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# Mesenchymal stem cells increase heme oxygenase 1-activated autophagy in treatment of acute liver failure

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ABSTRACT

In recent years, transplantation of mesenchymal stem cells (MSCs) has attracted much attention as a potential cell-based therapy for acute liver failure (ALF). As an inducible enzyme, heme oxygenase 1 (HO-1) has been reported to have cytoprotective, anti-apoptotic and immunoregulatory effects. Autophagy, a conserved catabolic process in cells, may be an important pathway for MSCs to treat ALF. In this study, we aimed to explore whether MSCs treat ALF by regulating autophagy and whether HO-1 was involved in the same pathway. Bone marrow-derived MSCs were isolated from Sprague-Dawley rats and cultured according to an established protocol. Co-culture systems of MSCs and hepatocytes were used to assess autophagy in the treatment of ALF. Meanwhile, MSCs were transplanted into rats with D-galactosamine (Gal)-induced ALF. Autophagy inhibitor (3-methyladenine, 3-MA), HO-1 inhibitor (zinc protoporphyrin, ZnPP) and PI3K specific inhibitor (LY294002) were employed in the study. Blood samples and liver tissues were collected before euthanasia. Survival rate, liver function, inflammatory factors, histology, Ki67 and TUNEL staining were determined. MSCs transplantation alleviated ALF both in vivo and in vitro. Autophagy and autophagy-related proteins were significantly up-regulated during MSCs treatment. 3-MA attenuated the therapeutic effect of MSCs. Administration of LY294002 before ALF induction inhibited hepatocyte autophagy. During the MSCs treatment, the HO-1 expression was increased, while inhibiting HO-1 attenuated the therapeutic effect of MSCs as well as hepatocyte autophagy. These findings suggested MSCs could alleviate ALF by increasing the HO-1 expression, which played an important role in activating autophagy through PI3K/AKT signaling pathway.



## 1. Introduction

ALF, which develops secondary to infection, toxin or immune-mediated attack, is a potentially fatal clinical syndrome characterized by rapid development of hepatocellular dysfunction with diffuse intrahepatic infiltration of inflammatory cells and massive multilobular necrosis [1]. Liver transplantation is the most effective treatment, but its widespread clinical application is limited by the shortage of donor liver, multiple post-operative complications and life-time immunosuppressant treatment [2]. An alternative approach, such as stem cell transplantation, has been suggested as an effective therapy for ALF [3]. Bone marrow MSCs have good application prospects for cell transplantation because they possess multiple differentiation potential, low immunogenicity and immune regulation, as well as the regulation of angiogenesis and apoptosis [4–6]. MSCs participate in not only responding to inflammatory cytokines, but also producing immune-regulatory secretors that mediate the process of inflammation [7]. During ALF, the balance of the liver immune microenvironment has been destroyed. MSCs can down-regulate inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-12p40 [8,9]. These properties confer MSCs a promising tool for the therapy of tissue repair. However, the mechanism of MSCs in the treatment of ALF remains largely unexplored.

Autophagy is a basic cellular homeostatic process that enables cells to eliminate portions of their own cytoplasmic contents. There

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are three types of autophagy, including macroautophagy microautophagy and chaperone-mediated autophagy [10]. Macroautophagy (often referred as autophagy) is the most common type of autophagy. In autophagy, autophagosomes transport cytoplasmic content into lysosomes (autolysosomes) for degradation of its cargo [11]. The autophagic pathway is positively regulated by starvation, growth factor deprivation and a multitude of immune-related signaling molecules [6]. The autophagy-related (Atg) genes, such as Atg3, Atg7 and so on, are essential for autophagosome formation in various cells, such as fibroblasts, macrophages and MSCs [12–14]. Autophagy has been reported to play important roles in regulating antigen presentation, lymphocyte homeostasis, cytokine production and differentiation of MSCs into hepatocyte-like cells [9,10,14–16]. However, the role of autophagy in regulating MSC-mediated immunomodulation remains elusive.

As a 32-kDa inducible enzyme, HO-1 belongs to the HO system, which catalyzes the degradation of the iron-containing molecule, and it exerts a wide range of cytoprotective, anti-inflammatory, anti-apoptotic and immunoregulatory effects in a variety of diseases [17–19]. It is up-regulated by pharmacological agents in D-Gal/lipopolysaccharide (LPS)-induced liver injury [20]. Previous studies have shown that MSCs can up-regulate HO-1, playing a therapeutic role [21]. However, the relationship between HO-1 and autophagy and the therapeutic effect of MSCs in ALF have not been illustrated. In the present study, we aimed to explore whether MSCs could be used to treat ALF by regulating autophagy and whether HO-1 was involved in the same pathway.

## 2. Materials and methods

### 2.1. Animals

Sprague-Dawley (SD) rats were purchased from the Laboratory Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School. Male SD rats (4–5 weeks old, weighting 120–140 g) were used as donors of mesenchymal stem cells (MSCs). Male SD rats (6–8 weeks old, weighing 180–200 g) were used as recipients of MSCs. This study was approved by the Institutional Animal Care and Use Committee of Nanjing University, China under the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number and suffering of animals.

### 2.2. Preparation of MSCs

MSCs were isolated from SD rats and cultured according to an established protocol [22]. Flow cytometry (FCM, BD FACS Aria II; , USA) was used to identify the MSCs using the antibodies against rodent CD29, CD34, CD44, CD45 and CD90 (BD Biosciences, CA, USA). Positive cells were counted and compared with corresponding immunoglobulin isotypes.

### 2.3. Experimental design and animal groups

Acute liver failure (ALF) was induced by an intraperitoneal injection of 1.0 g/kg D-galactosamine (D-Gal). After 24 h of ALF induction, the rats were randomly divided into six groups as follows: (1) control (saline); (2) D-Gal + phosphate-buffered saline (PBS); (3) D-Gal + MSCs; (4) D-Gal + MSCs+3-methyladenine (3-MA) (30 mg/kg body weight; Selleckchem, Houston, USA); (5) D-Gal + MSCs + zinc protoporphyrin (ZnPP) (50 μmoL/kg body weight; Sigma-Aldrich, St. Louis, MO, USA); and (7) D-Gal + MSCs + LY294002 (10 mM, Selleckchem, Houston, USA). A total of $1 \times 10^6$ MSCs suspended in 0.5 mL PBS were transfused into the caudal vein over a period of 3 min. Moreover, 3-MA, ZnPP or LY294002 was intraperitoneally administered to rats half an hour prior to ALF induction. All groups of rats ($n = 20$ in each group) were sacrificed after 1–7 days of D-Gal administration. The doses of D-Gal, 3-MA, ZnPP and LY294002 were established based on preliminary studies [21,23,24]. Rats were evaluated every 6 h and euthanized if they appeared moribund.

### 2.4. Survival study

The survival study was carried out with 20 rats in each group. Rats that survived more than 7 days after transplantation were considered to be survivors.

### 2.5. Collection of serum samples and hepatic tissue specimens

Rats were euthanized on days 1–7 post-transfusion. To detect serum cytokines on days 1–7, blood samples were collected from the tail vein 24 h before euthanasia. Liver tissues were excised and processed for Western blotting analysis. The remaining tissue was fixed and processed for histological and immunohistochemical analyses.

### 2.6. Enzyme-linked immunosorbent assay (ELISA) for inflammatory factors

To evaluate the anti-inflammatory effect of MSCs, the serum concentrations of following cytokines (TNF-α, IL-1β and U12p40) were determined using commercially available ELISA kits (Abcam Ltd., Cambridge, UK) after 3 days of ALF induction.

### 2.7. Histological and immunohistochemical studies

The liver samples were fixed in 4% paraformal-dehyde for 24 h prior to histological and immunohistochemical analyses. Fixed liver samples were dehydrated and paraffin-embedded. Three fragments, each measuring less than 3 mm in thickness, were obtained from each liver. Sections were stained with hematoxylin and eosin (H&E) for pathological assessment. Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) staining using a Cell Death Detection Kit (Roche, Mannheim, Germany). Cells positive for proliferating cell nuclear antigen (Ki67) were detected using specific antibodies (Aviva Systems Biology, Beijing, China). Images were captured, the labeled cell areas were manually quantified by two independent operators using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA), and five different fields were taken in each sample. Then the average number of the positive cells was calculated.

### 2.8. Isolation and characterization of hepatocytes

Primary hepatocytes were harvested by a two-step in situ collagenase perfusion procedure with our modifications [25]. The viability of isolated hepatocytes determined by trypan blue exclusion was more than 95%.

### 2.9. Establishment of co-culture system

Hepatocytes ($2 \times 10^5$) were co-cultured with MSCs ($2 \times 10^5$), which were separated by a porous membrane in six-well culture plates for 24 h. The total cell density remained at no more than $1 \times 10^6$ cells per well. The cells were randomly divided into six groups as follows: (1) control (homoculture of hepatocytes only); (2) D-Gal(0.01 g/ml); (3) D-Gal + MSCs; (4) D-Gal + MSCs+3-methyladenine (3-MA) (10 mmoL/L; Selleckchem, Houston, USA).

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### 2.10. Cell viability assay

The cytotoxicity of the D-Gal complexes against hepatocyte homoculture or co-culture medium was assessed using Cell Counting Kit 8 (CCK-8, Beyotime, China).

### 2.11. Cell apoptosis measurement by FCM

Hepatocyte cells ($10^5$ cells/mL, 2 mL/well) were allowed to adhere and reach 80% confluence. Cells were then treated with 10 g/L D-Gal for 24 h. One group was co-cultured with MSCs. To exclude the effect of DMSO solution, an equal volume of DMSO was added in the vehicle group, and an equal volume of RPMI-1640 medium was added in the blank control group. Cells were collected by centrifugation and washed twice with ice-cold PBS. The collected cells were then resuspended in 200 μL of binding buffer and incubated with 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) at room temperature for 15 min in the dark according to the manufacturer's instructions. The cells were immediately analyzed after staining using a FACScan flow cytometer. For each measurement, at least 20,000 cells were counted. Each experiment was conducted in triplicate.

### 2.12. Tandem mGFP-RFP-LC3B confocal microscopy

Primary hepatocytes stably transfected with GFP-RFP-LC3B were seeded into glass bottom cell culture dishes at a density of $1 \times 10^5$ cells per dish. After treatment, the cells were washed with PBS for three times and examined under a Nikon A1 confocal microscope system (Nikon, Japan). Images were processed with NIS Element Viewer software (Nikon). A total of 20 cells were randomly selected to evaluate the average number of mGFP-RFP-LC3B-positive puncta per cell.

### 2.13. Western blotting analysis

The total, cytosolic and nuclear proteins were extracted from frozen hepatic tissue samples according to the method described in the protein extraction kit (Active Motif, Carlsbad, CA, USA). Protein concentrations were determined using BCA protein assay kit. Protein extracts were subjected to SDS-PAGE on a 12% gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by incubation with a rabbit anti-LC3 polyclonal antibody (1:1000), rabbit anti-HO-1 polyclonal antibody (1:2000), rabbit anti-PI3K polyclonal antibody (1:1000), rabbit anti-AKT polyclonal antibody (1:1000) or anti-mTOR polyclonal antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA) at 4 °C, and the blots were then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) (Cell Signaling Technology, Danvers, MA, USA). Immunoreactive bands were visualized with an ECL chemiluminescence system on X-ray films (Kodak, Tokyo, Japan). Densitometric analysis of signal intensity was performed using Image J software (NIH, Bethesda, MD, USA).

### 2.14. Transmission electron microscopy (TEM)

To visualize autophagosomes and autolysosomes, hepatocytes were analyzed by TEM. Samples were concentrated at 1000 r/min and fixed with 1% glutaraldehyde in PBS at 4 °C for 3 h. They were washed five times with 0.1 M cacodylate buffer containing 0.1% $CaCl_2$. Samples were then post-fixed with 1% $OsO_4$ in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% $CaCl_2$ for 1 h. After rinsing with cold PBS, cells were slowly dehydrated in a graded series of ethanol and propylene oxide at 4 °C. After resin polymerization at 55 °C for 24–36 h, serial sections were cut with a diamond knife and mounted on formvar-coated slot grids. Finally, high resolution pictures were captured.

### 2.15. Statistical analysis

All data were presented as the means ± standard error of the mean. Pictures were compared via ANOVA, followed by Student's t-tests. High resolution pictures were analyzed by the Kaplan-Meier method and compared via the log-rank test. Statistical significance was defined as a two-sided P value $< 0.05$.

## 3. Results and discussion

### 3.1. MSCs relieve liver injury in ALF both in vivo and in vitro

To explore the effects of MSCs on ALF, hepatocytes from normal rats were incubated as controls. One group of hepatocytes was stimulated with D-Gal. The other group of hepatocytes in D-Gal treatment was co-cultured with MSCs, which were separated by a porous membrane in six-well culture plates for 24 h. CCK-8 and FCM were used to detect hepatocyte apoptosis(Fig. 1A and B). FCM analyses yielded cell histograms consisting of four quadrants, and the proportion of apoptotic cells in each group should be the sum of the upper right and lower right quadrants. As a result, the proportions of apoptotic cells in the blank control group, D-Gal control group and MSCs-treated group were 6.45%, 50.11% and 7.95%, respectively(Fig. 1B). These findings revealed that only a small proportion of cells underwent apoptosis in the blank control group, and there were significant differences between the D-Gal control group and MSCs-treated group, indicating promising effect of MSCs. Both results of CCK-8 assay and FCM showed a lower rate of apoptosis in the cell population cultured with MSCs compared with D-Gal groups, suggesting that MSCs possessed a therapeutic effect on damaged hepatocytes in vitro.

Similar results were found in vivo. MSCs were intravenously injected 24 h after D-Gal administration to determine the therapeutic efficacy of MSCs in ALF. We found that 80% of rats died within 7 days. In contrast, 70% of rats survived more than 7 days after ALF induction in the MSCs-treated group ($P < 0.05$) (Fig. 1C). Rats in the D-Gal group had signs of ALF, including loss of appetite, lazy activity, gait instability, urine yellowing and so on. Blood samples were collected and assessed, and the levels of ALT, NH3 and PT in the MSCs-treated group were dramatically decreased at each time point ($P < 0.05$) compared with the D-Gal group (Fig. 1D). Collectively, we concluded that MSCs could relieve ALF both in vitro and in vivo.

### 3.2. MSCs enhance autophagy and reduce the level of inflammation in injured hepatocytes

MSCs have been proved to have protective effects on acutely damaged hepatocytes [21,23]. Next, we explored the ways in which MSCs acted its functions. Autophagy (literally “self-eating”) is a cellular process responsible for the degradation of excess or aberrant long-lived cytosolic proteins and organelles within lysosomes in order to remove and eventually recycle the resulting macromolecules [14,16]. Subsequently, the implication of autophagy has been highlighted in various liver diseases. However, its role in the process of ALF remains unknown. We first established a co-culture system. In this system, we monitored the formation of autophagosomes in these hepatocytes stably transfected with tandem GFP-RFP-LC3B [26]. In these cells, early autophagosomes displayed both green signal and red signal. Autophagolysosomes displayed only

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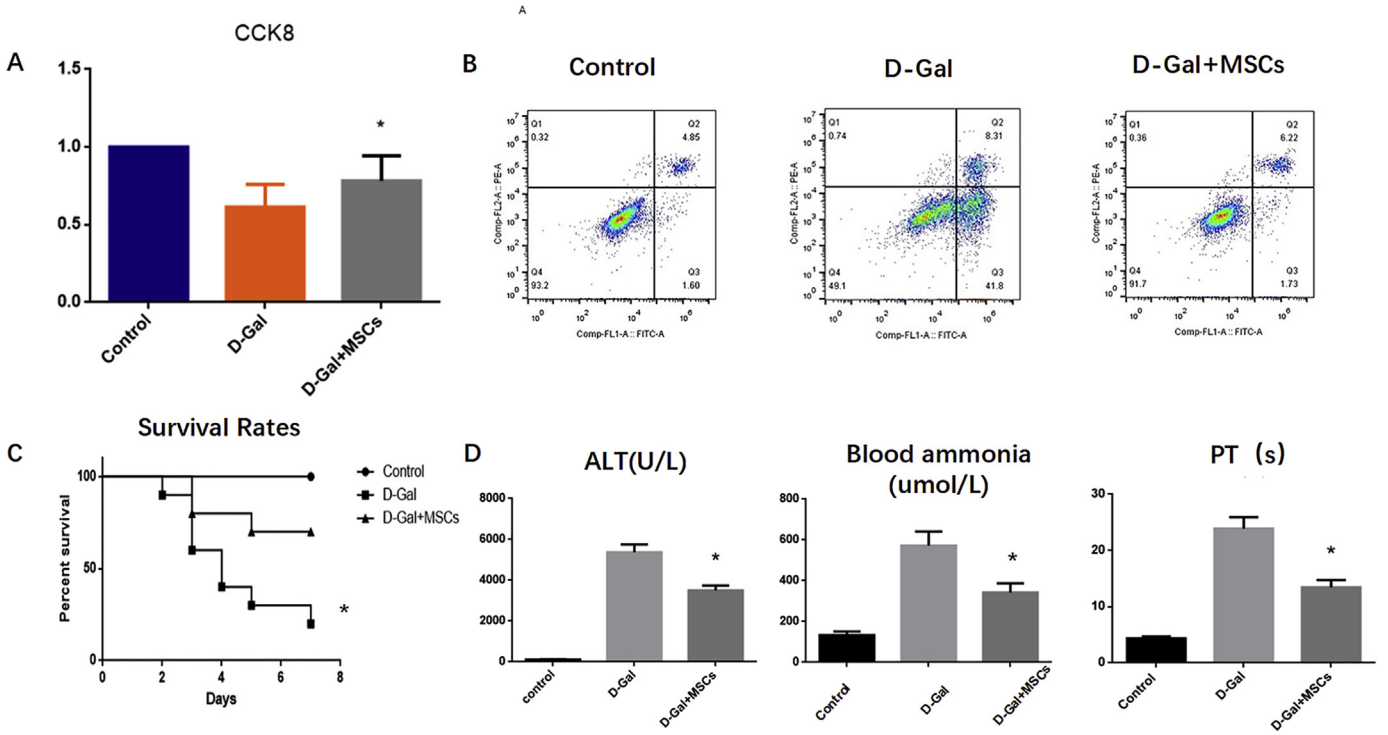


**Fig. 1. MSCs attenuated hepatocyte injury in vitro and has therapeutic effect on ALF in vivo. A &B** Cell Counting Kit 8 and FCM were conducted to detect the survival of damaged hepatocytes injured hepatocytes. The mortality was significantly reduced after co-culture of damaged hepatocytes($P < 0.05$). **C** Survival analysis of D-Gal-treated rats and MSCs transplatation($n = 20$ per group). The viability of MSCs treatment group was significantly higher than that of simple D-Dal-induced group($P < 0.05$). **D** Serum ALT, Blood ammonia and PT levels collected on the third day after D-Gal. *$P < 0.05$ vs. D-Gal controls. T-test, data are shown as mean ± standard error of the mean.

red fluorescence since the green signal was sensitive to the acidic conditions in the lysosomal lumen, while the red signal was more stable. Increased red spots appeared in the merged section of MSCs-treated cells, indicating the maturation of autolysosome in these cells (Fig. 2A). Autophagic vesicles containing engulfed organelles were detected in MSCs-treated cells by TEM, a standard technique used for autophagy detection (Fig. 2B). Then we examined the autophagy and formation of autophagy-related proteins in hepatocytes. The levels of LC3A/B, Atg3 and Atg7 in MSCs-treated heptaocytes were much higher than those in other groups(Fig. 3C and D).

Animal experiments also supported above-mentioned findings. The survival rate of MSCs-treated group was significantly higher than that of simple liver failure group(Fig. 2E). The mortality of rats was increased in the presence of autophagy inhibitor. Biochemical and histological parameters in the liver were tested on day 3 since the damage of liver function in rats was the worst at this time point. Consistently, D-Gal-treated group exhibited high levels of biochemical indicators, while MSCs-treated group showed a dramatic decrease (Fig. 2E). It has been reported that pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6and IL-12p40, are released in ALF [8,9,15]. We detected the expressions of these above-mentioned inflammatory factors in the liver by ELISA on day 3 after ALF induction. Pro-inflammatory molecules, such as TNF-α, IL-1β, IL-6and IL-12p40were significantly down-regulated after administration of MSCs(Fig. 2F). These data indicated that MSCs secreted anti-inflammatory cytokines in inflammatory response. H&E staining was conducted to investigate the liver histology of rats with ALF after transplantation of MSCs. Histological analysis showed a normal lobular architecture and cell structure of the liver in control animals, while extensive portal inflammation, hemorrhagic necrosis and severe lymphocyte infiltration were found in the ALF group, and the hepatocytes had swollen cytoplasm(Fig. 2Ga). MSC treatment restored normal histology. We conducted Ki67 and TUNEL staining to examine the proliferation and apoptosis of damaged heptaocytes. Immunohistochemical staining showed that the apoptosis of hepatocytes was decreased and proliferation was increased after injection of MSCs(Fig. 2Gb&c). These results suggested that MSCs played a therapeutic role by enhancing hepatocyte autophagy.

In order to further verify our conjecture, we employed the specific autophagy inhibitor (3-MA). We found that the inhibition of autophagy by 3-MA partly abolished the hepatoprotective effect of MSCs, as confirmed both in vivo and in vitro. In co-culture system, we introduced the mRFP-GFP-LC3 reporter to determine the role of MSCs in autophagic flux in the presence of 3-MA. We found a lot of yellow spots but not red spots in the presence of 3-MA (Fig. 2A), suggesting that 3-MA did not activate autophagy. TEM also showed that 3-MA reduced the formation of autophagosome(Fig. 2B). In animals, 3-MA was intraperitoneally injected into rats at 30 min before D-Gal administration. Pro-inflammatory molecules and apoptosis were up-regulated in hepatocytes(Fig. 2E and F&G). These findings indicated that the damage of liver function was aggravated in rats. Therefore, we concluded that MSCs alleviated ALF by promoting hepatocyte autophagy. However, the therapeutic effect was weakened after inhibition of autophagy.

### 3.3. MSCs enhance autophagy through PI3K/AKT pathway

Several previous studies have reported that autophagy can function in humans in a variety of pathways, such as PI3K/AKT signaling, MAPK/ERK1/2 signaling and p53/genotoxic stress pathways[27]. As the classical survival signaling pathway in most cells,

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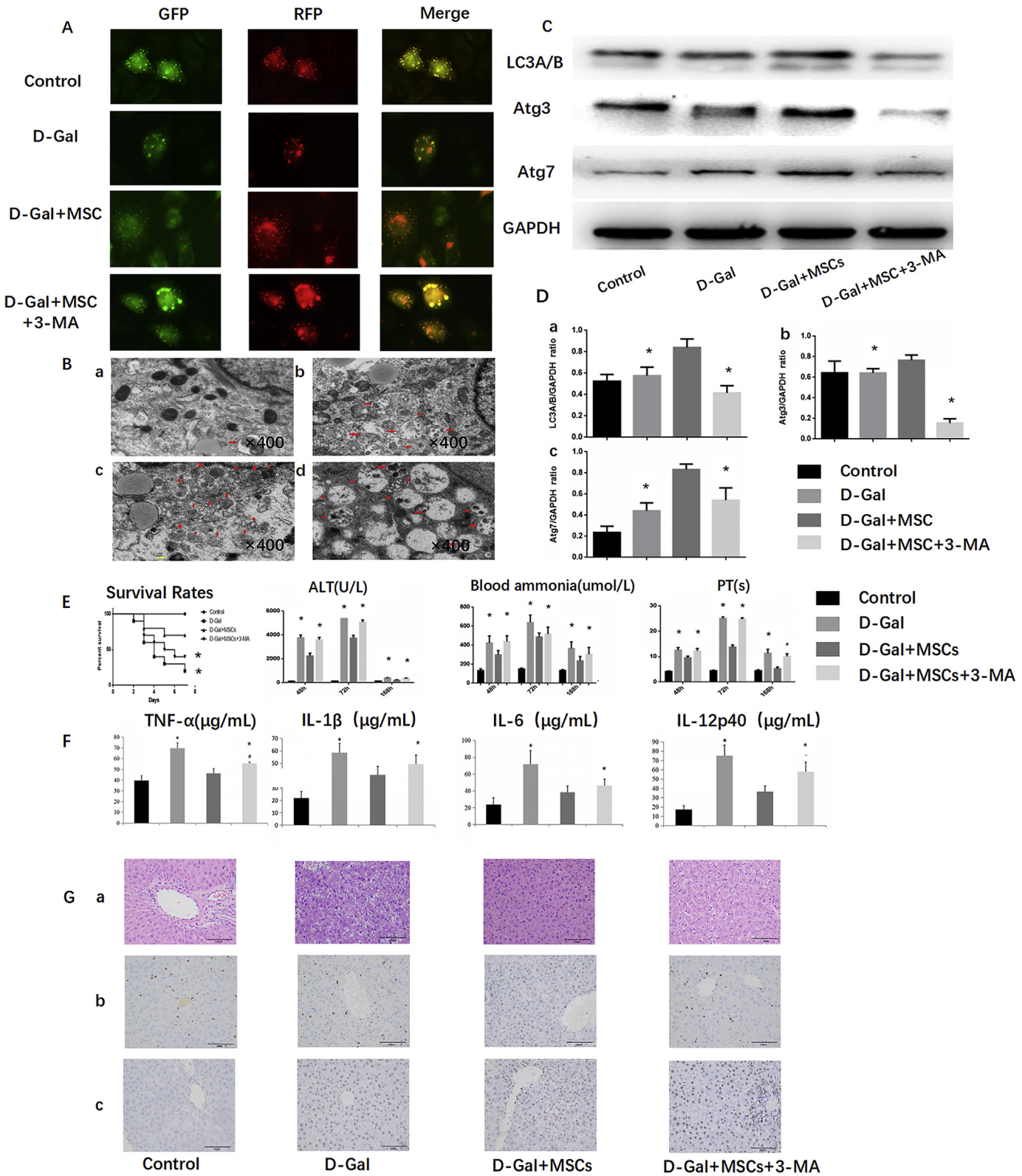


**Fig. 2. MSCs up-regulated the level of hepatocyte autophagy and inhibition of autophagy attenuated the therapeutic effect of MSCs.** **A** Primary hepatocytes stably transfected with mGFP-RFP-LC3B. The graphs show the average number of two kinds of LC3B puncta per cell. Green-positive, red-positive puncta (G + R+) are autophagosomes; green-negative, red positive puncta (G−R+) are autophagolysosomes**. B** Transmission electron microscope of four groups. (Red arrow was autophagosome). **C** Western blot of Atg3, Atg7 and LC3A/B protein levels (normalized to GAPDH standard). **D** Quantification of western blot bands. **E** The survival curve showed that adding 3-MA half an hour before the induction of ALF inhibited the therapeutic effect of MSCs($P < 0.05$). Serum ALT, Blood ammonia and PT levels collected after ALF-induced. **F** Liver tissue levels of pro-inflammatory cytokines at 3rd day after D-Gal injection. **G** Representative images of hematoxylin and eosin staining**(a)**, TUNEL (marking apoptosis) **(b)**and Ki67 (a proliferation marker)**(c)** in livers 72 h after D-Gal (magnification × 400). Treatment groups: control, ALF, ALF followed by intravenous MSCs (ALF + MSCs)24 h post-induction, ALF and 3-MA followed by MSCs (ALF + MSCs + 3-MA) 24 h post-induction.*$P < 0.05$ vs. D-Gal + MSCs controls. T-test, data are shown as mean ± standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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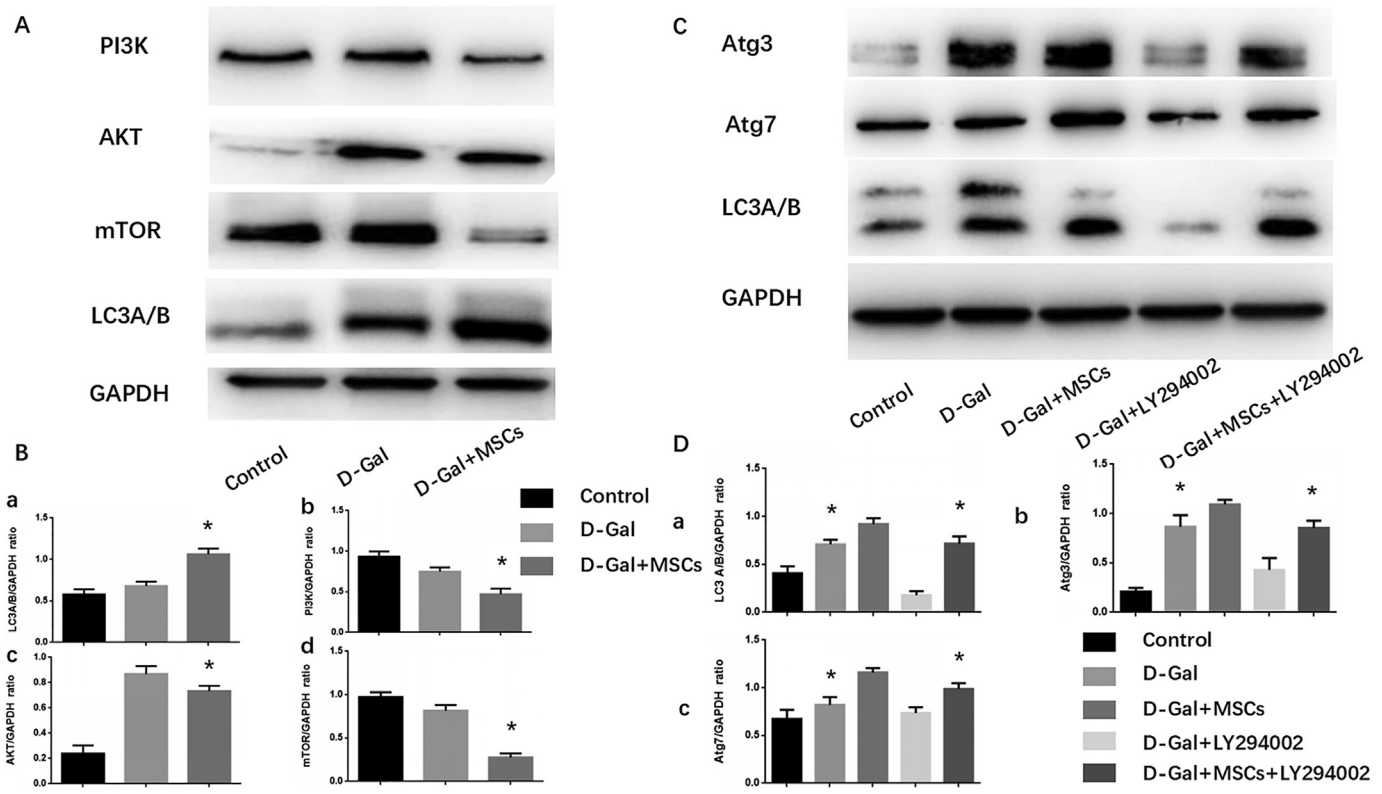


**Fig. 3. MSCs enhances autophagy by inhibiting PI3K/AKT signaling pathway A** Western blot of PI3K,AKT, mTOR and LC3A/B protein levels (normalized to GAPDH standard). Treatment groups: control, ALF, ALF followed by intravenous MSCs (ALF + MSCs)24 h post-induction. **C** Western blot of Atg3, Atg7 and LC3A/B protein levels (normalized to GAPDH standard). Treatment groups: control, ALF, ALF followed by intravenous MSCs (ALF + MSCs)24 h post-induction, ALF and LY294002 (ALF + LY294002), ALF and LY294002 followed by MSCs (ALF + MSCs + LY294002) 24 h post-induction. **B&D** Quantification of western blot bands.*P < 0.05 vs. D-Gal controls.#P < 0.05 vs. D-Gal + MSC controls. T-test, data are shown as mean ± standard error of the mean.

PI3K/AKT/mTOR is previously reported to function in MSCs. Therefore, we aimed to explore whether it played a role in the therapeutic effects of MSCs on ALF. The protein levels of PI3K/AKT were tested in each group. We found that the expression of PI3K/AKT/mTOR in the treatment group was significantly lower than that in the ALF group(Fig. 3A and B), indicating that PI3K/AKT/mTOR played a negative function in autophagy. As a specific PI3K inhibitor, LY294002 can reduce autophagosome formation. We intraperitoneally injected LY294002 at 30 min before D-Gal inducement. After the introduction of LY294002 into the ALF group, the expressions of LC3/Atg7/Atg3 at the protein level were decreased(Fig. 3C and D). The above-mentioned results indicated that MSCs alleviated liver injury by enhancing autophagy via inhibiting the PI3K/AKT signaling pathway.

### 3.4. MSCs induce autophagy in ALF via up-regulating HO-1

In previous studies, MSCs have been reported to produce HO-1 to reduce the production of inflammatory factors and the aggregation of neutrophils to relieve liver damage [21]. No relationship between HO-1 and autophagy has been demonstrated before. The up-regulation of HO-1 during the MSC treatment suggested that HO-1 was involved in the regulation of MSC-mediated hepatocyte autophagy(Fig. 4A and B). To further verify our conjecture, we established the D-Gal + MSCs + ZnPP control group. We treated rats with ZnPP at 30 min before D-Gal injection to suppress hepatic HO-1 activity. We found that the hepatoprotective effect of MSCs was partly reduced in the presence of ZnPP, which was confirmed by biochemical or histological parameters(Fig. 4C and D). Injection of ZnPP increased the mortality of rats. The 7-day survival rate of ALF rats treated without ZnPP before injection of MSCs was 70% compared with the group with inhibitor, which was 40%. Biochemical indicators, such as ALF, NH3 and PT, in ZnPP-treated group were much higher compared with the blank and D-Gal-treated groups(Fig. 4C), suggesting that rats were suffering from much more severe liver injury. Similar results were observed in pro-inflammatory molecules(Fig. 4D)H&E staining also showed that the introduction of HO-1 inhibitor aggravated the damage of hepatocytes. Ki67 and TUNEL staining further confirmed that inhibition of HO-1 could aggravate liver failure through promoting hepatocyte apoptosis and reducing hepatocyte proliferation(Fig. 4E). The elevated HO-1 activity suggested an increased enzymatic function after MSC treatment, and such increased HO-1 activity was reduced to the baseline level by ZnPP, indicating that HO-1 was involved in the regulatory process of MSC-mediated autophagy in the treatment of ALF.

To verify whether HO-1 was involved in the regulation of autophagy through the same signaling pathway as MSCs。We testd the level of pathway-related proteins.。It was found that after inhibiting HO-1, the protein level of PI3K, AKT and mTOR increased, while LC3 A/B decreased(Fig. 4F and G), suggesting that HO-1, like MSCs, was involved in the regulation of autophagy through the PI3K/AKT signaling pathway.

## 4. Conclusions

In the present study, we explored the mechanism by which MSCs regulated autophagy in the treatment of ALF and how HO-1

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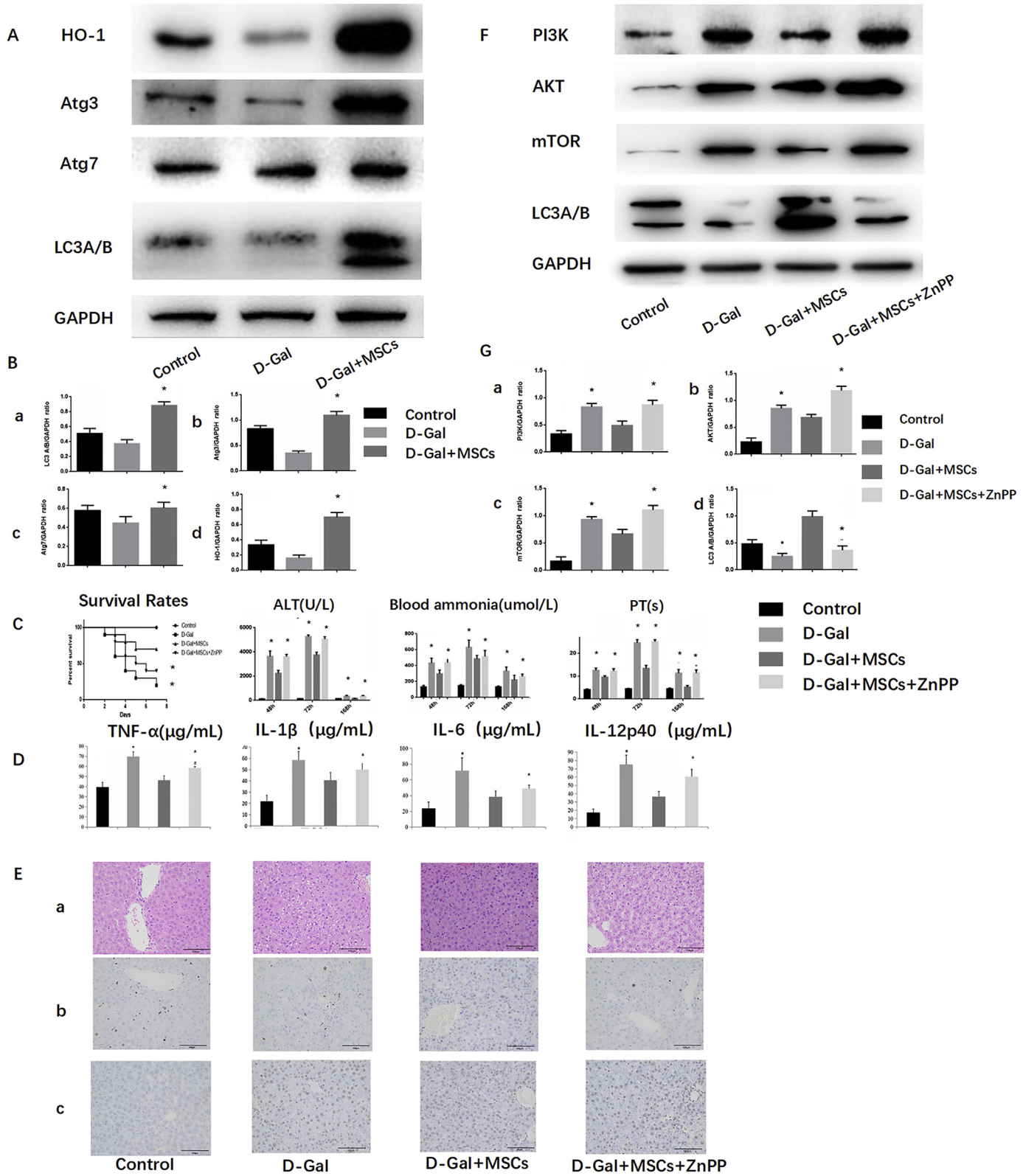


**Fig. 4. MSC regulated autophagy by up-regulating HO-1 and inhibiting PI3K/AKT signaling pathway A** Western blot of HO-1. Atg3, Atg7 and LC3A/B protein levels (normalized to GAPDH standard). Treatment groups: control, ALF, ALF followed by intravenous MSCs (ALF + MSCs)24 h post-induction. **C** Western blot of PI3K, AKT, mTOR and LC3A/B protein levels (normalized to GAPDH standard). **B&D** Quantification of western blot bands. **E** The survival curve showed that adding ZnPP half an hour before the induction of ALF inhibited the therapeutic effect of MSCs($P < 0.05$). Serum ALT, Blood ammonia and PT levels collected after ALF-induced. **F** Liver tissue levels of pro-inflammatory cytokines at 3rd day after D-Gal injection. **G** Representative images of hematoxylin and eosin staining**(a)**, TUNEL (marking apoptosis) **(b)**and Ki67 (a proliferation marker)**(c)** in livers 72 h after D-Gal (magnification × 400). Treatment groups: control, ALF, ALF followed by intravenous MSCs (ALF + MSCs)24 h post-induction, ALF and ZnPP followed by MSCs (ALF + MSCs + ZnPP) 24 h post-induction. *$P < 0.05$ vs. D-Gal + MSCs controls. T-test, data are shown as mean ± standard error of the mean.

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played a role in the treatment of MSCs. Cell-based therapy is a promising approach for liver regeneration[3]. So far, multiple cell types, including MSCs, have been tested preclinically and clinically. However, the mechanism of MSCs transplantation in the treatment of ALF remains unclear.

The first finding of our study was that MSC-mediated autophagy was an important pathway in the treatment of ALF. Autophagy and autophagy-related proteins were significantly increased after MSC treatment in vivo and in vitro. Autophagy inhibitor could weaken the therapeutic effect of MSCs. The expressions of proinflammatory cytokines were decreased after the enhancement of autophagy, suggesting that autophagy was also involved in the effect of MSCs on the intracellular inflammatory microenvironment. Then through detecting the related pathway proteins, we confirmed MSCs played its therapeutic role in ALF through PI3K/AKT signaling pathway. This finding provided a basis for the selection of pathways for the subsequent MSCs-based therapies in humans. Another important finding was MSCs could induce the over-expression of HO-1, and HO-1 participated in the regulation of autophagy and inflammatory microenvironment in the treatment of ALF which will provide a new target for the treatment of ALF. Overall, our findings confirmed that MSCs could alleviate ALF via regulating autophagy through PI3K/AKT pathway both in cells and rats, and paracrine HO-1 was also involved in autophagy regulation.

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## List of abbreviations

| | |
|---|---|
| ALF | Acute liver failure |
| ALT | Alanine aminotransferase |
| BMSCs | Bone marrow-derived mesenchymal stem cells |
| D- Gal | D-Galactosamine; |
| 3-MA | 3-methyladenine; |
| HO | Heme oxygenase |
| NO | nitric oxide; |
| IDO | indoleamine 2,3-dioxygenase |
| IL | Interleukin; LPS:Lipopolysaccharide; |
| MSCs | Mesenchymal stem cells |
| NF | nuclear factor |
| TNF | Tumor necrosis factor |
| TUNEL: | 2′-deoxyuridine 5′-triphosphatenick-end labeling |
| ZnPP | Zinc protoporphyrin |
| Atg | autophagy-related |
| CO | carbon monoxide; |
| SD | Sprague-Dawley |
| FCM | Flow cytometry |
| CCK-8 | Cell Counting Kit 8 |
| TBS | Tris-buffered saline; |
| TEM | Transmission electron microscopy |

## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.11.146.

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