

• BASIC RESEARCH •

# Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats

Dong-Chang Zhao, Jun-Xia Lei, Rui Chen, Wei-Hua Yu, Xiu-Ming Zhang, Shu-Nong Li, Peng Xiang

Dong-Chang Zhao, Rui Chen, Wei-Hua Yu, Xiu-Ming Zhang, Shu-Nong Li, Peng Xiang, Center for Stem Cell Biology and Tissue Engineering, Sun Yat-Sen University, 74# Zhongshan Road II, Guangzhou 510080, Guangdong Province, China  
Dong-Chang Zhao, Jun-Xia Lei, Wei-Hua Yu, Xiu-Ming Zhang, Shu-Nong Li, Department of Pathophysiology, Medical School of Sun Yat-Sen University, 74# Zhongshan Road II, Guangzhou 510080, Guangdong Province, China

Dong-Chang Zhao, Department of Pediatrics, Guangdong Province Maternal and Children's Hospital, 13# Guangyuan Xilu, Guangzhou 510010, Guangdong Province, China

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Correspondence to: Dr. Peng Xiang, Center for Stem Cell Biology and Tissue Engineering, Sun Yat-Sen University, 74# Zhongshan Road II, Guangzhou 510080, Guangdong Province, China. pxiang@gzsums.edu.cn

Telephone: +86-20-87335822 Fax: +86-20-87335858

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2.5 software. Male rats sex determination region on the Y chromosome (*sry*) gene were explored by PCR.

**RESULTS:** Compared to controls, infusion of MSCs reduced the mortality rates of incidence in CCl<sub>4</sub>-induced model (10% vs 20%) and in DMN-induced model (20-40% vs 90%). The amount of collagen deposition and alpha-SMA staining was about 40-50% lower in liver of rats with MSCs than that of rats without MSCs. The similar results were observed in fibrosis index. And the effect of the inhibition of fibrogenesis was greater in DMN10/MSCs than in DMN20/MSCs. The *sry* gene was positive in the liver of rats with MSCs treatment by PCR.

**CONCLUSION:** MSCs treatment can protect against experimental liver fibrosis in CCl<sub>4</sub>-induced or DMN-induced rats and the mechanisms of the anti-fibrosis by MSCs will be studied further.

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**Key words:** Mesenchymal stem cells; Liver fibrosis; Rat; Therapy

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## Abstract

**AIM:** Recent reports have shown the capacity of mesenchymal stem cells (MSCs) to differentiate into hepatocytes *in vitro* and *in vivo*. MSCs administration could repair injured liver, lung, or heart through reducing inflammation, collagen deposition, and remodeling. These results provide a clue to treatment of liver fibrosis. The aim of this study was to investigate the effect of infusion of bone marrow (BM)-derived MSCs on the experimental liver fibrosis in rats.

**METHODS:** MSCs isolated from BM in male Fischer 344 rats were infused to female Wistar rats induced with carbon tetrachloride (CCl<sub>4</sub>) or dimethylnitrosamine (DMN). There were two random groups on the 42<sup>nd</sup> d of CCl<sub>4</sub>: CCl<sub>4</sub>/MSCs, to infuse a dose of MSCs alone; CCl<sub>4</sub>/saline, to infuse the same volume of saline as control. There were another three random groups after exposure to DMN: DMN10/MSCs, to infuse the same dose of MSCs on d 10; DMN10/saline, to infuse the same volume of saline on d 10; DMN20/MSCs, to infuse the same dose of MSCs on d 20. The morphological and behavioral changes of rats were monitored everyday. After 4-6 wk of MSCs administration, all rats were killed and fibrosis index were assessed by histopathology and radioimmunoassay. Smooth muscle alpha-actin (alpha-SMA) of liver were tested by immunohistochemistry and quantified by IBAS

## INTRODUCTION

Liver fibrosis is the wound-healing response of the liver to chronic injury<sup>[1]</sup>. Following repeated injury, the liver undergoes a tissue remodeling and forms fibrosis. It is characterized by excessive accumulation of extracellular matrix, with the formation of scar tissue encapsulating the area of injury. This results in many clinical manifestations, including ascites, variceal hemorrhage, and encephalopathy. The prognosis of patients with the disease is poor, although liver transplantation is a good alternative treatment. Moreover, there are limited available donor livers for hundreds of millions of patients worldwide<sup>[2,3]</sup>. So, it is very important to investigate appropriate therapies for the disease by different treatments.

We<sup>[4]</sup> and others<sup>[5-9]</sup> have established that BM-derived MSCs could engraft injured tissue, such as, bone marrow, lung, liver, heart or brain, and recover its function. These results indicate that MSCs is an attractive cell source for regenerative medicine. Recent reports have shown the

capacity of MSCs to differentiate into hepatocytes *in vitro*<sup>[10,11]</sup> and *in vivo*<sup>[6,12,13]</sup>. Further, MSCs administration could repair injured lung, liver, or heart by reducing inflammation, collagen deposition and remodeling<sup>[5-7]</sup>. These imply that MSCs could not only have a possibility to repair acute damaged tissue but also have potentiality of reducing chronic fibrogenesis. Although Sakaida *et al*<sup>[14]</sup>, and Fang *et al*<sup>[6]</sup>, reported that BM or MSCs could reduce CCL<sub>4</sub>-induced liver fibrosis in mice, the mechanism by which MSCs repair the fibrosis is unclear and their results seem controvertible. Fang *et al*<sup>[6]</sup>, suggested that delaying MSCs administration by 1 wk after CCL<sub>4</sub> challenge did not prevent the disease progression, while Sakaida *et al*<sup>[14]</sup>, found that BM treatment to 4 wk CCL<sub>4</sub>-induced rats could get significantly reduced liver fibrosis. From the point of view of clinical practice, it is very important to select the model of liver fibrosis close to human disease for evaluating the effect of MSCs on the fibrosis. In the present study, we employed two models of fibrosis induced with CCL<sub>4</sub> or DMN to evaluate the effect of MSCs on the fibrosis. Our results showed that MSCs treatment could reduce mortality rates of rats and improve their fibrosis index, and earlier the MSCs were administrated, greater was the anti-fibrosis effect. We provide evidence here indicating that infusion of BM-derived MSCs should be therapeutic in liver fibrosis by blocking the fibrosis formation and progression.

## MATERIALS AND METHODS

### Preparing for BM-derived MSCs in rats

MSCs were prepared from rat bone marrow as described previously in our laboratory<sup>[15]</sup>. In brief, whole BM was flushed from the tibia and femur of Fischer 344 rats (6 wk old, male). MSCs preferentially attached to the polystyrene surface and were further purified by passages. MSCs were identified to express CD29 but not MHC class II antigens, costimulatory molecules CD80 and CD86 by flow cytometry<sup>[15]</sup>. The multiple differentiation potential of MSCs into neural cells, adipocyte and osteoblasts was also confirmed *in vitro*.

The multiple differentiation potential of MSCs into neural cells, adipocyte, and osteoblasts was also confirmed *in vitro*. In brief, to induce osteogenic differentiation, 8<sup>th</sup>-10<sup>th</sup>-passage cells were treated with osteogenic medium for 3 wk with medium changes twice weekly. Osteogenic medium consists of L-DMEM supplemented with 0.1  $\mu$ mol/L dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 10 mmol/L  $\beta$ -glycerol phosphate (Sigma-Aldrich), and 0.2 mmol/L ascorbic acid (Sigma-Aldrich). Osteogenesis was assessed by Chinalizarin staining. To induce adipogenic differentiation, 8<sup>th</sup>-10<sup>th</sup>-passage cells were treated with adipogenic medium for 3 wk. Medium changes were performed twice weekly. Adipogenic medium consists of H-DMEM supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1  $\mu$ mol/L hydrocortisone (Sigma-Aldrich), 0.1 mmol/L indomethacin (Sigma-Aldrich), and 10% rabbit serum (Sigma-Aldrich). Adipogenesis was assessed by Oil Red O staining. To induce neurogenic differentiation, 8<sup>th</sup>-10<sup>th</sup>-passage cells were pretreated for 24 h with neurogenic medium, which consists of H-DMEM

supplemented with 1 mmol/L  $\beta$ -mercaptoethanol ( $\beta$ -ME, Sigma-Aldrich). Then cells were treated with 5 mmol/L  $\beta$ -ME for 1 h. Neurogenesis was identified by antibody against neuron-specific enolase (NSE, SouthernBiotech, Birmingham, USA) staining.

Fischer 344 rats BM-MSCs in the 8-10<sup>th</sup> passage were suspended in sterile saline and used for further experiments as described below.

### CCL<sub>4</sub>- or DMN-induced liver fibrosis and MSCs administration

Female Wistar rats were 6-wk old, weighing between 140 and 160 g. Rats were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions, and were subjected to a 12:12-h daylight/darkness and allowed unlimited access to chow and water. The morphological and behavioral changes of rats were monitored everyday. Liver fibrosis was induced by CCL<sub>4</sub> or DMN administration as described<sup>[16]</sup>. The protocol was approved by Animal Care Committee of Sun Yat-Sen University.

### CCL<sub>4</sub>-induced model

On d 0, rats were injected by subcutaneous at a dose of 0.2 mL/100 g body weight of 40 mL/L CCL<sub>4</sub> (Guangzhou Chemical Factory, China) dissolved in paraffin oil (Guangzhou Chemical Factory, China). The injection was given twice a week for 6 wk. The same volume of paraffin oil alone was used as control. Liver fibrosis was determined by killing three to six rats with histopathology weekly. Twenty rats were randomly divided into two groups on d 42: CCL<sub>4</sub>/MSCs,  $n = 10$ , to infuse a dose of MSCs  $3 \times 10^6$  cells per rat by intravenous; CCL<sub>4</sub>/saline,  $n = 10$ , to infuse the same volume of saline as a control. Blank control were designed, paraffin/saline,  $n = 10$ , to infuse the same volume of saline on d 42 after paraffin administration. On d 70, all rats were killed and venous blood was collected and liver tissue harvested for analysis.

### DMN-induced model

On d 0, rats were injected intraperitoneally at a dose of 100  $\mu$ L DMN (Sigma, USA) (diluted 1:100 with 0.15 mol/L sterile saline) per 100 g body weight. The injection was given on three consecutive days of each week for 6 wk. Control animals received the same volume of 0.15 mol/L sterile saline. Three or six treated animals were killed weekly with determination of establishment fibrosis by histopathology. Sixty rats were randomly divided into three groups in different time points of DMN-induced: DMN10/MSCs,  $n = 10$ , to infuse a dose of MSCs  $3 \times 10^6$  cells per rat by intravenous on d 10; DMN20/MSCs,  $n = 10$ , to infuse the same dose MSCs on d 20; DMN10/saline,  $n = 40$ , to infuse the same volume of saline on d 10. We also designed saline,  $n = 10$ , to infuse the same volume of saline for blank control. On d 49, venous blood was collected and all rats were killed, and liver tissue harvested for analysis.

### Analysis of liver histopathology and immunohistochemistry

Liver samples were collected into PBS and fixed overnight in 40 g/L paraformaldehyde in PBS at 4 °C. Serial 5- $\mu$ m sections of the right lobes of the livers were stained with hematoxylin and eosin (HE) and Masson Trichrome (MT)

as rules in our laboratory. Additional sections were tested with the antibody against  $\alpha$ -SMA (1:200 dilutions, NeoMarkers) and immunoreactive materials were visualized by use of a streptavidin-biotin staining kit (UltraSensitive™ SP kit, Maixin-Bio, Fujian, China) and diaminobenzidine, as previously described<sup>[16]</sup>. MT and  $\alpha$ -SMA staining was quantified using IBAS 2.5 software (Germany) and the data expressed as percentage of positive staining. The average of the score taken from five random fields was used to generate a single per slide.

#### Hydroxyproline (Hyp) content assay

A colorimetric assay was performed as described<sup>[16]</sup>. Briefly, lyophilized liver sections (0.5 g) were hydrolyzed for 20 h in 6 mol/L HCl at 100 °C, redissolved in water, and centrifuged to remove any impurities. Samples were incubated for 10 min in 0.05 mol/L chloramine-T (Fisher, Fair Lawn, NJ, USA) at room temperature, followed by 15-min incubation in Ehrlich's-perchloric acid solution at 65 °C. Sample absorbencies were assessed at 561 nm and resulting values compared to a Hyp standard curve. Each sample was assayed in duplicate. The Hyp content was expressed as micrograms per gram of wet liver.

#### Examination of the level of laminin (LN), hyaluronic acid (HA), alanine aminotransferase aspartate (ALT) and total bilirubin in serum (TBIL)

ALT and TBIL were assessed using our routine laboratory methods. Serum HA and LN were also measured with the kit (Navy Medical Institute, Shanghai, China) by radioimmunoassay.

#### PCR detection of male-derived MSCs

Genomic DNA was prepared from liver tissue of the rats in each group using genomic DNA purification kit (G&T Biotech, MD, USA). The presence or absence of sex determination region on the Y chromosome (*sry*) gene of liver in recipient female rats was assessed by PCR. The presence of DNA in all tissues was assessed by analysis of the "house-keeping" gene  $\beta$ -actin. Primer sequences for *sry* gene (forward 5'-catcgaagggttaaagtcca-3', reverse 5'-atagtgtgtaggtgtgtgtcc-3') were obtained from published sequences<sup>[17,18]</sup> and amplify a product of 104 bp.  $\beta$ -actin

primers (forward 5'-tggtgtccctgtatgcctct-3', reverse 5'-taatgtcagcagcagattcc-3') were designed from GenBank (accession no. J00691) and amplify a product of 206 bp. The PCR conditions were as follows: incubation at 94 °C for 4 min; 35 cycles of incubation at 94 °C for 50 s, 60 °C for 30 s, and 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis, stained with ethidium bromide and imaged by Image Station 200R (Kodak, USA). Positive (male Fischer 344 rat genomic DNA) and negative (female Wistar rat genomic DNA) controls were included in each assay.

#### Statistical analysis

Data are expressed as mean $\pm$ SD. Significant differences were determined by using ANOVA in SPSS 9.0. Results were considered significant when  $P < 0.05$ .

## RESULTS

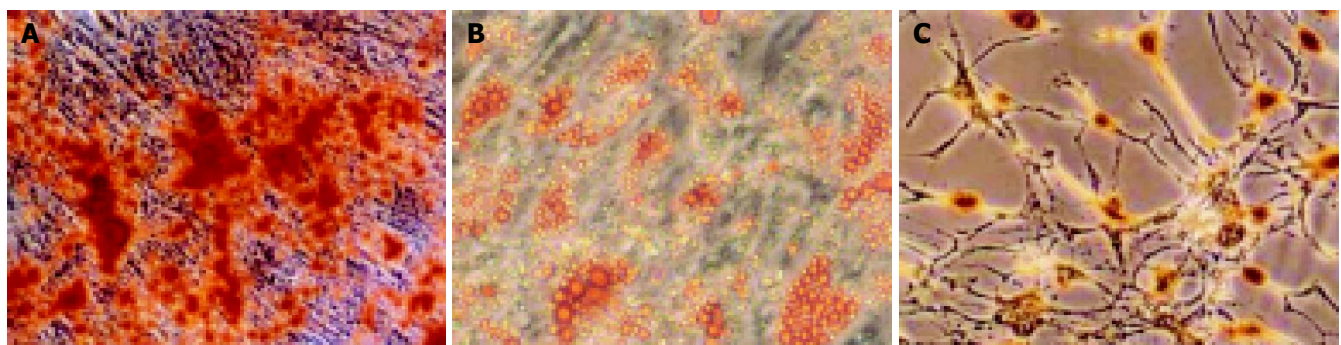
#### Multiple differentiation potential of MSCs into neural-like cells, adipocyte, and osteoblasts

Figure 1 shows that BM-derived MSCs had differentiated into neural-like cells, adipocyte, and osteoblasts in different conditions (Figures 1A-C).

#### CCl<sub>4</sub> or DMN administration induced fibrogenesis in rats

Rats given CCl<sub>4</sub> showed slower weight gain than rats with paraffin oil alone (Figure 2A). The average weight of rats with CCl<sub>4</sub> for 6 wk was 50-70 g lower than rats with paraffin oil alone. The mortality rates of incidence were about 10% in rats with CCl<sub>4</sub> after 6 wk, while the paraffin oil-treated rats were all alive. Up to d 21 of exposure to CCl<sub>4</sub>, the substantial fibrosis was not observed by histological examination. We found massive rearrangement of the hepatic architecture, including septal fibrosis, extensive bridging, inflammation and extensive fatty changes after 6 wk, while rats with only paraffin oil had normal liver architecture (Figures 4A1 and B1). These results were corresponding with previous findings<sup>[19]</sup>.

We also established the other fibrosis model induced with DMN, because there were differences of hepatotoxicity between CCl<sub>4</sub> and DMN<sup>[20]</sup>. The body weight was obviously lower in rats with DMN for 3 wk than that in rats with

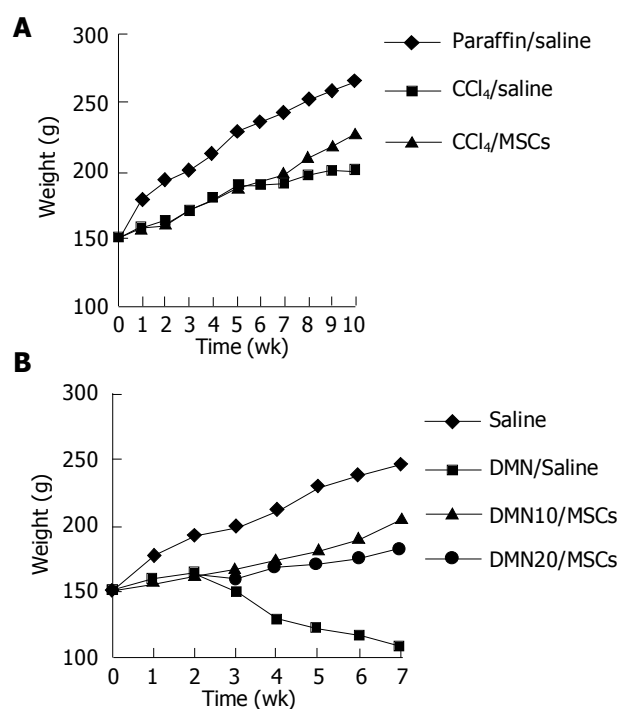


**Figure 1** MSCs are induced to differentiate into osteoblasts and stained red color by Chinalizarin (A). Under adipogenic conditions, MSCs accumulate lipid vacuoles, which are positively stained by Oil Red O assay (B). Under neurogenic

conditions, MSCs differentiate into neuronal-like cells and stained positively for NSE by immunocytochemical analysis (C). (Original magnification,  $\times 200$ .)

only saline (Figure 2B). If the body weight fell to less than 100 g, the rat was likely to die soon. The first rat died on d 10 after DMN administration. By d 21, 20% of rats died. Histopathology showed centrilobular congestion and hemorrhagic necrosis liver of rats after 1 wk. Centrilobular necrosis and intense neutrophilic infiltration were observed on d 14. By d 21, collagen fiber deposition could be observed, together with severe centrilobular necrosis and bridging necrosis, with bile duct proliferation and fibrosis surrounding the central veins. These results were corresponding with previous findings<sup>[16,21]</sup>.

To further verify fibrosis, we performed immunohistochemical staining of liver sections for  $\alpha$ -SMA, and examined levels of LN, HA, ALT, and TBIL in serum and liver Hyp contents (Figure 4E and Table 1).



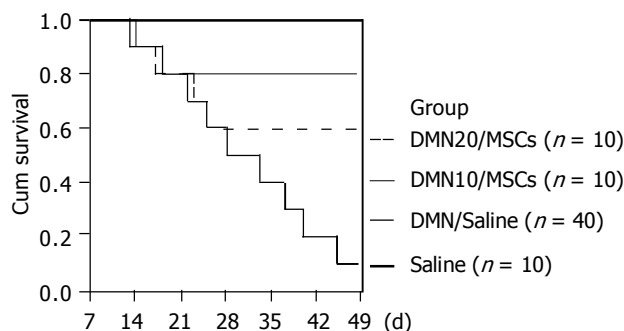
**Figure 2** Average weekly weight of rats induced with CCl<sub>4</sub> (A) or DMN (B). **A:** Paraffin/saline, *n* = 10; CCl<sub>4</sub>/saline, *n* = 10-8; CCl<sub>4</sub>/MSCs, *n* = 10-9; **B:** saline, *n* = 10; DMN/saline, *n* = 40-4; DMN10/MSCs, *n* = 10-8; DMN20/MSCs, *n* = 10-6.

### MSCs treatment improved the morphological and behavioral changes

On d 42 after CCl<sub>4</sub> treatment, rats were infused with MSCs or saline. We found that rats with MSCs had got good morphological and behavioral changes after 7<sup>th</sup> d, while rats with saline appeared extremely lethargic. Two rats with saline

died before the completion of the protocol, while rats with MSCs were all alive. There was a slight lift of weight in CCl<sub>4</sub>/MSCs compared with CCl<sub>4</sub>/saline (Figure 2A). We also observed that some rats in CCl<sub>4</sub>/saline had a large amount of ascites while being killed, which could be a reason for gain of weight while living.

There were dramatic changes of rats with MSCs after 7<sup>th</sup> d in DMN-induced model (Figure 2B). Rats exhibited grooming, but piloerection, their food and water intake were much increased and their weights increased faster. Rats without MSCs had bad good morphological and behavioral changes. Their weights gain stopped. Their eyes were pale and urines were yellow, and some animals had labored respiration. Thirty-six of forty rats in DMN/saline died of progressive liver fibrosis within 49<sup>th</sup> d after DMN administration. Life time of rats survived in DMN10/MSCs and DMN20/MSCs was significantly longer than that of rats in DMN/saline (Figure 3). Further, amounts of survival rats in DMN10/MSCs were more than those in DMN20/MSCs.



**Figure 3** Survival of rats in control group and DMN-induced group with or without MSCs administration. Analysis of survival was conducted by a log-rank test based on the Kaplan-Meier method.

### MSCs treatment decreased collagen deposition

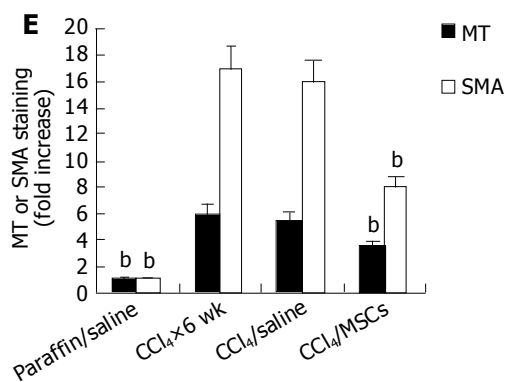
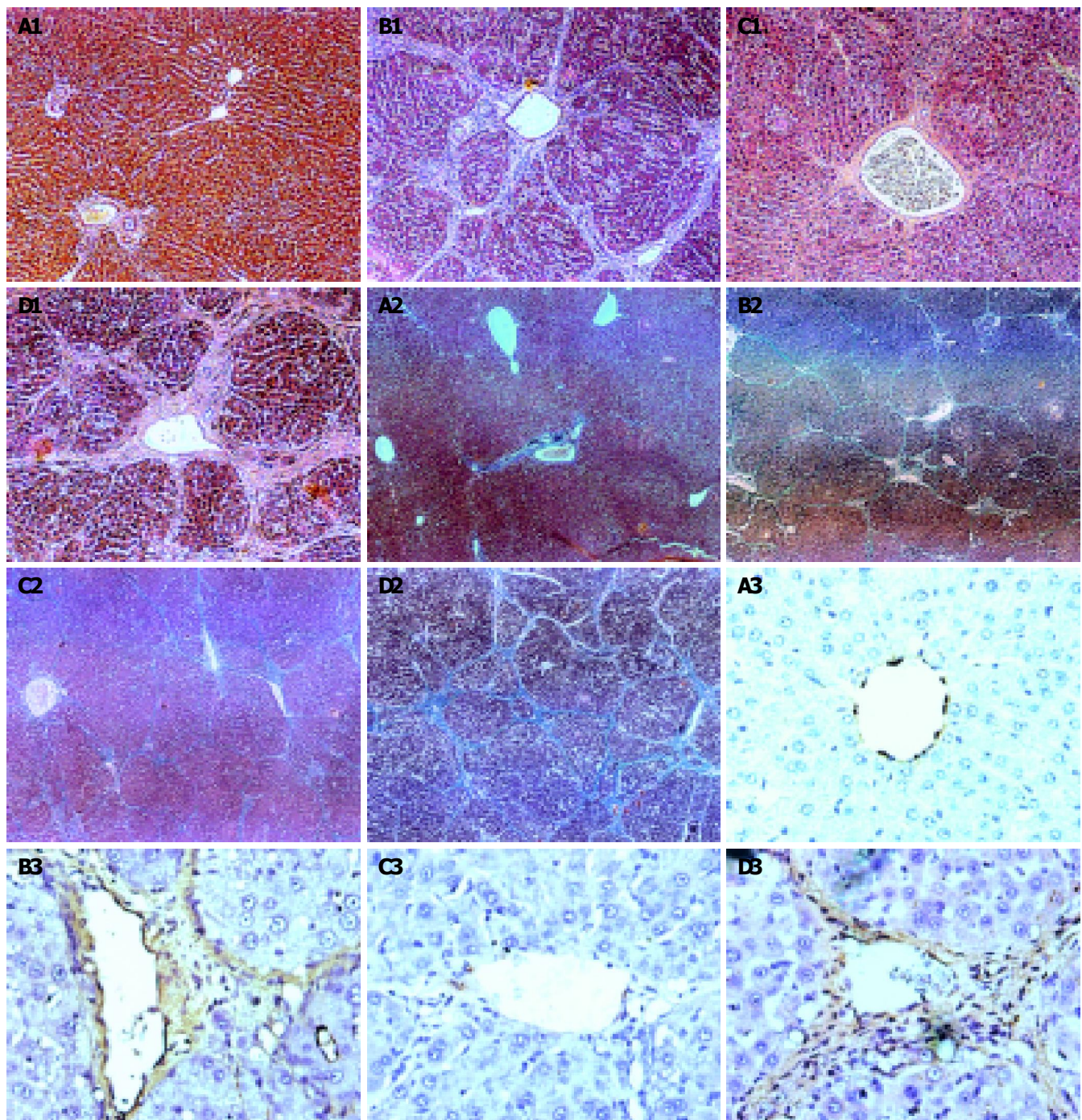
We analyzed liver histology in rats with MSCs in two models by HE staining and MT staining. HE staining showed that architecture of liver got better in CCl<sub>4</sub>/MSCs (Figure 4C1). Inflammation reduced and pseudolobules were resolved. Collagen accumulation and fatty degeneration were significantly lower in CCl<sub>4</sub>/MSCs compared with CCl<sub>4</sub>/saline (Figures 4C1, C2, D1, and D2). Quantification of MT staining demonstrated more than 40% decrease of collagen in liver of rats in CCl<sub>4</sub>/MSCs compared to that of rats in CCl<sub>4</sub>/saline (Figure 4E). Meanwhile, the liver histology also got improved in rats with MSCs treatment whether in DMN10/MSCs or in DMN20/MSCs (Figures 5C1 and D1). The

**Table 1** Effect of MSCs on levels of fibrosis index of rats induced with CCl<sub>4</sub>

Group	<i>n</i>	HA (μg/L)	LN (μg/L)	TBIL (mg/L)	ALT (U/L)	Hyp (μg/g)
Paraffin/saline	10	167.5±25.8 <sup>b</sup>	54.2±13.2 <sup>b</sup>	8.8±3.5 <sup>b</sup>	46.8±7.9 <sup>b</sup>	650±130 <sup>b</sup>
CCl <sub>4</sub> ×6 wk	10	883.5±109.6	224.2±56.2	22.8±5.9	132.7±42.9	2 500±330
CCl <sub>4</sub> /saline	8	783.5±89.6	215.8±61.9	21.6±5.5	110.1±58.3	2 100±430
CCl <sub>4</sub> /MSCs	9	454.1±98.8 <sup>b</sup>	85.2±43.9 <sup>b</sup>	9.8±6.3 <sup>b</sup>	51.3±11.9 <sup>b</sup>	1 150±510 <sup>b</sup>

<sup>b</sup>*P* < 0.01 vs CCl<sub>4</sub>/saline. Not significantly different between values of CCl<sub>4</sub>/saline and CCl<sub>4</sub>×6 wk.

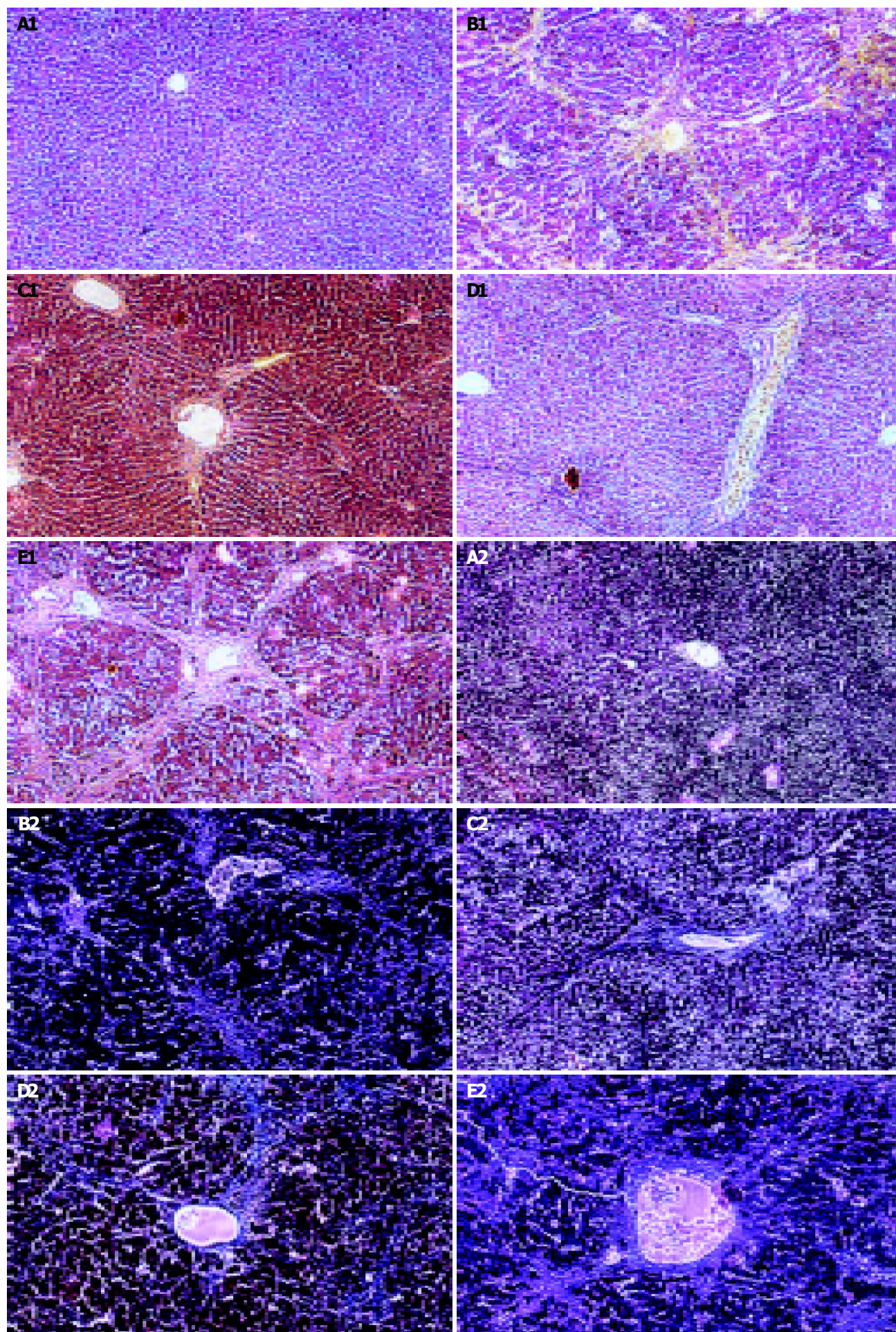




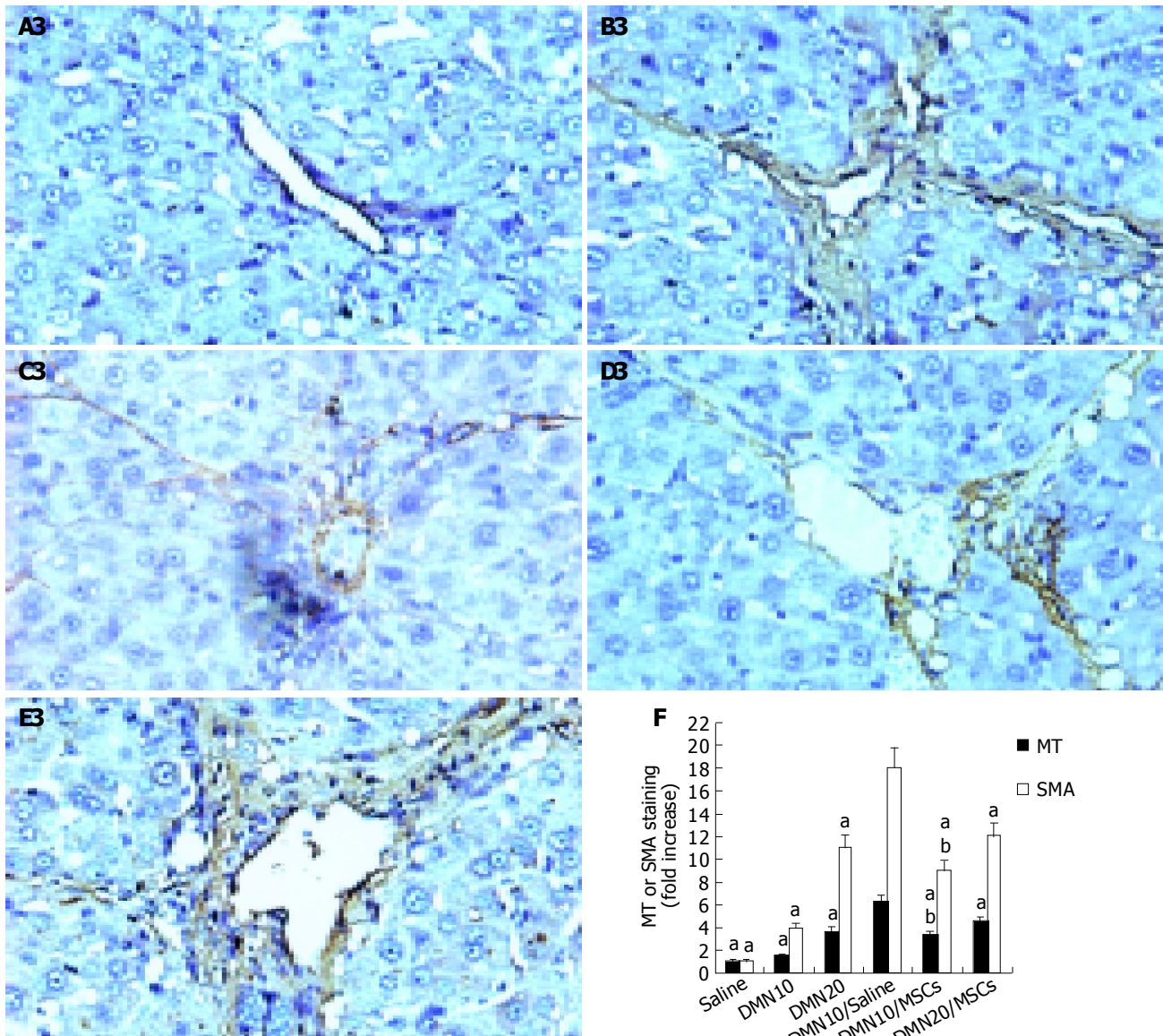
**Figure 4** Analysis of liver histopathology and immunohistochemistry in rats induced with CCl<sub>4</sub>. **A:** Paraffin/saline; **B:** CCl<sub>4</sub>×6 wk; **C:** CCl<sub>4</sub>/MSCs; **D:** CCl<sub>4</sub>/saline; 1: HE staining, original magnification ×100; 2: MT staining, original magnification ×40; 3: immunohistochemistry for α-SMA, DAB staining, original

magnification ×400; **E:** MT and α-SMA staining was quantified using IBAS 2.5 software. Data represent the fold-increase in positive staining vs paraffin/saline. Values are presented as mean±SD. <sup>a</sup>*P*<0.01 vs CCl<sub>4</sub>/saline. Not significantly different between values of CCl<sub>4</sub>/saline and CCl<sub>4</sub>×6 wk.









**Figure 5** Analysis of liver histopathology and immunohistochemistry in rats induced with DMN. **A:** DMN10; **B:** DMN20; **C:** DMN10/MSCs; **D:** DMN20/MSCs; **E:** DMN10/saline; 1: HE staining, original magnification  $\times 100$ ; 2: MT staining, original magnification  $\times 100$ ; 3: immunohistochemistry for  $\alpha$ -SMA, DAB

staining, original magnification  $\times 400$ ; **F:** MT and  $\alpha$ -SMA staining was quantified using IBAS 2.5 software. Data represent the fold-increase in positive staining vs saline. Values are presented as mean $\pm$ SD.  $^aP<0.05$  vs DMN20/MSCs,  $^bP<0.01$  vs DMN10/saline respectively.

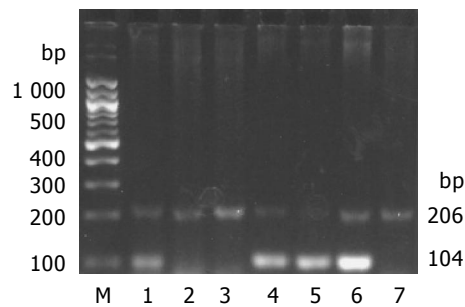
necrosis of hepatocyte was replaced by the regenerate. The thickened septal fibrosis became thinner or disappeared. Moreover, the degree of fibrosis was decreased in DMN10/MSCs than that in DMN20/MSCs by quantification of MT staining (Figure 5F). It implied that earlier the MSCs were treated, greater was the effect of MSCs on the inhibition of fibrosis.

We examined levels of HA and LN in serum and liver Hyp contents, which are biochemical markers of liver fibrosis. The result showed that the levels of markers significantly decreased in rats with MSCs than that in rats without MSCs in two models (Tables 1 and 2). And reduction of levels of Hyp and HA and LN was more obvious in DMN10/MSCs than that in DMN20/MSCs. These results

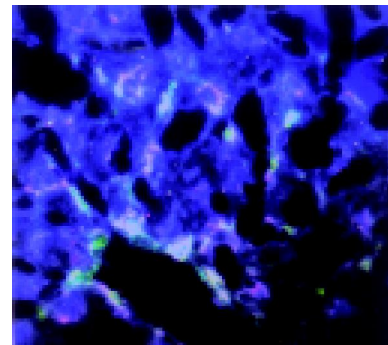
**Table 2** Effect of MSCs on levels of fibrosis index of rats induced with DMN

Group	n	HA ( $\mu$ g/L)	LN ( $\mu$ g/L)	TBIL (mg/L)	ALT (U/L)	Hyp ( $\mu$ g/g)
Saline	10	142.3 $\pm$ 18.9 <sup>a</sup>	44.5 $\pm$ 16.4 <sup>a</sup>	5.1 $\pm$ 1.5 <sup>a</sup>	32.7 $\pm$ 6.8 <sup>a</sup>	450 $\pm$ 110 <sup>a</sup>
DMN10	10	338.7 $\pm$ 60.3 <sup>a</sup>	82.3 $\pm$ 23.5 <sup>a</sup>	8.7 $\pm$ 1.8 <sup>a</sup>	85.3 $\pm$ 12.5 <sup>a</sup>	760 $\pm$ 210 <sup>a</sup>
DMN20	10	583.5 $\pm$ 59.6 <sup>a</sup>	114.9 $\pm$ 44.2 <sup>a</sup>	10.8 $\pm$ 3.5 <sup>a</sup>	162.7 $\pm$ 22.7 <sup>a</sup>	1 250 $\pm$ 450 <sup>a</sup>
DMN10/saline	4	981.4 $\pm$ 110.5	214.2 $\pm$ 56.5	21.6 $\pm$ 5.5	212.1 $\pm$ 51.4	2 250 $\pm$ 650
DMN10/MSCs	8	395.2 $\pm$ 43.1 <sup>a,b</sup>	83.1 $\pm$ 43.1 <sup>a,b</sup>	9.1 $\pm$ 4.7 <sup>a,b</sup>	67.7 $\pm$ 32.9 <sup>a,b</sup>	1 010 $\pm$ 580 <sup>a,b</sup>
DMN20/MSCs	6	517.8 $\pm$ 82.6 <sup>a</sup>	98.2 $\pm$ 57.2 <sup>a</sup>	11.1 $\pm$ 6.2 <sup>a</sup>	89.7 $\pm$ 38.6 <sup>a</sup>	1 150 $\pm$ 750 <sup>a</sup>

<sup>a</sup> $P<0.05$  vs DMN20/MSCs, <sup>b</sup> $P<0.01$  vs DMN10/saline respectively.



**Figure 6** PCR signals of *sry* gene and  $\beta$ -actin gene in liver of rats. *sry*, 104 bp;  $\beta$ -actin, 206 bp; M: 100-bp DNA maker; lane 1: CCl<sub>4</sub>/MSCs; lane 2: CCl<sub>4</sub>×6 wk; lane 3: DMN10/saline; lane 4: DMN10/MSCs; lane 5: DMN20/MSCs; lane 6: a male Fischer rat 344 as positive control; lane 7: a female Wistar rat as negative control.



**Figure 7** Signals of SRY by FISH with Rat 12/Y whole chromosome probes. Red color represent chromosome Y, green color represent chromosome 12, blue color represent nuclear. Original magnification ×40.

were consistent with the histology of liver.

We tested the level of ALT and TBIL in serum, which are primary indexes of liver function. Levels of these two indexes were closed to normal in CCl<sub>4</sub>/MSCs, but stayed higher in CCl<sub>4</sub>/saline. We also found that levels were lower in DMN10/MSCs and DMN20/MSCs than that in DMN/saline, although there were few high levels of ALT and TBIL in DMN20/MSCs.

#### **MSCs administration resulted in decreased $\alpha$ -SMA expression**

Immunohistochemistry of liver sections for  $\alpha$ -SMA expression showed intense staining patterns in CCl<sub>4</sub>/saline and DMN/saline (Figures 4B3, 4D3, 5A3, 5B3, and 5E3). However, the levels of  $\alpha$ -SMA staining in CCl<sub>4</sub>/MSCs, DMN10/MSCs, and DMN20/MSCs were significantly less (Figures 4C3, 5C3, and 5D3) compared to CCl<sub>4</sub>/saline and DMN/saline. Quantification of levels of  $\alpha$ -SMA staining of liver demonstrated more than 40-50% decrease in rats after MSCs administration in two experimental fibrosis models (Figure 5E). And there were higher levels of  $\alpha$ -SMA staining in DMN20/MSCs than that in DMN10/MSCs. This result was consistent with liver histopathology in two models.

#### **Expression of *sry* gene in livers of rats induced with CCl<sub>4</sub> or DMN**

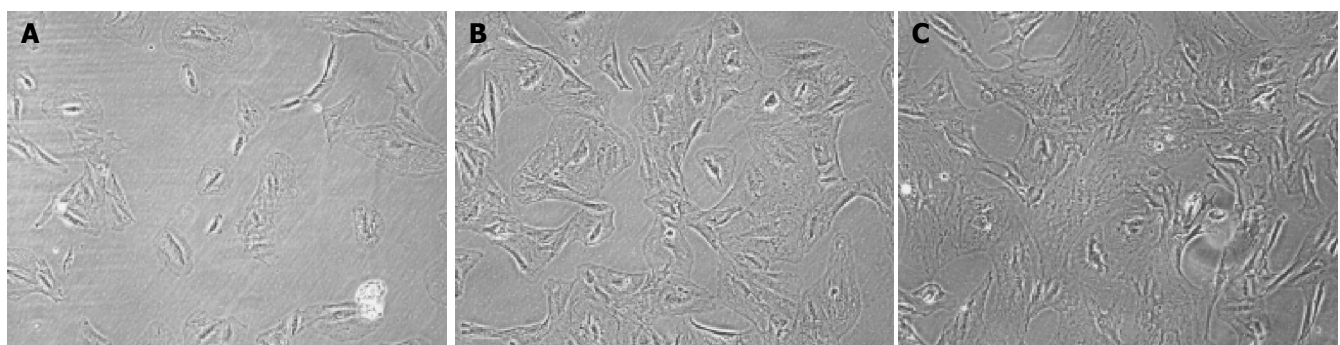
After 4 wk of MSCs treatment, the *sry* gene was positive

by using PCR analysis in the liver of female recipient induced with CCl<sub>4</sub>. And the similar result was seen in DMN10/MSCs and DMN20/MSCs after 29-39 d of MSCs treatment (Figure 6). These results suggested that BM-derived MSCs were capable of liver engraftment of rats induced with CCl<sub>4</sub> or DMN (Figures 7-9).

## **DISCUSSION**

In the current study, BM-derived MSCs were isolated and expanded in adult rats and their multiple differentiation potential was also confirmed *in vitro*. We evaluated the anti-fibrosis effects of infusion of BM-derived MSCs to rats with CCl<sub>4</sub>- or DMN-induced. The results showed that MSCs treatment could reduce mortality rates of rats and improve the fibrosis index.

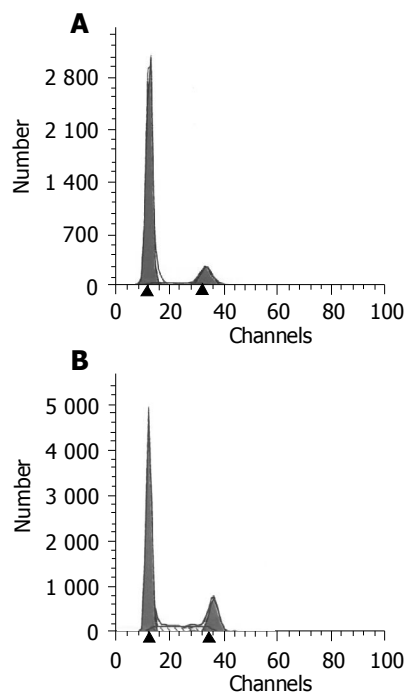
CCl<sub>4</sub>-induced liver fibrosis is a classical experimental fibrosis model. Although this model could undergo spontaneous resolution of fibrosis after stopping CCl<sub>4</sub> challenging in some reports<sup>[22]</sup>, we here did not find recovery of liver fibrosis in CCl<sub>4</sub>/saline within 4 wk of withdrawal of CCl<sub>4</sub>. In contrast, collagen accumulation and fatty degeneration were significantly lower in CCl<sub>4</sub>/MSCs compared with CCl<sub>4</sub>/saline. Further, we also confirmed the anti-fibrosis effects of MSCs by DMN-induced liver fibrosis model. DMN is a hepatotoxin, carcinogen and mutagen, and can cause liver fibrosis. It provides a suitable



**Figure 8** Morphology of hepatic stellate cells co-culture with or without mesenchymal stem cells by transwell *in vitro* for 72 h. **A:** Hepatic stellate cells co-culture with mesenchymal stem cells; **B:** hepatic stellate cells co-culture

with hepatocyte cells line; **C:** hepatic stellate cells only culture. Original magnification ×10.





**Figure 9** Analysis of hepatic stellate cells by FACS. **A:** Hepatic stellate cells co-culture with mesenchymal stem cells; **B:** hepatic stellate cells only culture.

rapid experimental fibrosis model as like in human liver fibrosis<sup>[21,23]</sup>. We investigated two groups with MSCs infusion in the different time of fibrosis development in this model. One group DMN10/MSCs was treated with MSCs on the 10<sup>th</sup> d after DMN administration, in which time it was in early stage of liver fibrogenesis. The other group DMN20/MSCs was infused to MSCs on the 20<sup>th</sup> d after DMN administration, in which time the fibrosis was forming. Our work demonstrated that infusion of MSCs administration can prolong the life time of rats and improve the liver function and reduce hemorrhagic necrosis collagen accumulation of liver in both groups. Moreover, fibrosis index got more improvement in DMN10/MSCs than that in DMN20/MSCs. These results suggest that infusion of MSCs could not only inhibit the fibrosis formation but also halt the progression of fibrosis in CCl<sub>4</sub>- or DMN-treated rats.

We<sup>[15,24]</sup> and others<sup>[25]</sup> have showed that BM-derived MSCs did not express MHC class II antigens, costimulatory molecules CD80 and CD86. This phenotype is regarded as nonimmunogenic and suggests that MSCs might be effective in inducing tolerance. It was identified that allogeneic or xenogeneic MSCs could persist into hosts after infusion<sup>[26]</sup>. We here employ BM-derived MSCs from Fischer 344 rats to infuse Wistar rats with liver fibrosis and get a good result. It implies that infusion of allogeneic MSCs is a great value for liver fibrosis in clinical practice.

In the current study the male donor cells was confirmed in the injured liver of female recipients by PCR for the *sry* gene. However, the mechanism by which MSCs repair the fibrosis is unclear. Fang *et al*<sup>[6]</sup>, found that although albumin-positive donor-derived cells were present at lower frequency in sections of CCl<sub>4</sub>-induced liver tissues, infusion of Flk1<sup>+</sup> murine MSCs might ameliorate liver fibrosis. Ortiz *et al*<sup>[5]</sup>,

observed that MSCs administration reduced the degree of bleomycin-induced inflammation and collagen deposition within lung tissue, but male donor DNA accounted for  $2.21 \times 10^{-50}$ % of total lung DNA in female recipient mice with MSCs treatment. Kinnaird *et al*<sup>[27]</sup>, and Silva *et al*<sup>[28]</sup>, thought that MSCs could secrete a wide array of arteriogenic cytokines and contribute to reducing fibrosis through paracrine mechanisms. These results suggested that MSCs themselves could not functionally rescue the recipients by substituting the damaged cells directly<sup>[29]</sup>. Although one limitation of the current study is that the quality or quantity of MSCs engraftment in liver was not clear, the fact that MSCs have anti-fibrosis effects in injured liver was clearly proved. We suggest that MSCs may protect against CCl<sub>4</sub>- or DMN-induced injury by altering the microenvironment of liver at sites of engraftment. In the present study, levels of  $\alpha$ -SMA positive staining of liver sections have more than 50% decrease after MSCs administration in two models (Figures 4E and 5F). This is an exciting event because the expression of  $\alpha$ -SMA in liver is an indicator of hepatic stellate cells (HSCs) activation, which is recognized as the key players in liver fibrogenesis<sup>[1]</sup>. For example, MSCs could secrete many cytokine and growth factors, such as hepatic growth factor<sup>[30]</sup>, which shows anti-apoptotic activity in hepatocytes and plays an essential part in the regeneration of the liver<sup>[21]</sup> and nerve growth factor<sup>[9]</sup>, which can induce apoptosis of cultured HSCs<sup>[31]</sup>. And recently, we have provided some evidences that *in vitro* coculture MSCs and HSCs can increase the number of HSCs in G<sub>0</sub> phase and reduce the number of HSCs in S phase (unpublished). Thus, MSCs may play a inhibition role in process of HSCs transition from the quiescent state to activated state.

In summary, this data reveals that infusion of BM-derived MSCs can protect against liver fibrosis and the mechanisms of the anti-fibrosis by MSCs will be studied further.

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