

Original Article

Comparison of the therapeutic effectiveness of human CD34⁺ and rat bone marrow mesenchymal stem cells on improvement of experimental liver fibrosis in Wistar rats

Hayam G Sayyed¹, Amany Osama², Naglaa K Idriss², Dina Sabry³, Azza S Abdelrhim⁴, Rania Bakry⁵

Departments of ¹Medical Physiology, ²Medical Biochemistry, Faculty of Medicine, Assiut University, Assuit, Egypt; ³Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt; ⁴Department of Anatomy, Faculty of Medicine, Sohag University, Sohag, Egypt; ⁵Department of Oncological Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assuit, Egypt

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Abstract: Background and objective: Human umbilical cord blood (UCB) cells and bone marrow mesenchymal stem cells (BM-MSCs) have numerous advantages as grafts for cell transplantation. We hypothesized differing impacts of human UCB cells and rat BM-MSCs on reversal of hepatic injury and revival of liver function in carbon tetrachloride (CCl₄)-induced liver fibrosis. Methods: Forty rats were divided into 4 groups; control group, CCl₄ group, CCl₄/CD34⁺ group and CCl₄/BM-MSCs group. Blood samples were driven from rats at 4, 8 and 12 weeks to measure serum concentration of albumin and alanine aminotransferase (ALT). Quantitative expression of collagen I α , TGF- β , α -SMA, albumin, MMP-2, MMP-9 and TNF- α were assessed by polymerase chain reaction. Histopathological examination of the liver tissue was performed. GFP labeled cells were detected in groups injected with stem cells. Results: Regarding liver function, CD34⁺ were more efficient than BM-MSCs in elevating albumin ($P < 0.05$) and reducing ALT ($P < 0.05$) concentrations. Concerning gene expression, CD34⁺ were more effective than BM-MSCs in reducing gene expressions of collagen I α ($P < 0.01$), TGF- β 1 ($P < 0.01$) and α -SMA ($P < 0.01$). Both CD34⁺ and BM-MSCs have the same efficacy in reducing TNF- α ($P < 0.001$ and $P < 0.01$, respectively). Furthermore, CD34⁺ were more valuable than BM-MSCs in increasing gene expression of albumin ($P < 0.05$) and MMP-9 ($P < 0.01$). Conclusion: Taken together; human UCB CD34⁺ stem cells were more efficient in improvement of experimental liver injury than BM-MSCs. This study highlighted an important role of human UCB CD34⁺ stem cells in liver fibrosis therapy.

Keywords: CCl₄ liver fibrosis, umbilical cord blood CD34⁺, bone marrow stem cell, stem cell therapy

Introduction

Liver fibrosis is a reversible progressive disease of hepatic tissue in response to chronic injury characterized by excessive accumulation of extracellular matrix (ECM) [1]. When injury crop up, damaged epithelial and/or endothelial cells secrete inflammatory mediators that attract inflammatory cells from the blood to the injured area and release mediators that persuade fibrosis such as TGF- β 1 and TNF- α , activate hepatic stellate cells and deposit ECM [2]. Furthermore, collagen, the main protein of connective tissues, represents a group of naturally occurring proteins, and comprises 25-35%

of the total body protein. It is predictable in liver fibrosis that there is extreme collagen creation [3].

The liver regenerative aptitude in response to acute injury is great. Mature hepatocytes can undergo several cell divisions to return the hepatic mass. Yet, in successive chronic injury the regenerative capacity of liver cells is lost and the liver become incompetent to preserve its functional mass; this is called "liver failure" [4]. As fibrotic liver diseases progress, the disease advances from collagen bands to bridging fibrosis then to frank cirrhosis. ECM deposition results from increased synthesis as well as

decreased degradation that due to decrease ECM removing activity of matrix metalloproteinases (MMPs) [5].

Until now, liver fibrosis management based on treating the cause, advising patients to stop alcohol and smoking, treating hepatitis infections and managing complications [6]. Liver transplantation has been used in the treatment of liver fibrosis. Nevertheless, the increasing patient's number and donors insufficiency, morbidity and mortality from liver fibrosis incessantly increased. Consequently, alternative therapies are immediately required [7, 8]. Cellular therapy is an essential treatment approach to enhance recovery of liver function [6]. Mesenchymal stem cells (MSCs) have involved interest as a probable cell source for repair of the damaged liver [7, 8]. Recently, stem cells, undifferentiated cells, hold promising therapeutic results since they undergo self-renewal and differentiation into several cell types, and are appropriate to human disorders [9]. Umbilical cord blood (UCB) contained mainly hematopoietic stem cells (HSCs) [10]. At birth, the UCB contained a large amount of HSCs whose levels about 100-fold greater than their levels in the adult peripheral blood. The stem cells and early progenitors expressed a surface membrane glycoprotein, CD34, and is the characteristic of HSCs. The number of CD34⁺ cells expressed HLA-DR (a major histocompatibility complex class II) and CD38 (a surface antigen on leukocytes) antigens. The number of CD34⁺ HLADR⁺ and CD34⁺ CD38⁺ cells in the UCB is greater than in adult bone marrow (BM). Thus, the UCB contains a higher amount of immature HPCs than adult BM [5].

In bone marrow (BM) there are numerous types of stem cell including "hematopoietic stem cells (HSCs), marrow mesenchymal stem cells (MSCs), and multi-potent adult progenitor cells". Many studies proposed that CD34⁺ cells and BM-MSC could alleviate chemically-induced liver fibrosis [3, 9], however, which cell type is more efficient in improving liver fibrosis and the mechanism of repair the fibrosis is unclear and their results appear notorious.

In the present study, we compared between the ameliorative effects of human umbilical cord CD34⁺ and rat bone marrow derived mesenchymal stem cells on functional restoration of the liver and improvement of injury in a word rat model of hepatic fibrosis. Also, we studied the

mechanism that induced the protective activity of stem cells.

Materials and methods

Experimental animals

Forty male Wistar rats, initially weighing from 150 to 200 grams, aged 6 weeks were obtained from the animal house of Faculty of Medicine, Assiut University, Egypt. Rats were housed in clean stainless steel cages, the cage size was 65 cm × 25 cm × 15 cm, and each cage contained 6 rats per cage. The cages contain wood chip bedding and nesting material. Rats were maintained on natural light/dark cycle (light on at 5 a.m.) in an aerated room under controlled hygienic conditions of 23 ± 1°C temperatures, 45 ± 5% relative humidity and provided with free access to food and water. All rats were acclimatised for 4 days before the experimental protocol was started. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [11] and were approved by the Ethical Committee for Scientific Research at the Faculty of Medicine, Assiut University, Assiut, Egypt.

Induction of liver fibrosis

Liver fibrosis was induced by subcutaneous injection of carbon tetrachloride (CCl₄) at a dose of 0.2 ml/100 gram body weight of 40 ml/L CCl₄ (Sigma, St Louis, USA) dissolved in equal volume of castor oil (Sigma, St. Louis, USA). The injection was given twice weekly for 6 weeks [12].

Experimental design

Rats were divided into the following groups (10 rats each): 1) Group 1 (Control group): rats received 0.2 ml/100 gram body weight of castor oil twice weekly for 6 weeks. 2) Group 2 (CCl₄ group): rats received 0.2 ml/100 g body weight of CCl₄. 3) Group 3 (CCl₄/CD34⁺ group): rats received CCl₄ as previous. The rats were infused with 10⁷ isolated CD34⁺ cells/rat intravenously (through tail vein) and scarified after 3 months. 4) Group 4 (CCl₄/BM-MSCs group): rats received CCl₄ as previous and followed by injection of 10⁷ BM-MSCs and scarified after 3 months.

At 4, 8 and 12 weeks from stopping CCl₄ and administration of stem cells, rats were held gently and anesthetized and venous blood was

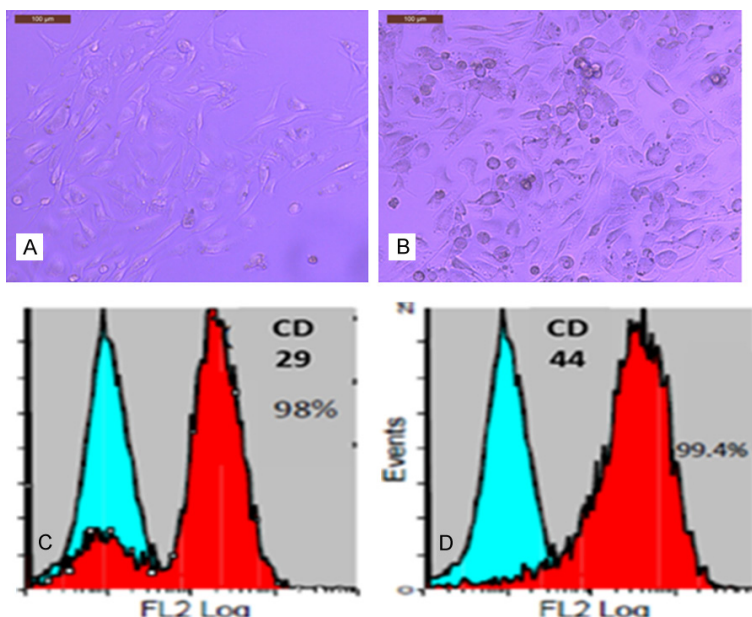


Figure 1. Rat Bone Marrow Derived Mesenchymal Stem Cell Culture and Identification. Rat BM-MSCs at one week of culture (A), rat BM-MSCs at two weeks of culture (approached 80-90% confluent) (B), Fluorescence-activated cell sorting (FACS) analysis for MSC positive cell marker. (C) FACS analysis for MSC positive cell marker CD29⁺ and (D) FACS analysis for MSC positive cell marker CD44⁺.

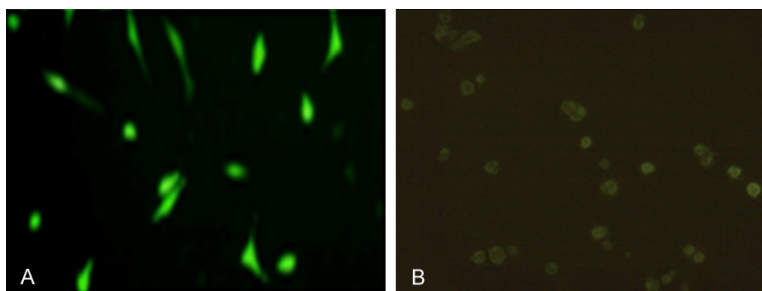


Figure 2. Fluorescence Tracked Green Fluorescent Protein (GFP)-Labeled Mesenchymal Stem Cells (MSCs) observed under Fluorescence Microscopy. Green blots were GFP labeled MSCs in vitro (A), homing of the injected labeled mesenchymal stem cells by detecting GFP fluorescent dye in the rat liver tissue after scarification (in vivo) (B).

collected from the retro-orbital vein to assess serum albumin and alanine transaminase (ALT). All rats were sacrificed with CO₂ narcosis, and the liver tissue was harvested for histopathological examination and real time PCR analysis.

Isolation and culture of BM-MSCs

Bone marrow cells were flushed from tibia and fibula of rat bones with phosphate-buffered saline (PBS) containing 2 mM EDTA. Over 15 ml

Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), 35 ml of the diluted sample was carefully layered, centrifuged for 35 minutes at 400 × g and the upper layer was aspirated leaving undisturbed mononuclear cell (MNC) layer at the interphase. This MNC layer was aspirated, washed twice in PBS containing 2 mM EDTA and centrifuged for 10 minutes at 200 × g at 10°C. The cell pellet was re-suspended in a final volume of 300 µl of buffer. Isolated MSCs were cultured on 25 ml culture flasks in minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and incubated for 2 hours at 37°C and 5% CO₂. Adherent MSCs were cultured in MEM supplemented with 30% FBS, 0.5% penicillin, streptomycin and at 37°C in 5% CO₂ in air [13]. Cultured MSCs were confirmed by morphology (Figure 1) and Fluorescent Analysis Cell Sorting (FACS) by detection of CD29⁺ and CD44⁺ specific to MSCs (Figure 2).

Collection of human umbilical cord blood

Human umbilical cord blood withdraws immediately after normal vaginal delivery within 24 hours after rupture of membranes and before separation of the placenta. Written

informed consent was obtained from each woman after full explanation of the study. Participants considered suitable for the study according to the following exclusion and inclusion criteria.

Women with the following criteria were excluded: Family history of gene based disorders or maternal fever during delivery.

The umbilical cord clamped one inch or less apart at the infant's abdomen. The umbilical

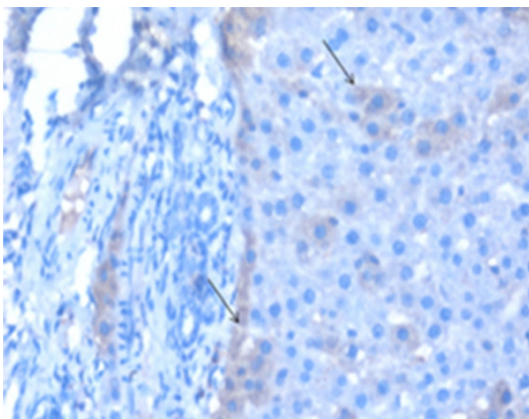


Figure 3. Immunohistochemistry of the Liver Tissue. Showed positive expression of CD34⁺ differentiated hepatocytes (arrow) in the portal area (400 ×). Some hepatocytes showed expression of CD34⁺ positivity around portal tract.

cord was sterilized with 70% alcohol followed by betadine at the needle insertion site. The needle inserted just above the clamp. Blood allowed pouring as much as possible. The collection normally took about 3-5 minutes.

Cell sorting of human UCB CD34⁺

Anti-coagulated human umbilical cord blood (UCB) was diluted 1:4 with PBS containing 2 mM EDTA (Gibco-Invitrogen, Grand Island, NY). As prescribed in our previous work [9]. Briefly, the MNCs were separated by centrifugation over a Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY) density gradient at 400 × g for 35 minutes at 10°C. The MNC fraction was washed first in PBS, then with MACS (magnetic cell sorting) buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2). CD34⁺ cells were isolated from MNCs, using the CD34⁺ positive cell selection kit (Mini-Macs; Miltenyi Biotec, Bergisch Gladbach, Germany). Percentage of isolated CD34⁺ cells was characterized by flow cytometry (**Figure 3**).

Labeling stem cells with GFP

At 4th passage, MSCs were harvested and labeled with GFP (amaxa GmbH, amaxa Inc. Europe/World USA Scientific Support). Human MSC were nucleofected using the human MSC Nucleofector Kit and a plasmid encoding the fluorescent protein GFP. Cells were centrifuged, washed twice in serum free medium, pelleted and suspended in nucleofector solutions. A

final concentration of $4-5 \times 10^5$ cells/100 µl nucleofector solutions was applied. The sample was placed in cuvette of electroporation transfection instrument at program U-23 (for high transfection efficiency) or C-17 (for high cell survival). 24 hours post-nucleofection cells were analyzed by light and fluorescence microscopy (**Figure 4**). Transfection efficiencies of around 80% can be reached with GFP. Labeled cells were injected intravenously in rat with CCL₄-induced liver fibrosis. After 12 weeks, liver tissue was examined with a fluorescence microscope (Leica, Germany) to detect and trace the cells stained with GFP.

Serum biochemical assessment

Blood sample was collected from rats at 4, 8 and 12 weeks. ALT and albumin were assessed using (CAT # ab105134, sensitivity: 10 mU/well and CAT # ab108789, sensitivity: 0.7 ng/ml, Abcam US, respectively) colorimeter kits according to manufacture instructions.

Histopathological examination

Liver tissues were collected and divided into two sections. The first section was assessed for tracing of injected labeled cells with GFP. The second section was washed with PBS and fixed overnight in 40 g/L paraformaldehyde at 4°C. Serials µm sections of the dissected liver tissues were stained with hematoxylin and eosin (H&E) by the method of Durry and Wallington [14] and Masson Trichrome [15] for evaluation of fibrosis.

Immunohistochemistry

Immunohistochemical staining was performed on 5-µm, formalin-fixed, paraffin-embedded sections using the CD34⁺, α-SAM and albumin antibodies (product No: M716529, M085129 and K067511, respectively, DAKO, Carpinteria, CA) at 1:50 dilution. Antigen retrieval was performed in all cases by steam heating the slides in a 1-mmol/L solution of EDTA (pH 8.0) for 30 minutes. After blocking of endogenous biotin, staining was performed using an automated immunostainer (product No: K150011, DAKO, Carpinteria, CA) followed by detection by using a streptavidin-biotin detection system (DAKO). Positive and negative control sections were used for each assay.

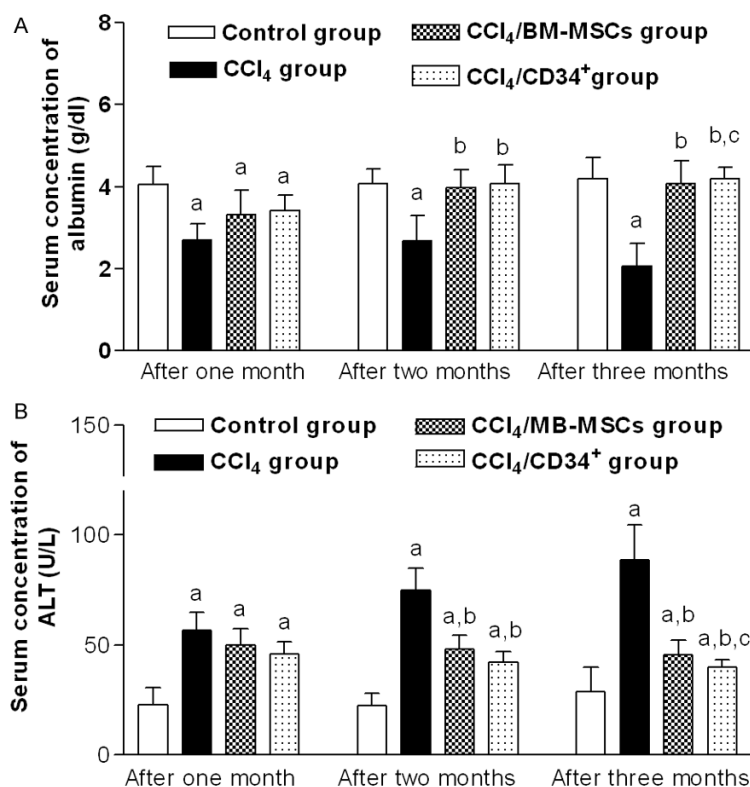


Figure 4. Effects of Human CD34⁺ and Rat Bone Marrow Mesenchymal Stem Cells on the Serum Concentration of Albumin and ALT in CCl₄-Induced Liver Fibrosis in Rats. Data were expressed as mean \pm standard deviation (SD). ALT: alanine transaminase. Analysis between groups was done using one way ANOVA test followed by Bonferroni test. ^aSignificantly different from the control group, ^bsignificantly different from the CCl₄ group, and ^csignificantly different from the CCl₄/BM-MSC group. *P* value of <0.05 was considered to be significant.

RNA extraction

Liver tissue of all studied groups was homogenized and total RNA was isolated with RNAeasy Mini Kit (CAT # 74116, Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA).

Real time PCR (qRT-PCR) for quantitative expression of collagen α , TGF- β , Albumin, MMP-2 and 9, TNF- α and α SAM

The mRNA expression level was quantified by qRT-PCR (Real time PCR). 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (CAT # 4368814, Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (CAT # K0251,

Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH house-keeping gene by the $\Delta\Delta$ Ct method. We used 1 μ M of both primers specific for each target gene. Primers sequence and annealing temperature specific for each gene demonstrated in **Table 1**.

Statistical analysis

The collected data was organized, tabulated and statistically analyzed using prism software statistical computer package version 5. Mean and standard deviation were calculated; one-way ANOVA (Analysis of variance) was used to test the difference about mean values of measured parameters among groups.

For interpretation of results of tests of significance, significance was adopted at $P < 0.05$.

Results

Histopathological analysis and immunohistochemistry

Rat BM-MSCs at one week of culture (**Figure 1A**) and at two weeks of culture (**Figure 1B**) showed 80-90% confluent cells and differentiated into a fusiform-shaped fibroblast like cells. The characteristics of cultured MSCs were confirmed by detecting cell surface phenotypings by using flow cytometry. 98% of BM-MSCs showed positive expression of the β 1-integrin CD29 (**Figure 1C**) and 99.4% of human UCB cells showed positive expression of the endoglin receptor CD44 (**Figure 1D**).

Table 1. Primers sequence for each gene

Target gene	Primer sequence: 5'-3'	Gene bank accession number
Collagen I α	Forward: AGAGCATGACCGATGGATTC	KJ696743.1
	Reverse: CCTTCTTGAGGTTGCCAGTC	
Albumin	Forward: TTTACGAGAAGCTTGGAGAG	FQ210445.1
	Reverse: TGTGCAGATATCAGAGTGGA	
TGF- β	Forward: TGCGCCTGCAGAGATTCAAG	NM_021578.2
	Reverse: AGGTAACGCCAGGAATTGTTGCTA	
MMP-2	Forward: CTATTCTGTCAGCACTTTGG	NM_031054.2
	Reverse: CAGACTTTGGTTCTCCAACCTT	
MMP-9	Forward: AAATGTGGGTGTACACAGGC	NM_031055.1
	Reverse: TTCACCCGTTGTGGAAACT	
TNF- α	Forward: AACTCGAGTGACAAGCCCGTAG	XM_008772775.1
	Reverse: GTACCACCAAGTTGGTTGTCTTTGA	
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC	XR_598347.1
	Reverse: GACATCAAGAAGGTGGTGAAGCAG	
α SAM	Forward: GTTTGAGACCTTCAATGTCCC	XM_003962516.2
	Reverse: CGATCTCACGCTCAGCAGTGA	

To explore cell homing, MSCs were labeled with GFP before transplantation in vitro (**Figure 2A**). After scarification, these transplanted GFP labeled cells were visualized by fluorescent microscope confirming that these cells were actually homed into rat's liver tissue as demonstrated in **Figure 2B**.

Immunohistochemistry of liver tissue showed expression of CD34⁺ differentiated hepatocytes in the portal area and some hepatocytes showed expression of CD34⁺ positivity around portal tract (**Figure 3**).

Biochemical assessment

Liver function was monitored by the serum level of albumin and alanine aminotransferase (ALT) as displayed in **Figure 4**. At the end of the 1st month, both CCl₄/BM-MSCs and CCl₄/CD34⁺ groups showed significant decrease in the serum level of albumin as compared to the control group ($P < 0.01$ and $P < 0.05$, respectively) and CCl₄ group ($P < 0.05$ and $P < 0.01$, respectively). At the 2nd and 3th months, serum level of albumin in rats injected with BM-MSCs and CD34⁺ increased significantly as compared to the CCl₄ group ($P < 0.001$ for each group). At the 3th month, serum concentration of albumin was significantly increased in the CCl₄/CD34⁺ group as compared to CCl₄/BM-MSCs group ($P < 0.05$). Regarding to the liver enzymes, ALT, at the 1st month there was significant increase of its con-

centration in the CCl₄/BM-MSCs and CCl₄/CD34⁺ groups as compared to the control group ($P < 0.001$ for each group). At 2nd and 3th months, it showed a significant decrease in comparison to the CCl₄ group ($P < 0.001$ for each group). However, its concentration was still significantly higher than the control level and there was significant decrease in the group that received CD34⁺ stem cells as compared to the group that received BM-MSCs stem cells ($P < 0.05$). These results indicated that CD34⁺ stem cells were more effective than BM-MSCs in improving liver function.

Quantitative expression of collagen I α , TGF- β , Albumin, MMP-2 and 9, TNF- α and α SMA by real time PCR

Concerning gene expression, the rat collagen I α , TGF- β , α -SMA and TNF- α gene were highly expressed in the CCl₄ group ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.01$, respectively) as compared to the control group and the expression of collagen I α was significantly decreased after stem cells administration ($P < 0.001$ for each) with less expression in the CCl₄/CD34⁺ group as compared to CCl₄/BM-MSCs group ($P < 0.01$). TGF- β was still significantly expressed in the CCl₄/BM-MSCs group ($P < 0.001$) and α -SMA still expressed in CCl₄/BM-MSCs group ($P < 0.05$) with less expression in the CCl₄/CD34⁺ group as compared to CCl₄/BM-MSCs group ($P < 0.01$). TNF- α gene expression was significantly reduced after BM-MSCs ($P < 0.01$) and CD34⁺ ($P < 0.001$) stem cells administration with no significant difference between BM-MSCs and CD34⁺ stem cells as shown in **Figure 5A, 5B, 5F and 5G**. These results pointed to those CD34⁺ stem cells were more efficient than BM-MSCs in alleviating liver fibrosis and inflammation.

As regard to gene expressions of albumin, MMP-2 and MMP-9, their expression in the liver tissues were significantly decreased in the CCl₄ group ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively) and the expression of albumin was significantly increased after BM-MSCs and CD34⁺

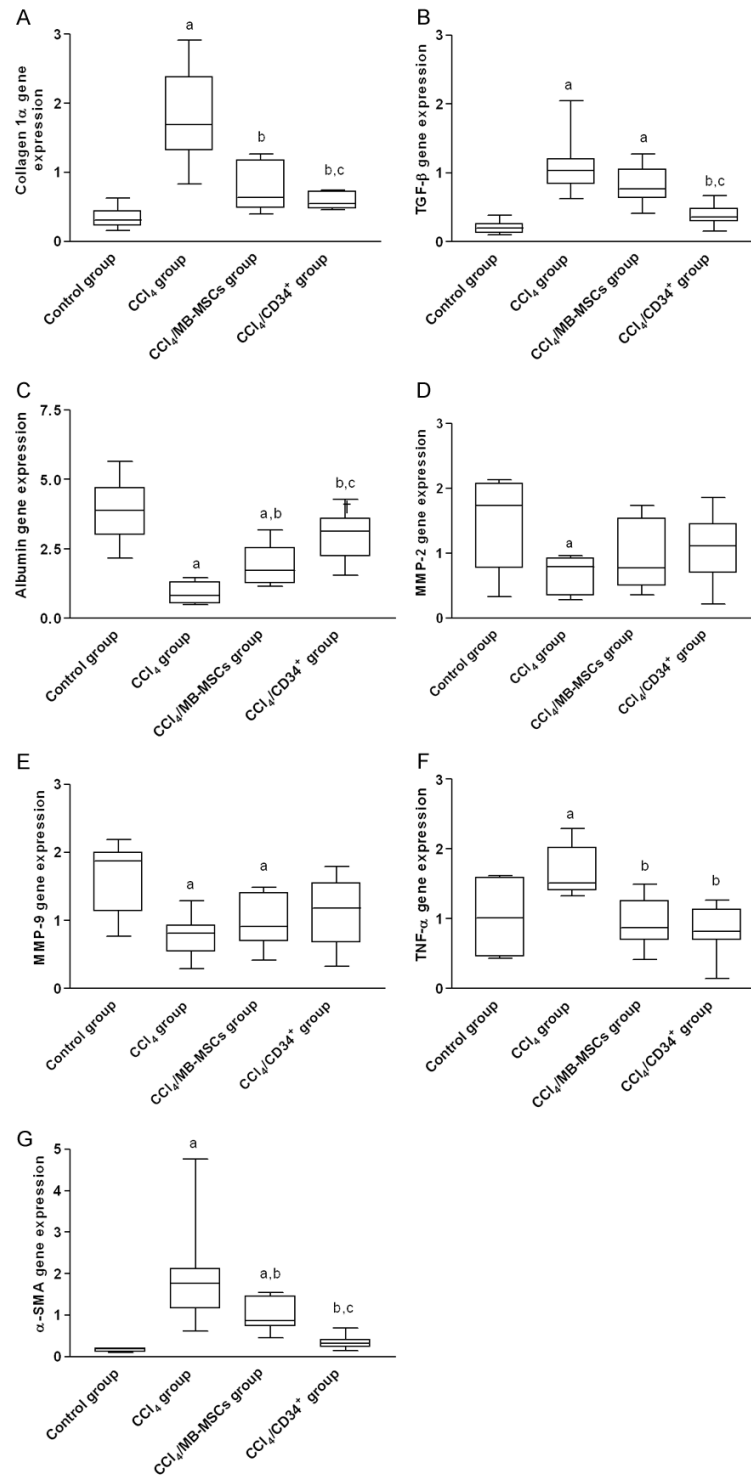


Figure 5. Box-Plot (Median Value, Lower and Upper Quartile) Representation of Gene Expression of Collagen 1 α (A), TGF- β (B), Albumin (C), MMP-2 (D), MMP-9 (E), TNF- α (F) and α -SMA (G) in CCl₄ Induced Liver Fibrosis. Transforming growth factor-beta, MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9, TNF- α : Tumor necrosis-alpha, α -SMA: alpha smooth muscle actin. Analysis between groups was done using one way ANOVA test followed by Bonferroni test. ^aSignificantly different from the control group, ^bsignificantly different from the CCl₄ group, and ^csignificantly different from the CCl₄/BM-MSC group. *P* value of <0.05 was considered to be significant.

stem cells administration (*P*< 0.05 and *P*<0.01, respectively) as compared with CCl₄ group with higher expression was in the CCl₄/CD34⁺ group in comparison to CCl₄/BM-MSCs group (*P*<0.05) (**Figure 5**). Furthermore, gene expression level of MMP-9 was increased after CD34⁺ administration as compared to CCl₄ (*P*<0.001) and BM-MSCs (*P*< 0.01) groups. However, the expression level of MMP-2 not increased to a significant level after stem cells administration as shown in **Figure 5C-E**. These results referred to those CD34⁺ stem cells were more valuable than BM-MSCs in improving synthetic liver function and ameliorating fibrosis.

Histopathological examination of the liver tissue

Histopathological examination using Hematoxylin and eosin (HE) staining and Masson's trichrome staining were performed to demonstrate the liver damage (**Figure 6**). For HE staining, liver tissue sections from the CCl₄ group exhibited mild focal fibrosis, suggesting the successful establishment of the animal model of liver fibrosis. In contrast, BM-MSCs and CD34⁺ treatment remarkably ameliorated the fibrosis compared to the CCl₄ group. For Masson's staining, the fibrosis and collagen were distinctly decreased by the BM-MSCs and CD34⁺ treatment compared to the CCl₄ group. Thus, mesenchymal stem cells had anti-fibrotic effect as evidenced by decreasing the liver collagen compared to the CCl₄ group together with improvement of liver histopathological picture.

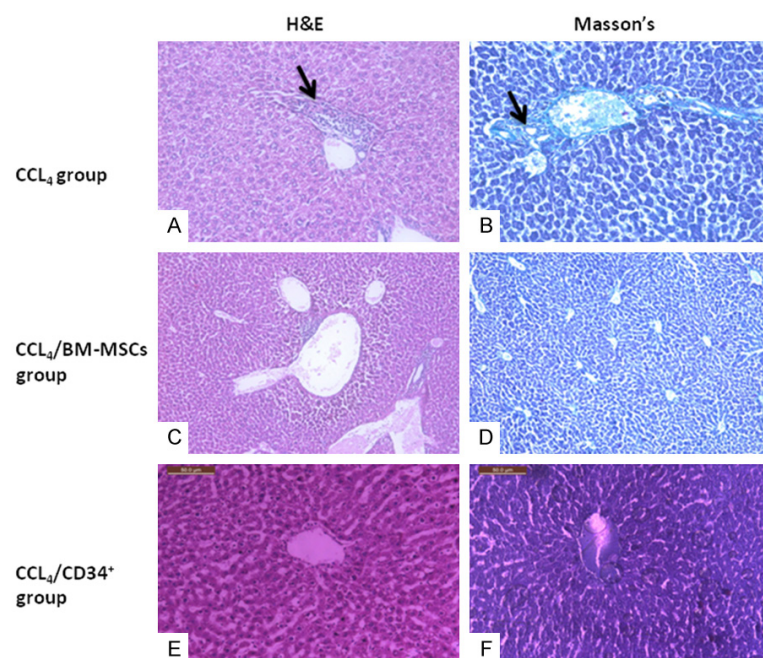


Figure 6. Histopathological Examination of Liver Tissue. Hematoxylin and eosin (HE) staining of liver sections (first column, HE staining $\times 200$) and Masson's trichrome staining (second column, Masson staining $\times 200$) showed mild focal portal fibrosis (black arrow) in CCL_4 -treated rats (A, B). Stem cells have anti-fibrotic effect as evidenced by decreasing liver collagen compared to the CCL_4 group and restored normal liver architecture after BM-MSCs (C: H&E $\times 40$ and D: Masson Trichrome $\times 40$) and CD34^+ injection (E: H&E $\times 200$ and F: Masson Trichrome $\times 200$).

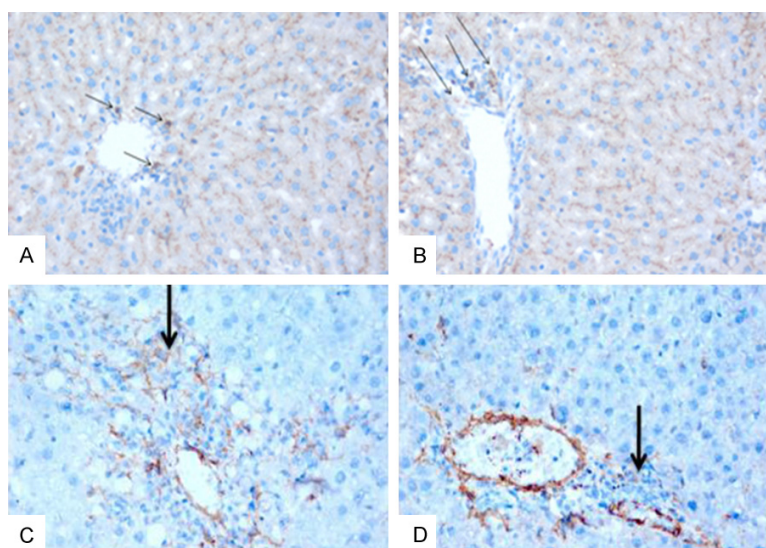


Figure 7. Immunohistochemistry. A: Showed positive expression of albumin for injected BM-MSCs (thin arrows) in the portal area (400 \times). B: Showed positive expression of albumin for injected CD34^+ (thin arrows) in the portal area (400 \times). C: Showed positive expression of α -SMA in the portal area (thick arrow) for injected BM-MSCs with positive blood vessels internal control (400 \times). D: Showed positive expression of α -SMA for injected CD34^+ in the portal area (thick arrows) with positive blood vessels internal control (400 \times).

Immunohistochemistry

Albumin was normally expressed by hepatocytes indicating functioning hepatocytes. It exhibited cytoplasmic expression. Bile ducts and portal mononuclear cells didn't express albumin. In this study, albumin expressing cells was detected in the portal areas indicating differentiation of injected BM-MSCs (**Figure 7A**) and CD34^+ cells into hepatocytes (**Figure 7B**). The α -SMA level by immunohistochemistry in CCL_4 /BM-MSCs group still expressed but lower expressed in CCL_4 / CD34^+ groups as shown in **Figure 7C, 7D**.

Discussion

Owing to a grave scarcity and shortage of liver donors and the high-risk of transplant rejection, a substitute therapeutic approach is desirable for patients with liver failure. With the dramatic development of stem cells, cellular therapy is a good way to solve the above problem. Stem cell therapy showed a great promising outcome for repair of tissues and organs injury, including the liver. Stem cells are undifferentiated cells that have the ability to self-renewal and multi-lineage differentiation [9]. The panorama of using stem cells to cure liver disease is exciting. Consequently, our study compared the ability of human umbilical cord blood derived CD34^+ and rat bone marrow-derived MSCs injection to ensure the functional revival of the liver and improvement of fibrosis in an experimental model of liver fi-

brosis in rats induced by CCl₄ and their possible mechanisms.

The main findings of our study were: 1) Both human CD34⁺ and rat BM-MSCs improved liver function in rats with CCl₄-induced liver fibrosis as indicated by increasing albumin and decreasing ALT serum concentrations and increasing albumin gene expression in the liver tissue and this confirmed by histopathological examination of the liver tissue. 2) Administration of human CD34⁺ and rat BM-MSCs exert a protective effect against fibrosis. This might attribute to decrease gene expression of profibrogenic factors as collagen I α , TGF β 1 and α -SMA and increase gene expression of anti-fibrogenic factors as MMP-9 in group received BM-MSCs. 3) Both human CD34⁺ and rat BM-MSCs were valuable in reducing inflammation by decreasing TNF- α gene expression. 4) Longevity of administered stem cells to treat liver fibrosis this indicated the feasibility of using stem cells to alleviate liver damage. 5) Human CD34⁺ was more efficient than rat BM-MSCs in ameliorating liver fibrosis. This effect might claim to the advantages of UCB stem cells over adult BM-MSCs in cell therapy. In comparison to BM-MSCs, umbilical cord stem cells have higher division, homing, differentiation and extension possibility and vulnerability to genetic handling, and they create a lesser frequency of graft-vs.-host disease [16].

Our work extended and confirmed the findings reported by other researchers for improvement of liver function with MSCs administration [17, 18]. Our work demonstrated that injection of human CD34⁺ and rat BM-MSCs obviously increase albumin and decrease ALT levels in the serum and this indicated normalization of liver function with stem cells injection and human CD34⁺ were more efficient in normalization of liver functions after 3 months. These findings implied that injection of human CD34⁺ is a better therapy for alleviation of liver fibrosis in clinical practice.

This occurred alongside with increased albumin gene expression with more expression with human CD34⁺ cells injection. The human albumin-expressing cells, blue stained, were detected to be discrete throughout some areas of the liver. Normally albumin expressed by hepatocytes indicating functioning hepatocytes. Bile ducts and portal mononuclear cells didn't ex-

press albumin. In our study, albumin expressing cells were detected in the portal areas indicating that injected stem cells (CD34⁺ and BM-MSCs) were transdifferentiated into functioning mature hepatocytes as detected by immunohistochemistry. This was reliable with the finding of Abdel Aziz et al. [19] who found increase albumin gene expression in the liver tissues after human CD34⁺ stem cells administration. Also, Ali and Masoud [20] demonstrated a marked increase in the albumin gene expression after transplantation of BM-MSCs.

Crosby et al. [21] had shown that c-kit and CD34⁺ positive cells isolated from human liver were able to differentiate into biliary epithelial cells and into endothelial cells. Thus, biliary cells and endothelial cells might also share some common precursors. Additionally, some antigens traditionally associated with hematopoietic cells (c-kit and CD34⁺) could also be expressed by oval cells. Our study showed positive staining CD34⁺ differentiated injected stem cells in the portal area and this explained that oval cells gradually transformed into small basophilic hepatocytes, which then became fully mature hepatocytes and replaced the lost liver mass. Thus, the precursor-product relationship between oval cells and basophilic hepatocytes had been proved in our study. Furthermore, human haemopoietic (CD34⁺) stem cells adhesion to human liver tissues is integrin and CD44 dependent and modulated by CXCR3 and CXCR4. Barely is the form of liver injury probable to be significant in settling on which stem cell repair mechanism is triggered, even though it is also probable to control how quickly liver repair or reconstitution happens. Our work proposed that in experimental chronic liver injury models, liver improvement is a slow process and may take more than 6 weeks. Cells conscripted to the site of regeneration might need to reveal a survival growth advantage over the endogenous liver cells that were affected by the disease process.

The capability of stem cells to recover liver function in rats with CCl₄-induced liver fibrosis was verified by histopathological examination of the liver tissue. Treatment with human CD34⁺ and rat BM-MSCs showed a return of liver architecture to normal and anti-fibrotic effect as evidenced by the decrease in liver collagen compared to the CCl₄ group together with improvement of the liver histopathological picture.

The development of liver fibrosis occurred as a result of ECM accumulation in a process included activation of the TGF- β 1 signaling pathway and hepatic stellate cells (HSC) that differentiated into ECM producing cells, myofibroblast-like cells, with resultant increased collagen production, [22] imbalance between metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), giving rise to collagenous ECM overproduction in the liver parenchyma [23, 24]. Our study demonstrated the effective therapeutic effect of human CD34⁺ against fibrosis was more than BM-MSCs. The documented reduction in the liver fibrosis might contribute to inhibition of collagen formation or enhancement of collagen breakdown. Many researchers observed the anti-fibrotic effect of BM-MSCs through inhibition of collagen formation [3, 9]. Moreover, Hong et al. [25] observed that human UCBMSCs capable to improve liver cirrhosis by breaking down collagen fibers. Zhao et al. [12] suggested that MSCs might have inhibitory role in the transition process of HSC from quiescent phase to the activated phase.

HSCs activation enhance not only the synthesis and deposition of the ECM but also the induction of α -SMA and these in turn promoted the growth of activated HSCs and contributed to the development of hepatic fibrosis. α -SMA is a cytoskeletal protein and it is an indicator of HSCs activation [26]. TGF β 1 believed to be the key fibrogenic cytokine in liver fibrosis that played a considerable role in the metabolism of ECM [25, 27]. Increased expression of TGF β 1 and α -SMA related to HSCs activation and ECM deposition during initiation and progression of fibrosis [28]. Furthermore, collagen I α , another pro-fibrotic factor produced by activated hepatic stellate cells, [20] is a chief component of liver fibrosis [22].

Our study revealed that treatment of CCl₄-induced rat liver fibrosis with stem cells resulted in a marked reduction in the gene expression levels of pro-fibrogenic factors, including collagen I α , TGF- β 1 and α -SMA with higher reduction in the CCl₄/CD34⁺ group. This is in accordance to a study conducted by Ali and Masoud [20] who's found reduction of collagen I α gene expression after 4 weeks of bone marrow derived MSCs transplantation in comparison with the CCl₄ treated group. Also, Li et al. [29] who demonstrated successful reduction

of TGF- β 1 level in the serum after transplantation of human umbilical cord MSC-exosomes into mouse liver fibrosis induced with CCl₄. Moreover, Bassiouny et al. [30] revealed that TGF- β and α -SMA mRNA levels were markedly decreased after cirrhosis treatment with human umbilical cord blood mononuclear stem cells in rats.

ECM breakdown controlled by matrix metalloproteinases (MMPs), the activity of which caught be inhibited by tissue inhibitors of metalloproteinases (TIMPs) [29]. MMP-2 (also knew as gelatinase-A) and MMP-9 (also knew as gelatinase-B), were the chief elements of MMPs family, were able to degrade various kinds of ECM [31]. MMP-2 secreted by HSCs [28], it degraded collagen IV, a chief constituent of the vascular basement membrane [32]. MMP-9 helped migration of bone marrow derived MSCs to the site of inflammation [20].

Our study showed that administration of stem cells enhanced gene expression of MMP-9 with high expression in the CCl₄/CD34⁺ group. This is consistent with the finding of Ali and Masoud [20] who found increased expression of MMP-9 after stem cells transplantation.

During progression of liver fibrosis diverse cytokines, chemokines and growth factors were secreted as part of the inflammatory reaction. TNF- α is a pro-inflammatory cytokines produced basically by macrophages [33].

In our study, administration of human CD34⁺ and BM-MSCs stem cells similarly reduced TNF- α gene expression. This effect claimed to anti-inflammatory and immunosuppressive characteristics of MSCs. This is in accordance with the finding of Huang et al. [34] who revealed that co-culture of MSC with neuron deprived from oxygen and glucose resulted in neuronal recovery with decreased TNF- α . Moreover, MSCs administration were efficient in declining the level of TNF- α and improving antigen-induced arthritis in mice [35] and sheep model of osteoarthritis [36].

In conclusion, our study revealed that human CD34⁺ is more effective in amelioration of CCl₄-induced liver injury in rat than BM-MSCs by reducing fibrosis, expressing liver-specific genes, decreasing gene expression of pro-fibrotic genes (collagen I α , TGF β 1, α -SMA), increasing

anti-fibrotic gene (MMP-9) and decreasing pro-inflammatory gene (TNF- α). This work also confirmed long term safety efficacy of human cord blood CD34⁺ and potential therapeutic capability of these cells supported by higher considerably higher synthetic effect of CD34⁺ for albumin than BM-MSCs.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hayam G Sayyed, Department of Medical Physiology, Faculty of Medicine, Assiut University, Assuit, Egypt. Tel: +2010-11826773; Fax: +20882332278; E-mail: hayam_said3@aun.edu.eg

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