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Research Article

Therapeutic Efficacy of Bone Marrow Mononuclear Induced Hepatocyte in a Model of Acute Liver Failure by Immunomodulation of IL-17 Pathway

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Abstract

Background: Previous studies reported the capacity of undifferentiated bone marrow mononuclear cells (BM-MNCs) to improve acute liver failure (ALF) animal model but little is known about the efficacy of BM-MNCs derived hepatocytes in ALF and its mechanism.

Materials and Methods: Human BM-MNCs (hMNCs) were separated. These cells without and with differentiation (HLCs) were injected into rats with ALF induced by CCL4. To assess its effect, transaminases were measured at different time points, survival rate was calculated. Engraftment of injected cells was evaluated by flowcytometry and qRT-PCR. Histopathological improvement by H and E, analysis of cell proliferation by PCNA and anti-human albumin were investigated. To explore the role of IL-17 pathway in therapeutic mechanism of hepatocytes, IL-17, RORC gene expression and serum IL-17 were evaluated.

Results: HLCs treated group showed significant engraftment, more proliferation by 2.3 fold, improvement in ALT by 2.2 fold and AST by 1.4 fold, survival rate, liver architecture, than hMNCs group ($p < 0.05$). In addition, HLCs group showed significant lower IL-17, RORC mRNA levels and serum level of IL-17 than hMNCs and control groups ($p < 0.001$).

Conclusion: BM-MNCs derived hepatocytes proved more therapeutic efficacy than undifferentiated cells in improvement ALF animal model by modulation of IL-17 pathway.

Keywords: *Acute Liver Failure Model; BM-MNCs Derived Hepatocyte; Immunomodulation; IL-17; RORC Gene*

Abbreviations

CCL_4: Carbon Tetrachloride; ALF: Acute Liver Failure; BMMNCs: Bone Marrow Mononuclear Cells; ALT: Alanine Transferase; AST: Aspartate Transferase; PCNA: Proliferating Cell Nuclear Antigen

Introduction

Orthotopic liver transplantation (OLT) is considered the best choice therapy for Acute liver failure (ALF) [1]. However, shortage of donor is the major limiting factor due to the rapid progression of the disease and mortality on the waiting list may be as high as 40% [2,3].

Cell-based therapies have emerged as an alternative to OLT. Cell transplantation offers advantages, being a less-invasive procedure when compared to whole organ transplantation and can be performed repeatedly if necessary [4].

Bone Marrow Mononuclear Cells (BM-MNCs) have emerged as potential candidates for cell therapy due to their ease of use. The two major populations of adult stem cells are bone marrow mesenchymal and hematopoietic stem cells (HSCs) [5].

The syngeneic transplantation of stem cell immediately after the administration of CCL_4 reduced the hepatic damage, the development of fibrosis, and accelerated liver regeneration [6].

Several studies reported on the safety and efficacy of undifferentiated bone-marrow-derived mononuclear cells to treat acute liver failure [7-11] but no attention toward the usage of pre-differentiated hepatocytes. Also, the mechanism by which transplanted *in-vitro* derived hepatocytes in recovery of ALF hasn't been fully elucidated.

So, in the present study for the first time the feasibility of bone marrow mononuclear derived hepatocyte like cells to improve recovery of induced acute liver failure animal model and the mechanism of action through IL-17 pathway have been investigated.

Materials and Methods

The study was done in Clinical Pathology and Pathology Departments and approved by ethical reviewer committee of faculty of medicine which in compliance with the national ethical guidelines for the care and use of laboratory animals (NO: Cp 409/ 2017).

Study design

Ninety six adult albino female rats which were divided randomly into three groups (each group constituted of 32 rats, 20 rats were maintained for 4 weeks for measurement of serum measurements, survival rate and histopathology, 12 rats from each group were sacrificed at 2w): Control group: the rats were subjected to a single intravenous injection of 0.9% saline in the rat tail vein 24h after CCL_4 injection, human mononuclear cells treated group (hMNCs group), the animals were infused with 10×10^6 undifferentiated hMNCs via tail vein 24 hours after CCL_4 injection. BM-MNCs derived hepatocyte like cells -treated group (HLCs group), the animals were infused with 10×10^6 induced hepatocyte 24 hours after CCL_4 injection. The treated groups were injected by 1 ml dexamethasone along with the injected cells to decrease any suspected inflammatory reaction.

Methods

Experimental acute liver failure in rats

Ninety six adult albino female rats of local strain aged 6-8 weeks, weighing 150 - 200 grams were used in this study. Rats were purchased from the animal house of the National Research Center. Rats were housed in a fully ventilated cages with free access to water and balanced diet 12-hour (h) light/dark cycles. Acute liver failure was induced by intraperitoneal (I.P.) injection of CCL_4 with a dose (3.0 ml/kg dissolved in vegetable oil 1:1).

Human MNCs preparation

The study involved patients who come for bone marrow aspiration. Informed consent was taken from the patients before sampling. The study was approved by ethical reviewer committee of faculty of medicine which in compliance with the Declaration of Helsinki, 2013 (NO: Cp 409/ 2017). The mononuclear cells (MNCs) were assessed for CD14, CD44, CD45 (BD immunocytometry system, San Jose, CA).

Differentiation of hMNCs into hepatocyte like cells

The MNCs were cultured into 25 cm^2 flask for 7 days with differentiation media constituted of DMEM L/G 10% FBS, penicillin/streptomycin, HGF 20 ng/ml and b-FGF 10 ng/ml. The hepatocyte-like cells were identified by morphology, hepatic gene expression of AFP, ALB and CK18 by qRT-PCR and measurement of AFP, albumin, transferrin and urea in the supernatant.

AFP, albumin, transferrin and urea measurement in the supernatant

The supernatant of cultured media of both hMNCs and HLCs on day 7 were collected and hold until measurement. AFP measured using a chemiluminescence immunoassay kit; for ALB production using immunoturbidimetric method by microalbumin kit; for urea produc-

tion using a colorimetric assay kit and transferrin was done using immunoturbidimetric method by cobas 6000 - module c501 analyzers. Cells used for the assays were trypsinized and counted. The results were normalized based on 10^6 live cells.

Transaminases measurement

The rats of each group were weighted and blood samples were collected from the retro-orbital venous plexus, using a fine heparinized capillary tube introduced into the medial epicanthus of the rat's eye**.** The serum was used for estimation of serum ALT (Alanine transferase), AST (Aspartate transferase) at 24h, 72h, 1 week, 2 week, 3 week and 4 week of CCL_4 injection using Cobasintegra 400 plus*.*

Survival rate measurement

The numbers of surviving rats as a function of time were estimated at 72 hours, 1 week, 2 week, 3 week and 4 week after injection of treated cells.

Engraftment of transplanted cells

Flowcytometric analysis

1 ml of rat blood at 4w was exposed for flowcytometry for detection of homing of h-MNCs by PECD34, FITCCD45 and PECD14.

Demonstration of chimerism by detection of human testis determining gene (SRY) in female rats by qRT-PCR

DNA was extracted from liver cells using Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, MA, USA) according to the attached instructions.

PCR assay was performed as previously described [13] using the specific primers and probes for human SRY gene and β-actin control gene (Thermo scientific). The PCR reaction was in a total volume of 25 µL containing 12.5 µL of taqman universal master mix (Applied Biosystem),PCR cycling conditions and Primers showed in table 1.

Gene	Primers and probes	Annealing T°
SRY	F: 5'-GCG ACC CAT GAACGCAT"-3 R:5'- AGT TTC GCA TTCTGGGATTCT CT-3 Probe: FAM-TGG TCT CGC GAT CAGAGG CGC-TAMRA	59°C
β-actin	F: TCACCCACACTG TGCCCATCTACG A R: CAGCGG AAC CGCTCATTG CCA ATG G Probe: FAM-ATG CCC TCC CCCATGCCATCC TGCGT-TAMRA	59°C
AFP	F: GATGCACCTGACCCACTTTATAAA. R: GAGATTGTCTGACCGATTCACT	58°C
Albumin	F: CAACTATGTCCGTGAGCTTCCA. R: GTGGTCGGTGCTGGTCTATATG	58°C
CK18	F: CCC GCT ACG CCC TAC AGAT. R: ACCACTTTGCCATCCACTATCC	58°C
IL-17	F: CTGTCCCCATCCAGCAAGAG R: AGGCCACATGGTGGACAATC	58°C
RORC	RORC F: GTGGGGACAAGTCGTCTGG R: AGTGCTGGCATCGGTTTCG	58°C

Table 1*: Primers of the selected genes.*

Quantitation of the male DNA in the recipient female rats by construction of standard curve which was done by serial eight dilutions from 1:10 to 1: 100,000 of starting concentration of DNA, each dilution was done in triplicate. Human male DNA was used as positive control, negative control was done from PCR mix without DNA and from female DNA.

Histopathological and immunohistochemical studies

After collection of blood samples, rats were anesthetized by I.P sodium phenobarbital (40 mg/kg B.W) then a midline abdominal incision (laparotomy) was done and the liver was rapidly excised and removed to be fixed in 4% paraformaldehyde and processed through paraffin embedding for later histopathological and immunohistochemical studies.

Study the architecture of the liver

The architecture of the liver was evaluated by Hematoxylin and Eosin. Necrosis was evaluated by counting the rows of necrotic hepatocytes from the perivenular zone. Steatosis, hydropic degeneration, inflammatory infiltrate expressed as the percent of animals in which such abnormalities were found comparing treated and untreated animals.

Assessment of hepatocyte proliferation

The mitotic index was counted in ten high-power fields. In addition, Sections of liver are subjected for immunohistochemical staining for expression of proliferating cell nuclear antigen (PCNA) (Thermo scientific).

Detection of distribution and survival of transplanted cells

To evaluate the distribution and survival of transplanted cells, human specific anti albumin (Novus biological) was assessed by immunohistochemical staining.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from cultured hMNCs without and with differentiation using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed as previously described [12]. Two-Step PCR was done, 8µL of RNA was reverse transcribed into cDNA using MultiScribe Reverse transcriptase according to manufacturer's protocol using GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystem). Volume of 5 µL of cDNA was added to a final PCR reaction mixture of 25 µL containing 12.5 µL Master Mix SYBR Green Dye (Applied Biosystem), 1.5 µL of each Primer, 4.5 µL RNase free water. Cycling conditions and primers are showed in table 1. The relative quantitation of the expression levels were measured using the $2^{-(\Delta\Delta CT)}$.

IL-17A measurement by ELISA

The serum from normal rats (before injection of CCL_4), injured control group and treated groups was collected and stored at -20°C for measurement of IL-17 by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

The data were collected, tabulated, and analyzed by SPSS (statistical package for social science) version 17.0 on IBM compatible computer. Two types of statistics were done, descriptive statistics [e.g. percentage (%), mean (x) and standard deviation (SD)] and analytic statistics which include the following tests: Chi-square test (χ^2) Mann Whitney U and Anova test.

Results

Flowcytometric analysis of hMNCs

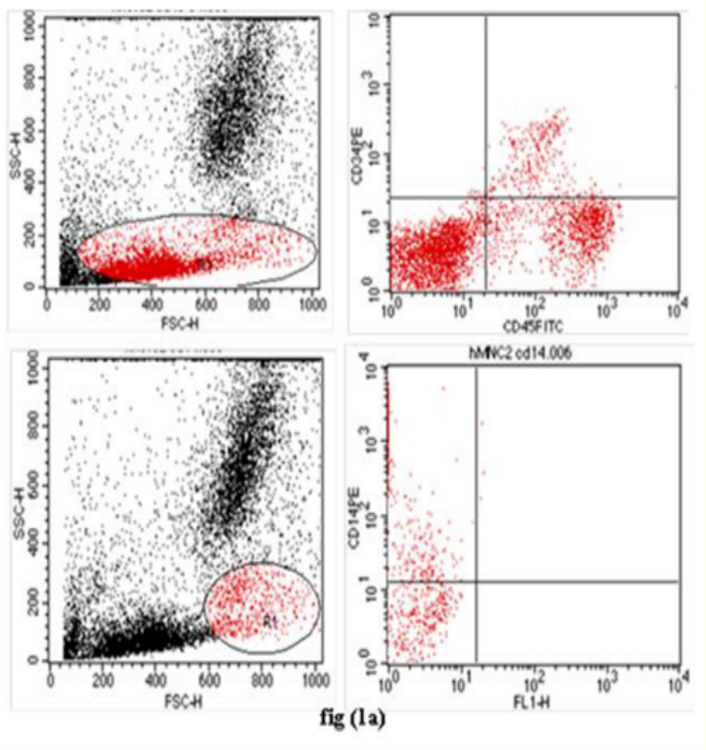
These cells showed immunophenotypic marker positivity for MNCs antigens CD45 (37.15 ± 9.84), CD34 (2.03 ± 0.97) and CD 14 (45.69 ± 14.94). Representative histograms for human MNCs are shown in figure 1a.

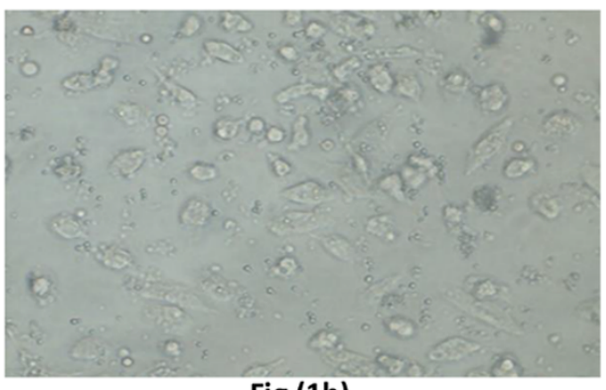
Characterization of hepatocyte like cells transformed from BM derived hMNCs

When the cultured cells were treated with hepatocyte differentiation medium, the cells progressively assumed the hepatocyte like morphology (Figure 1b). On molecular level, the differentiated hMNCs express hepatic specific genes AFP, ALB and CK18 by qRT-PCR (Figure 1c). The culture supernatant showed significantly higher levels of AFP, ALB, transferrin and urea among differentiated hMNCs (2.17 ± 1.30, 10.22 ± 3.58, 26.1 ± 6.3, 7.3 ± 1.4) than undifferentiated cells (0.52 ± 0.29 and 3.6 ± 1.07, 14.1 ± 2.9, 3.7 ± 0.7) with $p < 0.001$ respectively (Table 2).

	The studied cases N = 10		test	P value
	HLCs	hMNCs		
Albumin (mg/dl) Mean ± SD Range	10.22 ± 3.58 4.95 – 15.26	3.6 ± 1.07 2.2 – 5	U = 3.48	< 0.001
AFP (ng/ml) Mean ± SD Range	2.17 ± 1.30 0.5 - 4.2	0.52 ± 0.29 0.11 - 0.92	U = 3.33	< 0.001
Transferrin (mg/dl) Mean ± SD Range	26.1 ± 6.3 15.5 - 35.7	14.1 ± 2.9 10.6 - 19.1	t = 5.451	< 0.001
Urea (mg/ml) Mean ± SD Range	7.3 ± 1.4 4.9 - 9.5	3.7 ± 0.7 3 - 5.1	t = 7.152	< 0.001

Table 2: *Comparison between differentiated (hepatocyte like cells) and undifferentiated (hMNCs) regarding culture supernatant parameters.*





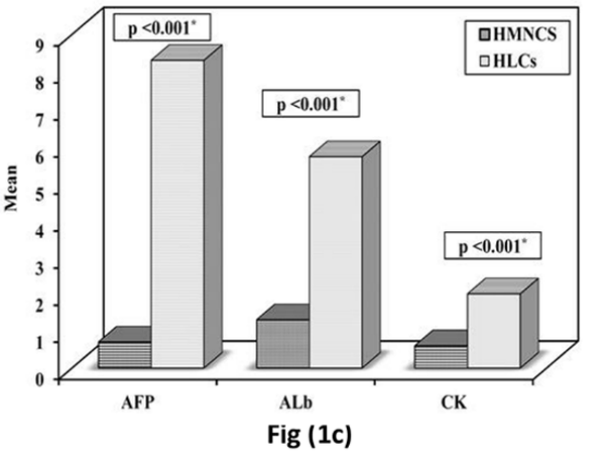


Figure 1: *Characteristics of BM- MNCs before and after differentiation Flowcytometric analysis of surface protein markers ofhMNCs (Figure 1a). The initially gated cells were analyzed for surface antigen markers CD45 , CD34 and CD14. Figure 1b: MNCs after differentiation (400x) by inverted microscope showing polygonal cells. Figure 1c: qRT-PCR of hepatic gene expression. mRNA level of AFP, ALB and CK18 was determined by relative qRT-PCR in studied groups. β-actin was used as housekeeping gene.*

Biochemical results

After 72h from injection of CCL_4, there was no significant difference as regarding the ALT and AST between undifferentiated hMNCs injected group and control group (p 0.18; 0.41) respectively. However, it was significantly lower in HLCs injected group than both control group ($p < 0.001$) and hMNCs injected group ($p < 0.001$) (Table 3 and 4).

After 1w from injection of CCL_4, there was no significant difference as regarding the ALT between hMNCs injected group and control group ($p = 0.77$). However, AST was significantly declined in hMNCs group than control group (0.04). Both ALT and AST were significantly lower in HLCs injected group than both control ($p < 0.001$) and hMNCs groups ($p < 0.001$).

After 2w, 3w and 4w from injection of CCL_4, the enzymes were significantly declined in both treated groups than the control. In addition, they were significantly lower in HLCs than hMNCs ($p < 0.001$, 0.02) for ALT and AST respectively (Table 3).

ALT (IU/L)	The studied groups			U test	P value
	Control N = 20	hMNCs N = 20	HLCs N = 20		
ALT (24h) X ± SD Range	N = 20/20 346.4 ± 75.65 222 - 492	N = 20/20 351.85 ± 86.52 212 - 493	N = 20/20 312.2 ± 57.3 218 - 425	t-test 0.21 1.71 1.61	0.83[a] 0.10[b] 0.12[c]
ALT (72h) X ± SD Range	N = 18/20 378.5 ± 75.59 285 - 497	N = 20/20 347.45 ± 65.56 200 - 451	N = 20/20 234.7 ± 38.01 168 - 301	t-test 1.36 7.28 6.65	0.18[a] <0.001[b] <0.001[c]
ALT (1w) X ± SD Range	N = 12/20 321.5 ± 61.81 245 - 401	N = 13/20 315.3 ± 47.16 220 - 380	N = 20/20 175.55 ± 31.44 124 - 220	U 0.29 4.67 5.40	0.77[a] <0.001[b] <0.001[c]
ALT (2w) X ± SD Range	N = 10/20 317.2 ± 81.33 216 - 457	N = 13/20 213.72 ± 59.79 112 - 309	N = 20/20 136.0 ± 39.57 80 - 210	U 3.29 4.32 3.82	0.001[a] <0.001[b] <0.001[c]
ALT (3w) X ± SD Range	N = 9/20 227.63 ± 42.68 190 - 294	N = 10/20 148.44 ± 39.6 82 - 198	N = 19/20 97.63 ± 35.81 50 - 159	U 3.56 3.92 3.25	<0.001[a] <0.001[b] 0.001[c]
ALT (4w) X ± SD Range	N = 9/20 215.5 ± 38.72 166 - 268	N = 10/20 103.43 ± 44.98 46 - 189	N = 19/20 46.86 ± 23.88 21 - 92	U 3.22 3.47 3.66	0.001[a] 0.001[b] <0.001[c]

Table 3a: *Comparison between the studied groups as regarding the liver enzymes (ALT).*

N = Number; X = Mean; SD = Standard Deviation; W = Week; U = Mann Whitney U test; P = Probability of error.

a = Comparison between control group and hMNCs.

b = Comparison between control group and HLCs.

c = Comparison between hMNCs and HLCs.

AST(IU/L)	The studied groups			U test	P value
	Control N = 20	hMNCs N = 20	HLCs N = 20		
AST (24h) X ± SD Range	N = 20/20 428.35 ± 58.73 301 - 498	N = 20/20 417.4 ± 71.86 253 - 510	N = 20/20 407.45 ± 57.2 306 - 560	t-test 0.53 1.14 0.48	 0.60[a] 0.26[b] 0.63[c]
AST (72h) X ± SD Range	N = 18/20 418.0 ± 56.37 313 - 516	N = 20/20 402.2 ± 60.0 280 - 487	N = 20/20 327.85 ± 36.72 270 - 392	t-test 0.83 5.9 4.73	 0.41[a] <0.001[b] <0.001[c]
AST (1 w) X ± SD Range	N = 12/20 431.83 ± 71.81 335 - 580	N = 13/20 373.05 ± 62.15 255 - 470	N = 20/20 265.55 ± 43.35 210 - 351	U 2.01 4.63 4.36	 0.04[a] <0.001[b] <0.001[c]
AST (2 w) X ± SD Range	N = 10/20 370.2 ± 73.74 294 - 518	N = 13/20 298.89 ± 67.72 188 - 397	N = 20/20 218.0 ± 42.45 168 - 300	U 2.33 4.27 3.77	 0.02[a] <0.001[b] <0.001[c]
AST (3 w) X ± SD Range	N = 9/20 323.5 ± 70.55 254 - 479	N = 10/20 212.56 ± 69.23 82 - 295	N = 19/20 160.44 ± 45.28 102 - 225	U 3.28 3.92 2.38	 0.001[a] <0.001[b] 0.02[c]
AST (4 w) X ± SD Range	N = 9/20 240.0 ± 95.21 160 - 422	N = 10/20 154.14 ± 49.74 87 - 213	N = 19/20 108.5 ± 42.34 53 - 172	U 2.06 3.14 2.30	 0.04[a] 0.002[b] 0.02[c]

Table 3b: *Comparison between the three studied groups as regards liver enzymes (AST).*

N = Number; X = Mean; SD = Standard Deviation; U = Mann Whitney U test; P = Probability of error; W = Week.

a = Comparison between control group and hMNCs.

b = Comparison between control group and HLCs.

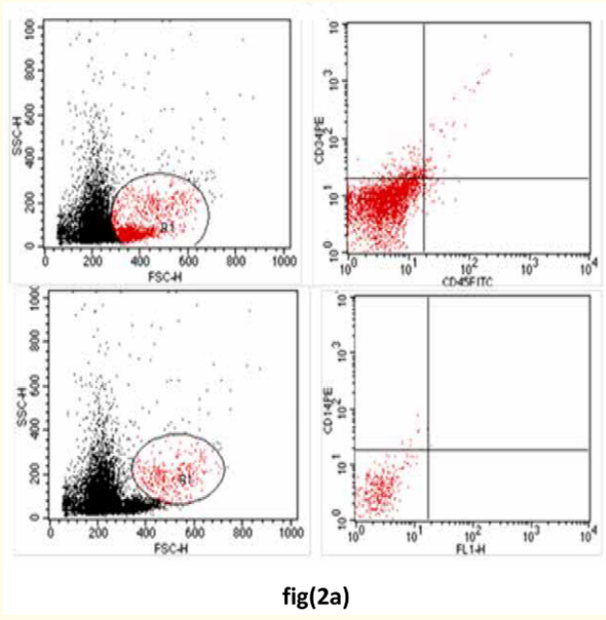
c = Comparison between hMNCs and HLCs.

Results of survival rate measurement

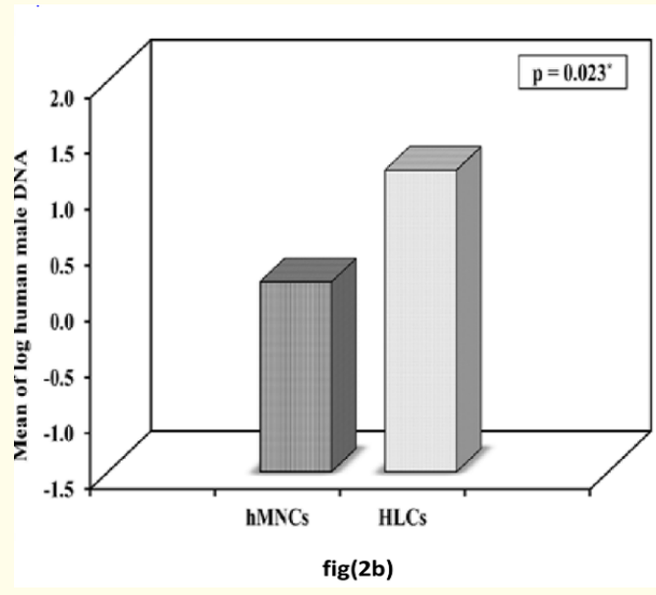
The survival of the different groups was measured as a function of time. At 72hrs of injection of CCL_4, there is no significant difference among the studied groups. Except for the 4th week, no significant difference found between the control and hMNCs group. However, HLCs group showed significantly higher survival rate all over the weeks of treatment (p = 0.006, 0.007, 0.012 and 0.008 for first week, 2ndw, 3rdw and 4thw) respectively (Figure 3c).

Analysis of human MNCs engraftment in rats

The blood from rats at 4thw was analyzed with flow cytometric analysis to determine the presence of human cells. Human CD14+, CD34+, CD45+Cells were found in peripheral blood of transplanted rats (4.67 ± 3.16, 3.21 ± 1.34, 3.41 ± 1.79) respectively (Figure 2a). In addition, liver tissue of animals injected with HLCs showed higher level of human male DNA than hMNCs by qRT-PCR (p = 0.023) (Figure 2b).



fig(2a)



fig(2b)

Figure 2: *Homing of hMNCs: Flowcytometric analysis of rat MNCs at 4 weeks (Figure 2a). Figure 2b: SRY gene by qRT-PCR. Level of log human male DNA was measured in liver tissues of female rats of all treated groups.*

Histopathological results

Hematoxylin and Eosin stained section for liver architecture

There was no significant difference between the studied groups regarding steatosis. Although some improvement in hMNCs group in liver architecture than the control group, this was non- significant. However, HLCs showed significant improvement in degeneration, necrosis and inflammatory infiltrate than hMNCs at 2w (p= 0.017, 0.013 and 0.013 respectively) but no significant difference at 4w (Figure 3b).

Proliferation rate of hepatocytes

In the present study, proliferation rate which reflect the liver regeneration was investigated and it was found that the liver tissue of HLCs injected group showed significantly higher level of cells with mitosis in 10 HPF and PCNA positive cells than both control ($p < 0.001$) and hMNCs injected group ($p < 0.001$) (Table 4 and figure 3a).

	The studied groups			Test of Sig.	p	Sig. bet. Grps
	Control	$HMNC_s$	HLCs			
Mitosis index	**(n = 9)**	**(n = 9)**	**(n = 9)**			$p_1 < 0.001^a$
Median (Min. - Max.)	2.6(2.2 - 3.5)	6.4(5.2 - 7.5)	15(11.5 - 18)	F = 157.397*	<0.001*	$p_2 < 0.001^b$
Mean ± SD	2.8 ± 0.4	6.3 ± 0.8	14.8 ± 2.4			$p_3 < 0.001^c$
PCNA (cells/ HPF)	**(n = 9)**	**(n = 9)**	**(n = 9)**			$p_1 < 0.001^a$
Median (Min. - Max.)	17(10 - 25)	38(33 - 46)	85(65 - 100)	F = 194.684*	<0.001*	$p_2 < 0.001^b$
Mean ± SD	17.8 ± 4.3	39.3 ± 4.1	85.4 ± 11.4			$p_3 < 0.001^c$

Table 4: *Comparison between the studied groups as regards mitosis index and PCNA.*

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey).

a = Comparison between control group and hMNCs.

b = Comparison between control group and HLCs.

c = Comparison between hMNCs and HLCs.

Distribution and survival of transplanted cells

In the present study, both hMNCs and HLCs were detected in the rat liver mainly around central vein by immunohistochemical stain of albumin which served as human specific marker and not react with rat hepatocytes. Moreover, transplanted HLCs injected group showed higher percentage of positive stain for anti- human albumin than hMNCs group (Figure 3c).

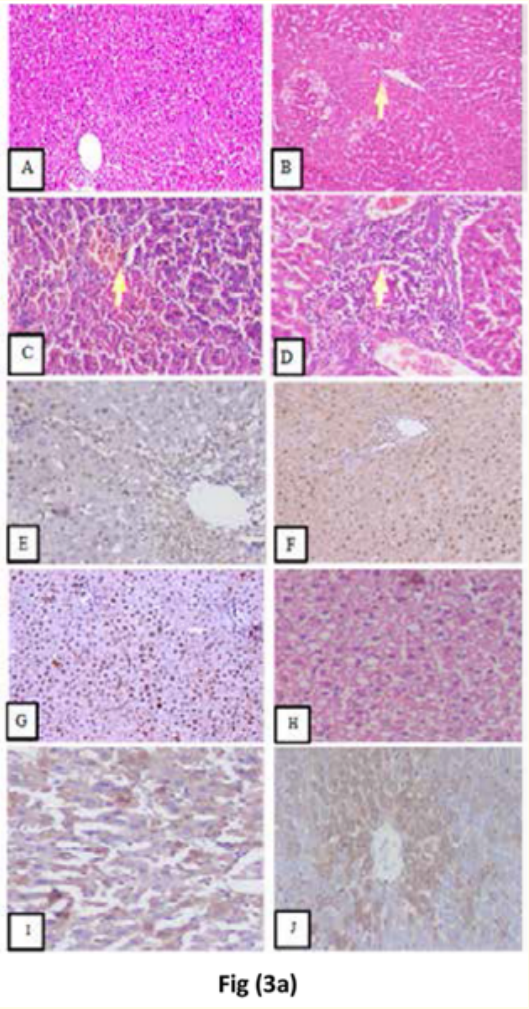


Fig (3a)

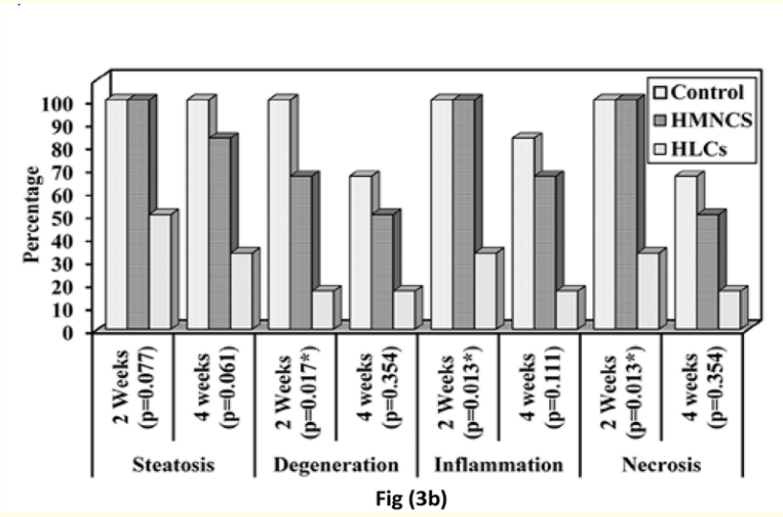


Fig (3b)

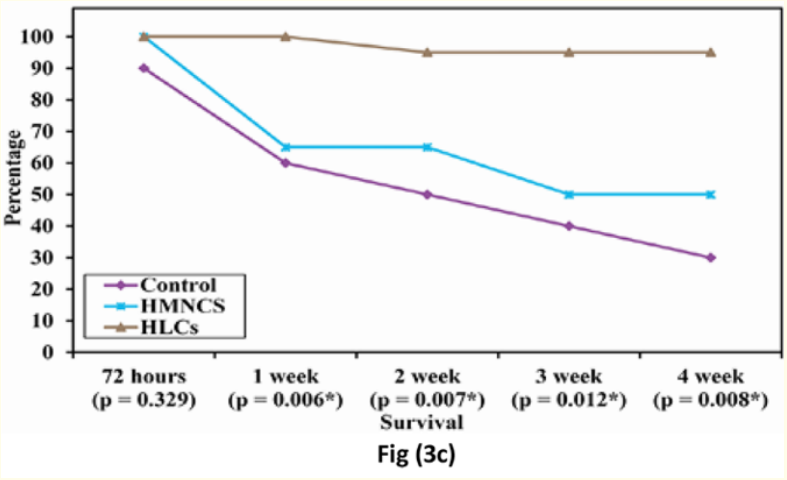


Fig (3c)

Figure 3: *Histological results and survival of the studied groups. Histopathological analysis (Figure 3a), A: normal liver tissue; B: bridging necrosis (H&E × 200);C: steatosis (H&E × 200); D: inflammatory cells in portal tract (H&E × 200); E: PCNA in control group (immunostaining × 200); F: PCNA in hMNCS treated group (immunostaining × 200); G: PCNA in HLCs treated group (immunostaining × 200); H: Expression of albumin in control group (immunostaining × 200); I: Expression of albumin in hMNCs- treated group (immunostaining × 100); J: Expression of albumin in HLCs- treated group (immunostaining × 100). Figure 3b: Comparison between the studied groups regarding liver architecture at 2w and 4w. Figure 3c: A curve showing survival rate in the studied groups at each time point.*

Effect of HLCs on IL-17 pathway

To determine if IL-17 involved in mechanisms of action in hMNCs and HLCs, the present study investigated the level of serum IL-17A and on the molecular level gene expression of IL-17 and RORC which is main inducing factor of TH17 differentiation. We observed that

the gene expressions of IL-17 and RORC in control group increased by 8.5 fold and 6.1 fold in comparison to the normal samples which significantly decreased to 4.7 and 3.1 folds in hMNCs group ($p < 0.001$ and 0.017 respectively). Moreover, the level of reduction in both IL-17 and RORC mRNA was significantly more in HLCs group (1.9 and 1.3 respectively) than hMNCs group with $p < 0.001$ and 0.009 respectively (Figure 4).

The serum level of IL-17 was significantly higher in injured control group than normal animals (24.5 ± 3 vs 8.7 ± 2, $p < 0.001$) which reduced in both groups treated with hMNCs and HLCs than control group (14.8 ± 2.7; 6.6 ± 2.3 and 24.5 ± 3respectively) with $p < 0.001$. In addition, HLCs showed significant lower value than hMNCs group ($p < 0.001$) (Figure 4).

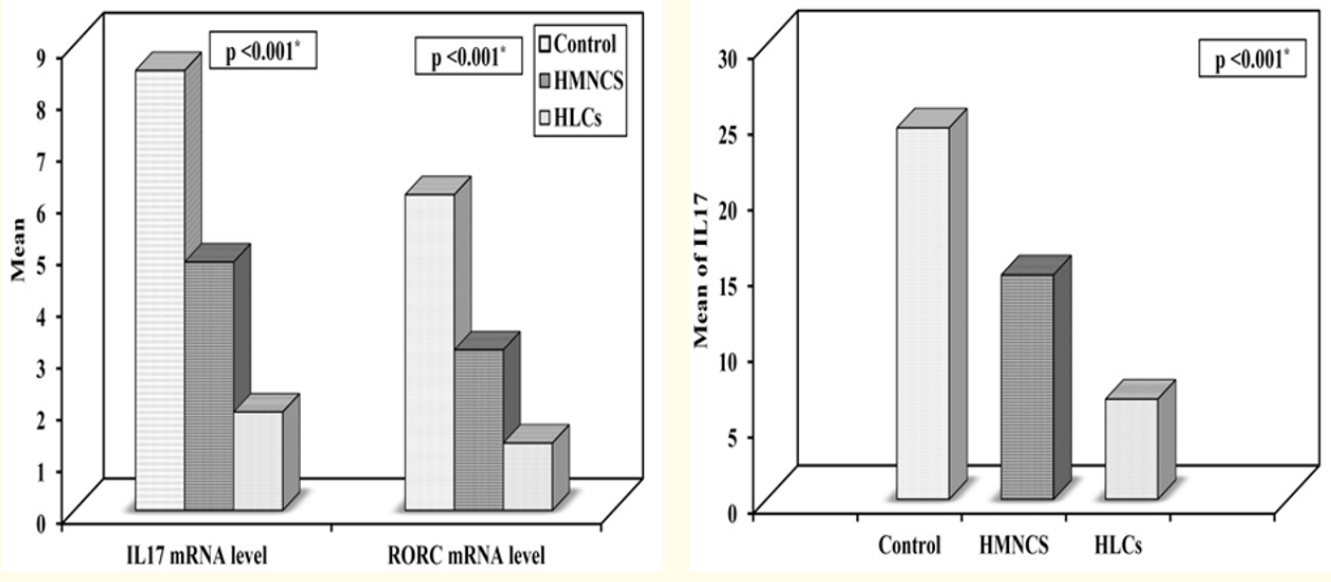


Figure 4: *Serum IL-17 in rats and qRT-PCR of IL-17 and RORC gene expression. mRNA level of was determined by relative qRT-PCR in studied groups. β-actin was used as housekeeping gene.*

Discussion

The present study used 2 types of cells in the therapy of ALF, undifferentiated hMNCs and differentiated hMNCs (HLCs). We used this approach to assess if differentiated cells more effective than undifferentiated in improvement of liver's detoxifying and synthetic activities resulting from the loss of functioning hepatocytes which is a rapid process in acute liver injury.

hMNCs induced into hepatocyte like cells showed change in morphology into polygonal cells and ovoid- like cells, secretion of ALB and AFP were detected in culture supernatant which are a specific markers of hepatocytes and this is agree with previous studies [14-16]. Gene expression of hepatic genes AFP, ALB and CK18 were determined to confirm the success of induction on molecular and protein level. Transferrin and detoxification of ammonia into urea were used to assess the metabolic function of the liver. All results indicated that hMNCs were successfully induced into functional hepatocytes.

To assess the homing of transplanted cells in blood rat, we used flowcytometric analysis of the markers after 4w of injection of the cells and proved that the peripheral blood of the rats contain the same human markers. Also, we detected the chimerism by SRY gene in the liver tissues by qRT-PCR in the both treated groups however, HLCs exhibit more level of human male DNA than hMNCs and this indicate that the differentiated cells have better homing and engraftment in the specific tissue microenvironment than the undifferentiated ones.

To assess the efficacy of bone marrow cells to improve the ALF, multiple parameters were investigated in this experiment. Transaminases were measured after 24h, 72h, 1w, 2w, 3w and 4w and there was significant decline in the mean values of serum ALT and AST in both treated groups when compared with the corresponding mean values in the non -treated group, this decline began at 72 h for ALT and

AST in HLCs treated group and 1w for AST, 2w for ALT in undifferentiated hMNCs treated group. Also, at all -time points, there was significant decline in the mean values of serum ALT and AST in HLCs group compared with hMNCs injected group. This better effect of differentiated cells than undifferentiated cells may indicate that the ability of regeneration of differentiated cells and its ability to act as functioning hepatocytes is more effective than undifferentiated cells [8,17] reported that transplanted BMMCs differentiated *in-vivo* into functional hepatocytes that can compensate for ALF in rats. The serum ALT and AST levels also markedly declined in response to transplantation of BMMCs, confirming that the liver deterioration can be ameliorated by transplanted BMMCs. The results indicated that BMMCs can locate to acutely injured livers and transform into hepatic-like cells and act like normal hepatic cells in rats.

The present study showed estimated the survival rate at each time point. Although it was higher in hMNCs treated group than control group, it was non- significant. However, it was significantly higher in HLCs group than hMNCs treated group. Other studies showed increase in survival rate in cell therapy for acute liver disease using toxic models [7,8] and in 90% partial hepatectomy model [11,18].

In the present study, H and E stained sections from acute liver failure showed no statistically significant improvement in histopathological changes between hMNCs treated group and control group. However, HLCs showed significant improvement at 2w for degeneration, necrosis and inflammatory infiltrate but not for steatosis. At 4w, there was no significant difference between the studied groups and this delayed improvement in the liver architecture in hMNCs and control groups may be reflected the lower survival of animals in these groups. Our results agree with [19] reported that livers of fulminant hepatic failure in mice showed serious hepatic congestion, hepatocyte degeneration, disordered hepatocyte cords, as well as multiple and extensive areas of cellular necrosis and inflammatory cell infiltration. This liver damage was ameliorated after stem cell treatment [9] reported that necrosis was reduced to almost null levels, but mild levels of steatosis and hydropic degeneration were still present.

In this study, the increase in mitotic index observed in both hMNCs and HLCs groups than the control group indicated that the transplanted cells engraft the liver and secrete mitotic cytokines in response to a specific damage. These results agreed with [8] who reported that liver can regenerate its self by increasing the rate of hepatocyte mitosis and differentiation of stem cells into hepatocytes or cholangiocytes. Stem cells are the main cell lineage for liver regeneration. The rate of mitosis is confirmed with PCNA by immunohistochemical staining which increased significantly after both differentiated and MNCs therapy. These results suggest that hepatocyte proliferation is induced by BMMCs therapy. Also, the expression of PCNA was significantly higher in HLCs group than hMNCs [7,8,17] who reported increasing expression of PCNA suggests that the cells may have subsequently undergone cell division. It is well known that PCNA, which exists and is synthesized in the cell nucleus, is a nuclear antigen related with the cell life cycle. Against to our result, Kieling., *et al.* [11] observed that BM-MNCs not improve the liver regeneration after partial hepatectomy induced ALF. So, from previous observations HLCs have better role in liver regeneration than hMNCs and this explain one of the mechanisms of significant improvement of survival rate in HLCs than hMNCs.

In the present investigation, immunohistochemical stained sections from acute liver failure-stem cell-treated groups showed positive brownish staining for the Anti-human albumin in the liver cells. This confirms that the livers of stem cell-treated rats contain human cells which homed to liver and differentiated into functional hepatocytes and this may also indicate another mechanism for improvement of survival in bone marrow treated group. These results agreed with [16,17] who reported that transplanted BMMCs to mice were found to populate the damaged liver and differentiated into albumin-producing hepatocyte-like cells.

The mechanisms underlying the therapeutic effects of stem cells transplantation in liver diseases could involve trans-differentiation, cell fusion, or paracrine bystander actions [5]. Previous studies prove the immunomodulatory properties of BM- MNCs [20,21]. Previous two studies reported modulation of IL6 and IL10 by BM-MNCs [11] and adipose derived mesenchymal stem cells [22] in improvement of ALF animal model. In this issue we investigated if IL-17 involved in the mechanisms of management of ALF by HLCs. IL17 was reported as a key factor in hepatic injury caused by neutrophil-induced inflammatory responses [23]. Increased expression of IL-17 in liver tissue

and serum IL-17 was correlated with TH17, NKT and progression of hepatic damage [24]. IL-17 was shown to be involved in progression of alcoholic hepatitis [25]. A recent study by Fischer., *et al.* [26] reported the role of IL-17 in acute on chronic liver failure.

RORγt is transcription factor of the RAR-related orphan nuclear receptor (ROR) family and it is known to be the main inducer of murine TH17 [27] and NKT17 which are major producer of IL-17 and major effector cells in the pathogenesis of acute liver failure [28], this is consistent with the present study in which IL-17 level was significantly higher in injured control group than its level before CCL_4 injection. Additionally, relative gene expression of RORC and IL-17 genes were highly elevated in control group after hepatic injury by CCL_4 in comparison to normal hepatic tissue (6.1 and 8.5 fold), their levels were significantly lower in hMNCs (3.1 and 3.7 folds) and in HLCs (1.3 and 1.9 fold). Also, the serum IL-17 was significantly lower in both treated groups than the control group. Additionally, HLCs showed much significant lower level than the hMNCs group. This indicated that HLCs mediated improvement of ALF by downregulation of IL-17 production through suppression of IL-17 gene expression and its transcription factor RORC.

The study is limited by the number of animals and inhibition of IL-17 pathway should be trialed to increase the efficacy of differentiated cells and this will be done in the next study.

Conclusion

Although many studies prove the role of BM- MNCs in attenuation of ALF, but no is known about BM- MNCs induced into functional hepatocytes. In this study, BM- MNCs derived hepatocytes have been proved to improve ALF through better engraftment of the liver tissue, hepatocyte proliferation and modulation of IL-17 pathway. So, it can be used as an alternative therapeutic approach to ameliorate ALF and we recommend to apply this procedure in addition to blocking IL17 pathway to increase its efficacy in treatment of ALF.

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