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Original Article

Therapeutic effect of human umbilical cord mesenchymal stem cell transplantation in rats with hepatic fibrosis

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Abstract: Objective: This study aimed to investigate the therapeutic effects of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) transplantation on hepatic fibrosis. Methods: CCl_4 was employed to induce Wistar rats to build a liver fibrosis model. The rats randomly fell to the normal control group (CCl_4/saline 0 wk groups); Liver fibrosis model (CCl_4/sline groups) 1, 2, 4 weeks group; MSCs transplantation (CCl_4/MSCs groups) 0, 1, 2, 4 weeks group. Hematoxylin and eosin (H&E) was conducted for morphological evaluation and Masson trichrome (MT) to evaluate the inflammation and degree of fibrosis. In these rats, the levels of following liver function indexes were detected: (e.g., serum glutathione aminotransferase (ALT), glutathione aminotransferase (AST), albumin (ALB), total bilirubin (TBIL) and direct bilirubin (DBIL)), as well as oxidative stress indexes (e.g., malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione (GSH)). Results: hUC-MSCs transplantation was capable of enhancing liver function, elevating pathological inflammation score and improving fibrous tissue deposition in rats with liver fibrosis. After the hUC-MSCs transplantation was conducted, the expressions of SOD, GSH and GPX in liver tissues were noticeably up-regulated, and the expression of MDA was significantly down-regulated in all transplantation groups, except for the 1-week transplantation group, the mentioned results were not significantly different from those of the corresponding model group. Conclusion: hUC-MSCs transplantation is capable of improving liver fibrosis, but fails to reverse liver fibrosis, it can only decelerate the process of liver fibrosis.

Keywords: Hepatic fibrosis, mesenchymal stem cells, oxidative stress

Introduction

Liver fibrosis refers to the liver's repair response to chronic inflammation, necrosis, or other injuries. In addition, cirrhosis is recognized as the end result of liver fibrosis [1]. Liver transplantation is now considered the only effective treatment for end-stage cirrhosis, whereas it is difficult to be extensively performed for numerous factors (e.g., lack of liver sources, complications, rejection reactions and high prices) [2]. Mesenchymal stem cells (MSCs) act as mesoderm-derived stem cells that exhibit multidirectional differentiation potential, which achieve several advantages (e.g., abundant source, low immunogenicity, low invasiveness and simple operation). These cells have been extensively reported in bone marrow, umbilical cord, umbilical blood, peripheral blood and adipose [3]. Promising progress has been achieved in clinical and subclinical trials to treat acute myocardial infarction [4], stroke, acute kidney injury [5] diabetes [6], fractures [7], pulmonary hypertension [8], as well as liver disease [9, 10]. Besides, it has been employed clinically to treat osteogenesis imperfecta and severe graft-versus-host disease [11]. However, the mechanism of action remains not perfectly defined [12]. Thus, the clarification of the effect of MSCs in liver injury-like diseases and the exploration of their specific regulatory mechanisms have been an urgent problem for basic research on stem cell transplantation. Human umbilical cord-derived MSCs (hUC-MSCs), a novel type of MSCs, have not been studied extensively. As revealed from our preliminary study, hUC-MSCs could differentiate into functional hepatocytes and then facilitate the biochemical and histopathological

variations in CCl_4-induced liver fibrosis model in rats [13]. However, oxidative stress and other related mechanisms have been rarely studied at home and abroad. In the present study, the therapeutic effects of hUC-MSCs transplantation on rats with liver fibrosis were investigated, as well as the effects of oxidative stress in a CCl_4-induced rat liver fibrosis model, as an attempt to lay an experimental and theoretical basis for clinically treating liver fibrosis.

Materials and methods

MSC isolation and culture

The umbilical cord was cut into 1-mm^3 pieces and then filtered via a 1.5 mm mesh. Subsequently, the seeding process was conducted in DMEM/F12 complete medium. The fresh medium was replaced per 3 to 4 day, and nonadherent cells were discarded. When the observed cells achieved 80% confluency, hUC-MSCs were separated by trypsin digestion, then cultured and subsequently identified by Alliancells Bioscience Co., Ltd.

Animal models

64 adult male Wistar rats, weighing 350-450 g, were obtained from the Experimental Animal Center of Hebei Medical University. This study was conducted by complying with the internationally accepted principles for laboratory animal use and care, as reported in the US guidelines (NIH publication #85-23, revised in 1985). The experiment was performed following the national ethical guidelines for the care and use of laboratory animals (Certificate No. 911102). All animals randomly fell to the following groups below. ① Normal control group (CCl_4/saline 0 wk groups) (n = 8). ② Liver fibrosis model (CCl_4/sline groups) 1, 2 and 4 weeks group (n = 8). The rats were executed at the 1st, 2nd and 4th week after the successful modeling by being injected with saline into the tail vein of the experimental rats. ③ hUC-MSCs transplantation (CCl_4/MSCs groups) 0, 1, 2 and 4 weeks group (n = 8), i.e., the rats were executed at the 1st, 2nd and 4th weeks after the successful modeling by tail vein injection of hUC-MSCs 5×10^6 rats each. While CCl_4/MSCs 0 wk group means successful modeling. CCl_4/sline groups and CCl_4/MSCs groups were set by the hypodermic injection of CCl_4 mixed with olive oil at the concentration of 40% (2 mL/kg) twice a week. Rats injected with saline was employed as a control group. As indicated from Masson trichrome staining, at the 4th week of the injection, the structure of liver lobules was disturbed, and increased fibrous deposits were observed around the central vein and in the confluent area, which suggested the successful establishment of a liver fibrosis model. Rats were continuously treated with CCl_4. After 1, 2, or 4 weeks of hUC-MSCs infusion, rats were sacrificed to evaluate the related index of liver fibrosis.

Serum parameter determination

The rats randomly fell to 8 groups. Besides, the rats were placed in metabolic cages before sampling, fasted without water, and then weighed after 24 h. The skin of the chest was disinfected, and the needle was inserted at the strongest apical pulsation. Moreover and 5 mL of blood was extracted from the left ventricular artery of the rats and then centrifuged at 900 r/min to extract the upper layer of serum. Under aseptic conditions, the skin was cut into the abdominal cavity, and the whole liver was rapidly separated, rinsed in saline, frozen in liquid nitrogen and subsequently stored in a refrigerator at -80°C for the further application. The supernatant was extracted, and the antioxidant indexes (e.g., SOD, MDA, GSH and GPx were measured separately by complying with the kit instructions, and ALT, AST, ALB, TBIL and DBIL were detected in the serum by employing BECKMAN COULTER CX9 automatic biochemical analyzer.

Histopathology

Liver specimens were fixed for 12-24 h in 4% phosphate-buffered paraformaldehyde (Huarui Scientific and Technological Co.) and subsequently embedded in paraffin for light microscopy examination. Next, tissue sections (3 μm thick) were stained with hematoxylin and eosin (H&E) for morphological evaluation and Masson trichrome (MT) to determine the degree of fibrosis.

Statistical analysis

Data were expressed as the mean ± SD and then analyzed with SPSS26.0 software. The performed statistical analyses consisted of

one-way ANOVA and the LSD test analysis. $P < 0.05$ was statistically significant.

Results

Characterization of hUC-MSCs

hUC-MSCs that exhibited a fibroblast-like morphology were isolated from the umbilical cord (**Figure 1**). As indicated from the results of flow cytometry, hUC-MSCs expressed high levels of MSCs-specific markers CD90, CD105 and CD73, whereas they expressed no or low levels of CD34, CD19, CD11b, HLA-DR and CD45, demonstrating that the cells isolated and cultured here were hUC-MSCs.

hUC-MSCs reverse the CCl_4-induced liver histopathological changes

The liver of normal control rats was dark red in color, lustrous and soft, which exhibited a fine and smooth surface and sharp edges. After CCl_4 was injected into the model group rats, the liver gradually swelled, the liver color tended to be dull, and the surface was rough and uneven. As compared with the model group, the liver in the hUC-MSCs transplantation group exhibited a slightly reddish color and a relatively smooth surface (**Figure 2**).

As indicated from the results of HE staining, the liver specimens of normal control rats had intact liver lobules with clear boundaries. In addition, hepatocytes did not exhibit any vacuole-like variations or degenerative necrosis; the liver plates were regularly arranged and neat, and bile duct hyperplasia and inflammatory cell infiltration were not identified in the confluent area. After CCl_4 was injected into the model group, the liver plates were disordered, hepatocytes were edematous, and inflammatory cell infiltration in the confluent area was significantly facilitated. As compared with the model group, in hUC-MSCs, the hepatocyte necrosis, vacuole-like variations and inflammatory cell infiltration were reduced in the transplanted group in comparison with the model group. The inflammation scores decreased significantly at all times compared with the model group, except for the CCl_4/MSCs 1 wk group, in which the inflammation scores were not significantly down-regulated (**Figure 2**).

As indicated from the results of Masson trichrome staining, fibrous tissue staining of the central venous canal wall of the liver lobules was identified in normal control rats, and a small amount of fiber deposition could be observed in the confluent area and lobular septum. In CCl_4/MSCs 0 wk (before MSCs transplantation), the structure of the liver lobules was disturbed, the improved fiber deposition was observed around the central vein and confluent area, and liver fibrosis was formed (**Figure 2**), thereby demonstrating the successful modeling of liver fibrosis. Compared with the model group, the fibrosis degree grading decreased significantly at all times, except for the CCl_4/MSCs 1 wk group, in which the fibrosis degree grading did not decline significantly. The results of quantitative analysis of intrahepatic fibrous tissue deposition also showed consistent results.

hUC-MSCs improve the biochemical indexes in the CCl_4-induced hepatic injury model

hUC-MSCs improve the biochemical indexes in the CCl_4 induced hepatic injury model in rats that has acted as a model system to study liver damage and fibrosis. Besides, this model was used to evaluate the therapeutic effect of hUC-MSCs. The hUC-MSCs were transplanted into a CCl_4-induced liver fibrotic rat model, and a significant reduction was observed in the serum levels of ALT, AST, TBIL and DBIL at 2 and 4 wk in the MSCs/CCl_4 groups ($P < 0.05$). In addition, the serum level of ALB tended to decline with the further CCl_4 induction in the saline/CCl_4 groups, whereas after the transplantation of hUC-MSCs, the serum levels of ALB markedly were up-regulated at 2 and 4 wk in the MSCs/CCl_4 groups, respectively ($P < 0.05$) (**Table 1**). This data in this section complied with a normal distribution, the data were expressed as mean ± SD, and one-way ANOVA and LSD tests were applied for statistics.

hUC-MSCs play an antioxidant role by up regulating SOD, GSH, GPx expression and down-regulating MDA expression

To detect the mechanism by which hUC-MSCs exert an antioxidant effect, the activity of SOD, the content of GSH, GPX and MDA in liver tissue of the respective group were detected by using the kit. With the extension of the CCl_4 induction time, in both the saline/CCl_4 groups and MSCs/CCl_4 groups, the activity of SOD and content of GSH, GPX tended to decrease, while the content of MDA tended to increase. After the identi-

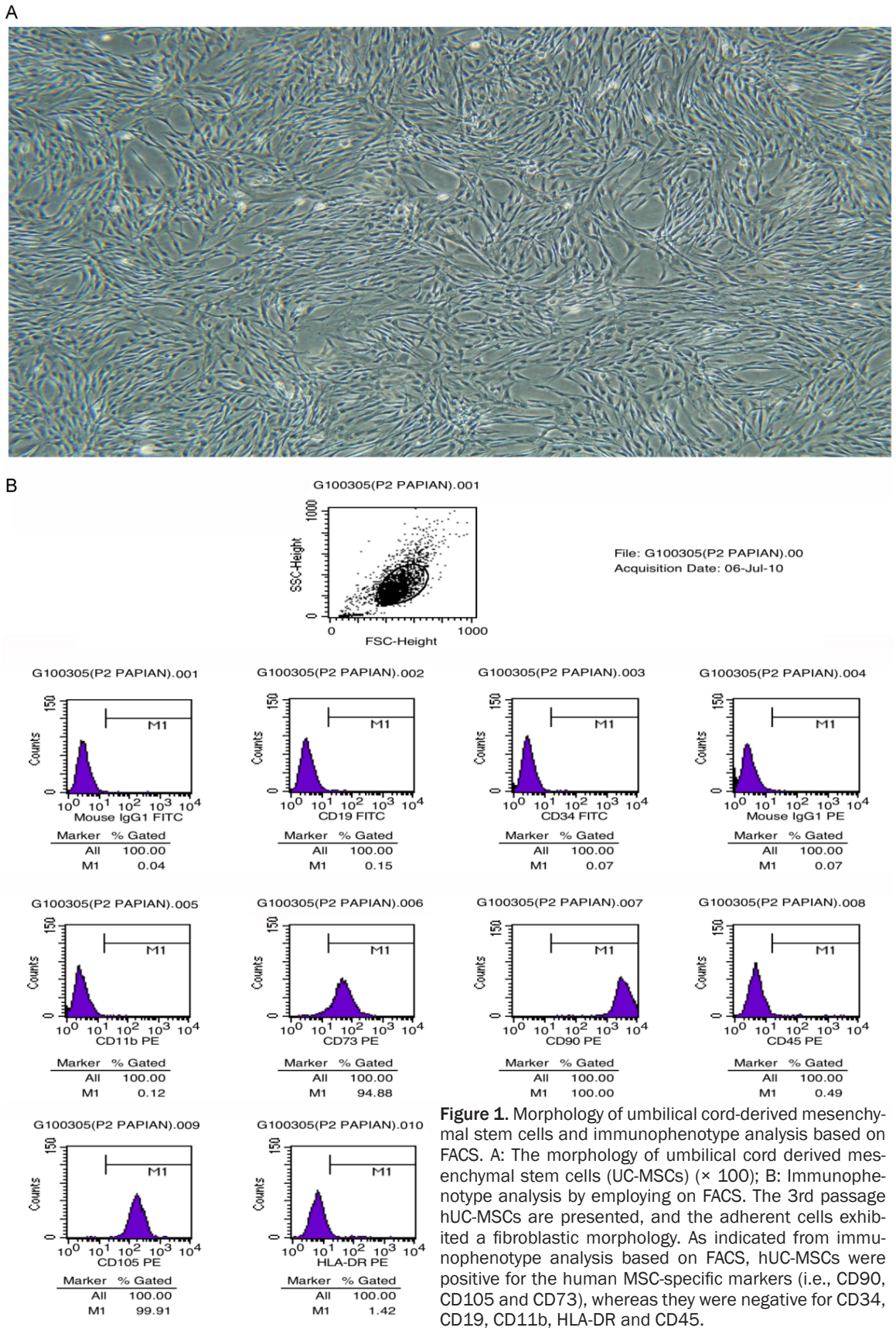

Figure 1. Morphology of umbilical cord-derived mesenchymal stem cells and immunophenotype analysis based on FACS. A: The morphology of umbilical cord derived mesenchymal stem cells (UC-MSCs) (× 100); B: Immunophenotype analysis by employing on FACS. The 3rd passage hUC-MSCs are presented, and the adherent cells exhibited a fibroblastic morphology. As indicated from immunophenotype analysis based on FACS, hUC-MSCs were positive for the human MSC-specific markers (i.e., CD90, CD105 and CD73), whereas they were negative for CD34, CD19, CD11b, HLA-DR and CD45.

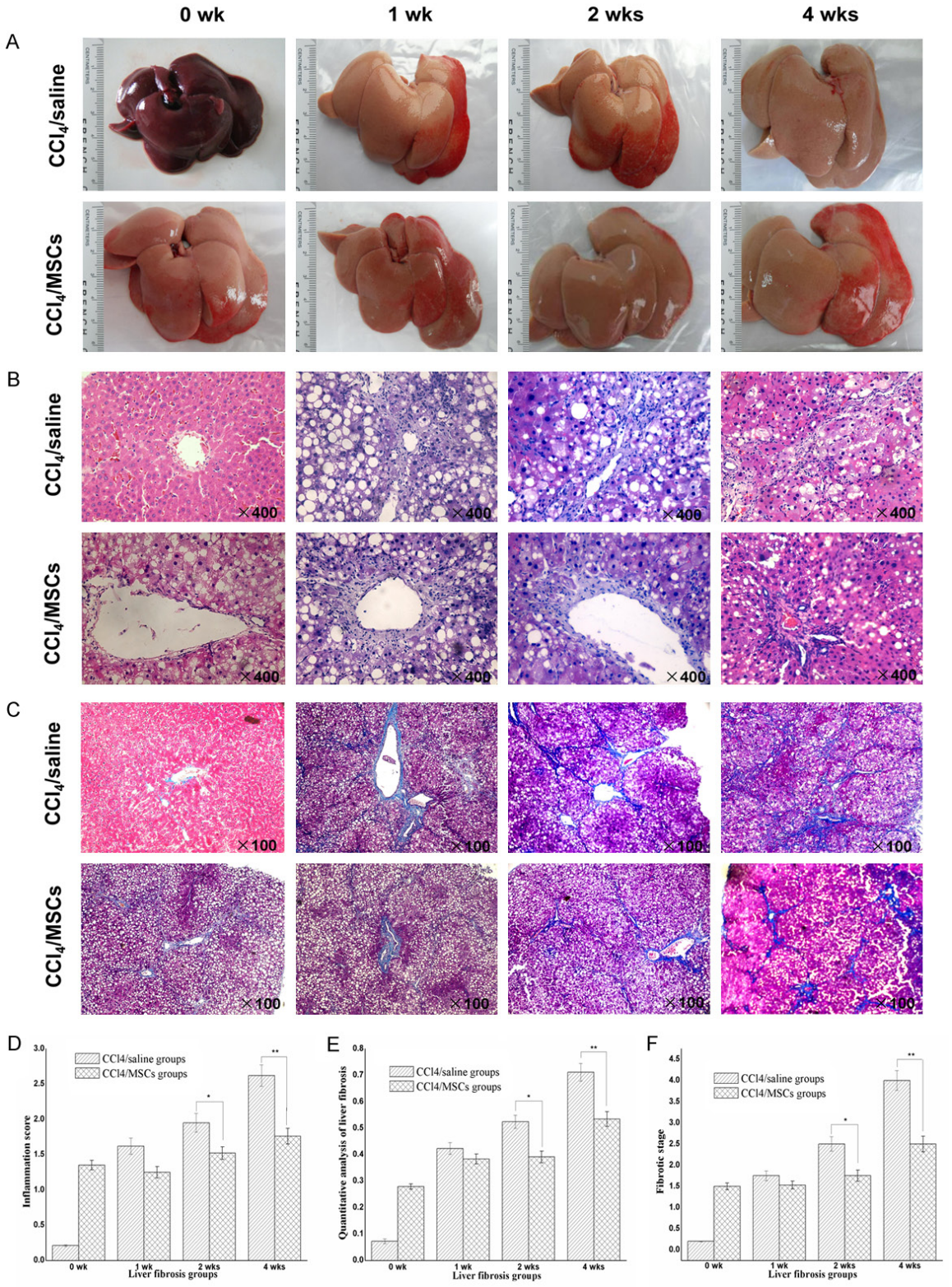

Figure 2. Histopathology of rats in the model group and hUC-MSCs transplantation group. A: Gross liver specimens of each group; B: Hematoxylin and eosin (H&E) for morphological evaluation of each group (× 400); C: Masson trichrome (MT) of each group (× 100); D: Inflammation score of each group; E: Quantitative analysis of fibrosis tissue in liver fibrosis; F: Fibrotic stage of the respective group. Notes: *P < 0.05 vs CCl_4/saline 2 wks, **P < 0.01 vs CCl_4/saline 4 wks.

Table 1. Effect of hUC-MSCs transplantation on liver function in CCl_4-induced hepatic fibrosis

Groups	ALT (U/L)	AST (U/L)	ALB (g/L)	TBIL (μmol/L)	DBIL (μmol/L)
CCl_4/saline 0 wk	52.34±3.88	66.07±4.16	40.12±2.89	0.54±0.10	0.32±0.04
CCl_4/MSCs 0 wk	124.78±9.56	127.44±7.65	33.27±2.11	0.88±0.11	0.51±0.05
CCl_4/saline 1 wk	141.00±16.33	150.76±15.13	29.99±1.53	0.97±0.15	0.70±0.02
CCl_4/MSCs 1 wk	130.32±15.35	142.32±14.24	32.17±2.25	0.93±0.08	0.60±0.04
CCl_4/saline 2 wks	152.35±14.56	172.22±19.34	26.60±2.34	1.51±0.21	0.90±0.07
CCl_4/MSCs 2 wks	132.88±13.23[a]	146.23±13.23[a]	28.12±2.13[a]	1.33±0.25[a]	0.70±0.05[a]
CCl_4/saline 4 wks	239.24±26.32	262.34±28.12	24.54±1.21	2.10±0.23	1.10±0.17
CCl_4/MSCs 4 wks	192.32±21.24[b]	204.52±23.24[b]	26.55±1.51[b]	1.57±0.15[b]	0.50±0.03[b]

Notes: [a]$P < 0.05$ vs CCl_4/saline 2 wks, [b]$P < 0.05$ vs CCl_4/saline 4 wks.

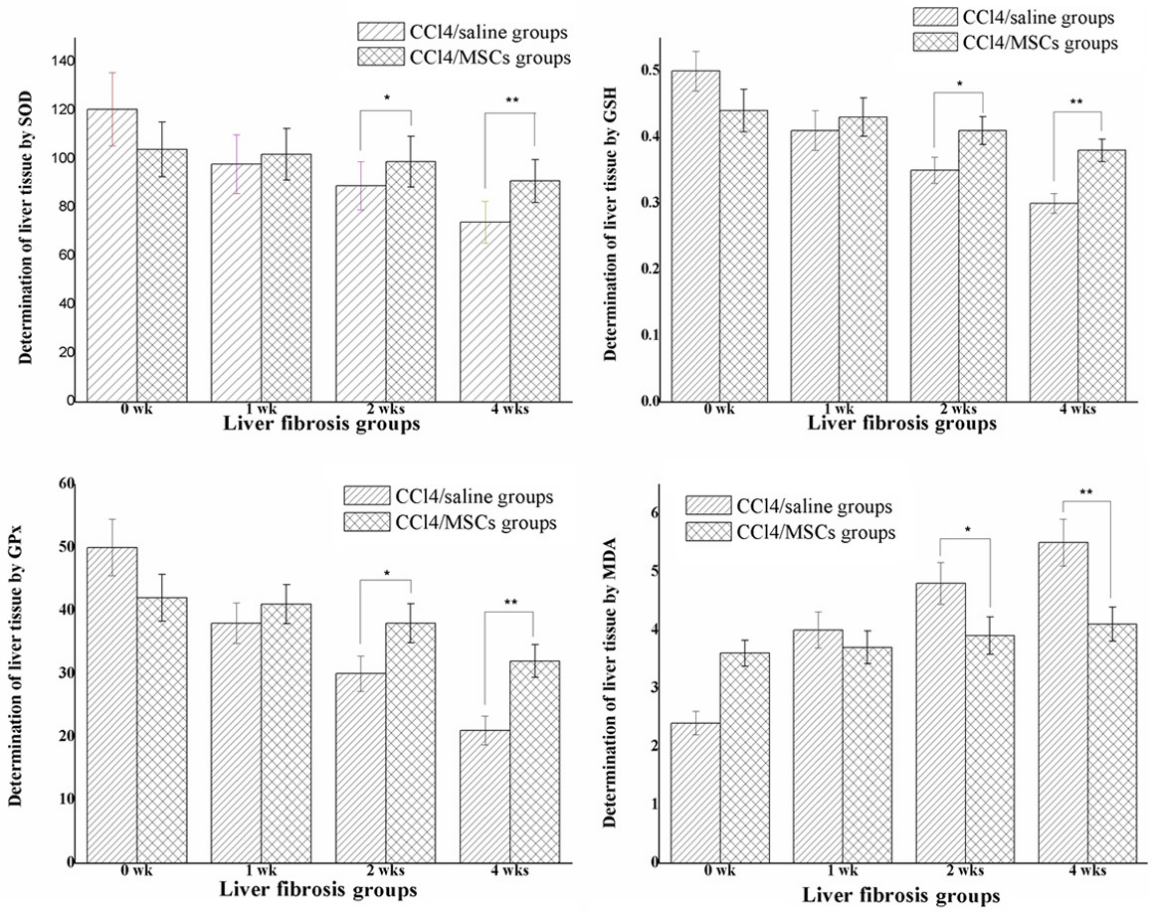

Figure 3. Determination of SOD, MDA, GSH and GPx in liver tissue. Notes: *$P < 0.05$ vs CCl_4/saline 2 wks, **$P < 0.01$ vs CCl_4/saline 4 wks.

cal CCl_4 injection, SOD, GSH and GPX in the MSCs/CCl_4 groups significantly increased ($P < 0.05$) as compared with the saline/CCl_4 groups. However, the expression of MDA was significantly down-regulated ($P < 0.05$) compared with the saline/CCl_4 groups. Except in the first week after the transplantation of hUC-MSCs in the MSCs/CCl_4 groups (**Figure 3**). The data in this section complied with a normal distribution, and the data were expressed as mean ± SD, and one-way ANOVA and LSD tests were applied for statistics.

Discussion

As revealed from the results of this study, after subcutaneous administration of CCl_4 olive oil solution was applied, the arrangement of liver plate was disturbed, hepatocyte edema and inflammatory cell infiltration in the confluent area were also significantly increased. The structure of liver lobules was disturbed, the improved fibrous deposition was identified around the central vein and in the confluent area, and liver fibrosis was formed, complies with similar studies reported [14]. Besides, a rat liver fibrosis model was successfully built. The results of liver pathological histological examination in rats showed that hUC-MSCs transplantation significantly improved hepatocyte necrosis and reduced inflammatory response and fibrous tissue deposition in rats with liver fibrosis, which complied with the therapeutic effect of bone marrow-derived MSCs transplantation [15, 16].

Liver function is recognized as a vital measure of hepatocyte damage, and ALT and AST levels can reflect the degree of liver tissue damage, and ALB levels can act as a sensitive indicator of liver function to assist in the diagnosis of liver disease. Some studies have shown that implantation of bone marrow-derived MSCs into mice with CCl_4-induced cirrhosis resulted in improved liver function and reduced mortality [15, 16]. In the present study, as indicated from the serological assays, hUC-MSCs transplantation resulted in a significant decrease in AST and TBIL and a significant increase in ALB, with statistically significant differences with those of the model group, demonstrating that hUC-MSCs transplantation has a therapeutic effect on liver fibrosis, which complies with the results of the above studies.

Existing studies concluded that oxidative damage is a vital pathological mechanism of liver injury, and the liver, as an important class of metabolic organs, is vulnerable to free radical attack. Parenchymal cells are the bearers of liver function, and mitochondria, microsomes and peroxisomes in parenchymal cells can produce large amounts of free radicals, leading to oxidative stress damage in parenchymal cells and eventually liver dysfunction [17]. The antioxidant defense system of hepatocytes comprises related antioxidant enzymes and relevant cytokines, among which superoxide dismutase (SOD) acts as an important enzyme involved in the construction of antioxidant defense system [18].

SOD refers to a vital antioxidant enzyme in living organisms. It exhibits a special physiological activity, and it is the critical substance for scavenging oxygen radicals. Besides, the level of SOD acts as a visual indicator of aging and death of human cells. It is generally known that oxygen free radicals can cause damage to tissue cells, and SOD can fight and block this damage. Thus, the damaged tissue cells can be further repaired in time, so the damage attributed to free radicals to cells could be restored. MDA is the main end product of lipid peroxidation, and its content reflects the intensity of lipid peroxidation in the body and indirectly reflects the severity of free radical attack on body cells [19].

It has been demonstrated that MSCs significantly increased SOD activity and inhibited ROS production in the injured liver [20, 21]. To demonstrate the oxidative stress response in rat liver after hUC-MSCs transplantation, the liver tissues of the respective group of rats were taken to detect the relevant oxidative stress indexes (e.g., SOD, MDA, GSH and GPx contents) after cell transplantation. It was reported that hUC-MSCs transplantation significantly up-regulated the expressions of SOD, GSH and GPx levels and down-regulated the expression of MDA content in liver tissues of rats in comparison with the model group, except for the CCl_4/MSCs 1 wk group, which complied with the above study. It was therefore suggested that hUC-MSCs transplantation could mitigate the oxidative damage in liver tissues of CCl_4-induced liver fibrosis, but hUC-MSCs need a period of time to function in vivo after transplantation, and the oxidative stress pathway may be one of the important mechanisms by which hUC-MSCs function.

In brief, CCl_4 is capable of successfully inducing liver fibrosis model. Moreover, chronic intervention of CCl_4 can induce oxidative damage in liver tissue. Besides, hUC-MSCs transplantation can improve liver function and inhibit liver fibrosis in rats with liver fibrosis, and hUC-MSCs transplantation is capable of up-regulating the expressions of SOD, GSH and GPx and down-regulating the expression of MDA. It is therefore suggested that hUC-MSCs transplantation

may elevate the degree of liver fibrosis in rats in terms of oxidative stress. However, it takes time for hUC-MSCs to function in vivo after transplantation, which may be associated with the hepatocyte-like differentiation of hUC-MSCs. However, the underlying mechanism requires in-depth investigation. Under persistent pathogenic factors, MSCs transplantation fails to reverse liver fibrosis, whereas it can only decelerate the process of liver fibrosis. It is therefore indicated that in clinical work, the pathogenic factors of liver fibrosis or cirrhosis should be detected and removed in time, and other aspects of treatment should be exploited to achieve better clinical results. For this reason, a question is raised that whether MSC transplantation can reverse liver fibrosis during the removal of the causative factors. Further, the need for re-transplantation of MSCs in clinical work and the timing of re-transplantation require in-depth studies on the ground.

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Disclosure of conflict of interest

None.

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