Cord blood-derived mesenchymal stem cells with hepatogenic differentiation potential ameliorate chronic liver affection in experimental models

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Abstract

Background. The liver is one of the major target organs for which cell-based therapies are very promising. The limitations of various cellular therapies, including bone marrow (BM)-derived mesenchymal stem cells (MSCs), urges the exploration of stem cell sources more suitable for transplantation. Human umbilical cord blood (HUCB) can overcome these drawbacks with a favorable reparative outcome.

Objectives. The aim of this study was to evaluate the therapeutic potential of MSCs in 2 groups of chronic liver injury experimental models.

Material and methods. Propagation and characterization of MSCs isolated from cord blood (CB) samples were performed and differentiation into osteogenic, adipogenic and hepatogenic lineages was induced. The 1st experimental model group (80 mice) included a negative control, a pathological control and 60 mice infected with Schistosoma mansoni (S. mansoni) and transplanted with MSCs. The 2nd experimental model group (30 hamsters) included 10 healthy hamsters serving as a negative control and 20 hamsters injected with repeated doses of carbon tetrachloride (CCl_4) to induce liver fibrosis; 10 of them were treated with an intrahepatic (IH) injection of 3×10^6 MSCs and the other 10 were untreated pathological controls. Mice and hamsters were sacrificed 12 weeks post-transplantation and their liver sections were stained immunohistochemically for the detection of human hepatocyte-like cells. Moreover, the sections were examined for the levels of fibrosis.

Results. In both models, the transplantation of CB-derived MSCs (CB-MSCs) resulted in the engraftment of the fibrotic livers with newly formed hepatocytes, as evidenced by positive immunohistochemistry staining with human Hepatocyte Paraffin 1 (Hep Par 1), alpha-fenoprotein (AFP), cytokeratin 18 (CK18), cytokeratin 7 (CK7), and OV6 monoclonal antibody. The transplanted liver sections showed markedly reduced hepatic fibrosis with a significantly lower fibrotic index, as well as significantly improved liver functions compared to the pathological control ($p < 0.001$).

Conclusions. This data provides hope that human CB-MSCs can be utilized as multipotent stem cells with unlimited potentiality in regenerative medicine and supports the concept of cellular therapy for the cure of hepatic fibrosis.

Key words: cord blood, mesenchymal stem cells, carbon tetrachloride, liver fibrosis

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Introduction

Liver fibrosis is the wound-healing reaction of hepatogenic cells to chronic liver injury.[1] Following repetitive injury, the liver undergoes a remarkable tissue remodeling and develops fibrosis. This fibrosis is characterized by an extensive accumulation of the extracellular matrix, with the disposition of scar tissue encapsulating the region of injury. Although liver transplantation is undoubtedly the most efficient therapy for cirrhosis, broad clinical application of this procedure is limited by the scarcity of donor organs.[2] Thus, it is crucial to investigate the efficacy of various treatments for hepatic cirrhosis.

The liver is one of the major target organs for which cell-based therapies are very promising. The limitations of various cellular therapies, including bone marrow (BM)-derived mesenchymal stem cells (MSCs), urges the exploration of stem cell sources more suitable for transplantation. Human umbilical cord blood (HUCB) can overcome these drawbacks with a favorable reparative outcome.[3,4] Human umbilical cord blood stem cells remain viable even after long periods of cryopreservation.[5–7] These cells are an ideal option for clinical applications due to their ready availability from cord blood (CB), ease of expansion in an in vitro culture, simple isolation via plastic adherence, ability to evade rejection, and their multipotentiality for differentiation.[8,9]

Over the past 2 decades, HUCB has been well established as a valued source of hematopoietic stem and progenitor cells. Besides hematopoietic cells, CB also contains cells with various stromal properties.[10,11] From the point of view of clinical practice, it is very important to select a model of liver fibrosis that is close to the human disease in order to evaluate the effect of stem cells on the fibrosis. In the present study, we employed 2 models of fibrosis, induced by Schistosoma mansoni (S. mansoni) infection in mice and by carbon tetrachloride (CCl_4) in hamsters, to evaluate the effect of the MSCs injected through diverse routes and doses.

Material and methods

Isolation and culture of mesenchymal stem cells

Cord blood samples were collected from full-term babies delivered by cesarean section after informed written consent was given by the mothers, as approved by the institutional review board at Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Samples were processed according to the procedure described by Broxmeyer et al.[10] Mononuclear cell fraction was isolated by the density gradient separation method and cultured according to the procedure described by Kögler et al.[11] The MSCs were initiated in cultures, passaged and propagated according to Kögler et al.[5]

Immunophenotyping

Cells from the 3rd passage were washed twice in phosphate-buffered saline buffer (PBS). Fluorescein isothiocyanate (FITC) mouse anti-human CD44, CD45 and HLA-DR, as well as FITC mouse anti-human CD105 coupled with PE (phycoerythrin) mouse anti-human CD90, and FITC mouse anti-human CD34 coupled with PE mouse anti-human CD73, were added to flow cytometry tubes.

One hundred microliters of the cell suspension was added to each tube and incubation was done for 20 min at 4°C. The cells were washed twice with PBS and then resuspended in PBS. The final analysis was done by flow cytometer.

Gene expression analysis

Molecular analysis of the MSCs was performed according to the protocols described by Zaibak et al. and Demerdash et al.[12,13] Total RNA was extracted from trypsinized cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Its concentration and purity were assessed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Reverse transcripts were prepared using a high capacity cDNA kit (Applied Biosystems, Foster City, USA). The expression of the following genes was detected by real-time PCR using a QuantiTect SYBR Green PCR kit (Qiagen): *Oct4* (5' TCTCGCCCCCTCCAGGT; 3'GCCCCACTCCAACCTGG), *Sox2* (5' AGCTACAGCATGATGCAGGACC; 3'CTGGTCATGGAGTTGTACTGCAGG); and *GAPDH* (5'ATGGAGAAGGCTGGGGCTC, 3'AAGTTGTCATGGATGACCTTG).

Osteogenic differentiation

Following the guidelines of Kögler et al., the MSCs were seeded at a density of 6×10^3 cells/cm^2 into 6-well plates and cultured in complete Dulbecco's modified Eagle's medium (DMEM).[5] When the cells reached approx. 80–90% confluency, the medium was replaced with osteogenic differentiation medium (Lonza, Basel, Switzerland). Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was added to negative control wells. The media were replaced every 3–4 days. On the 14th day, calcium deposition was assessed by alizarin red staining.

Adipogenic differentiation

Following the guidelines of Kögler et al., the MSCs were seeded at a density of 5×10^3 cells/cm^2 into 6-well plates and cultured in complete DMEM.[5] When the cells reached approx. 80–90% confluency, they were cultured in the adipogenic induction medium for 3 days, followed by an adipogenic maintenance medium for 3 days. Three cycles of induction/maintenance were carried out for optimal adipogenic differentiation. Dulbecco's modified Eagle's

medium supplemented with 10% FBS 1% penicillin/streptomycin, and 1% L-glutamine was added to negative control wells. On the 21st day, adipogenic differentiation was assessed by Oil Red O staining.

Hepatogenic differentiation

The induction of hepatogenic differentiation was done according to the protocol described by Waclawczyk et al.[14] Briefly, the MSCs of passage 3 were seeded at a density of 5×10^3 cells/cm^2 and cultured in 30% FBS/DMEM until reaching 80% confluency. The medium was then replaced with hepatogenic differentiation medium I (HDM I), consisting of hepatozyme supplemented with 0.5% FBS, hepatocyte growth factor (HGF) (50 ng/mL) (R&D Systems, Minneapolis, USA), fibroblast growth factor-4 (FGF-4) (20 ng/mL) (R&D Systems, Minneapolis, USA), epidermal growth factor (EGF) (20 ng/mL) (R&D Systems, Minneapolis, USA), 1% penicillin/streptomycin, and 1% L-glutamine. The cells were cultured for 2 weeks before exchanging the medium with HDM II, consisting of DMEM supplemented with HGF (50 ng/mL), FGF-4 (20 ng/mL), oncostatin-M (50 ng/mL), insulin transferrin selenium (ITS) (5 µL/mL), 10^{-7} dexamethasone, 1% penicillin/ streptomycin, and 1% L-glutamine. The HDM II was left for another 2 weeks. Cells were scraped, collected and slide-fixed for cytopathological diagnosis of hepatocyte markers (glycogen, alpha-fetoprotein (AFP) and cytokeratin 18 (CK18)) using periodic acid-schiff (PAS) staining (Sigma-Aldrich, St. Louis, USA) for glycogen detection and immunohistochemistry staining for AFP and CK18 detection.

Experimental model

All animals were raised and maintained at the animal house in TBRI in barrier units with a defined and regularly monitored health status. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Induction of an experimental model for hepatic fibrosis in mice by infection with Schistosoma mansoni cercariae

The S. mansoni-infected model consisted of 80 BALB/c mice, 8 weeks old and weighing 20–25 g, which were divided into the following groups:

- normal control group – 10 mice,
- infected control group – 10 mice infected with S. mansoni cercariae (60 cercariae/mouse) using the tail immersion method and left for 12 weeks to induce liver fibrosis, and
- MSCs-transplanted group – 60 mice infected with S. mansoni. This group was subdivided according to the method of MSCs transplantation into 2 subgroups (30 mice each): intravenous (IV) and intrahepatic (IH).

Both the IV and IH subgroups were further subdivided, according to the dose of transplanted MSCs, into 3 subgroups; IV1, IH1, IV2, IH2, IV3, and IH3, transplanted with 3×10^5 & 6×10^5 & 1×10^6 MSCs/mouse, respectively.

Mice from each group were sacrificed 4, 8, and 12 weeks after MSCs transplantation, respectively.

Induction of experimental model for hepatic cirrhosis by carbon tetrachloride

The CCl_4 model consisted of 30 hamsters weighing 60–65 g and divided into 3 groups:

- normal control group – 10 healthy hamsters;
- CCl_4 control group – 10 hamsters injected with CCl_4 at a dose of 100 µL/hamster (Al-gamhuria, Cairo, Egypt) mixed with olive oil (100 µL CCl_4 + 900 µL olive oil) intraperitoneally (IP) twice a week for 2 months.[15] Healthy control hamsters were injected with the same volume of olive oil;
- MSCs-transplanted group – 10 hamsters injected with CCl_4 as above and treated with MSCs (3×10^6 cells/hamster) IH 2 months after CCl_4 injection.

The cells were injected via the IH route because hamsters have no tail and the IV route was not feasible. The hamsters were sacrificed 12 weeks after MSCs transplantation.

Liver function analysis

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), and bilirubin were measured using a Synchron CX5 (Beckman Coulter, Brea, USA) analyzer.

Liver pathology and immunohistochemistry analysis

Liver sections from sacrificed animals were stained with hematoxylin and eosin (H&E) for histologic assessment and with Sirius red for assessment of fibrosis.[16] The CK18, cytokeratin 7 (CK7), Hepatocyte Paraffin 1 (Hep Par 1), AFP, and OV6 monoclonal antibodies were used (Santa Cruz Biotechnology Company, Santa Cruz, USA). In addition, negative controls in which the primary antibody was omitted and replaced by PBS were also used. Livers known to express markers were used as positive controls.

The intensity, distribution and pattern were analyzed and immunoexpression was evaluated. The percentage of positively stained cells was determined semiquantitatively by assessing the whole section.

Liver fibrosis analysis

Hepatic sections, 20 µm in thickness, were prepared from paraffin sections and stained with Picrosirius red. For the quantification of the collagen content, an Automatic Computer Image Analysis System (Zeiss, Oberkochen, Germany) was used. Image analysis was performed using

the computer software AxioVision v. 4.8, supplied by the manufacturer of the system. The sectional area of the red-stained fibrous tissue was measured in 5 consecutive microscopic fields (×5 magnification) to yield the fibrotic area (μm^2) and the proportion of fibrotic area relative to the total area examined was then calculated (fibrotic index in %).

Statistical analysis

The data were analyzed using the SPSS package v. 18.0 for Windows (SPSS Inc., Chicago, USA). Laboratory data of different groups were compared with one-way analysis of variance (ANOVA). At a p-value ≤0.05 differences were considered statistically significant.

Results

Immunophenotyping

The MSCs showed high expression levels of adhesion marker CD44, typical mesenchymal markers (CD90 and CD73), the endoglin receptor CD105, and dual expression of CD105/90; they were negative for HLA-DR, hematopoietic lineage marker CD34 and leukocyte common antigen CD45 expression (Table 1).

Gene expression analysis

The MSCs expressed *Oct4* and *Sox2*, which are considered to be core transcription factors that regulate the maintenance of the pluripotent state in embryonic and adult stem cells.

Osteogenic differentiation

When HUCB-MSCs were induced to differentiate into osteogenic lineage, the spindle shape of the HUCB-derived cells became less elongated and more polygonal in shape with the formation of aggregates (Fig. 1A).

Adipogenic differentiation

Under the influence of adipogenic differentiation conditions, the MSCs became large and rounded with an accumulation of neutral lipid vacuoles indicated by the Oil Red O stain (Fig. 1B).

Hepatogenic differentiation

Morphology

The fibroblastic-like morphology of the MSCs was lost. A broadened, flattened shape developed at first, while a round shape with a cuboidal morphology developed later. Slides coated with MSCs-derived cells and stained with H&E are shown in Fig. 1C.

Periodic acid-schiff staining

Smears were examined using a Zeiss Axio Scope A1 (Zeiss), under a magnification of ×400. Glycogen storage was determined by PAS staining. Positively stained glycogen granules were detected in the cytoplasm of differentiated MSCs, while undifferentiated MSCs were negative for PAS staining (Fig. 2).

Immunocytochemistry

Smears were examined using a Zeiss Axio Scope A1 (Zeiss), under ×400 magnification. With hepatocyte markers (AFP and CK18) being cytoplasmic markers, both AFP and CK18 were expressed in the cytoplasm of differentiated MSCs (Fig. 3 A,B), while undifferentiated MSCs were negative for both markers.

Schistosoma mansoni-induced fibrogenesis model

Only the results of transplantation with 1×10^6 MSCs after 3 months are presented here, as they were the most promising results.

Effect of intravenous transplantation of 1×10^6 MSCs/mouse on Schistosoma mansoni-induced fibrogenesis

Intravenous transplantation of 1×10^6 MSCs in S. mansoni-infected mice revealed a high, significant reduction ($p < 0.001$) in fibrotic index 3 months after MSCs transplantation, compared to the corresponding index in the infected control group (Table 1).

Table 1. Results of liver functions – aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin (ALB) levels – and % fibrosis in different studied groups of murine Schistosoma mansoni-infected mice (mean ±SE)

Parameter	Studied groups of murine Schistosoma mansoni-infected mice			
	negative control (n = 10)	infected control (n = 10)	infected & treated with IV 1×10^6 MSCs (n = 30)	infected & treated with IH 1×10^6 MSCs (n = 30)
Albumin [g/dL]	4.36 ±0.049	0.73 ±0.18	5.2 ±0.167	3.2 ±0.096
ALT [U/L]	24.66 ±0.421	136 ±3.65	51 ±0.96	66.82 ±3.06
AST [U/L]	68.67 ±0.76	350.67 ±19.33	79.67 ±7.02	101.76 ±0.66
% fibrosis	0.2 ±0.04	27.48 ±0.745	5.37 ±0.21	7.58 ±0.38

MSCs – mesenchymal stem cells; IV – intravenous; IH – intrahepatic; SE – standard error.

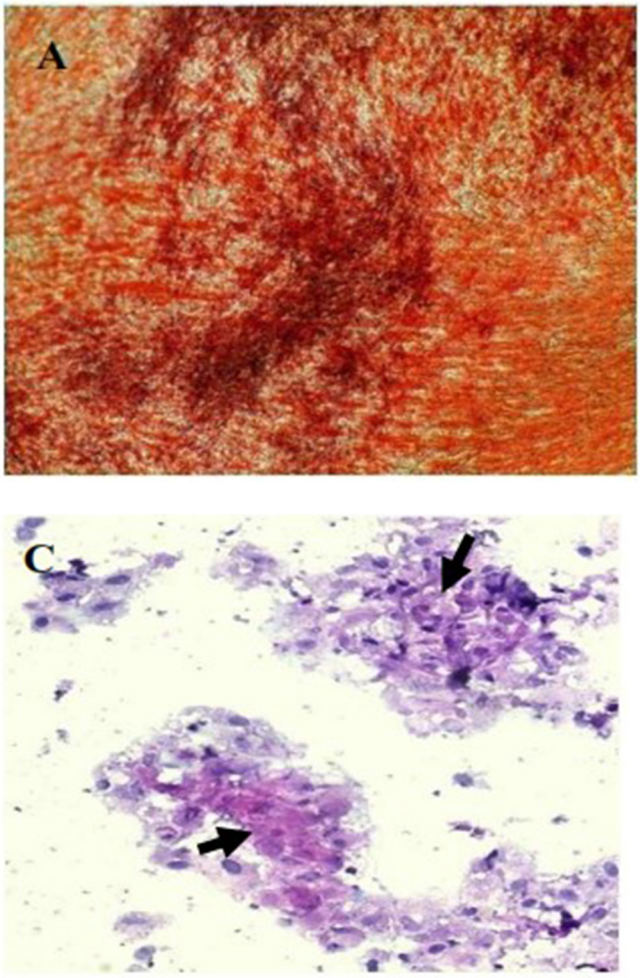

Fig. 1. Mesenchymal stem cells (MSCs) of P3 were induced to osteogenic, adipogenic and hepatogenic differentiation, which was confirmed A) by the formation of a mineralized matrix as evidenced by alizarin red staining, B) by the accumulation of neutral lipid vacuoles evidenced by the oil red O stain, and C) by the morphological changes towards hepatocyte-like cells showing large polyhedral cells with rounded nuclei in small sheets or separate hepatocyte-like cells

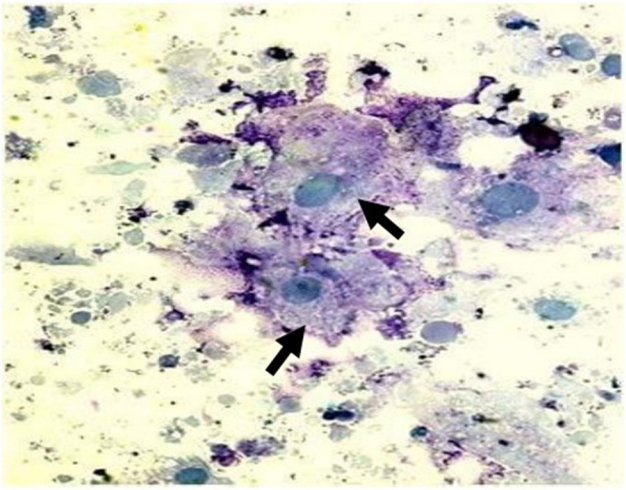

Fig. 2. Positively periodic acid-Schiff (PAS)-stained glycogen granules in the cytoplasm of differentiated mesenchymal stem cells (MSCs) showing large polyhedral cells with rounded nuclei in small sheets or separate hepatocytes-like cells positive for PAS stain (×400 magnification)

There was a significant improvement ($p < 0.001$) in the ALT, AST and ALB (Table 1) levels of the MSCs-transplanted group 3 months post-transplantation, when compared to the corresponding level in the infected control. It was observed that ALB reached a nearly normal level after the 3rd month.

In mice transplanted IV with 1×10^6 MSCs and sacrificed 12 weeks after transplantation, liver sections stained with H&E (Fig. 4 A–C) and Sirius red (Fig. 4 D–F) showed diminished granuloma size and a relative decrease in hepatic fibrosis. The cells were able to engraft into the fibrotic livers with some signs of regeneration, i.e., newly formed hepatocytes of human origin.

Livers of the MSCs-transplanted group showed engraftment with human hepatocyte-like cells, as proven by the cytoplasmic expression of AFP, Hep Par 1, CK18, CK7, and OV6 (Fig. 5 A–E) and by the negative staining of hepatocytes for desmin and vimentin. In addition, the livers of the MSCs-transplanted group showed less fibrosis than the pathological control group.

Effect of intrahepatic transplantation of 1×10^6 MSCs/mouse on Schistosoma mansoni-induced fibrogenesis

Almost same results were obtained as in IV route groups with less reduction in the fibrotic index (Table 1).

There was a significant improvement in ALT, AST and ALB levels (Table 1) 3 months after IH MSCs transplantation, compared to the levels in the infected control group. This improvement was less marked than that with the IV route groups.

In mice transplanted intrahepatically with 1×10^6 MSCs, liver sections stained with either H&E or Sirius red showed relatively diminished granuloma size and a relative decrease in hepatic fibrosis (Fig. 4), compared to the IV-transplanted group.

Immunoperoxidase staining of the liver sections from BALB/c mice infected with S. mansoni and injected IH

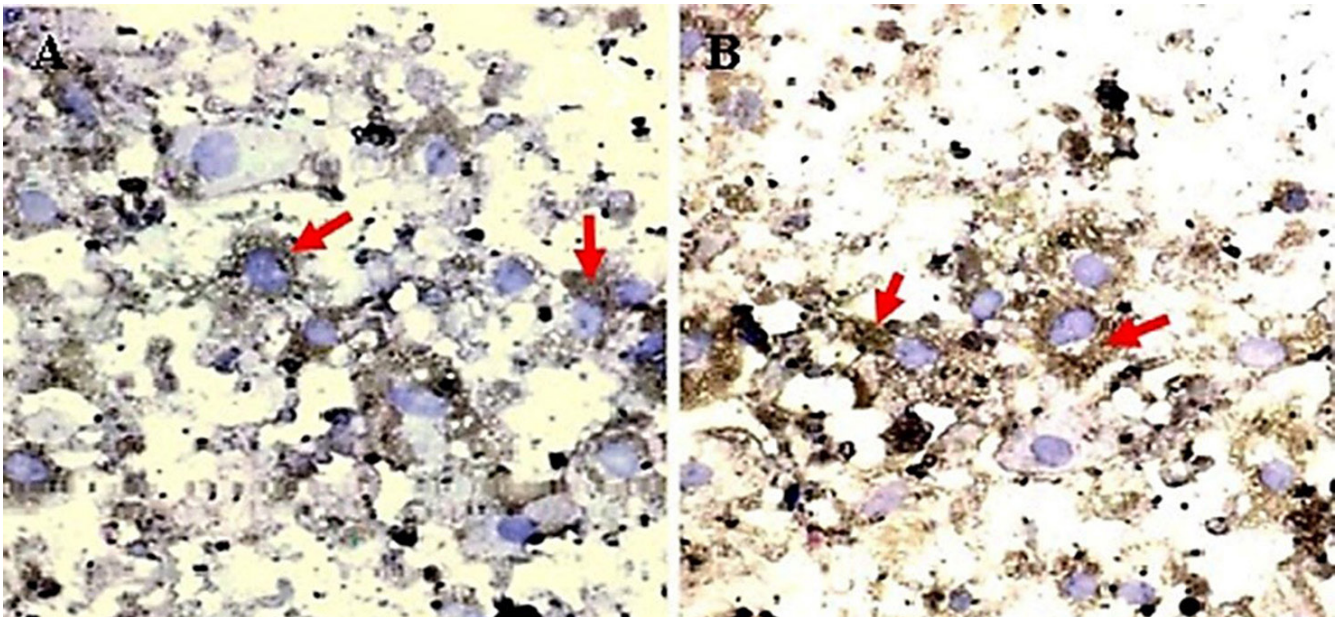

Fig. 3. Mesenchymal stem cells (MSCs)-differentiated hepatocyte-like cells positive for A) alpha-fetoprotein (AFP) and B) cytokeratin 18 (CK18), presented as cytoplasmic brownish stain (immunohistochemistry (IHC) ×400 magnification)

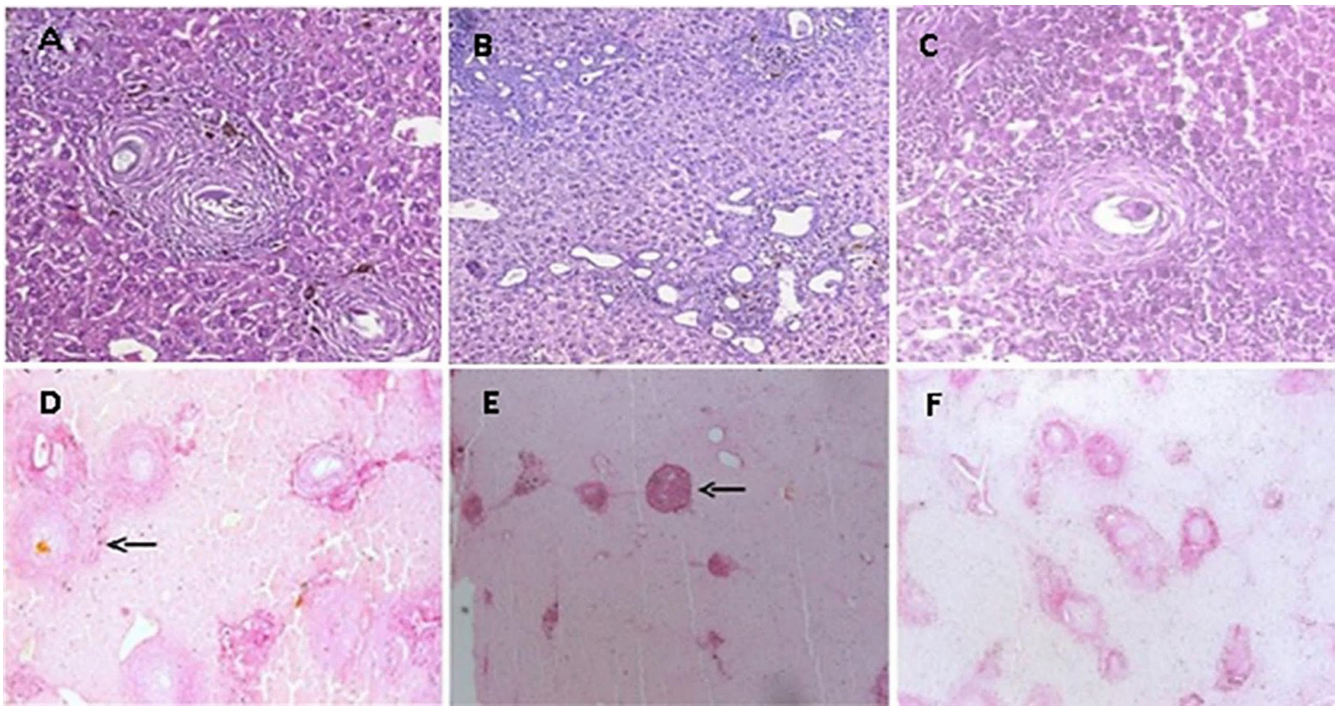

Fig. 4. Liver sections from BALB/c mice (hematoxylin and eosin (H&E) staining, ×400 magnification): A) infected control mouse showing a large fibrocellular granuloma 8 weeks post-infection; B) infected & intravenously (IV) 1×10^6 mesenchymal stem cells (MSCs)-treated mice 12 weeks post-treatment, showing a small regressed fibrocellular granuloma; C) infected and and intrahepatically (IH) 1×10^6 MSCs-treated mice 12 weeks post-treatment, showing moderate collagen fibrous bundles forming moderate size fibrocellular bilharzial granuloma. Liver sections from BALB/c mice infected with Schistosoma mansoni (Sirius red staining), showing: D) a large untreated fibrocellular granuloma 8 weeks post-infection; E) IV 1×10^6 MSCs-treated mice 12 weeks post-treatment with small regressed granuloma; F) IH 1×10^6 MSCs-treated mice 12 weeks post-treatment, showing moderate size fibrocellular bilharzial granuloma

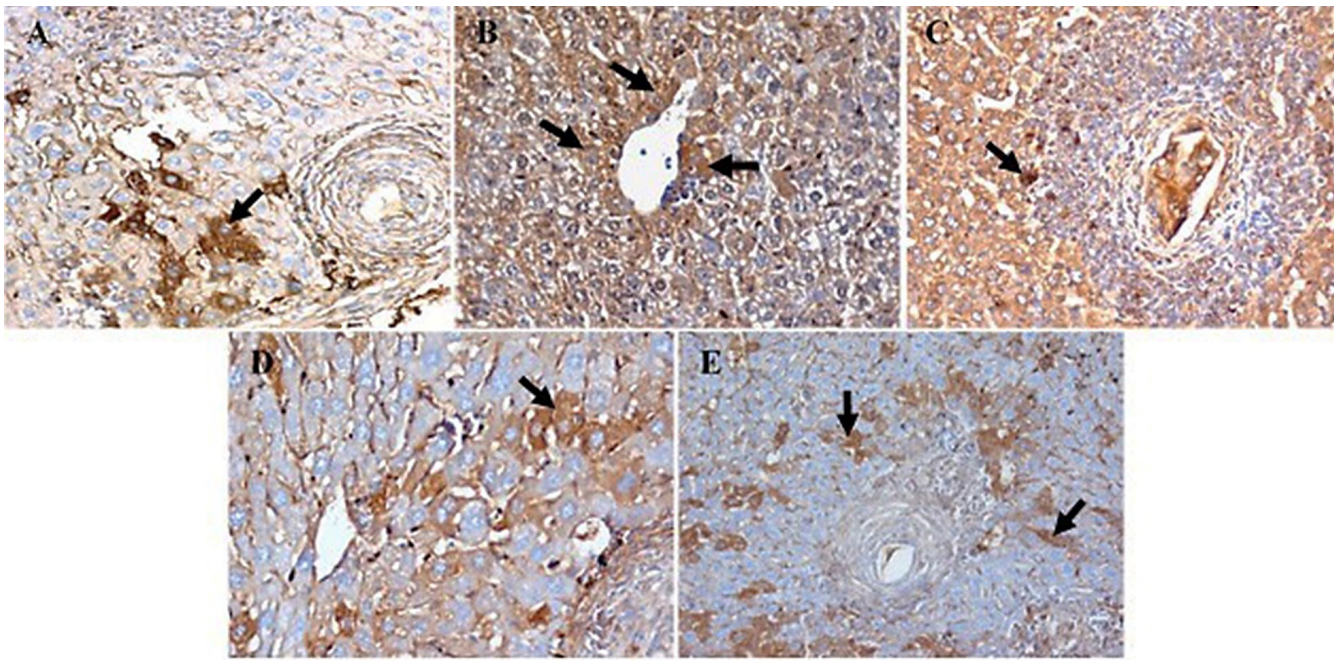

Fig. 5. Immunoperoxidase staining (×400 magnification) of liver sections of Schistosoma mansoni-infected BALB/c mice 12 weeks post-intravenous (IV) treatment with 1×10^6 mesenchymal stem cells (MSCs), showing: A) 50% new hepatocytes positive for alpha-fetoprotein (AFP), as a brownish cytoplasmic stain; B) 50% positivity for hepatocyte paraffin 1 (Hep Par 1); C) 50% positivity for cytokeratin 18 (CK18); D) 40% positivity for cytokeratin 7 (CK7); and E) 45% positivity for OV6

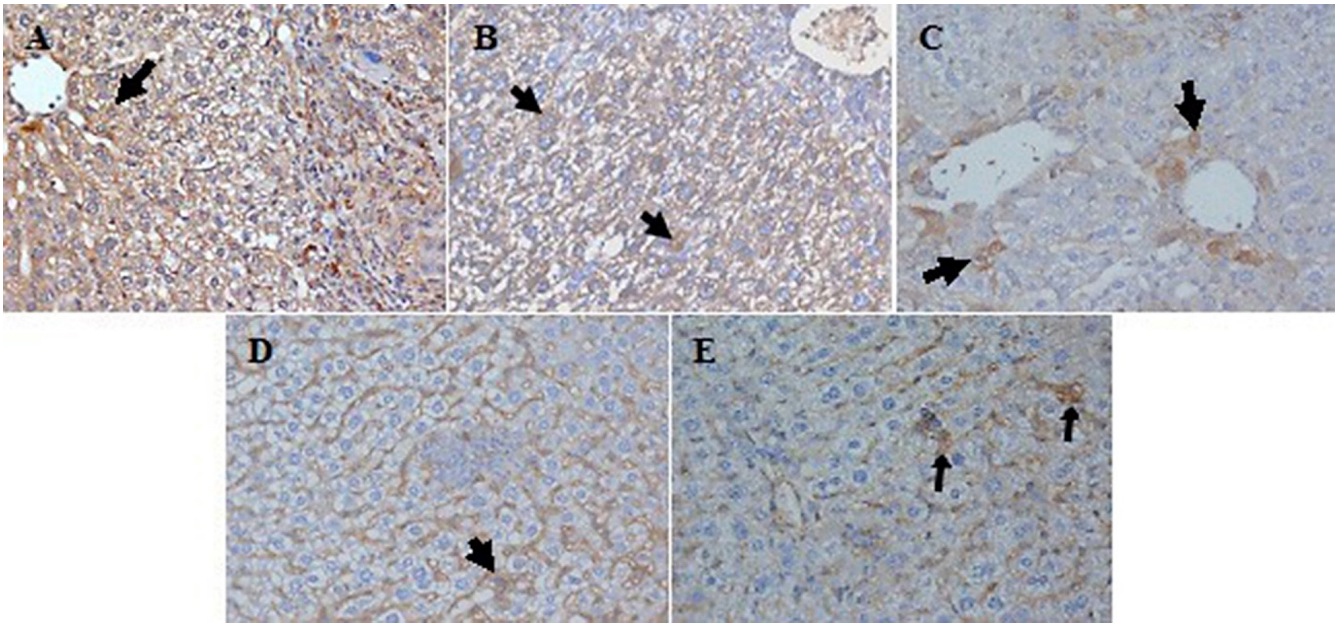

Fig. 6. Immunoperoxidase staining (×400 magnification) of liver sections of Schistosoma mansoni-infected BALB/c mice 12 weeks post-intrahepatic (IH) treatment with 1×10^6 mesenchymal stem cells (MSCs) showing: A) 10% new hepatocytes positive for alpha-fetoprotein (AFP), as a brownish cytoplasmic stain; B) 15% positivity for hepatocyte paraffin 1 (Hep Par 1); C) 20% positivity for cytokeratin 18 (CK18); D) 15% positivity for cytokeratin 7 (CK7); and E) 10% positivity for OV6

with 1 × 10^6 MSCs 12 weeks post-treatment showed new hepatocytes with brownish cytoplasmic stain positive for AFP, Hep Par 1, CK18, CK7, and OV6 (Fig. 6 A–E). Cells were able to engraft into the fibrotic liver with evidence of regeneration in the newly formed hepatocytes of human origin.

Carbon tetrachloride-induced liver cirrhosis model

Six trials over 3 months were performed to optimize the protocol for developing a model of cirrhosis. Two models were subjected to different doses and concentrations of CCl_4 to reach a sub-lethal dose which induced liver cirrhosis, yet avoided exposing the liver to severe toxicity ending in the death of the animal.

BALB/c mice were used as the 1st model. Carbon tetrachloride was dissolved in olive oil in ratios of 1:1, 1:2 and 1:5. The animals were then injected IP twice a week with 0.5 mL/kg, 0.5 mL/kg and 0.1 mL/kg of the prepared concentrations, respectively. Severe liver affection and death occurred within 1, 2 and 3 weeks, respectively.

The 2nd model used was hamsters. Carbon tetrachloride was dissolved in olive oil in ratios of 1:20, 1:5 and 1:10. The animals were injected IP twice a week with 0.01 mL/kg, 0.1 mL/kg and 0.0016 mL/kg of the prepared concentrations, respectively.

Liver specimens obtained from the animals injected with 0.01 mL/kg of the emulsion at a ratio of 1:20 did not show any evidence of liver affection or fibrosis after 1 month, while the hamsters subjected to a dose of 0.1 mL/kg at a ratio of 1:5 died as a result of severe liver affection within 2–3 weeks.

Histopathologic studies of liver specimens obtained from hamsters injected with 100 µL/hamster of the emulsion at a ratio of 1:10 showed that fibrosis started to be evident within 1 month and complete cirrhotic nodules were found after 3 months.

Carbon tetrachloride-induced fibrogenesis in hamsters transplanted intrahepatically with 3 × 10^6 mesenchymal stem cells

Transplantation of 3 × 10^6 MSCs in CCl_4-injected hamsters revealed a significant reduction ($p < 0.01$) in the fibrotic index 3 months after MSCs transplantation, compared to the corresponding index in the CCl_4 control group (Table 2).

There was a high significant improvement ($p < 0.001$) in ALT, AST and bilirubin levels (Table 2) 3 months after MSCs transplantation, compared to the corresponding levels in the CCl_4 control group.

The liver sections of the CCl_4 control hamsters stained with either H&E or Sirius red showed micro- and macrocirrhotic nodules (Fig. 7 A,B). Diminished cirrhotic nodules and a relative decrease in hepatic fibrosis were observed after MSCs transplantation. Cells were able to engraft into the fibrotic livers with newly formed hepatocytes of human origin as an evidence of some regeneration (Fig. 7 C,D).

The livers of the MSCs-transplanted group showed engraftment with human hepatocyte-like cells as proven by cytoplasmic expression of human AFP, Hep Par 1, CK18, OV6, and CK7 (Fig. 8 A–E).

Discussion

In the current study, CB-MSCs were isolated and expanded, and were positive for MSC phenotypic markers (CD73, CD105, CD90, and CD44), while being negative for HLA-DR and the hematopoietic stem cell phenotypic markers (CD34 and CD45). They showed high expression levels of *Oct4* and *Sox2* transcription factors, which regulate the maintenance of the pluripotency state in embryonic and adult stem cells.[17,18] Moreover, MSCs showed multilineage differentiation potential, being able to differentiate in vitro into mesodermal lineage cells (osteoblasts and adipocytes), and into the hepatogenic lineage of an endodermal origin. The expression of hepatic parenchymal markers – ALB, AFP, CK18, and glycogen – showed that our MSCs cultures contained functional hepatocytes, similar to the outcomes of the studies by Liang et al. and Li et al.[19,20]

We assessed the therapeutic effect of the transplantation of MSCs in 2 different experimental models: mice with S. mansoni infection (either IV or IH) and CCl_4-injected hamsters. In cell-based therapies, the dose of the transplanted cells is a key factor, as an appropriate cell count is vital for the survival of the injured experimental animals. In this study, we tested 3 doses (3 × 10^5, 6 × 10^5 and 1 × 10^6) of MSCs/mouse and a dose of 3 × 10^6 MSCs/hamster, in 0.5 mL DMEM. The recipient mice tolerated all doses and the best results were obtained when using the highest dose of 1 × 10^6/mouse. It was previously reported that the therapeutic effects of MSCs on liver cirrhosis gradually improved with increased cell dose.[21] A dose

Table 2. Results of liver functions (ALT, AST and total bilirubin) and % fibrosis in different studied groups of the CCl_4 model (mean ±SE)

Parameter	Studied groups of CCl_4 model		
	negative control (n = 10)	CCl_4 group (n = 10)	CCl_4 & treated with IH 3 x 10^6 MSCs (n = 10)
Total bilirubin [mg/dL]	0.28 ±0.02	5.97 ±0.23	0.52 ±0.01
ALT [U/L]	31.3 ±2.03	687.67 ±13.3	127.67 ±1.76
AST [U/L]	74.3 ±2.3	901.7 ±6.6	102.3 ±1.45
% fibrosis	1.27 ±0.19	17 ±0.82	2 ±0.21

AST – aspartate aminotransferase; ALT – alanine aminotransferase; CCl_4 – carbon tetrachloride; IH – intrahepatic; MSCs – mesenchymal stem cells; SE – standard error.

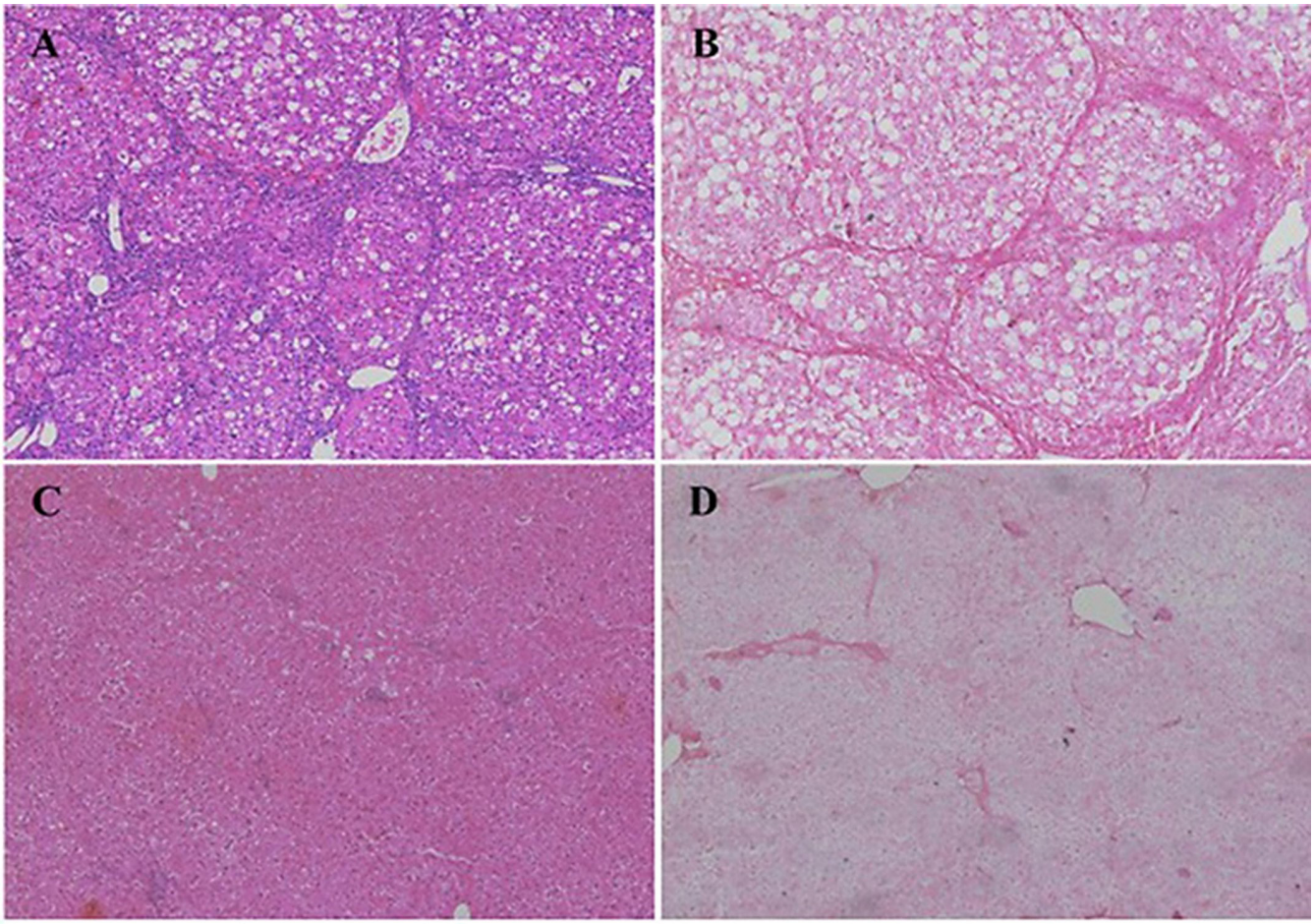

Fig. 7. Liver sections of a carbon tetrachloride (CCl_4) control hamster showing micro- and macrocirrhotic nodules: A) hematoxylin and eosin (H&E) staining, ×200 magnification; B) Sirius red, ×200 magnification. Liver sections of CCl_4 hamster 12 weeks post-intrahepatic (IH) treatment with 3×10^6 mesenchymal stem cells (MSCs), showing few fibrous bundles, liver with almost intact architecture; C) H&E, x100 magnification; and D) Sirius red, ×200 magnification

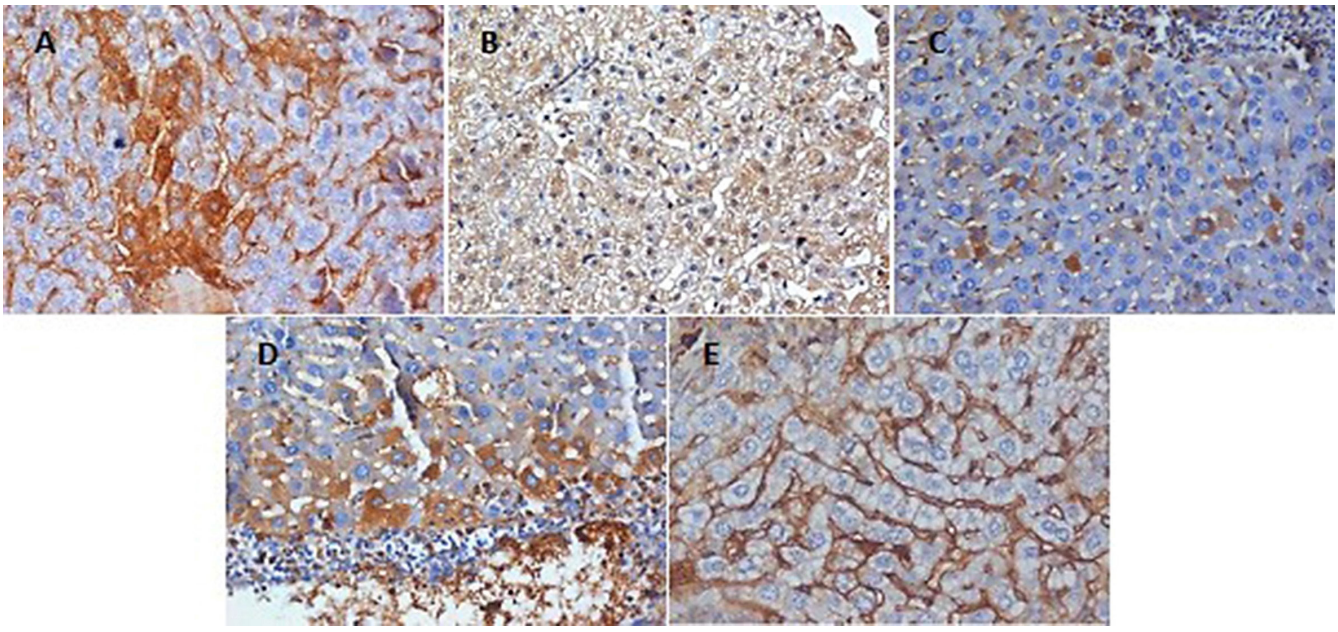

Fig. 8. Immunoperoxidase staining (×400 magnification) of liver sections of carbon tetrachloride (CCl_4) hamster 12 weeks post-intrahepatic (IH) mesenchymal stem cells (MSCs)-treatment, showing positive brownish cytoplasmic staining for A) alpha-fetoprotein (AFP), B) hepatocyte paraffin 1 (Hep Par 1), C) cytokeratin 18 (CK18), D) OV6, and E) cytokeratin 7 (CK7)

of 1×10^6 MSCs/mouse has been used in several mice experiments.[22,23] However, Yang et al. found that 1×10^6 cells/mouse dose was lethal for their mice, attributing that fact to vein embolism, so they used the 5×10^5 cells/mouse dose, while Park et al. recommended the use of an even higher dose – 2×10^6 MSCs/mouse.[24,25] We used triple maximum dose of MSCs/mouse to inject in a single hamster, according to their weight.

After transplantation, the livers of the MSCs-injected groups of both animal models showed engraftment with human hepatocyte-like cells, as proven by the cytoplasmic expression of human AFP, Hep Par 1, CK18, CK7, and OV6). Liver engraftment with human hepatocyte-like cells denoted the homing of MSCs to injured livers and the ability of MSCs to differentiate into hepatocytes.[24,25]

Salem and Thiemermann stated that the therapeutic potentialities of stem cells were based on their tendency to home to the sites of inflammation following tissue injury when introduced IV.[26] While the mechanisms driving this property were not fully understood, Ley et al. found that the injured cells were induced to express specific receptors or ligands that could facilitate trafficking, adhesion and infiltration of MSCs to the damaged sites, in a way very similar to leukocytes.[27] Furthermore, it has been demonstrated that MSCs express receptors for chemokines and ligands involved in leukocyte migration during inflammation, including SDF-1 and CXCR4.[28,29] Hepatic growth factor (HGF) secreted by liver-engrafted MSCs could promote their trans-differentiation into parenchymal hepatocytes.[30]

Our results showed a significant reduction in fibrotic index and improvement in liver function tests following MSC transplantation in both animal models in comparison to the corresponding pathological controls, which was more evident 12 weeks after injection. Many experimental reports showed a promising outcome of MSCs transplantation, as they have the potential to almost completely restore liver function, ameliorate symptoms and enhance survival rates in many hepatic disorders. [24,31]

Mesenchymal stem cells can prevent the hepatocytes from undergoing fibrogenesis by secreting a variety of cytokines, such as HGF, IL-6 and IL-10.[32] It has been proven that HGF has anti-apoptotic activity in hepatocytes and plays an essential role in liver regeneration.[33,34] Additionally, MSCs were found to have the potential to attenuate fibrosis in a CCl_4-induced liver fibrogenesis animal model by directly suppressing hepatic stellate cells.[35]

In the current study, the liver sections obtained from S. mansoni-infected mice showed large fibrocellular granuloma. Transplantation with 1×10^6 MSCs after S. mansoni infection showed a diminished granuloma size and a relative decrease in hepatic fibrosis, starting from the 4th week post-transplantation, and reaching its maximum improvement level 12 weeks after MSCs injection. Similar to our findings, Abdel Aziz et al. reported a significant reduction in the hepatic collagen content of hepatic fibrosis 4 weeks post-administration of MSCs and attributed this finding to a modulation in the expression of the *MMP* and *TIMP* encoding genes.[36] Several animal studies and clinical trials stated that MSCs have the potential to reverse the fibrogenesis process by inhibiting collagen deposition and TGF-β1 production.[9] Four weeks post-MSCs transplantation in the Schistosoma model, we detected the engraftment of MSCs into the fibrotic livers with evidence of some signs of regeneration, such as the appearance of newly formed hepatocytes, while the most prominent improvement in mice liver functions came 12 weeks after MSC injection. We found that the IV route was more effective in reducing the fibrosis index and improving liver function than the IH route, as did Kuo et al., who demonstrated that IV injection was more effective in rescuing liver failure than intrasplenic transplantation.[37]

It was observed that the improvement in both fibrosis index and liver function was more obvious in the CCl_4 model than in the S. mansoni-infected model. This could be attributed to the underlying pathology in each model, as CCl_4, a metabolite produced by cytochrome P-450 in hepatocytes, leads to lipid peroxidation and membrane damage, which results in a reversible acute centrilobular liver necrosis.[38] Meanwhile, in the S. mansoni model, most of the pathology is attributed to the host's reaction to the eggs, which reaches its peak by the 8th week of infection, and to the mice not receiving any treatment, so cumulative damage could have occurred. This balance is influenced by the varying competence of the host to kill worms, to inhibit worm fecundity and to destroy eggs and repair tissue damage.[39,40]

In conclusion, CB-MSCs transplantation succeeded in ameliorating liver fibrosis in both Schistosoma and CCl_4 chronic liver injury experimental models, as evidenced by the engraftment of the fibrotic livers with newly formed hepatocytes, diminished hepatic fibrosis and fibrotic index of the liver sections, and improvement in liver functions. Our results provide hope that CB-MSCs could be introduced as multipotent stem cells with great potentiality in cell-based therapy of liver fibrosis.

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