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Original Article

Therapeutic potential of human umbilical cord derived mesenchymal stem cells on rat model of liver fibrosis

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Abstract: End-stage liver disease is a worldwide cause of morbidity and mortality, which is associated with a considerable economic burden. As the disease progresses, fibrosis will replace the hepatic architecture and compromise liver functions. The regenerative approach for the injured liver can provide a hope for these patients; however, it is still facing many challenges. In the current study, we aimed at (1) assessing hepatic regenerative capacity of mesenchymal stem cells, isolated from human umbilical cord blood (HMSCs), in a rat model of carbon-tetrachloride (CCL4) induced liver fibrosis, (2) comparing the therapeutic effects with other cell populations derived from umbilical cord blood and (3) evaluating the host response to the human-derived cells. Fifteen rats received either the whole mononuclear cell fraction (HMNCs), CD34-ve subpopulation or HMSCs. A fourth group did not receive any treatment and another group was left without induction of fibrosis as positive and negative controls. All groups that received cellular treatment showed homing of the human cells and improvement of the liver architecture and functional capacity. The groups received CD34-ve cells and HMSCs had the most efficient improvement in liver functions, microscopic regenerative markers and histological appearance while the least immune reaction was noted with HMSCs. HUCB-MSCs showed significant immunemodulatory effect on rat immune cells. This study can provide a clue about a simple and effective method for the management of fibrotic liver diseases.

Keywords: Mesenchymal stem cells, CD34-ve cells, hepatic regeneration, liver fibrosis, immune response, cell therapy

Introduction

Chronic liver disease is a worldwide health problem that affects patients with various ethnic backgrounds. While non-alcoholic fatty liver disease is a major cause, the alcoholic variant is the second leading cause of chronic liver disease that ultimately ends with liver fibrosis [1]. The latter is currently considered as a serious health problem that represents an enormous challenge to health services, especially with the absence of effective cure [2]. Liver fibrosis is characterized by extensive deposition of extracellular matrix (ECM) proteins by activated myofibroblasts and the production of a fibrous scar in the injured liver [3].

Regenerative medicine represents a future hope for patients with this condition by generating normal hepatocytes to restore the functional capability of the liver. Mesenchymal Stem Cells (MSCs) are characterized by their ability of self-renewal, clonal expansion and multipotency [4]. Generally, there are two approaches to enhance the differentiation potential of MSC into a particular lineage; (1) genetic manipulation, in which lineage specific transcription factors can be overexpressed to induce lineage-specific MSC differentiation; (2) micro-environmental modification, where MSC are exposed to different mixtures of growth factors, hormones and extra-cellular matrix components to induce their differentiation through the induction of specific signaling cascades under the effect of the so-called stem cell niche [5]. This second approach is safer and more applicable for the future clinical use.

With this background, hepatic regeneration is considered as an attractive research goal that would provide a chance of cure for millions of patients with end-stage liver disease [6]. Unfortunately, there are many questions that yet to be answered, including the best type of cells that would be effective in regenerating the normal architecture of the liver, the immunological acceptance by the body of the cells from different donors, and the overall improvement of the liver functions upon receiving the cells. In this study, we aimed at answering some of these questions by assessing the efficiency of human umbilical cord derived cells and their subpopulations on the regenerative capacity of the liver in an animal model of liver fibrosis.

Materials and methods

Animals

A total of seventy-five adult albino rats weighing 150 ± 50 gm was included in the study. Rats were purchased from the Ophthalmology Research Institute (Giza, Egypt); and allowed to acclimatize for seven days before starting the experiment. Rats were kept on a standard diet regimen. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Suez Canal University. The "Principles of laboratory animal care" were strictly followed, as well as the related national laws and regulations. At the end of the experiments, animals were euthanized by inhalation of an overdose of ether and the livers were extracted and preserved in 10% buffered formalin.

Isolation of human mono-nuclear cells (HMNCs)

HUCB samples were collected after obtaining an informed consent from apparently healthy, non-smoker mothers who delivered full-term babies following normal vaginal delivery at the Obstetric Emergency Room of Suez Canal University Hospital. MNCs were isolated using Ficoll-Hypaque (Sigma-Aldrich) in order to isolate the buffy coat containing the low-density MNCs [7].

Cell sorting

MSCS were sorted using magnetic activated cell sorting technique (MACS). Briefly, the positive fraction for each marker was trapped in the magnetic field as the specific antibody was labelled with magnetic microbead (Miltenyi Biotec). The negative fraction for CD34 of MNCs was further sorted to isolate MSC by selecting the subpopulation of CD105+ve, CD45-ve cells, according to the manufacturer instructions. Cells were cultured on tissue culture plastic in α-MEM (Sigma-Aldrich) with 10% Fetal bovine serum (Sigma-Aldrich) and 1% Penicillin-Streptomycin-Amphotericin B (Lonza), and 1% Gentamicin Sulphate (Lonza).

Experimental design

Rats were randomly divided into the following five groups, each contained 15 rats; a) negative control group (CO-): age matched rats received only normal saline as vehicle, b) positive control group (CO+): Rats received CCl_4 (0.5 ml/kg 50% CCl_4 dissolved in corn oil solution) one subcutaneous injection twice weekly for 8 weeks [8], c) human mono-nuclear cells (HMNCs) Group: Rats received CCl_4 for 8 weeks and then treated with HMNCs in a dose of 10^6 cells\rat, d) CD34- stem cells group: Rats received CCl_4 for 8 weeks and then treated with HUCB CD34- cells in a dose 10^6 cells\rat and e) human mesenchymal stem cells (HMSCs) Group: Rats received CCl_4 for 8 weeks and then treated with HMSCs in a dose 10^6 cells\rat .

The cells were introduced by intravenous injection in the tail vein. All groups were followed up for 8 weeks after therapy, where 7 rats from each group were sacrificed 6th week after therapy (at 14th week), while the remaining 8 rats of each group were sacrificed 8th week after therapy (at 16th week). The timeline for the study was shown in **Figure 1**.

Assessment of soluble hepatic markers

Blood samples were collected through retro-orbital puncture. Serum was separated by centrifugation at 3500 RPM for 15 Min. Serum transaminases, ALT (Alanine transaminase), AST (Aspartate aminotransferase), and GGT (Gamma-glutamyltransferase), were quantified using kinetic assays (Chema Diagnostica) while serum albumin was assessed calorimetrically (Human Gesellschaft). These markers were evaluated at day zero, 6th week, 8th week (pre-therapy), 14th week, and 16th week, according to the manufacturer's instructions.

Serum marker of liver fibrosis (Procollagen III) was evaluated at day zero and at 10th week

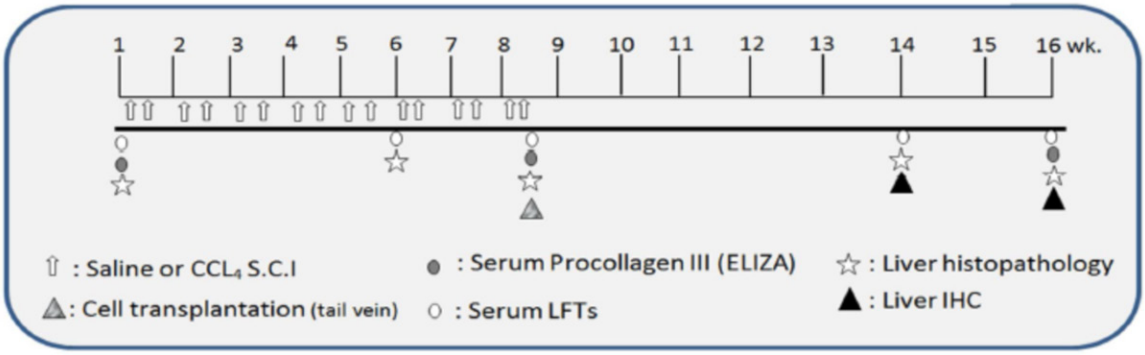

Figure 1. The time sequelae of the study investigations.

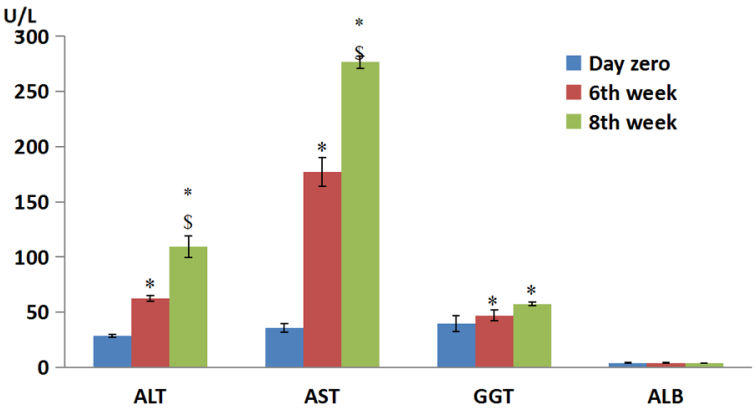

Figure 2. Comparison of ALT, AST, GGT & Alb pretherapy levels. All data ere expressed as means ± SE and were analyzed using one-way ANOVA and Bonferroni post-hoc test. ALT, AST, GGT were increased with the time course while Alb showed no difference. * in comparison to day zero at $P < 0.05$. $ in comparison to 6th week at $P < 0.05$ using Bonferroni test.

after induction and at 6th and 8th week after therapy using *N-terminal procollagen propeptide III (PIIINP) ELISA Kit,* according to the Glory Science Co., Ltd instructions.

Histopathological and immunohistochemical examination

Fixed rat livers were processed and sectioned at 5 µm. At least, two serial sections were stained with hematoxylin and eosin (H&E) or Masson Trichrome to assess the hepatic activity according to the METAVIR activity score [9].

For immunohistochemical studies (IHC), sections were stained with the following primary antibodies at the dilution of 1:50; a) anti-human albumin (R&D), b) anti-human cytokeratin19 (DAKO), c) antihuman human CD34 (Abcam), d) anti-rat CD68 (Abcam), e) anti-rat CD8 (Abcam) and anti-rat CD4 (Abcam).

Statistical analysis

STATA/SE 12 program was used for statistical analysis. The experimental unit in this study was the individual animal. The data was expressed as proportions or as mean ± SE. The significance of differences among proportions was evaluated by Chi square test. Differences among mean values of continuous variables were evaluated by ANOVA, and Bonferroni test. The significance level was considered as *P* value < 0.05.

Results

Assessment of liver function tests (LFTs)

Pre-therapy LFTs showed statistically significant difference of ALT, AST & GGT levels at day 0, 6th week and 8th week among rats after induction of liver fibrosis at day 0 ($P < 0.0001$, < 0.0001 & 0.04 respectively). Serum albumin showed no change over the time course as shown in **Figure 2**.

Post-therapy LFTs showed significant improvement of ALT, AST & GGT both at 14th week and 16th week using one-way ANOVA. Bonferroni test was used to found significant differences between groups, as shown in **Figure 3**. Liver enzymes were higher in liver fibrosis-induced groups in comparison to the negative control. All the treated groups showed significant reduction in ALT level in comparison to the positive control at week 14 irrespective of the cell type. However, there was no significant difference

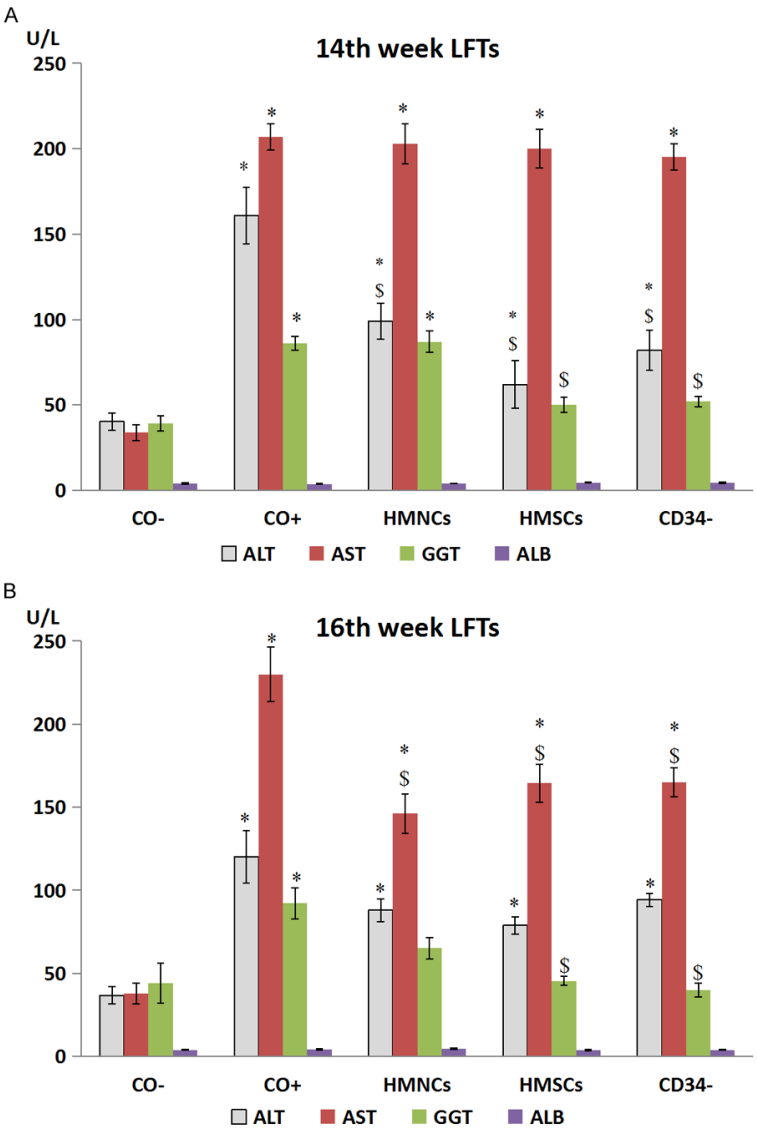

Figure 3. Comparison of ALT, AST, GGT & Alb. levels between different study groups at 14th week (A), and 16th week (B). All data ere expressed as means ± SE and were analyzed using one-way ANOVA and Bonferroni post-hoc test. * in comparison to CO-ve group at P < 0.05. $ in comparison to CO+ve group at P < 0.05.

between the three treated groups. AST and GGT levels show significant improvement in comparison to the positive control at week 16 in all treated groups. Albumin level did not significantly change across the groups in the two studied time points.

Assessment of procollagen III serum level

Pretherapy levels of procollagen III showed a trend of increase following the induction of liver fibrosis from day zero (165.1 ± 38.5) to 8th week (216.6 ± 25.6), with *p*-value of 0.07. Significant difference of Procollagen III levels was found among all research groups at 14th week (P = 0.0008). Also, there was a significant difference of Procollagen III levels among all research groups at 16th week (P≤0.0001). But, none of the research groups showed significant difference between 14th & 16th week level of Procollagen III (**Table 1**).

Histopathological assessment

Pretherapy histopathological assessment showed significant difference in both activity and fibrosis score (P = 0.007 & 0.03 respectively) according to METAVIR SCORE, represented in **Figure 4**.

The METAVIR activity score progress from A0 at day zero, to AI at 6th week, up to A2 at the 8th week of CCL_4 induction of liver fibrosis with no therapy. Concerning the METAVIR fibrosis score, it progresses from F0 at day zero, to F1 at 6th week, up to F2 & F3 (35%, and 65% respectively) at the 8th week of CCL_4 induction of liver fibrosis with no therapy.

Significant improvement in all therapy groups' METAVIR score was noticed both 14th & 16th weeks after therapy in comparison to the 8th week pretherapy level of METAVIR score, as shown in **Figure 5**. Histopathological assessment of the liver was done according to the METAVIR Scoring System, in form of two scores (activity score and fibrosis score), as shown in **Figure 6**.

Immunohistochemical assessment of homing and differentiation

Three antibodies used to identify the homing of transplanted human cells in rat liver, as seen in **Figure 7**, included the vascular differentiation

Table 1. Comparison of procollagen III levels in post therapy groups at 14th week & 16th week

Groups	Procollagen III (pg/ml) 14th week	Procollagen III (pg/ml) 16th week
CO-	165.13 ± 38.48	142.09 ± 34.57
CO+	308.30 ± 27.58$	370.89 ± 35.27$
HMNCs	278.51 ± 27.76	272.50 ± 23.22$,#
CD34-	189.19 ± 27.77#	221.44 ± 3.30#
HMSCs	168.49 ± 14.29#,θ	208.55 ± 11.53#
P value	0.0008*	< 0.0001*

Value expressed in mean ± SE. *P < 0.05 (Significant) using one-way ANOVA. $P < 0.05 (Significant) using Bonferroni test in comparison to Co-. #P < 0.05 (Significant) using Bonferroni test in comparison to Co+. θP < 0.05 (Significant) using Bonferroni test in comparison to HMNCs.

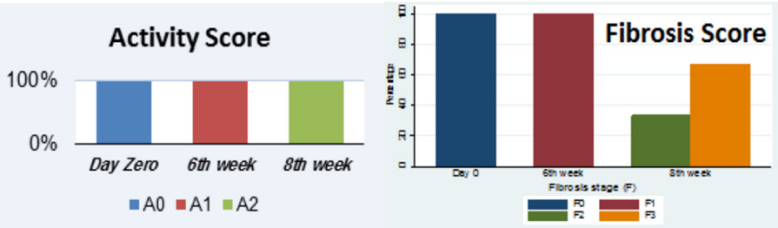

Figure 4. Bar chart showing pretherapy METAVIR SCORE in day zero, 6th week & 8th week (n = 5). In activity score; at day zero, all specimens were A0. At 6th week, all specimens were A1 while at 8th week, all specimens were A2. In fibrosis score; at day zero, all specimens were F0. At 6th week, all specimens were F1 while at 8th week, most of specimens were F3.

marker CD34, the differentiation marker of premature hepatocytes cytokeratin 19 and the mature hepatocytes marker of human Albumin.

At 14th week of research (6 weeks post therapy), although no statistical significance was found (P = 0.09), there was a trend of enhanced homing with HMSCs as shown by CD34 positivity indicating higher vascular differentiation. Both HMSCs and CD34-ve groups showed degree of positivity in all of the used homing human stains (CD34, CK19 & Albumin), indicating homing of injected human cells and different degrees of differentiation. The HMNCs showed 50% positivity in only CK19 stain. The CD34-ve group was the best group showing both CK19 stain & human albumin stain positivity indicating parenchymal differentiation & functional maturation of injected human cells (respectively), as shown in **Table 2**.

At 16th week, significant difference between groups in the positivity percentile of CK19 stain was noticed (P = 0.027), where HMSCs group showed significant difference in comparison to the other research groups (P = 0.025).

Although HMSCs was the only group showing CD34 & CK19 positive stains, it is the lowest group showing positivity of Human Albumin stain compared to CD34-ve & HMNCs groups, which may indicate the self-renewal of injected HMSCs so it still expresses the early marker of differentiation (CK19), while other groups undergo differentiation without self-renewal, so at 16th week the fully differentiated cells are the only remaining cells while no intermediate differentiating cells, as shown in **Table 3**.

Immunohistochemical assessment of immune response

Three markers were used to assess the rat immune response against the transplanted human cells, as shown in **Figure 8**, including specific markers CD4 & CD8 and the non-specific marker CD68.

CD4 stain: Significant difference in proportion of CD4 positive cells in both 14th week and 16th week was found between different research groups (P = 0.003 & < 0.0001 respectively). Although HMSCs group showed significant decrease in its CD4 cells compared to CO+ve group in both 14th & 16th weeks (P = 0.008), it was significantly higher than both CO-ve & HMNCs groups in both 14th (P = 0.014 & 0.003 respectively) & 16th weeks (P = 0.014 & 0.005 respectively). CD34-ve group simulates HMSCs in showing significant decrease in its 16th week proportion of CD4 positive cells compared to CO+ve group (P = 0.014), but significantly higher in it CD4 cells compared to both CO-ve & HMNCs groups (P = 0.025 & 0.008 respectively). No significant difference between 14th & 16th week CD4 cells between the research groups, as shown in **Table 4**.

CD8 stain: Significant difference in proportion of CD8 positive cells in both 14th week and 16th week between different study groups (P =

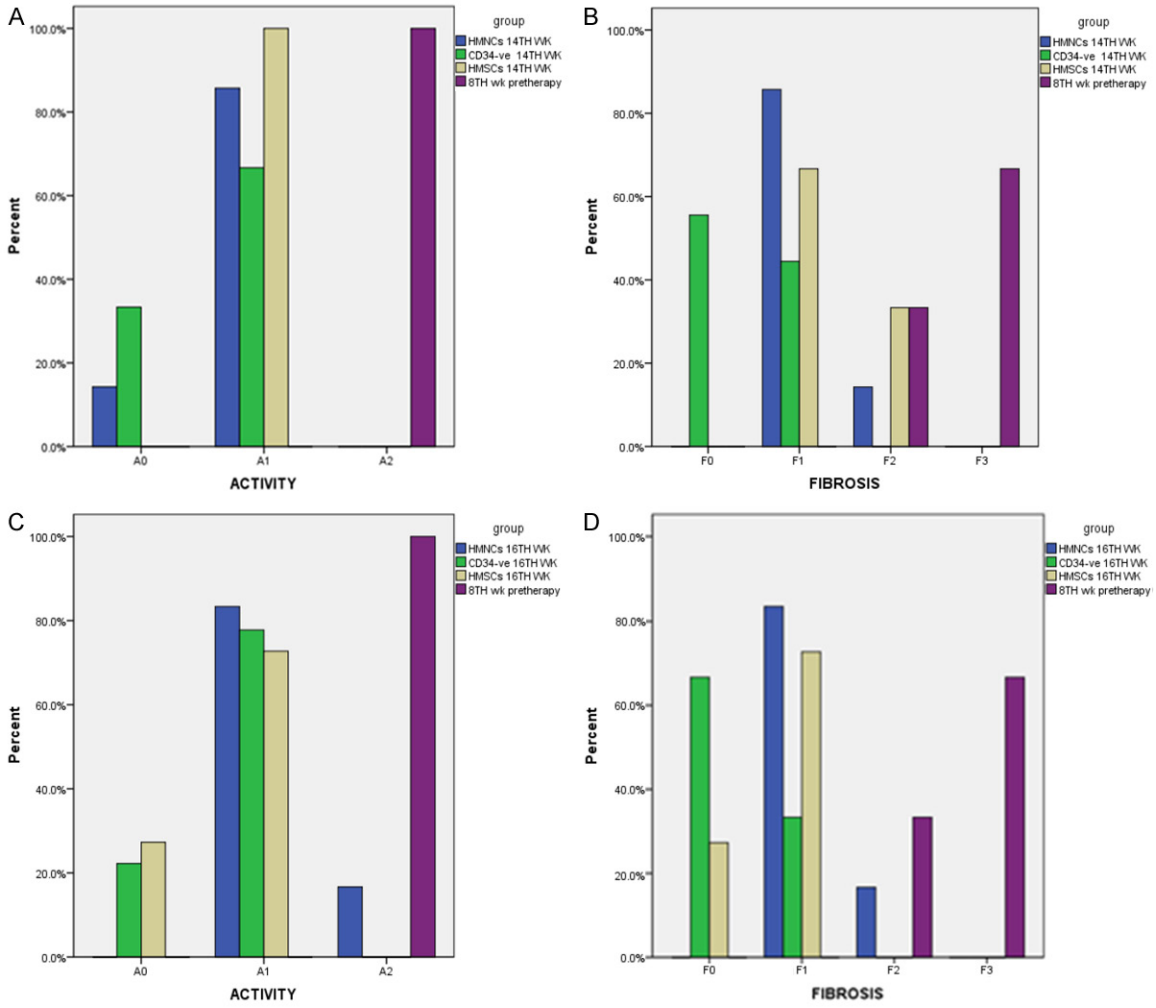

Figure 5. Bar charts showing comparison of therapy groups METAVIR score to the pretherapy 8th week. A: Activity score at 14th wk. B: Fibrosis score at 14th wk. C: Activity score at 16th wk. D: Fibrosis score at 16th wk. *P < 0.05 between HMNCs & 8th wk. pretherapy. #P < 0.05 between CD34-ve & 8th wk. pretherapy. $P < 0.05 between HMSCs & 8th wk. pretherapy. Chi square test was used. Pretherapy (n = 5), 14th wk. post therapy (n = 7), and 16th wk. post therapy (n = 8).

0.032 & 0.007 respectively). At 14th week: HMNCs group showed significant increase in its proportion of CD8 cells compared to both CO+ve & CO-ve groups (P = 0.04). At 16th week: HMSCs showed significant decrease in its proportion of CD8 cells compared to HMNCs group (P = 0.012).

HMSCs group showed significant decrease in its 16th week proportion of CD8 cells compared to its 14th week (P = 0.04), while the HMNCs group showed significant increase in its 16th week proportion of CD8 cells compared to its 14th week (P = 0.04), as shown in **Table 5**.

CD68 stain: Significant difference in proportion of CD68 positive cells in both 14th week and 16th week between different research groups (P = 0.011 & < 0.0001 respectively). At 14th week: CD34-ve group showed significant decrease in its proportion of CD68 in comparison to CO-ve, CO+ve & HMNCs groups (P = 0.028, 0.042 & 0.007 respectively).

At 16th week: CD34-ve group showed significant decrease in its proportion of CD68 cells compared to both CO+ve & HMSCs groups (P = 0.005 & 0.04 respectively).

No significant difference between 14th & 16th week proportion of CD68 cells was noticed in any of the research group, as shown in **Table 6**.

Discussion

Hepatic fibrosis is an ultimate outcome of various liver diseases, which is associated with

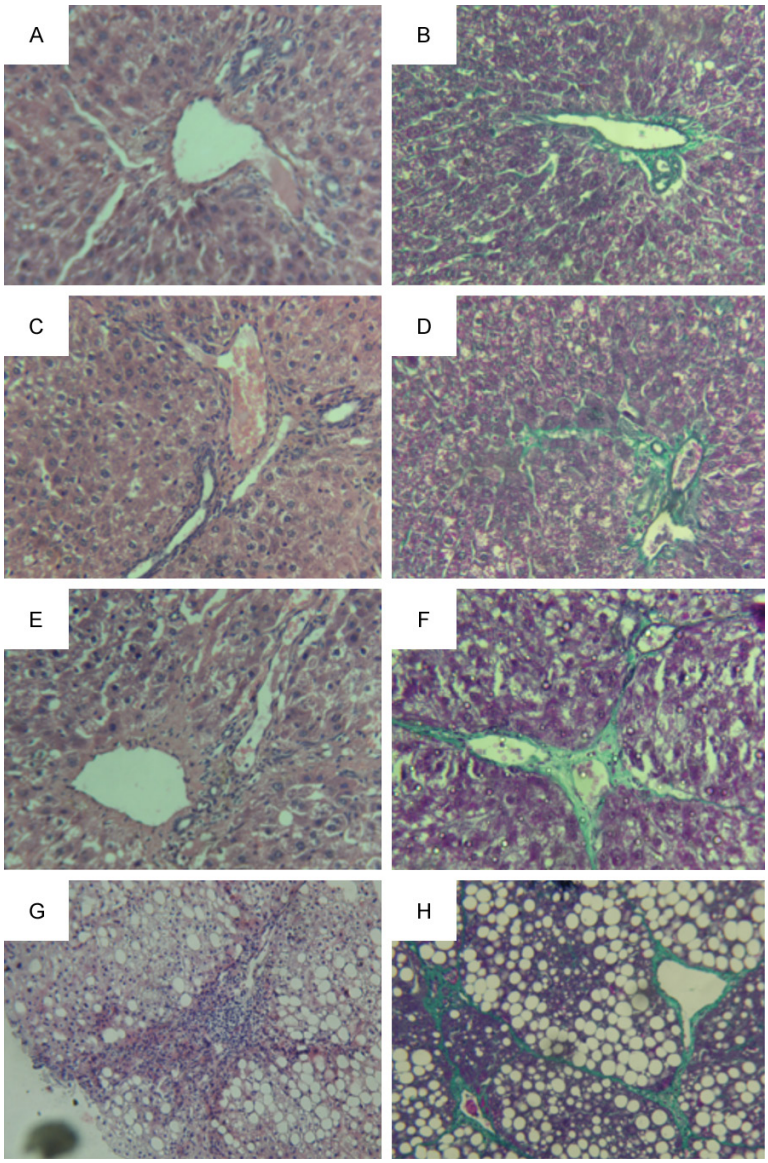

Figure 6. Microscopic photos showing the histopathological assessment of liver biopsies according to METAVIR Scoring System (×400). (A, C, E & G) refer to the METAVIR activity score using H&E stain, where (A) A0, (C) A1, (E) A2 & (G) A3. (B, D, F & H) refer to the METAVIR fibrosis score using masson stain, where (B) F0, (D) F1, (F) F2 & (H) F3.

worldwide morbidity and mortality and causes a huge economic burden for the health authorities [10]. Regenerative medicine can represent a hope for many refractory diseases and is an important field of investigations [11]. Umbilical cord blood is a very rich source of stem cells that is accessible, easy to obtain and of multipotential differentiation ability [12]. As regenerative medicine provides a hope for the management of refractory diseases, we investigated the potential of stem cells for restoring the liver parenchymatous histological and physiological features.

The current study assessed the effect of HMSCs, CD34-ve and HMNCs in the hepatic regenerative capacity of a rat model of liver fibrosis. The untreated groups were represented as normal matched rats or negative control, and liver fibrosis induced rats as positive control).

The induction of liver fibrosis using CCL4 was confirmed by the occurrence of hepatocytes and biliary epithelium necrosis which was reflected on the elevation of serum transaminases. The absence of any significant decrease of serum albumin level confirmed the induction of liver fibrosis rather than decompensated liver cirrhosis.

Liver regeneration was tested both structurally and functionally by the assessment of histopathological METAVIR activity score and LFTs (ALT, AST, GGT and Albumin) respectively. Homing and differentiation of transplanted cells were assessed by using IHC human antibodies (CD34, CK-19, and albumin). The inflammation and fibrosis of liver tissue were tested both serologically and histopathologically by the assessment of serum level of procollagen III and METAVIR fibrosis score respectively. The rat immune responses against transplanted human cells were studied using anti-rat CD4, CD8 and CD68 IHC stains.

The overall results were that introducing the rats with CD34-ve cells and HMSCs was associated with the best-obtained improvement in the liver architecture and restoration of the function. Administering HMNCs was associated with the least improvement.

Both groups injected with HMSCs or the subpopulation CD 34-ve cells showed significant functional improvement in LFTs in comparison

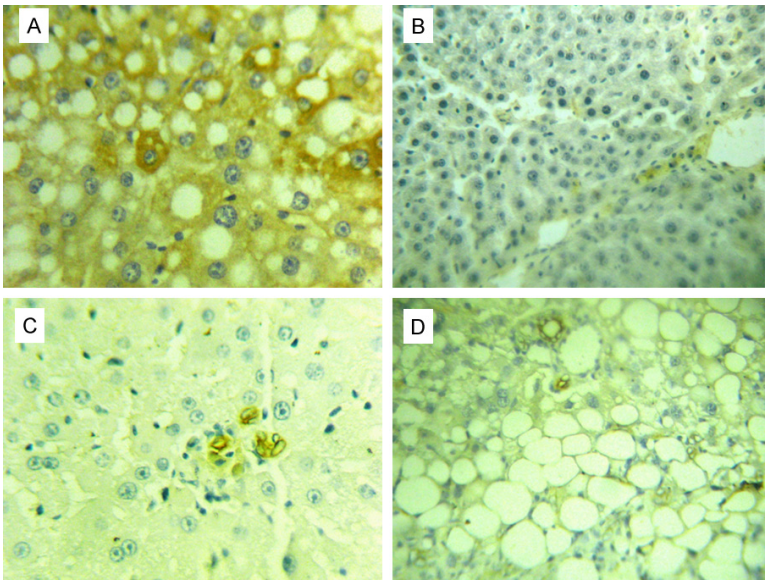

Figure 7. Microscopic photos showing positivity of homing stain in different liver sections, paraffin, DAB, Hx, ×400 IHC. A: Arrows refer to moderate Albumin positivity. B: Arrows refer to mild CD34 positivity. C: Arrows refer to moderate stained dispersed human CK 19 positive cells. D: Arrows refer to moderate human CK 19 positively stained bile canaliculi lining cells.

Table 2. Comparison of positivity % of homing stains between treated groups at 14th week

Groups	14th wk anti-human CD34 stain		14th wk anti-human CK19 stain		14th wk anti-human Albumin stain	
	Neg %	Pos %	Neg %	Pos %	Neg %	Pos %
HMNCs	100%	0%	50%	50%	100%	0%
CD34-	87.5%	12.5%	33.33%	66.67%	50%	50%
HMSCs	44.44%	55.56%	66.67%	33.33%	60%	40%
P value	0.091		0.441		0.49	

Table 3. Comparison of positivity % of human homing stains between treated groups at 16th week

Groups	16th wk anti-human CD34 stain		16h wk anti-human CK19 stain		16th wk anti-human Albumin stain	
	Neg %	Pos %	Neg %	Pos %	Neg %	Pos %
HMNCs	100%	0%	100%	0%	0%	100%
CD34-	100%	0%	100%	0%-	0%	100%
HMSCs	50%	50%	0%	100%$^{\theta,©}$	50%	50%
P value	0.188		0.027*		0.137	

*P value < 0.05 (significant) using chi square test. $^{\theta}$P < 0.05 (Significant) using chi square test in comparison to HMNCs. $^{©}$P < 0.05 (Significant) using chi square test in comparison to CD34-ve.

to the positive control, as shown by ALT in 14th week (62 ± 11.6 and 92.5 ± 13.9 respectively vs. 161 ± 16.5), AST in 16th week (165 ± 8.8 and 164.5 ± 17.9 respectively vs. 258.3 ± 9.2), and GGT in both 14th (58.5 ± 3.1 and 50 ± 7.4 respectively vs. 86 ± 4.1) and 16th weeks (258.3 ± 9.2 and 45.5 ± 2.8 respectively vs. 92.1 ± 16.3). The significant improvement of LFTs indicates that both HMSCs and CD34-ve cells are able to decrease not only hepatocytes necrosis, showed by the significant decrease of serum transaminases ALT and AST, but also by the decrease in the biliary epithelium destruction as shown by the decrease of serum GGT. These results are in agreement with the studies of other researchers [13-17]. Group 3 (HMNC) showed early transient improvement in the ALT serum level at 14th week compared to the group 2 (CO-) (99 ± 10.4 vs. 161 ± 16.5) indicating transient decrease of hepatocytes necrosis. Such effect was not present in the 16th week.

The improvement of LFTs in the group treated with HMSC was associated with a trend of consistent improvement in the METAVIR activity score (P=0.08) from 14th to 16th week, which is in accordance with a lot of studies searching the effect of HMSCs on liver fibrosis models [13, 18, 19].

The histopathological examination of the positive control liver biopsies showed a degree of regenerative hypersplasia, which is a type of irreversible cell injury, associated with absence of significant fibrosis, and elevated serum transaminases level [20]. On the other hand, the histopathological improvement of the study groups received HMSCs and CD34-ve subpopulation showed normal

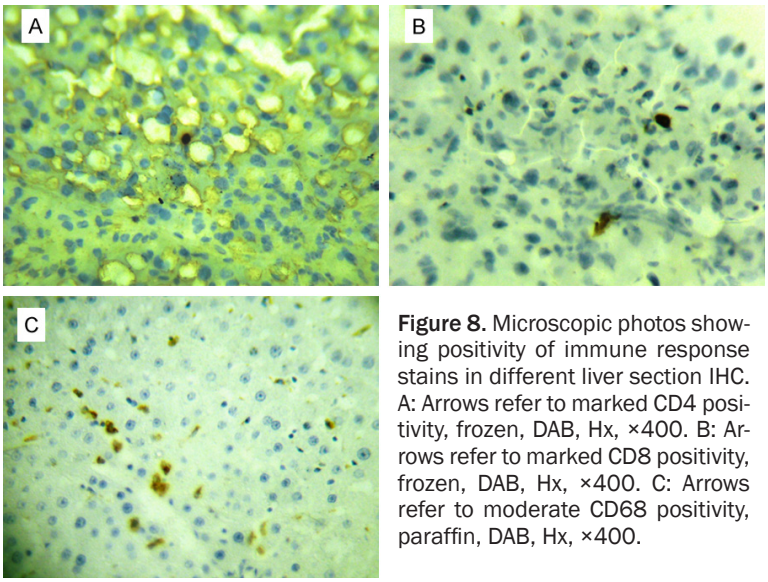

Figure 8. Microscopic photos showing positivity of immune response stains in different liver section IHC. A: Arrows refer to marked CD4 positivity, frozen, DAB, Hx, ×400. B: Arrows refer to marked CD8 positivity, frozen, DAB, Hx, ×400. C: Arrows refer to moderate CD68 positivity, paraffin, DAB, Hx, ×400.

hepatocytes with no regenerative hypersplasia and was associated with significantly decreased serum transaminases level.

Beside the functional and histological improvement, rats received HMSCs were the only group that showed positivity in all used IHC homing and differentiation markers (CD34, CK19 and albumin) in both 14th and 16th weeks and the best group showing human CD34 immunostaining. These results indicated higher homing, multipotential differentiation, vascular and parenchymal with its subtypes biliary and hepatic, and self-renewal (as the HMSCs group was the only group that still express the human CK19 at 16th week). CK19 is a marker of intermediate hepatoblast, then disappears in mature hepatocytes, while continue to be expressed in biliary epithelial lining. In the current study, human CK19 was positive in treated groups that appeared both as solitary positive cells within hepatic parenchyma, and as positive lining of the bile canaliculi, indicating presence of both intermediate hepatoblast, and biliary epithelium respectively.

The groups received CD34-ve cells and HMNCs expressed only human albumin, which is a marker of mature functioning hepatocytes, at 16th week. This result could indicate that all transplanted cells were fully differentiated at this timepoint. These results confirmed that HMSCs are capable of homing and multipotent differentiation (vascular, hepatic and biliary) in rat model of liver fibrosis, which is in accordance with the results reported by Abdel Aziz *et al.* 2008 who confirmed homing of male BM-MSCs by PCR detection of sex determination region in the Y chromosome (sry gene) in recipient female rats [7]. Zhou *et al.* concluded that HMSCs showed homing with hepatic and biliary differentiation (proved by positivity of hAFP, hHGF, and hCK 18 respectively) in mouse liver [21]. Kim et al confirmed the homing of transplanted human BM-MSCs in rat model of liver fibrosis by positive expression of human mitochondria specific antigen [22].

The transplantation of HUCB derived CD34-ve cells was associated with improvement of LFTs and improvement in METAVIR activity score compared to the 8th week pretherapy score. Also, this group showed early homing and multipotent differentiation at 14th week as shown by all studied homing markers, especially CK19 and human albumin. However, this multipotential differentiation was short term, as the homing and differentiation markers at 16th week showed positivity of only human albumin, indicating more preference toward hepatic parenchymal differentiation and absence of self-renewal of CD34-ve cells. The hepatic differentiation potential of CD34-ve cells was documented by Nonome *et al.* [23]. The positivity of homing and differentiation markers may explain the improvement of LFTs, via increasing regeneration rather than decreasing inflammation.

In accordance with the current study, Sato and colleagues proved that MSCs isolated from bone marrow were more potent in hepato-biliary differentiation than CD34+ve and non-MSCs CD34-ve cells based on IHC marker for human-specific AFP, albumin, CK19, and CK18 in all studied types of cells [24].

Regarding HMNCs treated group, although the brief improvement of in ALT was not associated with improvement in METAVIR activity score, it was associated with IHC positivity of human CK19 and human albumin stains (at 14th & 16th weeks respectively), which is mostly attributed to the well documented small stem cell fraction of MNCs. The latter represents between 0.071% and 0.39% of the HUCBMNCs. Also, Alvarez-Viejo *et al.* in 2013 reported that

Table 4. Comparison of 14th week & 16th week proportion of CD4 cells in different research group

Groups	14th week Proportion of CD4 cells						16th week Proportion of CD4 cells						*P* value
	< 1%	1%	2%	5%	10%	25%	< 1%	1%	2%	5%	10%	25%	
Co-ve	100%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	1
Co+ve	0%	0%	0%	100%$	0%	0%	0%	0%	0%	100%$	0%	0%	1
HMNCs	75%	0%	0%	25%	0%	0%	100%#	0%	0%	0%	0%	0%	0.408
CD34-	25%	75%	0%	0%	0%	0%	0%	100%$,#,θ	0%	0%	0%	0%	0.285
HMSCs	0%	100%$,#,θ	0%	0%	0%	0%	0%	100%$,#,θ	0%	0%	0%	0%	1
P value	0.003*						< 0.0001*						

*P < 0.05 (Significant) using chi square test. $P < 0.05 (Significant) using chi square test in comparison to Co-. #P < 0.05 (Significant) using chi square test in comparison to Co+. θP < 0.05 (Significant) using chi square test in comparison to HMNCs.

Table 5. Comparison of 14th week & 16th week proportion of CD8 cells in different research group

Groups	14th week Proportion of CD8 cells						16th week Proportion of CD8 cells						*P* value
	< 1%	1%	2%	5%	10%	25%	< 1%	1%	2%	5%	10%	25%	
Co-ve	100%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	1
Co+ve	0%	100%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	1
HMNCs	0%	0%	0%	100%$,#	0%	0%	0%	0%	0%	0%	33%	67%	0.04*
CD34-	0%	20%	0%	40%	40%	0%	0%	66.6%	0%	33.3%	0%	0%	0.31
HMSCs	0%	20%	0%	60%	20%	0%	0%	87.5%θ	0%	12.5%θ	0%	0%	0.04*
P value	0.032*						0.007*						

*P < 0.05 (Significant) using chi square test. $P < 0.05 (Significant) using chi square test in comparison to Co-. #P < 0.05 (Significant) using chi square test in comparison to Co+. θP < 0.05 (Significant) using chi square test in comparison to HMNCs.

Table 6. Comparison of 14th week & 16th week proportion of CD68 cells in different research group

Groups	14th week Proportion of CD68 cells						16th week Proportion of CD68 cells						*P* value
	< 1%	1%	2%	5%	10%	25%	< 1%	1%	2%	5%	10%	25%	
Co-ve	0%	0%	50%	25%	25%	0%	0%	0%	50%	50%	0%	0%	0.513
Co+ve	0%	0%	0%	63.8%	18%	18%	0%	0%	0%	45.4%	36.4%	18%	0.607
HMNCs	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	100%	0%	0.157
CD34-	44%$,#,θ	0%	0%	56%$,#,θ	0%	0%	100%#	0%	0%	0%	0%	0%	0.154
HMSCs	0%	0%	11%	56%	11%	22%	0%	0%	0%	33%©	17%©	50%©	0.58
P value	0.011*						< 0.0001*						

*P < 0.05 (Significant) using chi square test. $P < 0.05 (Significant) using chi square test in comparison to Co-. #P < 0.05 (Significant) using chi square test in comparison to Co+. θP < 0.05 (Significant) using chi square test in comparison to HMNCs. ©P < 0.05 (Significant) using chi square test in comparison to CD34-ve.

MSCs represent low highly variable percentage of BM-MNCs (0.0017% to 0.0201%) [25].

The current study assessed the rat immune response against xenotransplantation of human cells without immunosuppression. In general, the group received HMSCs emphasized the immune-modulatory, and hypo immunogenic effect of HMSCs-based therapy, as evidenced by significant decrease of the proportion of positive CD4 T-helper cells compared to the untreated group, both at 14th & 16th week. Also, the proportions of both positive CD8 T cytotoxic cells, and positive CD68 macrophages in HMSCs group were not significantly different from the untreated group, indicating that xenotransplantation of HMSCs did not only prevent the cytotoxic immune response, but also decreased the proliferation of effector CD4 T-helper cells. These results are in accordance with different studies supporting the immune-modulatory effect of HMSCs transplantation [18, 23, 26, 27].

The rat immune response against transplanted CD34-ve cells was an important parameter, which showed delayed decrease of the CD4+ve T-cells proportion (at the 16th week), compared

to untreated group, associated with non-significant difference in the proportion of CD8+ve T-cells from the control group. This delayed decrease in CD4+ve T-cells, is mostly due to the proportion of MSCs within the CD34-ve population. Interestingly, the group received CD34-ve cells showed decrease in the proportion of CD68+ve macrophages in comparison to the control group. This finding suggested that CD34-ve cell population may selectively modulate the naïve (non-specific) immune response, which, up to our best knowledge, was not previously reported. Further purification of the CD34-ve cell population with specific surface markers, such as CD45-ve and CD105+ve, may further enhance the immunomodulatory effect as MSCs treated group showed early decrease of CD4+T helper cells at 14th week. CD34-ve cells treated group lacked this significant effect on CD4+T helper cells.

However, the previous MSCs purification attenuates the immunomodulatory effect on non-specific immunity, as proved by the significant decrease of CD68+ve macrophages in CD34-ve treated group compared to MSCs treated group, which is mostly attributed to the effect of one of cell subset which are non-MSCs in the CD34-ve cells.

The short-term improvement of HMNCs treated group can also be explained by the fact that the HMNCs contain various subpopulations that can stimulate rat immune response. The increase of CD8+ T cytotoxic cells and CD68+ve macrophages proportion with HMNCs in comparison to the positive control could indicate stimulation of both non-specific and specific cellular rat immune responses, respectively.

However, the absence of significant increase of proportion of CD4+ T helper cells at 14th week is not well understood. One possible mechanism could be the earlier stimulation of CD4+ T helper cells, especially with the significant decrease of CD4+ve T-helper cells proportion by the 16th week. This finding can be confirmed in the future by earlier assessment of CD4+ T helper cells at 10th & 12th week (2 and 4 weeks post therapy). These data were in accordance with the studies of MSCs therapy for induced liver fibrosis [4, 13, 14, 21, 22, 28, 29, 30].

Conclusion

All the transplanted HUCB- derived cells (MNCS, CD34-ve cells, and MSCs) showed a degree of improvement of CCL4 induced hepatic injury. The best structural and functional improvement was in CD34-ve and MSCs treated groups, while HMNCs treated group showed transient improvement. Both CD34-ve and MSCs treated groups showed significant improvement of LFTs in comparison to the positive control. MSCs showed positive homing, multipotent differentiation and self-renewal, while HMNCs and CD34-ve treated groups showed less homing, and differentiation with no self-renewal.

Disclosure of conflict of interest

None.

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