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Research Article

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Case-control observational analytic study of Autologous Bone Marrow Derived Hepatocyte-like cell Transplantation in Child B Liver Cirrhosis Patients

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Abstract

Background/aims: liver transplantation is the sole definitive line of treatment for refractory liver diseases, which hampered by many obstacles. Cellular transplantation for chronic and metabolic liver disease have emerged as a convenient alternative to whole-organ transplantation We aimed at evaluating the efficacy of autologous bone marrow derived hepatocyte which injected intra-splenic in patients with liver cirrhosis grade B Child-Turcotte-Pugh score (CTP B). **Participants and methods**: 27 patients with liver cirrhosis CTP B score were divided into two groups according to the principle of treatment. Group (A) consisted of 13 patients, who received conventional treatment and hepatocyte derived from patients own (HSCs). Group (B) received regular conventional treatment. Both groups of patients were followed up for six months for assessment of liver functions. **Results**: There was a significant improvement in the degrees of ascites, lower limb edema, hepatic encephalopathy, CTP and Model of the end-stage liver disease scores in patients treated with hepatocytes derived from HSC. Also, we observed slight improvement in serum albumin, prothrombin concentration and international normalized ratio in this group. **Conclusion**: We demonstrated safety and short term efficacy of autologous bone marrow derived hepatocyte transplantation for the support of cirrhotic liver.

Keywords: HSCs; transplantation; liver cirrhosis.

Introduction

Decompensated chronic liver disease is a worldwide health problem affecting millions of patients. Liver transplantation, the sole definitive treatment at present, is hampered by the marked shortage of donors **(Dianat et al., 2013)**, invasive surgical procedure and risk of immune rejection **(Takamia et al., 2012).** Novel approaches for tissue repair are under research to overcome these problems.

Cellular transplantation for chronic liver disease and metabolic liver defects have emerged as a convenient alternative; either temporary or definitive, to whole-organ transplantation. Candidate sources used for cellular transplantation include fetal hepatocytes, bone marrow derived hemtopoeitic stem cells (BM –HSC), as well as induced pleuripotent stem cells (Yu et al., 2014). Hepatocyte-like cells (HLC) generated in-vitro from BM-HSCs (Pilat et al., 2013), by forced expression of specific transcription factors as OCT 4 (O), SOX2 (S), KLF4 (K) and c-MYC (M) (So called OSKM cocktail) or O, S, NANOG (N) and LIN28 (L) (So Called OSNL) (Dianat et al., 2013).

Previous publication reported restoration of liver mass and function, reduction of fibrosis and correction of inherited diseases following transplantation of BMSCs **(Pai et al., 2008).** Also these patients determined marked improvement in their laboratory data and quality of life with no complications related to the procedure **(Salama et al., 2012).**

There is controversy about the underlying mechanisms by which HSCs exert their beneficial effect in liver repair. Initially, several studies suggested that, due to plasticity of adult stem cells and their differentiation to hepatocytes **(Jang et al., 2004).** Other suggested that conversion to hepatocyte occur by fusion of adult stem cells with local hepatocytes **(Vassilopoulos et al., 2003)** or a paracrine proliferative effect on native hepatocytes **(Alison et al., 2009).** Another suggested its ability to restore fibrosis **(Zhao et al., 2005).**

Also, the results of using stem cells in the treatment of liver cirrhosis and hepatocellular carcinoma are conflicting **(li et al., 2013).**

Aim of the work:

The present study was designed to evaluate the therapeutic efficacy of hepatocyte-like cells generated from autologous bone marrow derived haematopoietic stem cells in patients with liver cirrhosis grade B Child-Turcotte-Pugh score (CTP B)

Subjects and Methods

A. Study design:

This study included 27 participants (6 females and 21 males) with liver cirrhosis grade B (CTP) score. Their ages ranged from 45-60 years. Participants were selected from the out patient's clinic and the internal medicine causality department, Cairo University hospital, from May 2013-May 2014.

All participants signed a consent form that was approved by our institutional ethics committee.

B. Subjects:

Exclusion Criteria: Individuals with HCC, concomitant renal or heart failure (HF) were excluded from the study as well as patients with severe bleeding tendency.

The diagnosis of liver cirrhosis was based on medical history, physical examination, laboratory finding and ultrasonogaphic examination. The degree of liver decompensation was evaluated using CTP and Model of the end-stage liver disease (MELD) scores. Patients with grade B CTP were included in the study. Grading of ascites was defined according to International Ascites club **(Moore et al., 2003)** and assessment of conscious level with detection of hepatic encephalopathy clinically. These participants didn't received antiviral treatment for HCV infection. Blood samples were withdrawn for routine laboratory investigations.

Patients were randomly divided into 2 groups:

Group (A) 13 participants (23.08% females and 76.92% males, their mean age was 51.08±4.46 years). This group of participants received regular conventional treatment of lactulose, Ursodeoxcholic acid , sylimarin & spironolactone plus transfusion of hepatocyte derived from patient's own HSCs. We injected an average of $5x10^7$ hepatic lineage-committed cells in a total of about $2x10^8$ mononuclear cells; the viability was at least 90%.

Group (B) 14 participants (21.43% females and 78.57% males, their mean age was 51.43±4.67 years), received regular conventional treatment only.

Both groups of patients were on salt restriction diet and liver supportive drugs. Participants presented with edema or developed it throughout followed-up period received spironolactone (100-200mg/day) with regular follow-up of body weight and urine output. Lactulose, metronidazole and frequent enemas were given for patients with hepatic encephalopathy. We divided our patients into two groups according to type of therapy:

C. Intervention:

1. Sample Collection:

Under aseptic conditions, 80-100 ml of bone marrow were aspirated from the posterior superior iliac spine, under local anesthesia, the marrow was collected on preservative-free heparin.

2. Cell Separation:

Under aseptic conditions, bone marrow was layered over Ficol-Hypaque (density 1017) and centrifuged for 20 minutes at 1800rpm. The mononuclear cell fraction (MNCs) was collected and counted.

3. Flowcytometric Evaluation of the Cell Population:

The MNC population was evaluated for cell populations using EPIC Elite Flowcytometer and monoclonal antibodies against CD 133, 34, 90 (R&D).

4. Short -term liquid culture:

MNCs were cultured in complete medium (DMEM, 100ul/ml penicillin/streptomycin, 100ul amphotericin) with the addition of 20ng/ml hepatocyte growth factor (R &D) for 5-7 days.

5. Cell Harvest:

The liquid culture system was left for seven days; the medium was refreshed every other day. At day seven, evaluation of hepatic lineage commitment was done by:

A- Evaluation of the cell size by cytospin preparation stained with hematoxyline/Eosin (Hx/Eo) and by cell size analysis using Beckman Coulter (Fig.1).
B- B- Immunohistochemical stain use α fetoprotein (AFP) antibody to demonstrate AFP production in these cells (Fig.2).
C- Functional evaluation by measurement of albumin concentration in the culture supernatant using nephlometry (Fig.3).

6. Cellular Transplantaion:

We injected an average of $5x10^7$ hepatic lineage-committed cells in a total of about $2x10^8$ mononuclear cells; the viability was at least 90%.

7. Follow-up:

All participants were followed-up for six months; each participant came for followed-up every month. In each visit, they examined clinically for the presence of lower limb edema and grading of it, assessment of conscious level and recording of hepatic encephalopathy and evaluation of ascites by ultrasonograpgy. Laboratory investigations were done in each visit included liver function test, complete blood count, coagulation profile, blood urea, creatinine and AFP.

The statistical methodology

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Student *t* test for independent samples. Non parametric test (Mann Whitney U) was used for analysis of two quantitative data as data was not symmetrically distributed. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. *p* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 13 for Microsoft Windows.

Results

Demographic data of the studied participants are shown in Table 1.

Table 1: Demographic data of the study groups.

Group	Serum Albumin (g/dl)	Total protein (g/dl)	INR	Total Bilirubin (mg/dl)	AST (IU/l)	ALT (IU/l)	AFP (µg/l)
Group A Mean ± SD	2.56±1.48	6.32±0.34	1.7±0.4	2.2±1.7	74.2±24.0	67.2±23.8	20.5±17.1
Group B Mean± SD	3.06±0.19	6.45±0.45	1.3±0.1	1.2±0.6	61.7±7.9	65.4±13.0	20.014±12.5
P value	0.250	0.403	0.004*	0.064	0.095	0.812	0.934

**Significant difference.*
INR international normalized ratio, AST aspartate aminotransferase, ALT alanine aminotransferase and AFP α fetoprotein.

All participants had HCV related liver cirrhosis. (Group A) patients showed significant improvement in the degrees of ascites and lower limb edema starting from the 4th month from initiation of therapy; as well as significant improvement in the grade of hepatic encephalopathy starting from 5th month post therapy and onwards (Table 2, 3 & 4).

Table 2: Comparison between study groups in grade of ascites

	Baseline	1m	2m	3m	4m	5m	6m
Group A							
No	23.1%	76.9%	76.9%	69.2%	69.2%	84.6%	84.6%
Mild	76.9%	23.1%	7.7%	15.4%	15.4%	0.0%	0.0%
Moderate	0.0%	0.0%	15.4%	7.7%	15.4%	15.4%	7.7%
Large	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Group B							
No	35.7%	57.1%	50.0%	50.0%	0.0%	0.0%	0.0%
Mild	64.3%	42.9%	50.0%	50.0%	78.6%	57.1%	57.1%
Moderate	0.0%	0.0%	0.0%	0.0%	21.4%	42.9%	42.9%
Large	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
P value	0.67	0.42	0.023	0.121	0.00*	0.00*	0.00*

** Significant difference.*

Table 3: Comparison between study groups in grade of lower limb edema

	Baseline	1m	2m	3m	4m	5m	6m
Group A							
No	0.0%	15.4%	76.9%	76.9%	53.8%	53.8%	53.8%
Mild	92.3%	84.6%	23.1%	15.4%	38.5%	38.5%	30.8%
Moderate	7.7%	0.0%	0.0%	7.7%	7.7%	0.0%	15.4%
Severe	0.0%	0.0%	0.0%	`0.0%	0.0%	7.7%	0.0%
Group B							
No	0.0%	57.1%	57.1%	57.1%	0.0%	0.0%	0.0%
Mild	100.0%	42.9%	42.9%	42.9%	92.9%	85.7%	0.0%
Moderate	0.0%	0.0%	0.0%	0.0%	7.1%	14.3%	100.0%
Severe	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
P value	0.481	0.209	0.420	0.209	0.000*	0.000*	0.000*

** Significant difference.*

Table 4: Comparison between study groups in grade of hepatic encephalopathy

	Baseline	1m	2m	3m	4m	5m	6m
Group A							
No	92.3%	100.0%	84.6%	76.9%	69.2%	61.5%	69.2%
Mild	7.7%	0.0%	15.4%	23.1%	30.8%	38.5%	30.8%
Group B							
No	100.0%	100.0%	100.0%	100.0%	92.9%	35.7%	64.3%
Mild	0.0%	0.0%	0.0%	0.0%	7.1%	64.3%	35.7%
P value	0.481	--------	0.222	0.098	0.297	0.046*	0.015*

** Significant difference.*

Total serum bilirubin levels showed improvement in (Group A) from a mean of 2.2 mg/dl to a mean of 1.5 mg/dl after 6 months, while the control group (Group B) maintained the same serum total bilirubin levels (Table 5).

Table 5: Different parameters of liver functions in study groups over the follow-up period (mean)

	Albumin		PT		PC		INR		Total Bilirubin		AST		ALT	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
Baseline	2.56± 0.45	3.06± 0.19	15.3± 2.43	12.5± 0.53	57.9%± 11.77	78.7% ±5.29	1.7± 0.37	1.3± 0.08	2.2± 1.69	1.2± 0.63	74.2± 24.00	61.7± 7.94	67.2± 23.83	65.4± 12.97
P value	0.002		0.001		0.000		0.003		0.064		0.095		0.812	
1m	2.96± 0.46	3.00± 0.15	14.37± 2.35	12.7± 0.44	64.0%± 17.73	74.7%± 5.86	1.6± 0.44	1.3± 0.1	1.8± 1.17	1.1± 0.33	57.2± 17.41	52.0± 0.00	55.5± 25.42	71.9± 11.76
P value	0.769		0.027		0.057		0.031		0.057		0.303		0.050	
2m	2.97± 0.32	3.00± 0.05	13.82± 2.15	12.9± 0.27	67.8%± 14.88	73.0%± 3.01	1.5± 0.37	1.4± 0.07	2.0± 1.14	1.5± 0.08	38.4± 12.38	39.6± 5.88	46.5± 19.19	51.1± 0.54
P value	0.744		0.151		0.240		0.353		0.141		0.755		0.404	
3m	3.07± 0.59	2.79± 0.24	16.1± 5.64	13.3± 0.63	61.7%± 21.29	69.5%± 4.65	2.1± 1.51	1.4± 0.05	2.7± 1.79	2.3± 0.00	43.7± 21.46	36.0± 0.00	42.7± 32.98	34.0± 0.00
P value	0.132		0.101		0.219		0.121		0.436		0.220		0.360	
4m	3.23± 0.71	2.83± 0.15	14.5± 3.54	13.3± 0.70	68.2%± 22.49	67.9%± 6.47	1.6± 0.56	1.4± 0.12	1.7± 0.26	1.8± 0.13	53.2± 12.60	51.9± 0.54	29.7± 15.98	23.0± 0.00
P value	0.070		0.253		0.964		0.229		0.234		0.717		0.157	
5m	3.23± 0.57	2.84± 0.29	14.1± 3.50	14.6± 1.54	70.2%± 17.81	60.3%± 10.3	1.6± 0.68	1.6± 0.33	1.52± 0.73	1.2± 0.16	51.5± 18.81	56.0± 0.00	28.1± 9.47	26.0± 0.00
P value	0.041		0.642		0.097		1.000		0.146		0.405		0.440	
6m	3.37± 0.21	2.75± 0.29	12.7±0.00	15.00± 2.23	74.00%± 0.00	58.44%± 12.21	1.30± 0.00	1.73± 0.56	1.05 ± 0.34	1.42 ± 0.76	71.11 ± 5.67	74.67 ± 5.77	73.67 ± 4.00	74.92 ± 0.29
P value	0.000*		0.002*		0.000*		0.014*		0.115		0.119		0.283	

** Significant difference.*

PT prothrombin time, PC prothrombin concentration, INR international normalized ratio, AST aspartate aminotransferase, ALT alanine aminotransferase and m month.

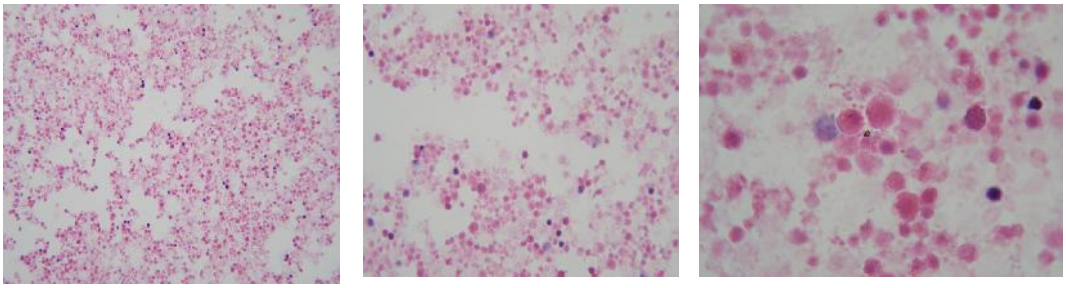

Fig. 1: cytospin preparation of the cultured cells stained by Hx/Eo.

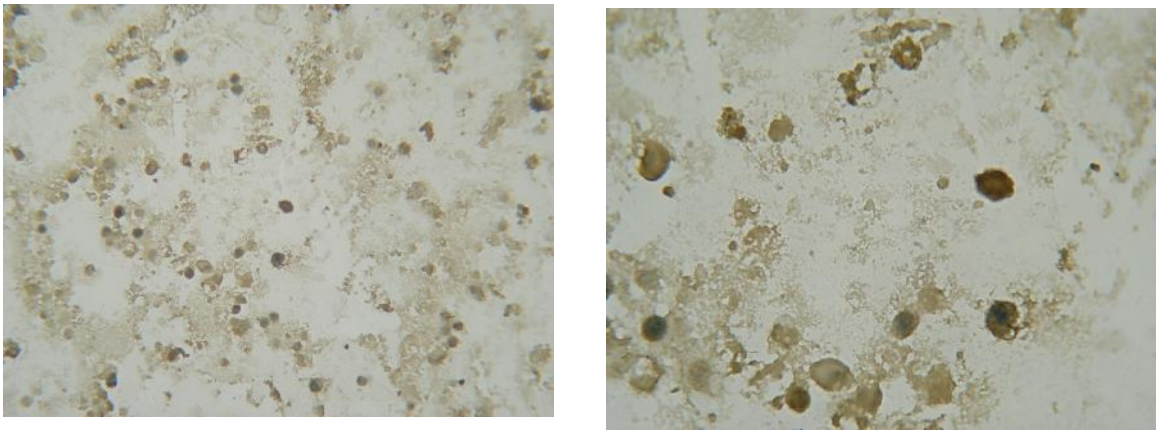

Fig. 2: immunohistochemical staining of cytospin preparation using AFP antibodies

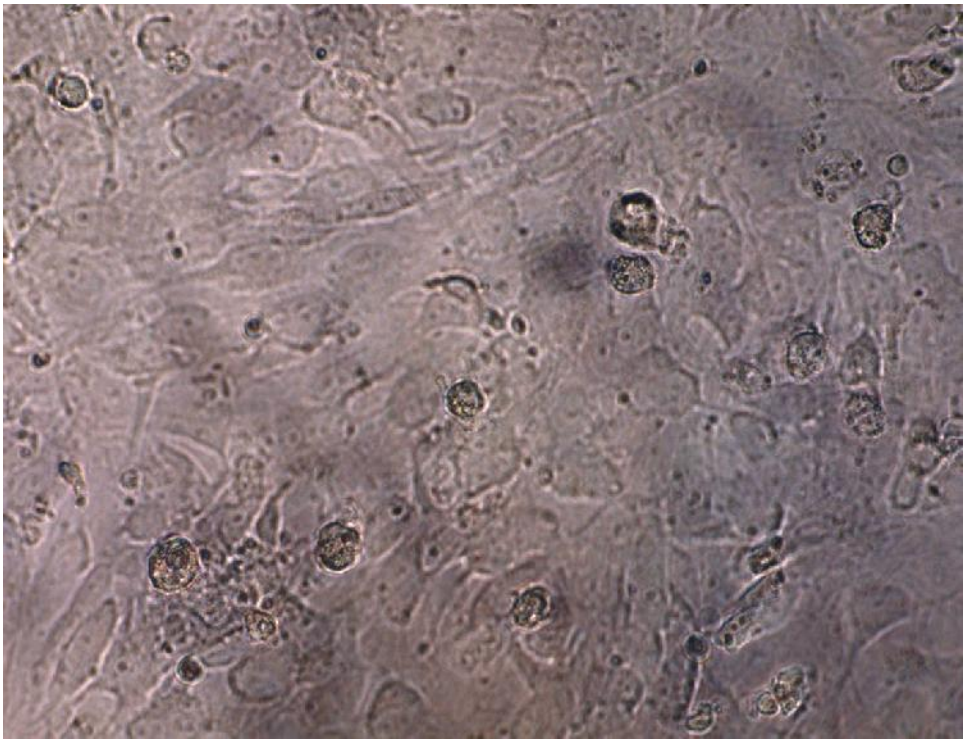

Fig. 3: inverted microscope image x400 Day 8 of differentiation in group D, about 90% of cells displayed hepatocyte like morphology.

However CTP and MELD scores were initially high in (Group A) patients, but it showed significant improvement throughout following up period with maximum improvement in MELD that occurred at 2 months (Fig.4 and Fig. 5).

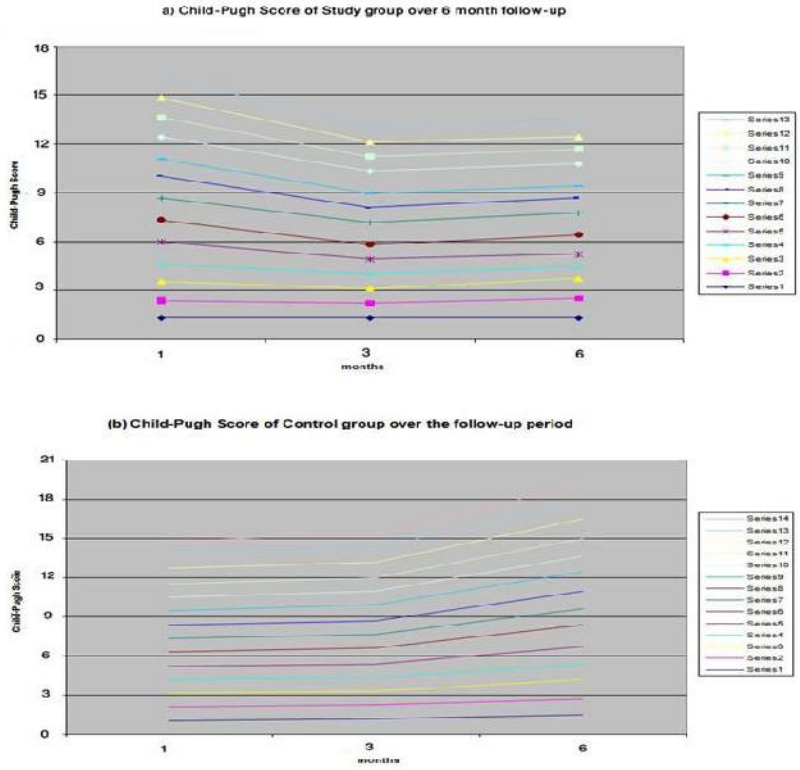

Fig.4: a. Child-Turcotte-Pugh score (CTP) scores of stem cell treated group, b. Child-Turcotte-Pugh score (CTP) scores of control group over 6 month follow-up

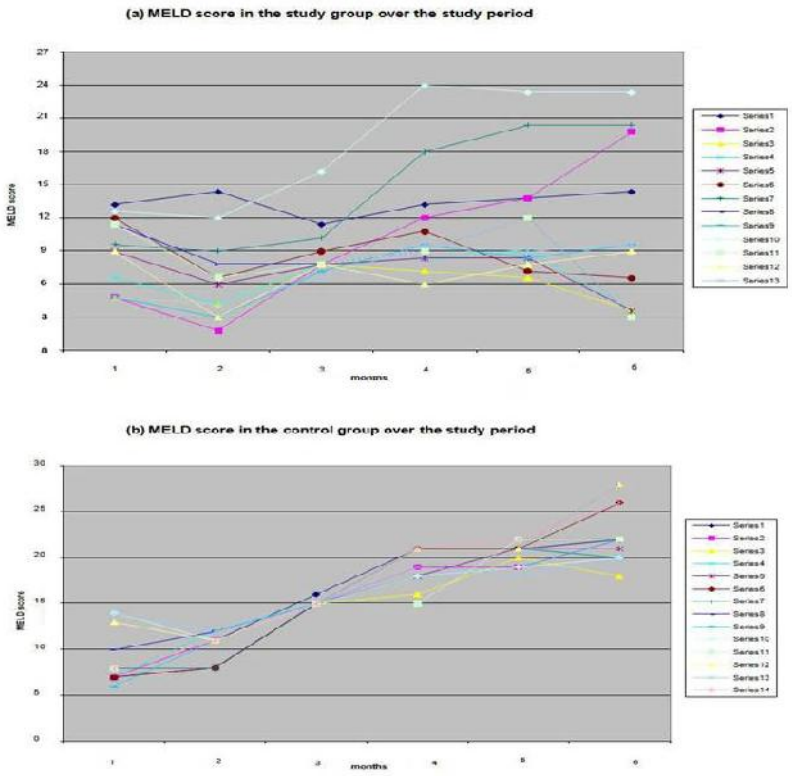

Fig.5:a. Model of the end-stage liver disease (MELD) scores of stem cell treated group, b. Model of the end-stage liver disease (MELD) scores of control group over the study period

No significant difference in the incidence of hematemesis or melena was observed between both groups.

Although patients in (Group A) hadn't received an albumin infusion or plasma, we observed a slight improvement in serum albumin level, prothrombin concentration and international normalized ratio (Fig. 6).

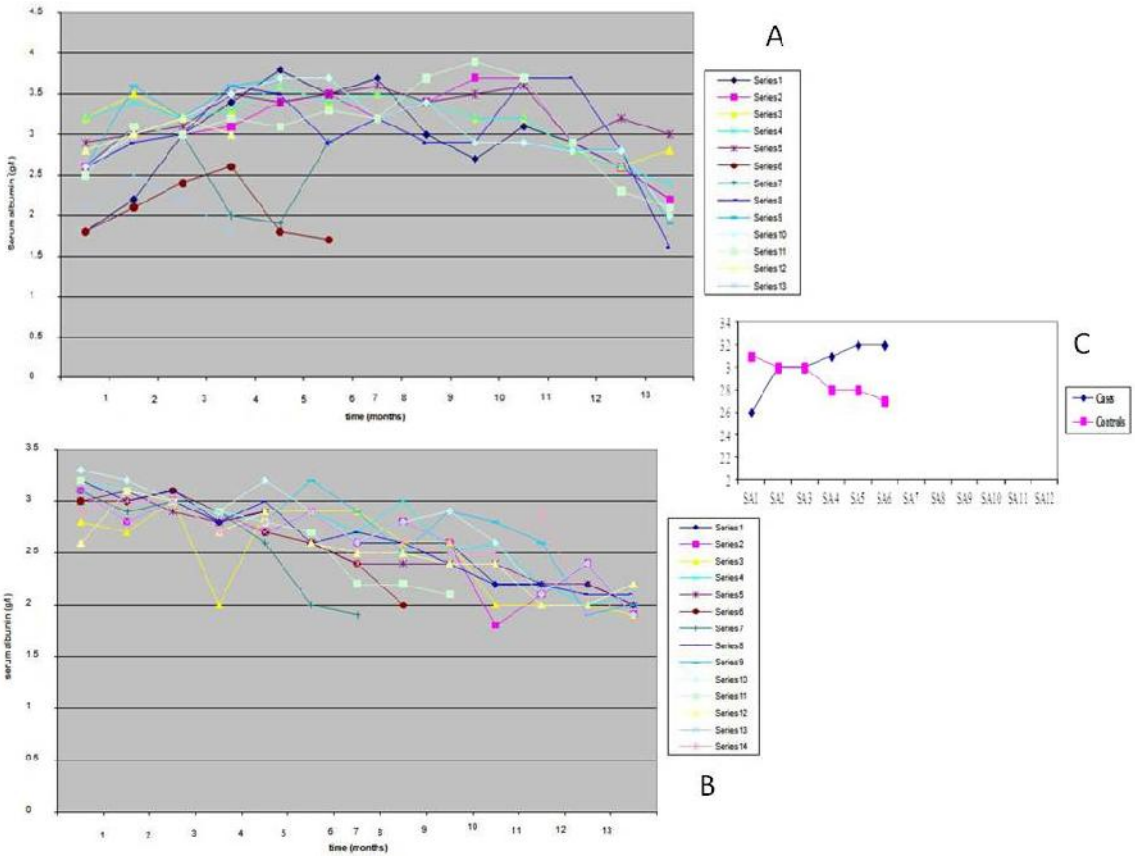

Fig. 6: A. Serum albumin in the stem cell treated group, B. Serum albumin in the control group, C. Mean serum albumin of both groups over the study period

Discussion

The liver is the lone internal human organ having the capacity of natural regeneration after damage **(Starzl, et al, 1993).** Liver regeneration is a three-tier process, with mild injury repaired by raising the rate of hepatocyte mitosis and by differentiation of stem cells into hepatocytes and cholangiocytes [8]; moderate injury repaired by endogenous hepatic stem cells , and severe injury necessitating exogenous stem cell contribution (Cantz et al.,2008).

Stem cells are the cells of origin of all human tissues with a capacity for self renewal which allows them to regenerate damaged tissues in the body**.** Stem cell therapy is rapidly progressing as a potential mode of regenerating damaged and atrophic tissues and organs **(Kakinuma et al., 2009).**

Several studies have been reported that both rodent and human embryonic stem cells (ESCs), bone marrow HSCs, mesenchymal stem cells (MSCs), umbilical cord stem cells, fetal and adult liver progenitor cells and mature hepatocytes are capable of self-renewal, producing daughter hepatocytes both in vivo and in vitro **(Bae, 2008).** These results were in agreement with our study, in which we documented transdifferentiation of HSCs into hepatocyte. This produced cells proved to be hepatocytes by different methods, namely cell size, detection of α fetoprotein and functional production of albumin.

Yu et al., (2014) demonstrated that transplantation of hepatocyte-like cells (HLCs) derived from hiPSCs may be considered as alternatives to liver transplantation for the treatment of the following conditions (acute liver cell failure, liver cirrhosis, viral hepatitis and inherited metabolic liver diseases) **(Yu et al., 2014)**.

On the contrary studies done by **Wang, et al., (2003) and Vassilopoulos et al., (2003)** reported that the production of donor derived hepatocytes is due to the fusion of host hepatocytes with BM derived cells and is not due to transdifferentiation (**Wang et al., 2003 and Vassilopoulos et al., 2003).**

Terai et al., (2006) used an intravenous infusion of undifferentiated bone marrow mononuclear cells in liver cirrhosis patients. They demonstrated a significant improvement in liver function after transfusion (**Terai et al., 2006). Furst et al., (2007)** used bone marrow derived selected CD133+ve for liver regeneration after surgical resection **(Furst et al., 2007).** Both approaches depend on homing property of circulating stem cells to injured areas in addition to the microenvironment-dependent transdifferentiation of homed stem cells into hepatocyte. In the present work, we hypothesized that the cirrhotic microenvironment of the liver is not optimal for supporting homing and differentiation. For this reason, we examined the efficiency of ex-vivo differentiation of stem cells before infusion.

Follow up of our patients throughout a 6 months period post transplantation showed improvement of liver functions, especially serum albumin, total protein, CPS and MELD scores with significant improvement of ascites, hepatic encephalopathy and bleeding tendency.

Our results were in agreement with the study done by **Terai et al., (2006)** in which they infused $5.2\pm0.63x10^9$ autologous bone marrow cell from the peripheral vein in patients with decompensated liver cirrhosis. At 24 weeks following transplantation, they observed a significant improvement in mean serum albumin levels, total protein levels and CTP scores, in addition to improvement in ascites (**Terai et al., 2006).**

Peng et al., (2011) reported that transplantation of autologous bone-marrow derived mesenchymal cells (BMMC) in 53 patients with liver cell failure due to hepatitis B showed favorable short-term efficacy with improved levels total bilirubin, prothrombin time and MELD score of patients 2-3 weeks following transplantation **(Peng et al., 2011)**.

Case report by **Gasbarrini et al., (2007)** described the use of autologous unsorted bone marrow derived stem cells (BMSCs), which were injected into the portal vein as salvage treatment for hepatic failure in a 67-year-old man ineligible for liver transplantation. There was an apparent rapid improvement in synthetic function of the liver and liver biopsy performed 20 days after injection of cells showed increased hepatocyte replication around necrotic foci **(Gasbarrini et al., 2007).**

Salama et al., (2010) documented that near normalization of liver enzymes in 54% of 90 patients who presented with end stage liver disease and received autologous CD34+ and CD133+ stem cell infusion in the portal vein which was preceded by the administration of GSF for 5 days **(Salama et al., 2010)**. These results were to some extent in agreement with our results, as we found a reduction in the serum level of alanine aminotransferase (ALT) which reached normal values at 6 months following transplantations with significant difference at 2 months while, aspartate aminotransferase (AST) levels reached near normal values but this was statistically not significant.

In the present study, we infused $50x\ 10^6$ cells showing hepatocyte lineage commitment. The discrepancy in the number of cells infused is due to the low percentage of bone marrow mononuclear cells which are able to undergo hepatocyte differentiation.

Lyra et al., implied the safety of autologous bone marrow-derived cells for patients with chronic liver disease injected through the hepatic artery (**Lyra et al.,2007 &2010). Levicar et al., (2008)** also confirmed the safety and beneficial effect lasting around 12 months of the procedure of using autologous infusion of mobilized adult bone marrow derived CD34+ cell without granulocyte colony stimulating factor (GCSF) in patients **(Levicar et al., 2008).**

Comparison between hepatic and splenic routes of stem cell injection, showed no significant difference, except in the first month following injection **(Esrefoglu, 2013).** However, splenic route was preferable, because its technique was easy; moreover, it is associated with increased incidence of mild complications as fever and transient shivering **(Esrefoglu, 2013).** In the present study, we observed that ten out of thirteen patients following transplantation experienced fever within the first twenty four hours, which subsided by regular antipyretics. Three patients showed transient shivering. No other side effects were observed.

Conclusions

We demonstrated the safety and short term efficacy of autologous bone marrow derived hepatocyte transplantation for the supporting cirrhotic liver. Further studies are needed for the production of standardized protocol to standardize the cell dose, the life span of injected cells, tolerability, pretreatment of liver, the appearance of long term complications and which group of patients will benefit most.

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