**Human umbilical cord mesenchymal stem cells protect against ferroptosis in acute liver failure through the IGF1-Hepcidin-FPN1 axis inhibiting iron loading**

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Human umbilical cord mesenchymal stem cells protect against ferroptosis in acute liver failure through the IGF1-hepcidin-FPN1 axis, inhibiting iron loading

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Abstract

Acute liver failure (ALF) is a significant global issue with elevated morbidity and mortality rates. There is an urgent and pressing need for secure and effective treatments. Ferroptosis, a novel iron-dependent regulated cell death, plays a significant role in multiple pathological processes associated
with liver diseases, including ALF. Several studies have demonstrated that mesenchymal stem cells (MSCs) have promising therapeutic potential in the treatment of ALF. This study aims to investigate the positive effects of MSCs against ferroptosis in an ALF model and explore the underlying molecular mechanisms of their therapeutic function. Our results show that intravenously injected MSCs protect against ferroptosis in ALF mouse models. MSCs decrease iron deposition in the liver of ALF mice by downregulating hepcidin level and upregulating FPN1 level. MSCs labelled with Dil are mainly observed in the hepatic sinusoid and exhibit colocalization with the macrophage marker CD11b fluorescence. Serum ELISA demonstrates a high level of IGF1 in the CCL4+MSC group. Suppressing the IGF1 effect by the PPP could block the therapeutic effect of MSCs against ferroptosis in ALF mice. Furthermore, disruption of IGF1 function results in iron deposition in liver tissue due to the impaired inhibitory effects of MSCs on hepcidin levels. Our findings suggest that MSCs alleviate ferroptosis induced by disorders of iron metabolism in ALF mice by elevating IGF1 level. Moreover, MSCs have been identified as a promising cell source for ferroptosis treatment in ALF mice.

**Key words:** mesenchymal stem cell; acute liver failure; ferroptosis; iron loading; insulin-like growth factor-1

**Introduction**

Acute liver failure (ALF) represents a potentially life-threatening condition characterized by sudden onset, rapid deterioration, numerous complications, and alarmingly high mortality rates[1]. ALF worldwide is estimated to affect approximately 1 to 6 individuals per million annually[2]. While liver transplantation remains the most effective treatment for ALF, its accessibility is hindered by the severity and rapid progression of patients’ illnesses, coupled with the scarcity of available donor organs. Furthermore, even after successful liver transplantation, the disease mortality rate can still reach up to 30%[3]. Regrettably, recent years have witnessed minimal progress in improving the survival rates of ALF patients[4, 5]. Therefore, there is an imperative need to explore high-efficiency nonsurgical treatment to improve the overall prognosis of patients.

Ferroptosis is a recently discovered iron-dependent nonapoptotic programmed cell death mechanism[6]. Recent studies have shown that the characteristics of ferroptosis are found in different stages of liver diseases, encompassing iron metabolism disorder, imbalance of the antioxidant system
and lipid peroxide accumulation[7]. Brent R. Stockwell identified metabolism, ROS, and iron biology as pivotal regulators of ferroptosis[8]. Moreover, a plethora of studies have also demonstrated that intracellular iron-loading status is necessary for intracellular ferroptosis[9, 10]. Furthermore, it has been discovered that intracellular iron overload, which is mediated by hepcidin and ferroportin (FPN1), is the key link to the occurrence of ferroptosis[8, 11]. Hepcidin, a circulating hormone primarily produced by the liver, strictly regulates systemic iron metabolism in mammals[12]. FPN1 is currently recognized as the exclusive iron export channel from cells into the plasma. The structure of hepcidin-bound FPN1 unveils iron homeostatic mechanisms, hepcidin binds FPN1 in an outwards-open conformation to negatively regulate FPN1, and it completely occludes the iron efflux pathway by inhibiting iron transport[13-15]. A deficient hepcidin response to iron loading may result in systemic iron overload[16], whereas transgenic mice overexpressing hepcidin experienced severe iron deficiency anemia[17]. In humans, juvenile hemochromatosis, caused by loss-of-function mutations in hepcidin, results in severe iron deposition and multiple organ damage, including afflictions of the liver, heart, and endocrine tissues[18]. In mice with subarachnoid hemorrhage treated with heparin (an inhibitor of hepcidin), downregulating the expression of hepcidin and increasing FPN1 could exert protective effects against ferroptosis following subarachnoid hemorrhage[15]. Furthermore, several studies have indicated a negative association between FPN1 and ferroptosis. For example, FPN1 knockout in the brain microvasculature decreased brain iron levels and inhibited ferroptosis in ischemic stroke mice[19]. Moreover, activation of the NRF2/FPN1 pathway mitigated myocardial ischemia-reperfusion injury in diabetic rats by regulating iron homeostasis and ferroptosis[20].

Stem-cell therapy has great promise and has been revealed as a therapeutic option for ALF[21, 22]. After investigating different types of stem cells, it has been determined that human umbilical cord-derived mesenchymal stem cells (H-uc MSCs) are an appropriate treatment option, as these cells can be easily obtained and are characterized by their high quality, purity, and abundance. Simultaneously, MSCs have emerged as highly viable alternatives in clinical practice. Meanwhile, stem cell-derived hepatocyte therapy could also effectively treat ALF[23, 24]. Currently, 163 ongoing clinical trials use MSCs to treat different liver diseases, including ALF (https://clinicaltrials.gov/; Accessed 13 December 2021). In patients with ALF, the therapeutic effect of MSCs is satisfactory in the short term. Furthermore, it has been reported that MSCs have a protective role against ferroptosis by maintaining SLC7A11 function[25]. Furthermore, bone marrow-derived mesenchymal stem cells
have been shown to alleviate ischemia-reperfusion injury following steatotic liver transplantation by inhibiting ferroptosis[26]. Currently, the primary mechanism by which MSCs protect against ferroptosis is through inhibiting lipid peroxidation rather than promoting intracellular iron export[26, 27]. However, the focus of our study was mainly on MSCs that could inhibit ferroptosis by reducing intracellular iron levels.

Several reports have underscored that the in vivo biological function of MSCs is closely associated with specific growth factors, such as insulin-like growth factor-1 (IGF1). In murine models of colitis, MSCs have been demonstrated to enhance colon epithelial integrity and regeneration through the elevation of hepatic secretion of IGF1[28]. Likewise, H-uc MSCs have exhibited the capacity to attenuate severe burn-induced multiple organ injury by increasing protective cytokine IGF1 level[29]. IGF1 also plays a crucial role in the pathological state of the liver. For example, in a liver injury model, treatment with a low dose of IGF1 has been demonstrated to repair the development of liver injury and reduce the severity of liver fibrosis[30, 31]. Furthermore, IGF1 treatment could improve the biochemistry, histology, and genetic expression of proregenerative and cytoprotective factors in healthy and IGF1-deficient miceIGF1 with acute liver damage[32]. However, there is limited experimental evidence to support the role of IGF1 in mediating the molecular mechanism of MSC therapy for ALF by protecting against ferroptosis.

This study aimed to delineate the effects of H-uc MSCs on ferroptosis in CCL4-induced hepatotoxicity and elucidate the intricate mechanism involving the IGF1-hepcidin-FPN1 axis. Our study provides a valuable resource for deciphering the molecular mechanism that underlies the therapeutic effect of H-uc MSCs on ALF.

Materials and methods

Extraction and identification of H-uc MSCs

Human umbilical cords were obtained through full-term caesarian section deliveries with the informed consent of the parents. MSCs from the umbilical cord were isolated and cultured using the method previously described by the Sung research group[33]. During MSC quality control, cultured cells at the fourth passage (P4) were assessed using osteogenic and adipogenic differentiation medium for differentiation capacity. The phenotype of MSCs was identified using flow cytometry (FACS Celesta; BD, New Jersey, USA). Oil red and alizarin red S staining were used to detect osteoblasts
and adipocytes. The following antibodies obtained from BD were employed for flow cytometry: CD90 (519007657), CD44 (519007656), CD73 (519007649), CD105 (519007648), and H-uc MSC Negative Cocktail (519007661). H-uc MSCs were labelled with a Dil fluorescent probe (C1036, Beyotime, Shanghai, China) following the protocols provided by the manufacturer, and the labelling efficiency was detected by flow cytometry.

**Animal models and experimental design**

Eight-week-old C57BL/6J male mice were purchased from Vital River Laboratory Animal Technology (Beijing, China) and randomly divided into three groups \((n = 10\) mice per group). These groups were designated the negative control group (NC), ALF group \((CCL_4+PBS)\) and MSC treatment group \((CCL_4+MSC)\). In the NC group, mice were given 100 \(\mu\)L PBS through the tail vein. In the ALF group, mice were administered 2.5 mL/kg \(CCL_4\) via intraperitoneal (i.p.) injection, and then 100 \(\mu\)L PBS was injected via the tail vein after 3 h. In the MSC treatment group, mice were administered 2.5 ml/kg \(CCL_4\) via i.p. injection and \(5\times10^5\) MSCs in 100 \(\mu\)L PBS via the tail vein after 3 h. Mice were sacrificed on day 3, and liver and serum samples were collected for further analysis.

For the IGF1 receptor inhibitor experiment, the IGF1 receptor inhibitor picropodophyllin (PPP; MedChemExpress, New Jersey, USA) was utilized. The mice were randomly divided into four groups \((n = 10\) mice per group): the negative control group (NC), the ALF group \((CCL_4+PBS+vehicle)\), the MSC treatment group \((CCL_4+MSC+vehicle)\), and the PPP group \((CCL_4+MSC+PPP)\). Mice in each experimental group received 1.25 ml/kg \(CCL_4\) through i.p. injection. Mice in the PPP group \((CCL_4+MSC+PPP)\) were treated with daily intraperitoneal administration of 30 mg/kg PPP and a single MSC injection of \(5\times10^5\) cells. Mice in the ALF group \((CCL_4+PBS+vehicle)\) and the MSC group \((CCL_4+MSC+vehicle)\) were treated with daily intraperitoneal administrations of inhibitor vehicles. Mice were sacrificed on day 3, and liver and serum samples were collected and stored at -80°C.

**Measurement of serum malonaldehyde (MDA) and glutathione (GSH)**

Serum was isolated from whole blood samples by centrifugation at 3500 rpm for 15 min. Serum MDA and GSH were examined using the MDA assay kit (TBA method) and the Reduced GSH assay kit (Nangjing Jiancheng Bioengineering Institute, Nangjing, China). The results were tested using a microplate reader (SpectraMax190; Molecular Devices, Sunnyvale, USA).

**Measurement of tissue iron**

The liver proteins were extracted by the tissue homogenate method and detected by the BCA Protein
Assay Kit (PC0020; Solarbio, Beijing, China). Iron levels in the liver were measured using a tissue iron assay kit (Nanjing Jiancheng Bioengineering Institute). The results were measured using the microplate reader.

**ELISA for serum IGF1**

Serum IGF1 concentrations were measured using the Mouse IGF1 ELISA kit (JL12620; Jianglaibio, Shanghai, China) following the manufacturer’s protocols. The results were measured using the microplate reader.

**Hematoxylin-eosin (HE) staining**

The left lobes of the liver were collected and fixed with 4% paraformaldehyde and then embedded in paraffin. Each tissue sample was sectioned into 4-μm-thick slices. The HE staining procedure was performed following the instructions of the Hematoxylin-Eosin Stain Kit (G1120; Solarbio).

**Immunohistochemistry (IHC)**

The slices were subjected to dewaxing and rehydration. Subsequently, permeabilization was performed using 1% Triton X-100 (#T8200, Solarbio) and blocking with 10% horse serum. Next, the sections were labelled with the following primary antibodies: Ptgs2 (1:200, ab283574; Abcam, Cambridge, UK), Gpx4 (1:200, ab125066; Abcam), hepcidin (1:200, AF7003; Affinity, Liyang, China), IGF-1R (1:200, AF6125; Affinity), P-IGF-1R (1:200, AF3123; Affinity), P-AKT (1:200, AF0016; Affinity), ferritin (1:100, ab287968; Abcam) and TfR1 (1:2000, ab269513; Abcam) and incubated overnight at 4 °C. Then, the secondary antibody was incubated at 37 °C for 20 min. Reaction products were visualized by using 3,3'-diaminobenzidine (DAB) staining. The photos were taken with a Motic PA53 biological microscope.

**Prussian blue iron staining (Perl’s staining)**

The slices were subjected to dewaxing and rehydration. The Prussian blue iron staining procedure was performed following the instructions of the Prussian Blue Iron Stain Kit (G1428; Solarbio). The results were measured using a microplate reader (SpectraMax190, Molecular Devices, US).

**Immunofluorescence staining**

The sections were subjected to dewaxing and rehydration and then permeabilized with 0.1% Triton X-100 for 10 min. Then, the sections were blocked for 1 h with 10% horse serum. The sections were incubated overnight with a FITC-conjugated anti-mouse/human CD11b primary antibody (1:100, 101205; Biolegend, California, USA) at 4 °C. Finally, the nucleus was visualized by staining with DAPI, which contained an anti-fluorescence attenuation sealant (BL739A; Biosharp, Hefei, China).
Western blot analysis

Liver proteins were isolated and collected using RIPA buffer (R0010, Solarbio) supplemented with 1X protease inhibitor mixture (P6730; Solarbio). The total protein was quantified using the BCA Protein Assay Kit (PC0020; Solarbio). The proteins were separated through 10% SDS-PAGE and subsequently transferred onto PVDF membranes. The target protein was probed using the primary antibodies hepcidin (1:500, AF7003; Affinity), BMP6 (1:500, AF5196; Affinity) and FPN1 (1:200, DF13561; Affinity). Subsequently, the membrane was incubated with the corresponding HRP-conjugated secondary antibody (1:10,000 in TBS-T buffer) for 2 h at room temperature. The protein bands were visualized using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, USA) and the ECL Kit (Boster, Wuhan, China). GAPDH was used as the internal control.

Statistical analysis

The data are represented as the mean ± SEM. The intergroup comparison was assessed by the t test in GraphPad Prism 8.0.1 (GraphPad Software, San Diego, USA). P < 0.05 was considered statistically significant.

Result

Characterization of H-uc MSCs

Under an optical microscope, the P4 H-uc MSCs were found to have adherent growth ability and a spindle-shaped appearance (Figure 1A). To identify the pluripotency of H-uc MSCs, we effectively induced their differentiation into both adipocytes and osteoblasts using specific differentiation media. Alizarin red S staining verified the deposition of red calcium salt and the formation of mineralized nodules (Figure 1B). The formation of lipid droplets was verified by Oil red O staining (Figure 1B). Subsequently, flow cytometry was employed to identify the phenotype markers of H-uc MSCs. As shown in Figure 1C, the percentages of CD105−, CD90− and CD73-positive cells were 100%, 99.5%, and 99.9%, respectively, and the percentages of CD34−, CD11b−, CD19−, CD45- and HLA-DR-negative cells were less than one percent. H-uc MSCs could be effectively labelled by Dil, and the labelling efficiency was approximately 99% (Figure 1D).

Ferroptosis occurred in CCL4-induced ALF mice

As shown in Figure 2A, compared with the NC groups, typical liver histopathological changes in ALF, such as moderate and heavy hepatic steatosis and diffuse hepatic necrosis, were observed in the liver tissues from the CCL4-treated groups under an optical microscope. The liver function indexes...
AST and ALT were measured, and it was found that the levels of AST and ALT increased as the concentration of CCL4 increased (Figure 2B). Survival curves were used to describe the survival status of the CCL4-treated and NC mice. The results showed that the survival rate of mice decreased with increasing CCL4 concentration, and there was an approximate 50% survival rate in the mice treated with 2.5 ml/kg CCL4 (Figure 2C). Therefore, 2.5 ml/kg CCL4 was selected for subsequent experiments. To investigate the impact of ferroptosis on CCL4-induced ALF, we examined markers of lipid peroxidation and the antioxidant system. We found that after CCL4 treatment, serum levels of MDA and liver tissue levels of Ptgs2 were significantly increased (Figure 2D–F). In contrast, serum levels of GSH and liver tissue levels of Gpx4 were robustly decreased (Figure 2D–F). Iron deposition in tissues is a distinct marker of ferroptosis. Our findings indicated that treatment with CCL4 resulted in more noticeable iron deposition in the liver compared with the NC group (Figure 2G). Hepcidin expression in the liver was consistently upregulated in CCL4-treated mice (Figure 2E,F). Taken together, CCL4-induced ALF changes the variables associated with ferroptosis and increases hepcidin protein levels.

**Intravenous MSC treatment protected against ferroptosis in ALF mice**

To enhance our understanding of the relationship between ferroptosis and MSCs, we employed H-ucMSCs for intervention in CCL4-treated mice. Hepatomegaly is the most common clinical symptom in patients with acute liver disease[34, 35]. Gross examination showed that under the treatment of MSCs, the liver surface of mice changed from gray-white to a ruddy hue, and the size of the liver went from large to small. This indicated that MSCs effectively reversed the increased liver injury in CCL4-treated mice (Figure 3A). We also observed that treatment with MSCs could accelerate the amelioration of liver function in ALF mouse models (Figure 3B). As shown in Figure 3C, HE staining revealed limited areas of hepatic steatosis and hepatic necrosis in the MSC intervention group. There were also significant changes in the protein levels of classic biomarkers associated with ferroptosis. We found that serum levels of MDA and liver tissue levels of Ptgs2 were significantly decreased, while serum levels of GSH and Gpx4 were robustly increased in the MSC intervention group (Figure 3D–F). These findings demonstrated that intravenous MSC treatment rapidly restored liver damage and inhibited ferroptosis progression in CCL4-induced ALF mice.

**MSCs diminished liver iron deposition via the hepcidin-FPN1 axis in ALF mice**

Iron homeostasis plays an essential role in the occurrence of ferroptosis. Transporting iron from cells
to the extracellular space is an important mechanism for controlling and maintaining iron homeostasis. The iron storage protein ferritin and ferric uptake regulator transferrin receptor 1 (TfR1) were detected by immunohistochemistry. Iron absorption and storage were enhanced in the liver in ALF mice, leading to liver iron deposition (Supplementary Figure S1). As shown in Figure 4A,B, MSC treatment reduced the iron content of liver tissue compared with that in the PBS-treated CCL<sub>4</sub> group. The regulation of hepcidin protein dynamically determines the transport of iron in the body. Immunohistochemical results and western blot analysis indicated that hepcidin protein levels were enhanced in the CCL<sub>4</sub> group treated with PBS, but these levels were restored after MSC treatment (Figure 4C,D). It should be noted that the increased expression of hepcidin protein directly leads to a decrease in the FPN1 protein level, resulting in a barrier to the output of intracellular iron. Our study also revealed that the correlation between hepcidin and FPN1 expression was increased in response to MSC treatment compared to that in the PBS-treated CCL<sub>4</sub> group, which was contrary to the change in hepcidin levels (Figure 4F). The findings indicated that MSCs reduce liver iron content in ALF mice through the hepcidin-FPN1 axis.

**Circulating IGF1 is needed for the inhibitory effect of MSCs on ferroptosis in ALF mice**

Subsequently, we investigated and examined how MSCs exerted their protective potential against CCL<sub>4</sub>-induced ferroptosis. To assess the liver distribution of intravenously injected MSCs, immunofluorescence staining of the macrophage marker CD11b was performed. In the liver, Dil-MSCs were distributed in the hepatic sinusoid and showed colocalization with CD11b fluorescence. This result indicated that intravenously injected MSCs could migrate to the liver tissue but were engulfed by macrophages (Figure 5A). Therefore, we hypothesized that MSC treatment exerts a potent therapeutic effect by elevating the release of some factors in the injured body. Considering the significant role of IGF1 in the early stages of repair and regeneration of the liver, our research focused on the IGF1 protein. Serum samples were collected from mice in each group, and IGF1 levels were measured using ELISA. Our results showed significantly higher levels of serum IGF1 in the MSC treatment model than in the CCL<sub>4</sub>-treated groups (Figure 5B). Subsequently, we assessed the expression changes of related molecules in the IGF1-IGF-1R system after MSC treatment. Immunohistochemical staining of the liver revealed increased expression of IGF-1R in the group treated with MSCs compared to the group treated with PBS (Figure 5C). Next, we administered the IGF1 receptor inhibitor PPP to mice treated with MSCs for three consecutive days via intraperitoneal injection.
injections and confirmed the role of IGF1 in MSC treatment efficacy by blocking the role of IGF1. The ALF with MSC treatment exhibited significantly higher survival rates than the ALF with MSC and PPP treatments (Figure 5D). Furthermore, we observed further exacerbation of ferroptosis in the PPP groups. As shown in Figure 5E–G, serum levels of MDA and liver tissue levels of Ptgs2 were downregulated in the CCL₄-MSC-vehicle group compared with the CCL₄-MSCs-PPP group, while the changes in serum levels of GSH and liver tissue levels of Gpx4 were significantly reversed between the CCL₄-MSCs-PPP group and the CCL₄-MSC-vehicle group. These findings indicated that IGF1 plays an essential role in the treatment of MSCs against ferroptosis in ALF mice.

**MSCs diminished liver iron deposition via the IGF1-hepcidin-FPN1 axis in ALF mice**

To investigate the potential of MSCs in reducing liver iron deposition by modulating circulating IGF1 levels, we employed the PPP again and measured the total iron content and hepcidin expression. Iron deposition in the liver was significantly exacerbated in the CCL₄-MSCs-PPP group when the role of IGF1 was blocked (Figure 6A,B). As shown in Figure 6C,D, immunofluorescence staining and WB analysis revealed that hepcidin expression increased in the inhibitor group even after receiving MSC injections. Furthermore, FPN1 expression in the inhibitor group exhibited an inverse correlation with hepcidin protein levels (Figure 6C,D). This study showed that MSCs decreased liver iron content by regulating the IGF1-hepcidin-FPN1 axis in ALF mice.

**Discussion**

Multiple forms of cell death, including necrosis, apoptosis, and necroptosis, take part in the occurrence and progression of liver injury. For example, Guo et al. demonstrated that the absence of MLKL alleviates liver fibrosis by suppressing hepatocyte necroptosis[36]. Moreover, apoptosis and autophagy also play significant roles in CCL₄-induced hepatocellular injury [37-40]. Since the liver is the main organ of the human body for storing iron, we primarily focused on the mechanistic basis of ferroptosis in ALF. Sun et al. demonstrated that targeting ferroptosis was a new challenge and opportunity as a potential strategy for treating ferroptosis-related diseases[41]. Upregulating the expression of GPX4 could diminish ferroptosis in hepatocellular carcinoma[42, 43]. Wei et al. demonstrated that aqueous extracts of M. mori fructus attenuate CCL₄-induced liver injury by inhibiting ferroptosis via the Nrf2 pathway [44]. In this study, an acute and critical ALF mouse model was established. In addition to the increasing region of hepatic steatosis and hepatic necrosis, ALF
livers also experienced significant iron deposition, imbalances in the anti-redox system, and accumulation of lipid peroxides. Furthermore, the therapeutic effect of MSC treatment was observed in reducing lipid peroxide accumulation and iron deposition while increasing the antioxidant system level in ALF mice. Our results showed that ferroptosis played a crucial role in CCL₄-induced ALF, and MSCs had a protective role in resisting ferroptosis.

Excessive iron levels lead to significant cellular dysfunction, especially in liver tissue, which is regarded as the primary organ for iron storage[45]. Ferroptosis has been observed in lipopolysaccharide-induced liver injury, and retinoic acid can protect against liver injury by regulating iron homeostasis[46]. Iron overload exacerbated lipid metabolism disorder and liver injury in rats with nonalcoholic fatty liver disease[47]. Hepcidin is a main regulator of iron homeostasis within hepatocytes and can trigger the degradation of FPN1. FPN1 is the exclusive nonheme iron export protein in mammals[48]. Numerous studies have shown that the dysregulation of hepcidin secretion and altered iron homeostasis impact the progression of liver diseases[49]. Bao et al. found that iron overload in hereditary tyrosinemia type 1 induced liver injury through the Sp1/Tfr2/hepcidin axis[50]. Many hepcidin antagonists have been explored and reported to have promising therapeutic potential in regulating iron homeostasis[51, 52]. In the present study, we found that iron overload existed in the CCL₄-induced ALF mice. Elevated levels of hepcidin and reduced FPN1 expression were associated with iron overload in ALF mice. Furthermore, our results showed that MSC treatment played an important role in reducing iron deposition through the downregulation of hepcidin expression and the upregulation of FPN1 expression in ALF mice. Thus, we concluded that MSCs protected against CCL₄-induced liver ferroptosis through the hepcidin-FPN1 axis.

Numerous studies have reported that IGF1 could promote tissue regeneration and improve the recovery function of tissue by preventing oxidative stress[53, 54]. Kokoszko et al. found that IGF1 can prevent iron-induced oxidative damage in iron-sensitive tissues[55]. Our study unequivocally confirmed that blocking IGF1 still exacerbated oxidative damage in ALF mice despite the intravenous injection of MSCs. Additionally, numerous studies have shown that IGF1 positively regulates erythropoiesis by increasing erythroid mass, erythropoietin synthesis, and iron bioavailability[56, 57]. Insulin/IGF1 signaling could regulate iron homeostasis in Caenorhabditis elegans. Kucic et al. showed that MSCs genetically engineered to overexpress IGF1 could enhance cell-based gene therapy for renal failure-induced anemia[58]. In our study, we found that inhibiting IGF1 could increase iron loading status in the livers of ALF mice. Therefore, it is suggested that IGF1 could be
related to liver iron homeostasis. The hepcidin level reflects the bioavailability of Fe in the body[59, 60]. An increasing number of cytokines, including growth hormone, sex hormones, growth differentiation factor 15, hepatocyte growth factor, and epidermal growth factor, have been shown to influence hepcidin levels. Only a limited number of studies have demonstrated a negative correlation between the levels of IGF1 and hepcidin in acromegaly patients and healthy people[61]. Our study confirms that blocking IGF1 could increase hepcidin levels. Additionally, IGF1 may function as one of the suppressors of hepcidin. Although it has been proven that IGF1 has no effect on the induction of hepcidin mRNA by BMP6 in vitro[62], hepcidin expression is also regulated by JAK-STAT signaling in addition to BMP-SMAD signaling[63, 64]. Therefore, it is necessary to enhance the experimental design to firmly establish this point in subsequent stages.

In conclusion, our study demonstrates an increased hepcidin level in CCL4-induced ALF mice compared to NC mice, which may initiate liver iron deposition and lead to ferroptosis in CCL4-induced ALF mice. Furthermore, it has been demonstrated that MSCs possess antiferroptotic effects by increasing IGF1 levels in ALF. However, the specific mechanism remains to be further studied.

Supplementary Data

Supplementary data is available at Acta Biochimica et Biphysica Sinica online.

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Conflict of Interest

The authors declare that they have no competing interests.

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**Figure Legends**

Figure 1. Characterization of H-uc MSCs. A. Optical micrographs of morphological characterization of MSCs at passage 4 (P4). B. Osteogenic differentiation of MSCs assessed by alizarin red staining. Adipogenic differentiation of MSCs assessed by Oil Red O staining. The magnification is ×200. C. Flow cytometry analysis for specific phenotype markers of MSCs at P4. D. Flow cytometry analysis of the labelling efficiency of Dil-labelled MSCs.

Figure 2. Effects of CCL4 exposure on liver histoarchitecture and iron homeostasis in mice. A. Representative images of the liver subjected to H&E staining. The magnification is ×200 and ×400. B. The levels of the liver function indexes AST and ALT were measured. C. Survival curves for mice treated with different concentrations of CCL4. D. The levels of MDA and GSH in the liver were measured. E. Immunolocalization of Ptgs2, Gpx4 and hepcidin proteins after CCL4 exposure. The magnification is 100×. F. The mean optical density (MOD) of Ptgs2, Gpx4 and hepcidin proteins was calculated from 3 stained pictures of three mice per group. G. The levels of total iron content in the liver were measured. Data are expressed as the mean ± SE. ns: no significant difference. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 3. MSC treatment prevented CCL4-induced ferroptosis in ALF. A. Representative gross morphology images of the liver. B. The levels of the liver function indexes AST and ALT were
measured. C. Representative images of the liver subjected to H&E staining. The dashed boxes indicate the necrotic area of the liver. The magnification is ×100 and ×400. D-E. Immunolocalization of Ptgs2 and Gpx4 proteins in the NC, CCL4 + PBS, and CCL4 + MSC groups (n = 3). The magnification is 100×. The MOD of Ptgs2 and Gpx4 proteins was calculated from 3 stained pictures of three mice per group. F. The levels of MDA and GSH in the liver were measured. Data are expressed as the mean ± SE. ns: no significant difference. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 4. MSC treatment diminished liver iron deposition by regulating the hepcidin-FPN1 axis in ALF mice. A. The levels of iron in the liver were measured. B. Representative images of Prussian blue iron staining in the liver. The magnification is 100×. C. Immunolocalization of hepcidin protein in the NC, CCL4 + PBS, and CCL4 + MSC groups. The magnification is 100×. The MOD of hepcidin protein was calculated from 3 stained pictures of three mice per group. D. WB analysis of the protein levels of hepcidin, BMP6 and FPN1 per group (n = 3). Data are expressed as the mean ± SE. ns: no significant difference. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 5. The role of IGF1 in ALF mice with MSC treatment. A. Immunofluorescence staining of the macrophage marker CD11b in the liver. The magnification is ×1000 and ×3000 B. Serum IGF1 expression was detected by ELISA. C. Immunohistochemical staining of IGF-1R proteins in the NC, CCL4 + PBS, and CCL4 + MSC groups (n = 3). The magnification is 100×. D. The survival curves of mice in each group. E-F. The levels of MDA and GSH were measured. G. Immunolocalization of Ptgs2 and Gpx4 proteins in the NC, CCL4 + PBS, and CCL4 + MSC groups (n = 3). The magnification is 100×. Data are expressed as the mean ± SE. ns: no significant difference. *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 6. The role of IGF1 in iron deposition in ALF mice treated with MSCs. A. The levels of iron in the liver were measured. B. Representative images of Prussian blue iron staining. The magnification is 100×. C. Immunolocalization of hepcidin protein per group. The magnification is 100×. The MOD of hepcidin protein was calculated from 3 stained pictures of three mice per group. D. WB analysis of hepcidin and FPN1 protein levels per group (n = 3). Data are expressed as the mean ± SE. ns: no significant difference. *P < 0.05, **P < 0.01, and ***P < 0.001.
A  H-ec MSCs

B  Adipogenesis
Osteogenesis

C

D

143x119mm (300 x 300 DPI)
For Peer Review

150x149mm (300 x 300 DPI)
A

铁浓度变化

B

Perls stain

C

Hepcidin

D

Hepcidin

GAPDH

FPN1

相对蛋白水平

199x146mm (300 x 300 DPI)
164x105mm (300 x 300 DPI)
Fig. S1 MSC treatment prevented CCl\textsubscript{4}-induced iron absorption and storage in ALF. Immunolocalization of ferritin and TfR1 proteins in NC, CCL\textsubscript{4} + PBS, and CCL\textsubscript{4} + MSC groups (n = 3). The magnification is ×100.
The findings in present study elucidate that H-uc MSCs could inhibit iron loading through the IGF1-Hepcidin-FPN1 axis in ALF mice, thereby protecting against ferroptosis and reducing the mortality of ALF mice induced by CCl₄.

1. Mesenchymal stem cells (MSCs) can reduce the mortality of acute liver failure (ALF) induced by CCl₄.
2. Intravenous MSCs treatment protect against ferroptosis through diminishing liver iron deposition in ALF mice.
3. MSCs diminish liver iron deposition via the IGF1-hepcidin-FPN1 axis in ALF mice.
160x148mm (300 x 300 DPI)