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Original Paper

Treatment Efficiency of Different Routes of Bone Marrow-Derived Mesenchymal Stem Cell Injection in Rat Liver Fibrosis Model

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Key Words
BM-MSCs • Liver fibrosis • Transplantation route • Rats

Abstract
Background/Aims: The most appropriate route for bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation in the management of liver fibrosis remains controversial. This study investigated the therapeutic efficacy of intravenous and intrasplenic BM-MSC transplantation on carbon tetrachloride (CCl_4)-induced rat liver fibrosis. ***Methods:*** Fifty rats were divided into 5 groups (n = 10 rats per group): healthy control group, CCl_4 group, CCl_4/recovery group, CCl_4/BM-MSC intravenous group, and CCl_4/BM-MSC intrasplenic group. BM-MSCs were isolated, labeled with green fluorescent protein (GFP), and injected into fibrotic rats either intravenously or intrasplenically. Gene expression of interleukins (IL-1β and IL-6), interferon (INF)-γ, hepatic growth factor, and the hepatocyte-specific marker cytokeratin 18 was estimated by quantitative real-time reverse transcription-polymerase chain reaction. Vascular endothelial growth factor and connective tissue growth factor was detected by western blot analysis and enzyme-linked immunosorbent assay, respectively. At 2 weeks after intravenous and intrasplenic BM-MSC injections, GFP-positive cells were detected in liver tissue. ***Results:*** Both routes achieved a similar enhancement of liver function, which was confirmed by histopathological examination. The intravenous route was more effective than the intrasplenic route in reducing gene expression levels of IL-1β, IL-6, and INF-γ. However, fibrotic changes were still observed in the recovery group. ***Conclusion:*** Intravenous BM-MSC injection was an efficient and appropriate route for BM-MSC transplantation for the management of liver fibrosis.

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Introduction

Liver fibrosis is a reversible reaction to repetitive liver damage induced by various factors such as hepatitis, alcoholic hepatic disease, and non-alcoholic steatohepatitis [1]. It is identified by the intense deposition of extracellular matrix (ECM) in a process that includes activation of hepatic stellate cells (HSCs), which are transformed to myofibroblast-like cells [2]. Furthermore, activated HSCs produce inflammatory cytokines and profibrogenic factors that result in the overproduction of ECM and liver inflammation [3]. Liver transplantation provides an efficient approach to manage liver fibrosis [4]. However, given the rising number of patients and limited number of donors and the increase in morbidity and mortality of liver fibrosis, alternative therapies are needed [5]. Mesenchymal stem cells (MSCs) show potential therapeutic value since they have high self-renewal ability, are capable of multipotent differentiation, and have low immunogenicity [6]. However, the therapeutic efficacy of bone marrow-derived (BM)-MSCs in managing liver fibrosis is still controversial. BM-MSCs can ameliorate liver fibrosis through their differentiation into hepatocytes and by secreting diverse growth factors and cytokines that suppress the inflammatory reaction, inhibit apoptosis of hepatic cells, and can efficiently recover liver function [7]. However, others have proposed that BM-MSCs can aggravate hepatic fibrosis [8]. The various delivery routes used for BM-MSC transplantation to cure liver fibrosis appear to be related to these controversies in the action of BM-MSCs [9].

To assess the effectiveness and possible mechanisms of BM-MSC transplantation on the management of carbon tetrachloride (CCl_4)-induced liver fibrosis, this study compared the therapeutic efficacy of intravenous and intrasplenic injection of BM-MSCs, and elucidated the possible mechanisms underlying differences in the efficacy of different transplantation sites.

Materials and Methods

Experimental animals

Fifty male 6-week old white albino rats, initially weighing 150–200 g, were acquired from the Faculty of Medicine Animal House. The rats were housed in clean stainless steel cages of 65 cm × 25 cm × 15 cm (6 rats per cage) containing woodchip bedding. The rats were kept on a 12-h light/dark cycle in an aerated room and supplied with nutrition and water ad libitum. Altogether, the rats were kept for 1 week before the experimental procedures were started. All procedures were conducted in agreement with the Guide for the Care and Use of Laboratory Animals [10] and were approved by the ethics committee of our institution.

Liver fibrosis induction

To induce liver fibrosis, 0.2 mL CCl_4/100 g body weight of 40 ml/L CCl_4 (Sigma Corp., St. Louis, MO) liquefied in castor oil (Sigma) was injected subcutaneously twice weekly for 6 weeks [11]. Liver fibrosis was detected on histopathology examination of rat liver samples. The control group (n = 10) was injected subcutaneously with castor oil (0.2 mL/100 g body weight) twice weekly for 6 weeks.

Experimental design

On day 42, the fibrotic rats were divided randomly into 4 groups (10 rats per group): CCl_4 group (euthanized immediately at the end of 42 days); CCl_4 group/recovery group (received no treatment); CCl_4/BM-MSC intravenous group (1.0×10^7 BM-MSCs were infused intravenously through the tail vein once); CCl_4/BM-MSC intrasplenic group (1.0×10^7 BM-MSCs were injected intrasplenically under ultrasound guidance). After 12 weeks, the rats of the last 3 groups were euthanized with CO_2 narcosis, and liver tissue was used for histopathological examination and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analyses.

BM-MSC preparation, isolation, and culture

Tibia and fibula bone marrow was flushed out with phosphate-buffered saline (PBS) containing 2 mM EDTA for isolation and culture of BM-MSCs. Then, 35 mL of the sample was layered carefully on 15 mL Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), centrifuged for 35 min at 400 × g, and the superior layer was aspirated without disturbing the mononuclear cell layer at the interphase. Then, the mononuclear cell layer was removed, washed twice with PBS, and centrifuged at 200 × g at 10°C for 10 min. The cell pellet was resuspended in 300 µL PBS-EDTA buffer and the isolated BM-MSCs were cultured in 25-mL culture flasks in minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and incubated for 2 h at 37°C with 5% humidified CO_2. Lastly, a culture of adherent MSCs was maintained in MEM augmented with 30% FBS, 0.5% penicillin and streptomycin at 37°C with 5% CO_2 and air [12]. Cultured MSCs were recognized by their morphology and fluorescence activated cell sorting (FACS) using the surface markers $CD29^+$ and $CD44^+$, which are specific to MSCs.

Labeling BM-MSCs with green fluorescent protein (GFP)

BM-MSCs were collected and labeled with GFP (Amaxa Inc., Gaithersburg, MD). BM-MSCs were nucleofected using a Human MSC Nucleofector Kit and a GFP-encoding plasmid; cells were centrifuged, washed twice in serum-free medium, pelleted, and finally applied to the nucleofector solution ($4.0–5.0 \times 10^5$ cells/100 µL nucleofector solution). The sample was placed in a cuvette of the electroporation transfection instrument and the program U-23 (for high transfection efficiency) or C-17 (for high cell survival) was used. At 24 h after nucleofection, BM-MSCs were examined by light and fluorescence microscopy. With GFP, transfection efficiencies can reach 80%. Finally, GFP-labeled BM-MSCs were infused into fibrotic rats. After 12 weeks, liver tissues were observed using a fluorescence microscope (Leica, Wetzlar, Germany) to confirm the liver-specific homing of GFP-labeled BM-MSCs.

Biochemical assessment

The rats were anesthetized and blood was taken from the retro-orbital vein to measure albumin and alanine transaminase (ALT) using colorimeter kits (CAT # ab105134, sensitivity: 10 mU/well and CAT # ab108789, sensitivity: 0.7 ng/mL, respectively, Abcam Inc., Cambridge, MA) according to the manufacturer's instructions.

Histopathological assessment

Immediately after euthanasia, liver tissue was excised and divided into 2 sections. The first section was used for tracing the injected GFP-labeled BM-MSCs. The second section was washed with PBS and fixed in paraformaldehyde (40 g/L) at 4°C overnight. Five-micrometer sections were stained with hematoxylin and eosin by the method of Durry and Wallinngton [13] for general examination and Masson's trichrome [14] for the evaluation of fibrosis.

RNA extraction and qRT-PCR

Liver tissues of all groups were homogenized with a universal laboratory homogenizer (aid type 309). Then, total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA and assessed with a dual spectrophotometer (Beckman Coulter Inc., Fullerton, CA). qRT-PCR was used for quantitative analysis of gene expression of the fibrogenic markers transforming growth factor (TGF)-β1 and α-smooth muscle actin (SMA), inflammatory markers interleukin (IL)-1β, IL-6, and interferon (INF)-γ, hepatocyte-specific marker cytokeratin 18 (CK18), and hepatic growth factor (HGF). For cDNA synthesis, 1000 ng isolated total RNA was reverse transcribed with a High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). Subsequently, cDNA was amplified using a SYBR Green I PCR Master Kit (Fermentas | Thermo Fisher Scientific, Vilnius, Lithuania) on a Step One instrument (Applied Biosystems) as follows: enzyme activation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 55°C, and 30 s at 72°C. Changes in gene expression were normalized relative to the mean critical threshold value of the GAPDH housekeeping gene. The primers for each gene are listed in Table 1.

Table 1. Primer sequences for each gene

Target gene	Primer sequence: 5′–3′	GenBank accession number
IL-1β	Forward: GCTGTGGCAGCTACCTATGTCTTG Reverse: AGGTCGTCATCATCCCACGAG	NM_031512.2
IL-6	Forward: CCACTTCACAAGTCGGAGGCTTA Reverse: GTGCATCATCGCTGTTCATACAATC	NM_012589.2
INF-γ	Forward: AGGCCATCAGCAACAACATAAGTG Reverse: GACAGCTTTGTGCTGGATCTGTG	NM_138880.2
α-SMA	Forward: GTTTGAGACCTTCAATGTCCC Reverse: CGATCTCACGCTCAGCAGTGA	XM_003962516.2
TGF-β	Forward: TGCGCCTGCAGAGATTCAAG Reverse: AGGTAACGCCAGGAATTGTTGCTA	NM_021578.2
CK18	Forward: GGACCTCAGCAAGATCATGGC Reverse: CCACGATCTTACGGGTAGTTG	XM_008760823.1
HGF	Forward: TGGTGTTTCACAAGCAATCCAGA Reverse: CCGTTGCAGGTCATGCATTC	NG_016274.2
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC Reverse: ACATCAAGAAGGTGGTGAAGCAG	XR_598347.1

Enzyme-linked immunosorbent assay (ELISA)

Connective tissue growth factor (CTGF) levels (in pg/mL) were assessed in the liver tissue homogenates of the studied groups using an ELISA kit (Wuhan USCN Business Co., Ltd. Wuhan, China) according to the manufacturer's instructions.

Western blot analysis

Western blotting was used to measure the protein levels of vascular endothelial growth factor (VEGF) in liver tissues. Tissues from all groups were lysed using RIPA lysis buffer at 4°C. Extracts were centrifuged at 16, 000 rpm at 4°C (Eppendorf AG, Hamburg, Germany) for 30 min, and the supernatants were collected and utilized for protein quantification, according to the Bradford method. Proteins were denaturation by boiling the samples in Laemmli sample buffer. Lysate supernatants (30 μg/sample) were separated by 10% SDS–PAGE and transferred to an NC membrane (Amersham Biosciences Corp., Piscataway, NJ) by means of a semi-dry transfer apparatus (Turbo; Bio-Rad Laboratories Inc., Hercules, CA). Then, the membrane was blocked with 5% milk in a buffer containing 10 mmol Tris–HCl (pH 7.4), 150 mmol NaCl, and 0.05% Tween 20 (TBST) at 4°C overnight. The membranes was washed with TBST and incubated with a 1/2000 dilution of a rat anti-VEGF antibody (R&D Systems, Inc., Minneapolis, MN) for 1 h at 4°C with constant shaking. The NC membrane was then washed with TBST and subsequently probed with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham) at a dilution of 1/2000. Chemiluminescence detection was performed using an Amersham ECL Detection Kit. The membrane was exposed and stained for the housekeeping protein β-actin. Membrane autoradiographs were attained by detecting the protein bands. The results from the blots are presented as a direct assessment of the area of the superficial bands in autoradiographs and quantified by densitometry using Bio-Rad Image software.

Statistical analysis

Collected data were organized, tabulated, and analyzed by Prism software statistical computer package version 6 (GraphPad Software, San Diego, CA). Mean and standard deviation (SD) were calculated; one-way analysis of variance (ANOVA) was used to examine differences among the groups. Significance was set at $P < 0.05$.

Results

Characterization and identification of BM-MSCs

BM-MSCs proliferated, became elongated and spindle-shaped, and reached 80–90% confluence after 14 days of culture (Fig. 1A). GFP was visualized *in vitro* after cell labeling (before transplantation) using a fluorescent microscope (Fig. 1b). At 2 weeks after BM-MSC transplantation, GFP-positive cells were detected in rat liver tissue (Fig. 1c). Surface phenotype analysis of BM-MSCs was performed by flow cytometry. More than 90% of BM-MSCs were positive for β1-integrin CD29 (Fig. 2a) and endoglin receptor CD44 (Fig. 2B).

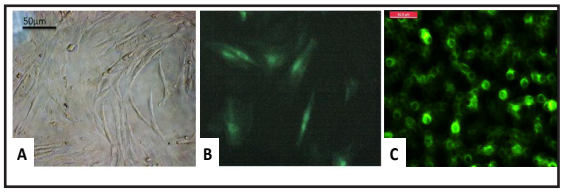

Fig. 1. Rat BM-MSC culture and identification. (A) BM-MSC culture at 2 weeks (80–90% confluent). (B) Labeling of BM-MSCs with the fluorescent marker GFP (in vitro). (C) Homing of GFP-labeled BM-MSCs in liver tissue after scarification (in vivo).

Biochemical assessments

As demonstrated in Fig. 3, the rats in the CCl_4 and CCl_4/recovery groups presented with significantly increased levels of serum ALT and decreased levels of serum albumin compared with the control group ($P < 0.001$ for each). Raised serum ALT is an indicator of hepatocyte damage and loss of cell membrane functional integrity. Furthermore, reduced serum albumin reflects the impaired synthetic function of the liver. However, serum ALT levels were also diminished significantly in the BM-MSC transplanted groups (intravenous and intrasplenic) compared with the CCL_4 and CCl_4/recovery groups ($P < 0.001$ for each), with no difference between intravenous and intrasplenic injections. This indicated the successful differentiation of the transplanted cells, which could function as mature hepatocytes.

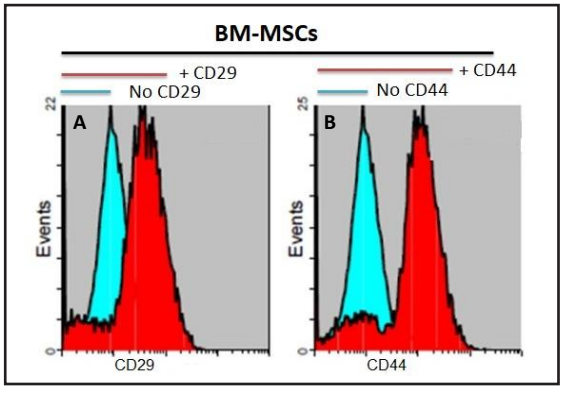

Fig. 2. FACS analysis of BM-MSC-positive cell markers. (A) FACS analysis for the BM-MSC-positive cell marker $CD29^+$. (B) FACS analysis for the BM-MSC-positive cell marker $CD44^+$.

Effects of intravenous and intrasplenic BM-MSC injections on the gene expression of inflammatory markers

Fig. 4 shows gene expression of inflammatory markers (IL-1β, IL-6, and INF-γ) using qRT-PCR. Compared with the control group, IL-1β, IL-6, and INF-γ expression was increased significantly in the CCl_4 and CCl_4/recovery groups ($P < 0.001$ for all). However, a significant decline in the expression of all of these markers was observed after intravenous BM-MSC injection ($P < 0.001$ for every parameter vs. CCL_4 and CCl_4/recovery groups) and intrasplenic BM-MSC injection ($P < 0.001$

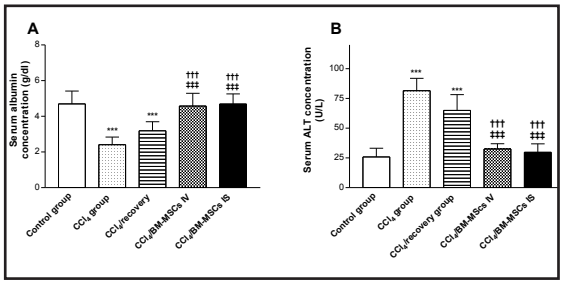

Fig. 3. Effects of intravenous (IV) and intrasplenic (IS) BM-MSC transplantation on albumin and ALT serum concentrations in CCl_4-induced liver fibrosis. Data are stated as the mean ± SD, n = 10 rats. Comparisons among groups were made using one-way ANOVA followed by Bonferroni's test. ***P<0.001, significantly different from the control group; †††P<0.001, significantly different from the CCl_4 group; and ‡‡‡ P<0.001, significantly different from the CCl_4/recovery group.

for each parameter vs. CCl_4 group and $P < 0.5$ vs. CCl_4/recovery group for IL-1β and IL-6). Instead, there was a significant reduction of IL-1β, IL-6, and INF-γ gene expression following intravenous administration of BM-MSCs compared to their intrasplenic administration ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively). Thus, intravenous BM-MSC injection has a more pronounced anti-inflammatory effect than intrasplenic BM-MSC injection.

Effects of intravenous and intrasplenic BM-MSC injections on fibrogenic markers (TGF-β1, α-SMA, and CTGF)

As shown in Fig. 5, the gene expression of TGF-β1 and α-SMA (an indicator of activated HSCs) and CTGF concentration showed a remarkable rise in the CCl_4 and CCl_4/recovery groups compared with the control group ($P < 0.001$ for each). Intravenous and intrasplenic injections of BM-MSCs resulted in a similar reduction in the expression of fibrogenic marker genes ($P < 0.001$ for each) compared with the CCl_4 and CCl_4/recovery groups.

Effects of intravenous and intrasplenic BM-MSC injections on CK18 and HGF gene expression

Fig. 6 displays CK18 (a specific marker of biliary epithelial cells) and HGF (a potent supporter of liver cell mitosis) gene expression. Compared with the control group, CK18 and HGF gene expression was decreased significantly in the CCl_4 and CCl_4/recovery groups ($P < 0.001$ for each). BM-MSC transplantation resulted in a significant rise of CK18 and HGF gene expression ($P < 0.001$ for each vs. CCl_4 group and $P < 0.01$ and $P < 0.001$ vs. CCl_4/recovery group, respectively), with no significant difference between the transplantation routes.

Western blot analysis

VEGF protein was decreased significantly in the CCl_4 group ($P < 0.001$) compared with the control group. However, intravenous and intrasplenic BM-MSC injections increased VEGF protein levels similarly ($P < 0.001$ for each vs. CCl_4 group and $P < 0.01$ vs. CCl_4/recovery group) as shown in Fig. 7.

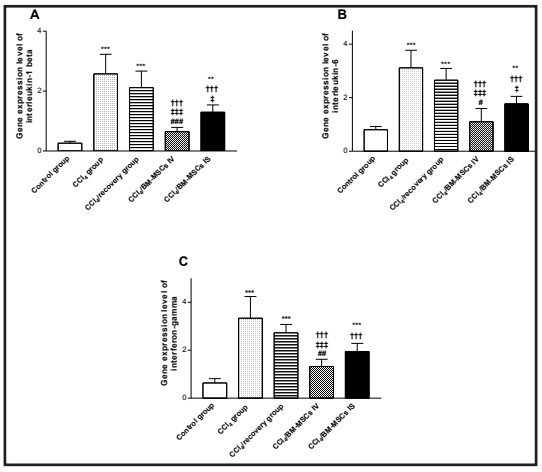

Fig. 4. Effects of intravenous (IV) and intrasplenic (IS) BM-MSC transplantation on the gene expression of inflammatory markers: interleukin-1 beta (A), interleukin-6 (B), and interferon-gamma (C). Data are presented as the mean ± SD, n = 10 rats. Comparisons among groups were made using one-way ANOVA followed by Bonferroni's test. **P<0.01 and ***P<0.001, significantly different from the control group; †††P<0.001, significantly different from the CCl_4 group; ‡P<0.05 and ‡‡‡P<0.001, significantly different from the CCl_4/recovery group; and #P<0.05, ##P<0.01, and ###P<0.001, significantly different from the BM-MSC intrasplenic group.

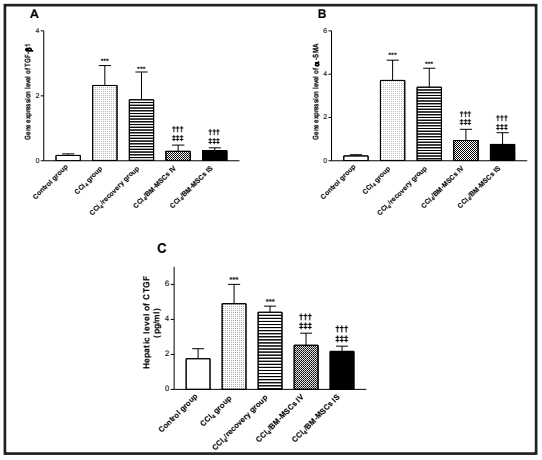

Fig. 5. Effects of intravenous (IV) and intrasplenic (IS) BM-MSC transplantation on fibrogenic markers: TGF-β1 (A), α-SMA (B), and CTGF (C). Data are presented as the mean ± SD, n = 10 rats. Comparisons among groups were made using one-way ANOVA followed by Bonferroni's test. ***P<0.001, significantly different from the control group; †††P<0.001, significantly different from the CCl_4 group; and ‡‡‡P<0.001, significantly different from the CCl_4/recovery group.

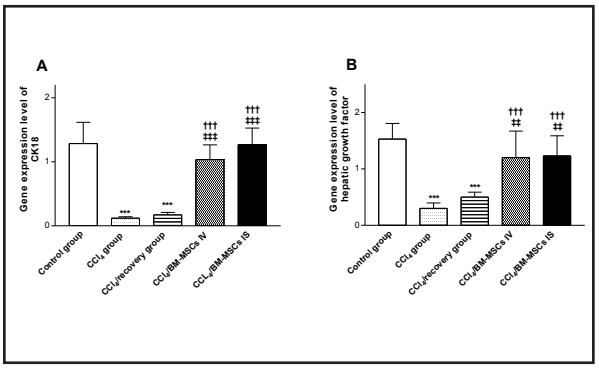

Fig. 6. Effects of intravenous (IV) and intrasplenic (IS) BM-MSC transplantation on CK18 gene expression (A) and hepatic growth factor (B). Data are expressed as the mean ± SD, n = 10 rats. Comparisons among groups were made using one-way ANOVA followed by Bonferroni's test. ***P<0.001, significantly different from the control group; †††P<0.001, significantly different from the CCl_4 group; and ‡‡P<0.01 and ‡‡‡P<0.001, significantly different from the CCl_4/recovery group.

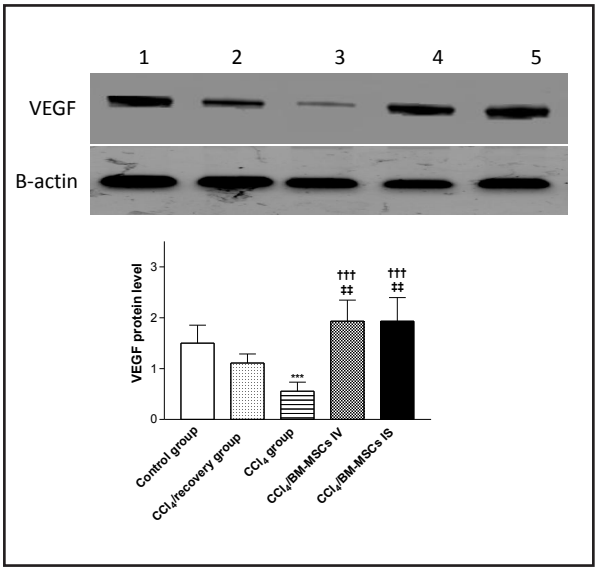

Fig. 7. Effects of intravenous (IV) and intrasplenic (IS) BM-MSC transplantation on VEGF protein levels in liver tissue. Control group (1), CCL_4/recovery group (2), CCl_4 group (3), CCL_4/BM-MSC intravenous (4), and CCL_4/BM-MSC intrasplenic (5). The corresponding densitometric values (mean ± SD), which are expressed as arbitrary units calculated for each group, are normalized on the basis of the respective value for β-actin. Data are expressed as the mean ± SD, n = 10 rats. Comparisons among groups were made using one-way ANOVA followed by Bonferroni's test. ***P<0.001, significantly different from the control group; †††P<0.001, significantly different from the CCl_4 group; and ‡‡P<0.01, significantly different from the CCl_4/recovery group.

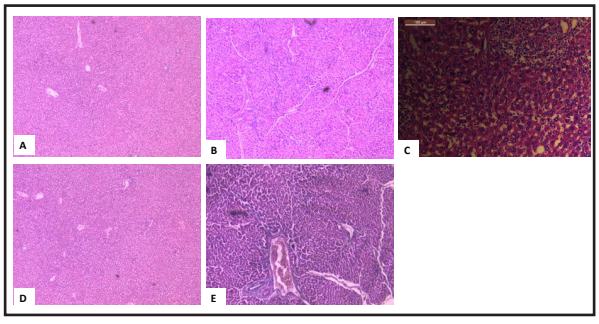

Fig. 8. Hematoxylin and eosin staining of liver tissue. (A) The control group shows regular hepatic architecture. (B) The CCl_4 group shows mild focal portal fibrosis. (C) The CCl_4/recovery group reveals inflammatory cell infiltration and hepatocyte degeneration. BM-MSC administration intravenously (D) and intrasplenically (E) restored normal liver architecture.

Histopathological examination

Liver sections stained with hematoxylin and eosin revealed typical lobular architecture in the control rats. Mild focal portal fibrosis was seen in the CCl_4 group. Abnormal morphology, with inflammatory cell infiltration and hepatocyte degeneration was seen in the CCl_4/recovery group (Fig. 8). Intravenous and intrasplenic BM-MSC administration decreased this abnormal morphology efficiently. Masson's trichrome staining confirmed the establishment of hepatic fibrosis in the CCl_4 group via collagen deposition (bright blue) within the liver architecture (dark purple and pink) and revealed decreased collagen deposition following intravenous and intrasplenic BM-MSC administration. The CCl_4/recovery group showed a nodular appearance with foci of hepatocyte dysplasia (Fig. 9).

Discussion

To date, only a few studies have compared different BM-MSC transplantation routes for the treatment of liver fibrosis. Moreover, the relationship between transplantation route and transplantation efficacy is unclear [15]. In the present study, we injected BM-MSCs through two different routes (intravenous and intrasplenic) and compared their efficacy for the management of CCl_4-induced liver fibrosis and also assessed the conceivable mechanisms underlying this effect.

The main conclusions of this study were: 1) both intravenous and intrasplenic BM-MSC injections ameliorated rat liver function by elevating serum albumin levels and reducing serum ALT levels; 2) intravenous BM-MSC administration was more efficient than the intrasplenic route in decreasing inflammation by reducing the gene expression of pro-inflammatory cytokines (IL-1β, IL-6, and INF-γ); 3) both intravenous and intrasplenic BM-MSC injections had an anti-fibrotic effect, which was demonstrated via the reduction of pro-fibrogenic factors (repressing the gene expression of TGF-β1 and α-SMA and reducing the hepatic levels of CTGF) and the increase of anti-fibrogenic factors (CK18 and HGF), and this was verified by liver tissue histopathological examination; 4) the increase of VEGF protein levels was identical after intravenous and intrasplenic BM-MSC administration; 5) prolonged presence of transplanted BM-MSCs to improve liver fibrosis, thereby reducing the need to transplant additional stem cells for the treatment of liver damage; and 6) pathological and biochemical examination revealed that the liver was still affected in the CCl_4/recovery group.

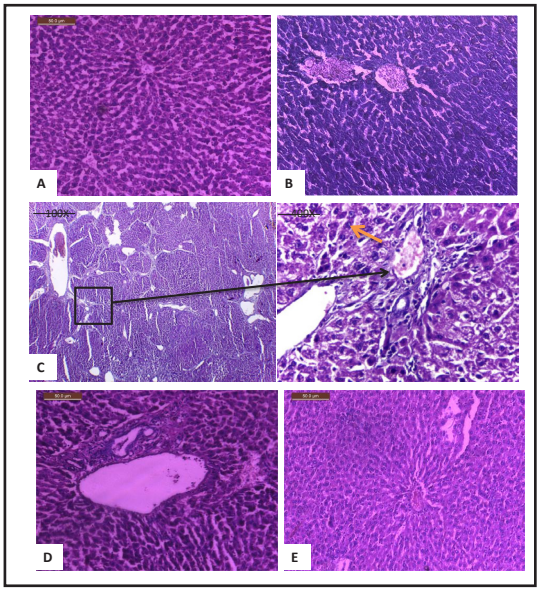

Fig. 9. Masson's trichrome staining of liver tissue. (A) The control group shows regular hepatic arrangement. (B) The CCl_4 group shows focal portal fibrosis. (C) The CCl_4/recovery group reveals nodular appearance of the liver due to fibrous septation, and the magnified rectangular shape shows a fibrous nodule, while the orange arrow indicates foci of hepatocyte dysplasia. Intravenous (D) and intrasplenic (E) BM-MSC administration decreased the number of collagen fibers compared with the CCl_4 group.

The current study demonstrated the equivalent therapeutic efficacy of the two routes of BM-MSC transplantation (intravenous and intrasplenic) for the recovery of liver function. Furthermore, this hepatic functional recovery was combined with liver parenchymal healing and hepatocyte regeneration, as shown by histopathological assessments of hepatic tissue. In partial agreement with the present study, Truong et al. [16] established the equivalent functional recovery of the liver between intravenous and portal vein injection of BM-MSCs.

Liver injury results in inflammation and the release of inflammatory mediators from inflammatory cells and liver parenchyma, which further trigger HSCs and increase their multiplication and survival, thereby worsening ECM deposition with an exacerbation of fibrosis [3] In the present study, the inflammatory response was blunted, as detected by the reduced gene expression of IL-1β, IL-6, and INF-γ, more by the intravenous administration of BM-MSCs than by their intrasplenic administration. This is in partial agreement with the findings of Zhao et al. [4] who demonstrated that intravenous injection of BM-MSCs reduced IL-1β and IL-6 expression more than their intraperitoneal or intrahepatic injection. The immunosuppressive effect BM-MSCs is driven by the suppression of T lymphocyte proliferation, B cell function, and dendritic cell maturation [17].

Following liver injury, HSCs, which are dormant with low mitotic activity under basal

conditions, are activated and change to myofibroblast-like cells, the chief producer of ECM protein in the liver, and increase their expression of α-SMA, which is a hallmark of activated HSCs [3]. Activated HSCs secrete TGF-β1 and CTGF to achieve their auto-activation [18]. TGF-β1 is a primary agent in the liver fibrogenic response, which is the result of an imbalance between ECM synthesis and degradation with the overproduction of ECM components [19]. Moreover, Dooley and Ten Dijke [20] described the fibrogenic influence of TGF-β1 through the initiation of inflammation. CTGF is a crucial profibrogenic cytokine produced by activated HSCs, and TGF-β increases the buildup of ECM in the liver [17]. Paradis et al. [21] reported CTGF over-expression in rat and human hepatic fibrosis. The present study revealed that both routes for BM-MSC transplantation had a comparable anti-fibrotic effect by inhibiting the expression of pro-fibrogenic factors. The current study concurred with the data of Abdel Abdel Aziz et al. [22] and El-Mahdi et al. [23] who found reduced TGF-β1 and α-SMA gene expression after BM-MSC transplantation and extended this finding to CTGF.

CK18 is a specific marker of mature hepatocytes and is expressed by biliary cells and hepatic cells [24]. HGF has a vital role in liver regeneration [4] and exerts an anti-fibrotic effect by inhibiting the expression of collagen and TGF-β1, thereby repressing the activity of HSCs [6]. Consistent with the findings of Li et al. [25], the present study revealed that both intravenous and intrasplenic administration of BM-MSCs had similar effects in increasing CK18 and HGF gene expression.

In liver fibrosis, ECM deposition distorts liver vasculature and shunts portal and hepatic arterial blood, thus impeding oxygen and nutrient exchange between hepatic sinusoids and hepatocytes and at last leads to cell apoptosis [26]. Therefore, promoting angiogenesis can increase vascularity, and consequently decrease fibrogenic progression [27]. VEGF, a pro-angiogenic factor, is vital for the propagation and passage of endothelial cells and the formation of novel vascular networks [28].

Consistent with the study of Li et al. [25], we revealed the increased levels of VEGF protein in hepatic tissue after BM-MSC transplantation, and this effect was prolonged for both delivery routes. Consequently, BM-MSCs seemed to accelerate angiogenesis by increasing VEGF protein levels. Numerous studies have demonstrated the increased expression of VEGF after BM-MSC transplantation in ischemic stroke [29] and chronic renal failure [30]. In contrast, Xi et al. [28] suggested that inflammation-associated angiogenesis could contribute to progressive liver fibrosis. Furthermore, Al-Rasheed et al. [3] proposed that an immature vascular plexus appeared around regenerating hepatocytes, impeded the exchange of oxygen and nutrients between sinusoids and hepatocytes, decreased parenchymal perfusion, and finally induced the progression of fibrosis.

In conclusion, this study demonstrated that intravenous BM-MSC transplantation inhibited the inflammatory response more effectively than intrasplenic transplantation, indicating that intravenous BM-MSC transplantation is an efficient, safe, and suitable method for reversing liver fibrosis. Additionally, this study supported earlier findings suggesting the beneficial effect of BM-MSCs in managing liver fibrosis. This effect occurred via the suppression of inflammation (by decreasing IL-1β, IL-6, and INF-γ gene expression), reduced production of pro-fibrogenic factors (TGF-β1, α-SMA, and CTGF), increased production of anti-fibrogenic factors (CK18 and HGF), and up-regulation of the angiogenic factor VEGF.

Disclosure Statement

The authors declare no conflicts of interest. The authors are responsible for the content and writing of the paper.

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