with acute liver failure is closely related to the occurrence of hepatic coma, which usually caused by endotoxemia. Indeed, endotoxemia are observed in 75-93.3% of acute liver failure and severe hepatitis [23]. The common treatment such as reducing blood ammonia, artificial liver support system and transjugular intrahepatic portosystemic shunt (TIPS) can only relieve the symptoms, but cannot delay the progression of the delicate disease [24]. Up to now, with inspiring march in stem cell technology, an enthusiasm with cell-based therapy has been accumulated with the hope that the tragic outcome of liver failure can be saved. In recent years, with the welling up of studies on MSCs therapy for liver failure, solid evidences have consistently been showed that MSCs can effectively treat liver failure. In an open-label randomized controlled study, the administration of allogeneic BMSCs reduced serum total bilirubin (TBIL) and model for end-stage liver disease (MELD) scores in 56 patients with acute-on-chronic liver failure (ACLF) [25]. Some studies proved that MSCs could differentiate into hepatocyte-like cells (HLCs) *in vivo*, considered as one of the mechanisms of MSCs in the treatment of liver failure [26, 27]. While some experiments have suggested that MSCs cannot differentiate into HLCs after engrafting even if the improved survival, reduced alanine aminotransferase (ALT) and relieved liver injury [28]. These findings demonstrate that the effective performance of MSCs in the treatment of liver failure is not just dependent on the supplementing exogenous hepatocytes. The mechanism behind this therapeutic effect of MSCs may not have much to do with the liver. The similar effects between uninduced BMSCs and HLCs on the treatment of liver failure also confirms the above deduction from the side [29].

It is worth mentioning that some inflammatory factors including IL-6, TNF- α , and CD8⁺T cells, which are induced by bacteria and endotoxins (lipopolysaccharide, LPS) from gut, are significantly decreased after

the BMSCs application in liver failure [30]. Previous studies have indicated that gut plays a significant role in endotoxemia based on liver failure. The level of plasma endotoxin in rats colectomized before administration of TAA had no difference from that in control group, the content of inflammatory factor and fatality were much lower [31]. We further investigated the role of BMSCs in gut and endotoxemia based on acute liver failure. The findings suggested that an appropriate dose of TAA could induce severe acute liver failure with damaged gut. After intragastric infusion of TAA, the BMSCs (1×10^6) were injected into caudal vein, the levels of plasma endotoxin and DAO were remarkably decreased compared to the TAA group. Also, excretion of IL-6 and TNF- α decreased markedly, with the marked increase of survival rate. Histology with intestine from groups showed that the better intestinal mucosal barrier was observed after the BMSCs application. Moreover, the levels of IL-6 and TNF- α in intestine were strongly reduced in rats with BMSCs. These findings suggested BMSCs were indeed effective in the treatment of liver failure and had important effects on intestinal injury and endotoxemia.

In this study, we used a fluorescence tracing approach to exhibit that BMSCs targeted migrated to intestinal injury sites. In order to confirm that BMSCs can differentiate into enterocytes and explore the possible pathway mechanism, we carried out the co-culture of BMSCs and IEC-6 in vitro using transwell culture medium. The data presented in this study with cocultivation medium, demonstrating the possibility of intestinal epithelioid differentiation of BMSCs with CK and villin overexpression, and the blocking of PI3K/AKT/mTOR signal pathway using LY294002 could interfere with this differentiation. PI3K/AKT/mTOR signal pathway has traditionally been considered a common mechanism of proliferation, differentiation and apoptosis. Previous studies have also mentioned the role of this pathway in stem cell differentiation, which is mutually verified with our research. Meanwhile, some previous studies have stated that the therapeutic effects of BMSCs are potentially based on its release of trophic and immunomodulatory factors such as transforming growth factor-beta (TGF- β) and interleukin 10 (IL-10) [32]. For example, BMSCs can inhibit cytotoxic CD8⁺ T lymphocyte (CTL) and NK cells through intercellular contact and paracrine factors including TGF- β and IL-10, which have the immunosuppressive effects to alleviate inflammation. While endotoxemia and intestinal damage were strongly reduced in our model, we did not have a clear idea of the treatment mechanism behind this migration. In order to determine whether supplementing exogenous hepatocytes or protective cytokines secretion or even both, is the main treatment mechanism of BMSCs, further research is needed.

Of note, in recent studies about BMSCs therapy, the application of BMSCs could reduce the burden of liver failure in line with our results and attesting to the translational relevance, repair of intestinal mucosa reduced endotoxemia and mortality in a rat model of acute liver failure. Thus, it is beneficial to the treatment of acute liver failure and prevention from developing to endotoxemia using BMSCs. Clearing intestinal endotoxins and repairing intestinal damage should be paid more attention when against liver failure.

Conclusions

BMSCs from rats have a favorable impact on the application of stem cell-based therapy on TAA-induced acute liver failure. The two obvious advantages of this method lie in the repair of intestinal injury and the improvement of endotoxemia. Furthermore, the PI3K/AKT/mTOR signaling pathway is associated with the differentiation of BMSCs into enterocytes. These valuable data not only show us the role of intestinal endotoxemia, but also inspire us to build an effective therapy in the treatment of acute liver failure.

Abbreviations

MSCs: Mesenchymal stem cells; ESCs: Embryonic stem cells; iPSCs: Induced parthenogenetic stem cells; BMSCs: Bone marrow mesenchymal stem cells; PH: Partial hepatectomy; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; PBS: Phosphate buffer saline; PI: Propionyl iodide; TAA: Thioacetamide; H&E: Hematoxylin and eosin; BrdU: Bromodeoxynucleoside uracil; DAO: Diamine oxidase; ELISA: Enzyme-linked immunosorbent assay; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor- α ; CK: Cytokeratin; IEC-6: Small intestinal crypt in rats; PI3K: Phosphoinositide kinase-3; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride; ANOVA: Analysis of variance; TIPS: Transjugular intrahepatic portosystemic shunt; TBIL: Total bilirubin; MELD: Model for end-stage liver disease; ACLF: Acute-on-chronic liver failure; HLCs: Hepatocyte-like cells; ALT: Alanine aminotransferase; LPS: Lipopolysaccharide; TGF- β : Transforming growth factor-beta; IL-10: Interleukin 10; CTL: Cytotoxic T lymphocyte

Declarations

Acknowledgements

None

Authors' contributions

TJ, GX and BY conceived and coordinated the study, designed, performed, analyzed the experiments, and TJ wrote the paper. HZ and YY carried out data collection, data analysis, and revised the paper. CT and JY designed the study and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Animal care and all experimental procedures were performed in accordance with the protocol approved by Animal Experimental Ethical Committee of Kunming Medical University (Approval number KMMU2018020).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Histological criteria for the assessment of liver and intestine damage

Score	Liver	Intestine
0	no damage	no damage
1	minimal congestion and vacuolization, single-cell necrosis	mild focal epithelial oedema and necrosis
2	mild congestion and vacuolization, ≈30 % necrosis	moderate diffuse swelling and necrosis of the villi
3	moderate congestion and vacuolization, 30 %–60 % necrosis	severe necrosis with evidence of neutrophil infiltration in the submucosa
4	severe congestion and vacuolization, ≈60 % necrosis	major widespread necrosis with massive neutrophil infiltration and evidence of hemorrhage

Figures



Figure 1

Morphology of the BMSCs. (a) Passage 0 (P0) BMSCs at $\times 40$ magnification. (b) P3 BMSCs at $\times 40$ magnification. (c) and (d) The adipogenic differentiation of BMSCs and oil red O staining: control group (c) and adipogenic induction group of BMSCs (d), differentiation to adipocyte was demonstrated by Oil Red O staining, $\times 100$ magnification. (e) Cell cycle detection of BMSCs. (f) Most of BMSCs (80.68%) were in G1 phase, which means that they have strong ability of cell proliferation. (g) FACS analysis of surface markers. (h) Typical hematologic lineage CD29 and CD90 were expressed in BMSCs, while CD45 and CD34 were not.

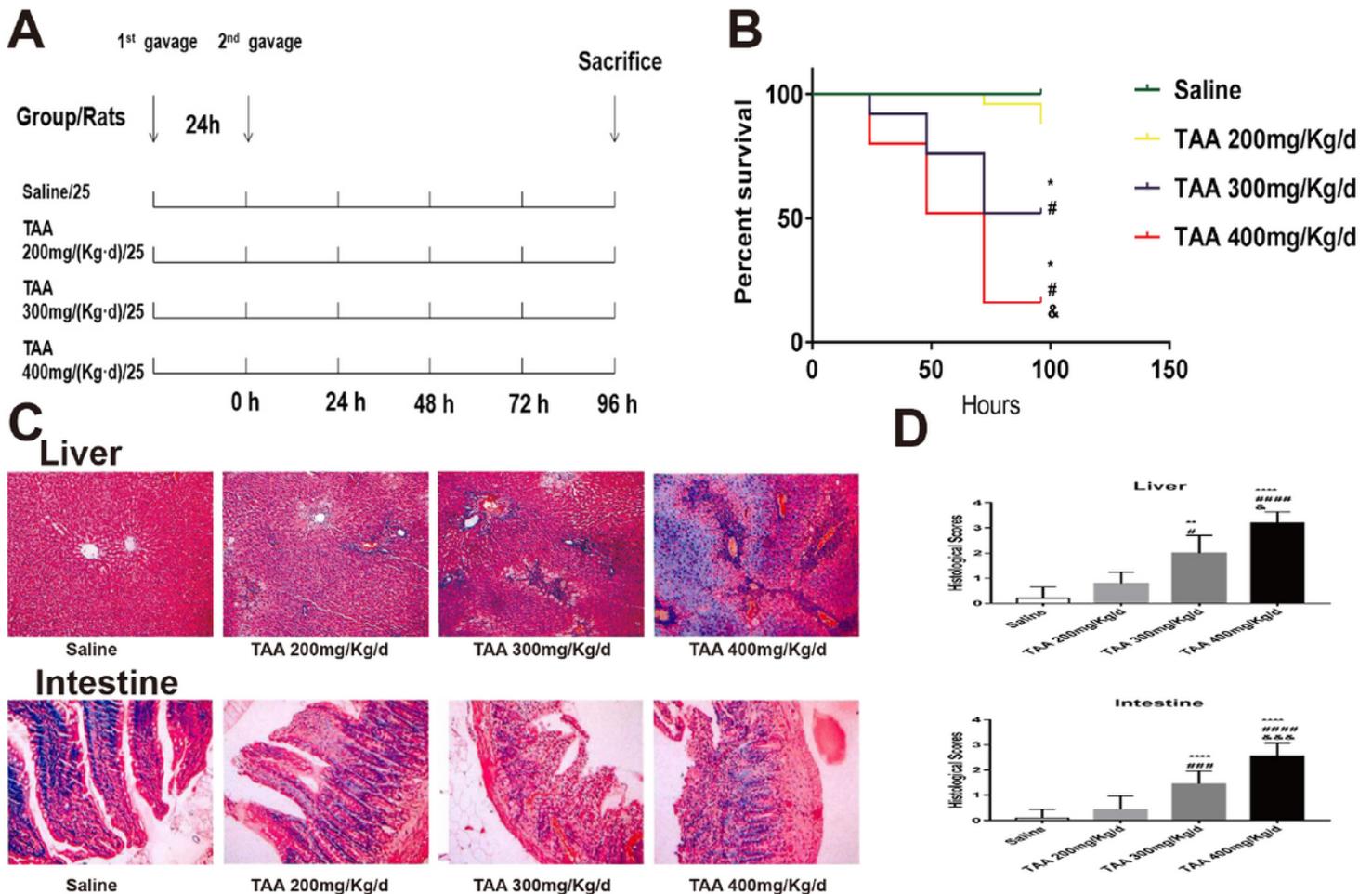


Figure 2

Animal model of acute liver failure induced by TAA. (a) Grouping and administration. (b) Survival analysis. * $p < 0.05$ vs. saline; # $p < 0.05$ vs. TAA 200mg/Kg/d; & $p < 0.05$ vs. TAA 300mg/Kg/d. (c) H&E staining, $\times 100$ magnification. (d) Histology analysis of four groups. ** $p < 0.01$, **** $p < 0.0001$ vs. saline;

$p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ vs. TAA 200mg/Kg/d; & $p < 0.05$, && $p < 0.001$ vs. TAA 300mg/Kg/d.

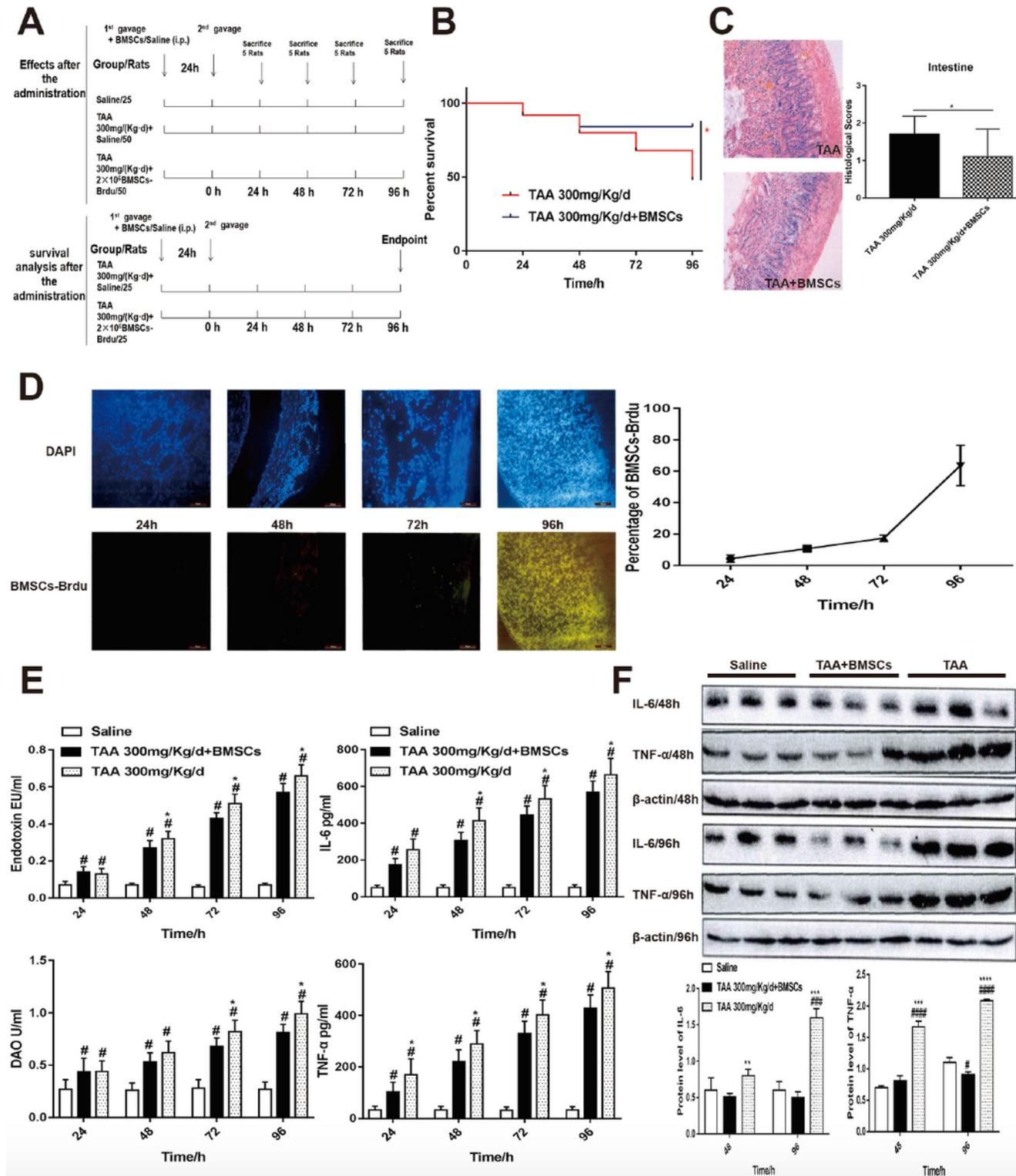


Figure 3

BMSCs administration alleviated acute liver failure in vivo. (a) Grouping and administration; Therapeutic experiment(upper) and mortality observation(lower). (b) Survival analysis. * $p < 0.05$. (c) H&E staining and histology analysis of intestines. * $p < 0.05$, $\times 100$ magnification. (d) Tracing of BMSCs in rat

intestines. DAPI-blue, BMSCs-Brdu-green. × 20 magnification. (e) Differences of plasma endotoxin, DAO, IL-6 and TNF- α in rats at different time points after BMSCs administration. # $p < 0.05$ vs. the saline group at the same time. * $p < 0.05$ vs. the TAA+BMSCs group at the same time. (f) Differences of intestinal IL-6 and TNF- α in rats at different time points after BMSCs administration. ### $p < 0.001$, #### $p < 0.001$ vs. the saline group at the same time. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. the TAA+BMSCs group at the same time.

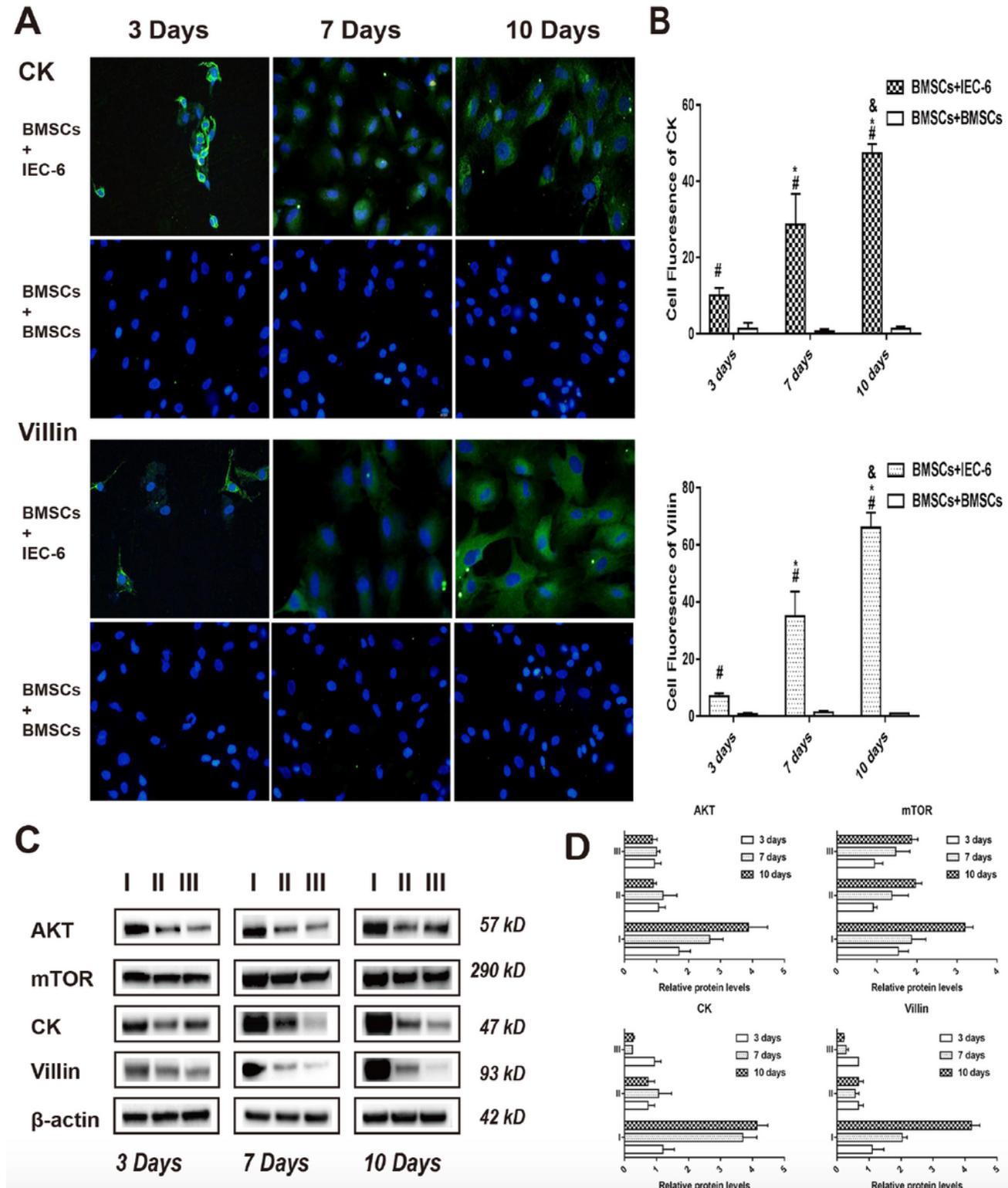


Figure 4

PI3K/AKT/mTOR signaling pathway was involved in the differentiation of BMSCs into enterocytes in vitro. (a) Immunofluorescence staining was performed to exhibit CK and Villin expression (green staining) in the co-culture system at different 3 time points. DAPI-blue. × 40 magnification. (b) Differences of CK and Villin in two co-culture systems. # $p < 0.05$, vs. the BMSCs + BMSCs group at the same time. * $p < 0.05$, vs. the BMSCs + IEC-6 group at the 3 days. & $p < 0.05$, vs. the BMSCs + IEC-6 group at the 7 days. (c) Western blot showed that blocking the PI3K/AKT/mTOR signal pathway interfered with the intestinal epithelial differentiation of BMSCs. Group I: IEC-6+BMSCs; Group II: IEC-6+LY294002+BMSCs; Group III: BMSCs+BMSCs. (d) Differences of AKT, mTOR, CK and Villin in 3 groups at different time points after PI3K blocker LY294002 administration.