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Efficacy of autologous mesenchymal stem cell transplantation in patients with liver cirrhosis

LIVER

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ABSTRACT

Background/Aims: Because of several limitations and complications of liver transplantation, new alternative treatment modalities are required for patients with liver cirrhosis. Many study results encourage the use of autologous bone marrow-derived mesenchymal stem cells for liver diseases. In this study, we assessed the impact of autologous mesenchymal stem cell transplantation on liver tissue and liver chemistry.

Materials and Methods: Twenty-five patients with biopsy-proven liver cirrhosis were enrolled in the study. Patients received 1×10^6 autologous mesenchymal stem cells/kg via a peripheral vein. Biochemical parameters were checked monthly. Periodical radiological screening and liver biopsies before mesenchymal stem cell transplantation were performed after 6 months. Liver specimens were assessed by a pathologist.

Results: No side effect was observed and the mesenchymal stem cell transplantation procedure was well tolerated. Twelve patients completed the study. In 8 patients, improvements in Model for End-Stage Liver Disease (MELD) scores were observed. Serum albumin levels markedly increased in the third month. In patients with non-responder hepatitis C, HCV RNA levels both became negative after mesenchymal stem cell transplantation. Histopathological examinations of liver tissues before and at 6 months after transplantation revealed no change in liver tissue regeneration or fibrosis. However, in 5 patients, hepatitis activity index scores decreased.

Conclusion: Autologous mesenchymal stem cell transplantation via peripheral vein is safe and feasible. Consecutive liver biopsy examinations suggested that mesenchymal stem cells could not reach the liver in a sufficient amount. Improvement in patients and clearance of HCV RNA may have occurred through immunomodulatory mediators secreted by transplanted mesenchymal stem cells, namely the "endocrine" effect.

Keywords: Mesenchymal stem cells transplantation, liver cirrhosis, liver biopsy

INTRODUCTION

Although liver transplantation (Tx) is the gold standard of treatment for patients with decompensated liver cirrhosis, well-known challenges of Tx, such as a shortage of allografts, high cost of and complications due to surgery, and long-term immunosuppression, hinder the feasibility of this process (1). These challenges urge scientists and clinicians to perform new research to establish an alternative treatment, which every patient can benefit from and access easily. Use of mesenchymal

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stem cells (MSCs) appears to be the strongest candidate for the alternative approach mentioned above.

The liver has been shown to have its own stem cell population, which has immunomodulatory and regenerative properties (2). In rodents, the locations of these stem cells were demonstrated to be present in stem cell niches at the canal of Hering (proximal biliary tree) and intralobular bile ducts (3). Liver tissue stem cells appear to maintain the viability and functional capacity of hepatocytes and biliary epithelial cells, particularly under prolonged liver damage (4). Bone marrow (BM) contains MSCs that are capable of differentiating into multiple cell lineages and have immunomodulatory effects (5). These MSCs are proposed to participate in the response process of the liver to injury (6).

In the last decade, data from several experimental and some clinical trials raised the hope for using extrahepatic stem cells as tools for liver regeneration. For example, Y chromosome-positive hepatocytes were observed in female patients' liver biopsy specimens who had received BM transplants from male donors. In addition, using the same method, Y chromosomepositive hepatocytes were observed in female livers that were grafted into male patients (7). These findings indicate an extrahepatic source of stem cells for these hepatocytes. In fact, stem cells have been shown to differentiate into functional hepatocytes in the laboratory environment (8), and when systemically delivered, their homing behaviors, particularly toward the injury site, have been demonstrated (5). In mice with carbon tetrachloride-induced liver fibrosis, BM stem cell Tx was shown to reduce liver fibrosis and improve survival (9). Beneficial data obtained from laboratory and experimental studies paved the way for human applications. For this purpose, either using autologous BM itself (10) or BM-derived stem cells by systemic or liver-targeted infusions, phase I and II studies for end-stage liver disease have been performed (11). The results of these studies have indicated that the safety and feasibility of autologous MSC Tx and data are encouraging. Thus far, approximately 18 clinical trials targeting liver cirrhosis have been performed with BM-derived stem cells (12). Although their study designs, cell dosages, cell types, administration routes, and end points are not identical, most of the studies involved time-limited beneficial outcomes, such as improvements in prognostic scores and laboratory values with no or negligible side effects. However, the majority of the clinical trials lack biopsy control.

In this study, we intended to evaluate the efficacy of autologous MSC Tx in patients with established liver cirrhosis.

MATERIALS AND METHODS

This prospective open-labeled study was performed between 2009 and 2011 in the Gülhane Military Medical Academy Department of Gastroenterology, Ankara. The study's protocol was approved by the Institutional Review Board of Academy Hospital and the Stem Cell Transplantations Scientific Advisory Board at the Turkish Republic Ministry of Health. The study was registered at Clinicaltrials.gov (NCT01499459). The project was financed by the Republic of Turkey's Ministry of Science, Industry and Technology.

Twenty-five patients with liver cirrhosis whose platelet count was more than 30,000 /μL and international normalized ratio (INR) levelswere below 2 were enrolled regardless of their etiologies. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (revised in Edinburgh in 2000) as reflected in *a priori* approval by the institution's human research committee. Patients with metabolic liver diseases, alcohol abuse, coexisting hepatocellular carcinoma and other malignancies, respiratory and cardiovascular comorbidities, and/or insulin-dependent diabetes and those in the first year of antiviral hepatitis treatment were excluded. The etiologies of the 25 enrolled patients were as follows: 4 had autoimmune hepatitis, 7 were cryptogenic, 1 was an alcoholic, 3 had hepatitis C, 7 had hepatitis B, 2 had primary biliary cirrhosis (PBC), and 1 had primary sclerosing cholangitis (PSC). Patients with hepatitis C were non-responders and patients with hepatitis B were taking oral antiviral drugs (lamivudine, adefovir, or entecavir). All patients continued receiving their prescribed medications, such as diuretics, beta-blockers, and antihypertensive drugs.

BM aspiration and MSC production and transplantation:

All patient BM samples were aspirated by a hematologist and sent to Aticell® in Trabzon, Turkey, a validation-certificated, commercial stem cell company. All manufacturing and production steps for the generation of clinical-grade autologous MSCs were performed under good manufacturing practice conditions (13). BM (20–40 mL) was aspirated from the posterior iliac crest of patients using sterile injectors containing 1:10 diluted heparin sodium, (FLUKA, United States Pharmacopeia Reference Standard, Sigma-Aldrich, St. Louis, MO, USA) under local anesthesia and sedation. BM mononuclear cells were isolated by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). After isolation, mononuclear cells (4-5×10^5 cells/cm^2) were plated in a 75-cm^2 cell culture flask (CytoOne T75 TC USA Scientific, Inc., Orlando-Florida, USA) with low-glucose Dulbecco's modified Eagle's medium with L-glutamine (Life Technologies Ltd., Paisley, UK) containing autologous serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) and cultured at 37°C in a 5% CO_2 atmosphere. When the cultures approached 70% confluence, the cells were harvested by treatment with a trypsin/EDTA solution (Gibco, Grand Island, NY, USA) and replaced at a density of 4-5×10^5 cells/cm2 in 162-cm^2 CorningR CostarR cell culture flasks (Sigma-Aldrich St. Louis, MO, USA). After one primary culture and two passages, MSCs were isolated and controlled for quality (Results shown in Table 1) Then, MSCs were cryopreserved with physiological serum containing 7.5% $DMSO_4$ (Sigma-Aldrich,

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Table 1. Quality control test results

Quality Control Test	Result
Cell Viability (%)	>95% vitality
Purity	CD73, CD105, CD90 expression >90% (positive); CD34, CD 45, HLA-DR expression <1% (negative)
Sterility	Sterile
Mycoplasma	Negative
Pyrogenity	Apyrogen
Differentiation Analysis	Rate of differentiation into adipocyte, chondrochyte, osteoblast ≥30%
Tumorogenity	RTA <1 (0.8±02)

CD: cluster of differentiation
RTA: relative telomere enzyme activity
HLA: human leukocyte antigen

St. Louis, MO, USA), 2.5% hydroxyethyl starch (FLUKA, European Pharmacopoeia Reference Standard, Sigma-Aldrich, St. Louis, MO, USA), and 2% autologous serum and then stored until use.

The autologous MSCs were transplanted via A peripheral vein in Gülhane Military Medical Academy Gastroenterology Clinic. For the Tx process, all patients were hospitalized and kept under observation for 24 h.

Liver biopsies and histopathology examination:
After achieving good growth of MSCs from BM (1×10^6 cells/kg), liver biopsies were performed at Gülhane Military Medical Academy Department of Radiology using an ultrasound-guided trucut biopsy gun. In patients with INR levels higher than 1.5, despite fresh frozen plasma transfusions, transjugular liver biopsy was preferred. Later, liver biopsies were performed, 6 months after MSC Tx.

All tissue samples were fixed in 10% neutral buffered formalin and processed routinely. Upon completion of overnight processing, tissue pieces were embedded in paraffin and then standardized 4-µm-thick sections were obtained. At least 2 serial sections, which were stained with H&E, were used to assess the liver parenchyma. One section was stained with Masson trichome to evaluate the degree of fibrosis and the other was impregnated with silver to highlight the reticulin framework of the parenchyma in all cases.

In addition, cells having the characteristics of stem cells (both native and infused stem cells) were identified using the known immunohistochemical markers, CD133 and MOC31. The overall degree or rate of hepatocyte regeneration was highlighted using the Ki-67 antibody in the tissue sections.

Light microscopic examination was performed by an experienced pathologist who was unaware of the specific cases. At the end of the assessment, the pathologist gave an overall decision based on the modified histological activity index modified HAI or Ishak system) for both necroinflammatory activity (grade) and extent of fibrosis (stage).

Follow-up:
All patients underwent ultrasonography liver examinations every 3 months and abdominal dynamic computed tomography or dynamic magnetic resonance imaging at the beginning and end of the study. They all were monitored using blood chemistry, complete blood cell counts, and prothrombin time monthly to track the changes in Child–Pugh and Model for End-Stage Liver Disease (MELD) scores. Patients with non-responder HCV infection had monthly HCV RNA real time PCR tests. The assay and quantification were performed as described before (14).

Statistical analysis
Continuous variables were expressed as mean±standard deviation, whereas categorical variables were denoted as numbers or percentages where appropriate. The Wilcoxon signed-rank test was used to compare MELD, Child-Pugh, and modified HAI scores of the patients before and after Tx. Comparisons of the variables [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), total bilirubin (TBIL), INR, and albumin levels] during the treatment time (at 0, 3, 6, 9, and 12 months) were performed using the Friedman test. Because of extreme values and skewed distribution on the original scale, HCV RNA levels were transformed to log 10 values. The analyses were performed by Statistical Package for Social Sciences version 22.0 (SPSS Inc., Chicago, IL, USA). A two-tailed p value less than 0.05 was accepted to be statistically significant.

RESULTS
Throughout the study, 13 patients were excluded from the study group. Two patients on the Tx waiting list had liver transplanted from cadaveric donors. Three patients died due to complications of end-stage disease. In 8 patients, AticellR declared that they were unable to grow MSCs to the desired amount due to technical reasons, and MSC administration was canceled in cases where the MSC products did not pass the quality control tests (Table 1). Twelve patients (7 male and 5 female) between the ages of 22 and 73 (39±15) years completed the study. All patients had MSC Tx without any acute or chronic side effects. The procedure was well tolerated by all patients. The majority of the patients had a sense of well-being after MSC Tx, which could not be distinguished from the placebo effect.

Patients' etiologies, ages, gender, MELD scores, Child-Pugh scores, and modified HAI scores in liver biopsy specimens before and 1 year after MSC Tx are presented in Table 2. Wilcoxon signed-rank test results, regarding MELD, Child-Pugh, and modified HAI scores, are presented in Table 3. Liver chemistry data of patients throughout the follow-up period, including AST, ALT, ALP, GGT, TBIL, INR, and albumin levels, are summarized in Table 4.

Although statistically non-significant, 8 of 12 patients had a decrease in their MELD scores. In contrast, 2 patients with PBC, 1

Table 2. Patients' etiologies, age, gender, MELD, Child–Pugh, and modified HAI scores before and 1 year after MSC Tx

Patient no	Etiology	Age	Gender	MELD Scores		Child-Pugh Scores		Modified HAI Scores	
				Before	After	Before	After	Before	After
1	Hepatitis C	73	Female	13	11	6	6	12	10
2	Hepatitis C	29	Male	14	12	6	6	10	9
3	Alcoholic Liver Disease	43	Male	15	14	7	7	11	7
4	Cryptogenic	27	Male	10	9	5	5	9	8
5	Primary Sclerosing Cholangitis	24	Male	7	12	5	5	9	9
6	Primary Biliary Cirrhosis	48	Female	10	12	7	9	12	12
7	Primary Biliary Cirrhosis	43	Male	9	13	6	5	9	9
8	Hepatitis B	48	Male	14	16	7	7	7	8
9	Hepatitis B	50	Male	9	8	5	5	5	5
10	Autoimmun Hepatitis	24	Female	15	14	7	6	7	5
11	Autoimmun Hepatitis	35	Female	14	12	6	6	8	9
12	Autoimmun Hepatitis	22	Female	14	11	6	7	11	IS*

*IS: insufficient sample
MELD: model for end-stage liver disease
HAI: histological activity index
MSC: mesenchymal stem cells

Table 3. MELD, Child-Pugh, and modified HAI scores

		Scores (Mean±SD)	p*
MELD Score	Before	12.0±2.7	0.874
	After	12.0±2.1	
Child-Pugh Score	Before	6.0±0.7	0.705
	After	6.1±1.1	
Modified HAI Score	Before	9.1±2.1	0.121
	After	8.2±2.0	

*Wilcoxon signed-rank test
MELD: model for end-stage liver disease
HAI: histological activity index

with hepatitis B, and 1 with PSC had increased MELD scores. The prominent increase with 5 score belonged to the patient with PSC. In the overall assessment of patients' recorded liver function tests over the following year, changes were not significant, except concerning the increase in albumin levels (Table 4), and none of the patients received albumin throughout the follow-up period. However, some individual changes were observed, which may be related to etiology. Therefore, beyond statistical non-significance, case-based evaluation of data warrants future trials.

Clearance of HCV RNA:

Two patients with HCV hepatitis were non-responders to previous PEGylated interferon and ribavirin treatments. They had slightly high blood transaminase levels and positive HCV RNA levels. After MSC Tx, patients 1 (at 4 months) and 2 (at 3 months) showed negative HCV RNA levels. At 6 months, patient 1's HCV RNA became positive till 9 months, where it became undetectable again. For the final 2 months of follow-up time, HCV RNA was positive again. Patient 2 had a more stable trace, where

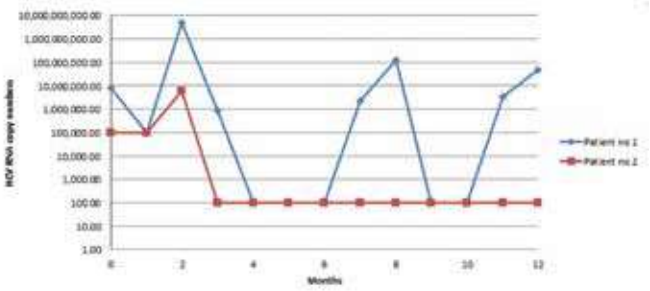

Figure 1. HCV RNA levels at the time of follow-up.

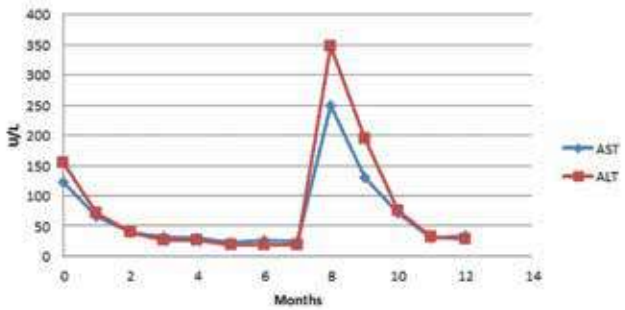

Figure 2. Transaminase levels of patients during the follow-up period.

after the third month, his monthly HCV RNA levels were undetectable throughout the follow-up period (Figure 1).

Efficacy in autoimmune hepatitis:

Patient 11 was diagnosed with BM suppression due to azathioprine before recruitment and she was not receiving any immunosuppressant medication. She had 8×10^7 cells infused via a peripheral vein. At 2 months of follow-up, her elevated transaminase levels normalized (Figure 2). High prothrombin time and INR decreased (from 21.3 s to 16.8 s and from 1.62 to

Table 4. Liver chemistry

	Months (Mean±SD)					
Liver Chemistries	**0**	**3**	**6**	**9**	**12**	**p***
AST	72.2±58.3	70.4±45.4	75.0±73.8	67.9±54.5	71.3±57.8	0.911
ALT	66.2±69.7	57.6±55.8	76.7±85.8	75.0±69.0	75.0±83.0	0.723
ALP	199.8±149.9	224.7±183.2	202.0±138.2	218.6±160.9	191.50±113.9	0.453
GGT	75.5±50.9	74.1±49.1	92.1±72.6	104.6±112.4	77.6±52.8	0.132
TBIL	1.64±0.77	1.82±0.98	1.74±0.82	2.09±1.04	1.99±1.32	0.060
INR	1.40±0.30	1.35±0.26	1.36±0.19	1.31±0.21	1.32±0.21	0.816
ALB	3.40±0.49**	3.69±0.50**	3.63±0.61	3.63±0.60	3.41±0.68	0.013

*Friedman Test
**Wilcoxon Signed Ranks Test (p<0.005)
AST: aspartat aminotransferase
ALT: alanine aminotransferase
ALP: alkaline phosphatase
GGT: gamma-glutamyl transferase
T.BIL: total bilirubine
INR: international normalized ratio
ALB: albumin

1.22, respectively) and low albumin levels elevated to physiological ranges (from 3.16 g/dL to 3.69 g/dL) at the 5th month. Before her 6-month visit, she had been prescribed 450 mg Diosmin + 50 mg Hesperidin, 2 tablets/day, by a vascular surgeon for varicose veins at lower extremities. At 6 months, her liver enzyme levels were abnormal, and after cessation of the prescribed drug, the enzyme levels became normal again at 11 months.

Histopathological examination:

All patients' extent of the fibrosis stage was the same before and after treatment of the liver biopsy specimens, which was 6/6. Although statistically non-significant, 5 of 12 patients had a decrease in their modified HAI score. A prominent decrease was observed in a patient with alcoholic cirrhosis (Table 2). Immunohistochemical staining with CD133 and MOC31 for existing stem cells was negative in all samples. In addition, Ki-67 staining was not highly prominent, suggesting no change in the rate of hepatocyte regeneration.

DISCUSSION

The study group comprised patients with different etiologies in which most had priority for Tx. Encouraging evidence, such as sexmismatched hepatocytes observed in chronically damaged liver tissues of patients who received sex-mismatched BM Tx, and homing behavior of transplanted stem cells provided a rationale for the possibility of regenerating liver tissue via MSC Tx (7). We did not recruit patients with metabolic liver diseases because their BM-derived MSCs would have expressed the same genetic mutations as of the disease. Eight patients were excluded from the study group because of a low growth of MSCs or cell viability below 95%. Some factors, such as HBV infection and cirrhosis, may have a negative impact on MSC proliferation. Zhong et al. (15) reported that compared with normal donors, BM samples of patients with cirrhosis and hepatitis B showed impairment in the proliferation of MSCs which may constitute a reasonable explanation for our exclusion.

Our preferred transplantation route was via a peripheral vein, which appeared to be minimally invasive. Recent data and comments about usage of peripheral vein as a route the for MSC Tx are conflicting. Eggenhofer et al. (16) reported that in their experimental study, MSCs transplanted via the intravenous route were trapped in the capillary bed of the lungs and they did not migrate beyond. In contrast, in another study, rats with carbon tetrachloride-induced liver cirrhosis were transplanted with MSCs via 3 different routes: intravenous, intrahepatic, and intraperitoneal injections. The best result was reported to be in favor of the peripheral vein route (17). MSCs can be labeled and tracked by nuclear pharmaceuticals in human beings. In a clinical trial performed by Gholamrezanezhad et al. (18), patients with advanced cirrhosis were transplanted In^{111}-oxine-labeled MSCs via peripheral vein, and the time dependent biodistribution was monitored. The detected radioactivity was reported to gradually increase in the liver and spleen. Previously, in an experimental study, we labeled rat BM-derived MSCs with fluorodeoxyglucose (^{18}F-FDG) and tracked their homing behavior toward esophageal sites with caustic injury via positron emission tomography. The duration of the process was 4 h because of ^{18}F-FDG halftime (19). In this study, MSCs were not labeled. Hence, we could not directly track and assess the fate of transplanted cells. Instead, liver biopsy was the only tool for indirect evidence. However, unfortunately, consecutive liver biopsy examination results had no significant differences in terms of liver tissue regeneration. These findings suggest that MSCs probably could not reach the liver and were possibly held in the lung capillary bed or were incapable of homing in properly on the cirrhotic liver.

The research and knowledge regarding stem cells and their characteristics are increasing rapidly. However, there is still a lack of consensus concerning which stem cell to use for which disease. We have selected BM, which is the most studied and well known source for MSCs. An article, which was published after our study's termination, indicated the superior differentiation capacity of various placenta-derived stem cells to functional hepatocytes compared with other types of adult stem cells (20). Therefore, we propose that using adult stem cells of non-BM origin may have exhibited different outcomes and altered liver tissue histopathological examination results in terms of proliferation.

Regarding our data from patients with established cirrhosis, another suggestion should be to treat liver diseases in their early phases, instead of the cirrhosis state. A nearly complete distortion of the microarchitecture, as in end-stage cirrhosis, probably has no impact on MSC homing or differentiation.

Reviewing our follow-up data reveals the subgroups comprising patients with autoimmune hepatitis and hepatitis C. Although less than sufficient number of patients for statistical analysis were studied, a decrease in patients' MELD scores was observed in both the subgroups. Three of these patients also had a decrease in the modified HAI scores. This decrease in scores in patients with autoimmune hepatitis is attributable to well-known immunoregulatory effects of MSCs through a series of immune cells such as suppressing T- and B-cell proliferation and dendritic cell maturation and modulating natural killer cells and macrophages (21). In fact, considering the abovementioned features, MSC administration in acute steroid-refractory graft-versus-host disease is an effective treatment modality and can be used in routine practice (22). Autologous MSC Tx may be a potential treatment modality for patients with steroid-resistant autoimmune hepatitis because it has no side effects compared with immunosuppressant drugs.

By checking the serum HCV RNA levels of 2 patients with hepatitis C monthly, we obtained a surprising amount of data regarding MSCs' immunomodulatory effect, which is the clearance of viral RNA. During the follow-up period, it was difficult to explain these findings. Nevertheless, under the context of rapidly growing research and new data, we can better understand the underlying mechanism. Factors other than antiviral drugs playing a role in viral clearance through innate immunity are recently being discovered. Pan et al. cocultured MSCs with the HCV-infected Huh-7 hepatoma cell line; they reported that MSCs inhibit HCV replication with a maximum 90% ratio in a dose-dependent manner. They also revealed an association between the anti-HCV activity of MSCs and an interferon-inducible trans-membrane protein, IFITM3 (23). In a case report, Kniazev et al. (24) declared that the same effect was observed in a patient with ulcerative colitis who had a cytomegalovirus infection, where complete virus elimination occurred after MSC Tx, without administrating any antiviral therapy. A clinical trial performed by Zhang et al. (25) with an immune non-responder patient group infected with HIV-1 raised hope for establishing an alternative treatment modality besides highly active antiretroviral therapy. Umbilical cord MSC Tx to these patients resulted in an increase in circulating naïve and central memory CD4 T-cell counts and restoration of HIV-1-specific IFN-γ and IL-2 production.

Some other recent findings regarding innate immunity describe small interfering RNAs (siRNAs) in mammalian host cells infected by viruses, where they exert anti-viral activity (26). Choi et al., in their experimental study, demonstrated the role of microRNA-27a in modulating HCV infectivity. They have shown that in microRNA-27a-transfected differentiated hepatocyte-like cells, HCV infectivity was suppressed (27). Some patients with liver cirrhosis due to hepatitis C had been recruited to previously established MSC Tx trial groups, and the latest study in literature was performed by Salama et al. (28). Unfortunately, we could not obtain any data regarding patients' HCV RNA levels in all previously performed studies. Composing new MSC Tx study groups, particularly from HCV hepatitis patients who are non-responders to current drug therapies, would open a new gate for novel antiviral innate immunity-related treatment modalities by revealing the molecular mechanisms.

MSC Tx appears to be beneficial in patients with alcoholic cirrhosis. In a clinical trial performed by Jang et al. (29), 11 patients with biopsy-proven alcoholic cirrhosis were twice transplanted BM-derived autologous MSCs via hepatic artery (29). They reported that in 6 of 11 patients, histological improvement was obtained. Similar to these findings, our patient with alcoholic cirrhosis had a decrease in MELD and HAI scores. In contrast, 2 patients with PBC and 1 patient with PSC had no benefit at all in terms of MELD and modified HAI scores.

Our data and recent findings in the literature shadow out the enormous knowledge waiting to be discovered in the context of stem cell research, which seems to provide novel treatment opportunities. We propose that patient groups with homogeneous etiologies for MSC Tx should be established and that multiple infusions with a higher number of cells should be performed.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Institutional Review Board of Gülhane Military Medical Academy Hospital (03.05.2007/1491-424-07) and the Stem Cell Transplantations Scientific Advisory Board at the Turkish Republic Ministry of Health (10.04.2008/B.10.0.THG.0.14.00.03-2120/6401).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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