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CIRRHOSIS AND LIVER FAILURE

Histological improvement following administration of autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: a pilot study

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Keywords
bone marrow-derived mesenchymal stem cell – cirrhosis – hepatic fibrosis – liver function – portal hypertension

Abbreviations
AC, alcoholic cirrhosis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BM, bone marrow; BM-MSCs, bone marrow-derived mesenchymal stem cells; collagen-1, type 1 collagen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; MELD, model for end-stage liver disease; MTC, masson's trichrome; γGT, gamma glutamyl transferase; SD, standard deviation; TGF-β1, transforming growth factor-beta 1; α-SMA, α-Smooth muscle actin.

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Abstract

Background: In experimental models, bone marrow-derived mesenchymal stem cells (BM-MSCs) have the capacity to differentiate into hepatocytes and exhibit antifibrotic effects. However, there have been no studies in humans with alcoholic cirrhosis. *Aim:* The aim of this study was to elucidate the antifibrotic effect of BM-MSCs in patients with alcoholic cirrhosis, as a phase II clinical trial. *Methods:* Twelve patients (11 males, 1 female) with baseline biopsy-proven alcoholic cirrhosis who had been alcohol free for at least 6 months were enrolled. BM-MSCs were isolated from each patient's BM and amplified for 1 month, and 5×10^7 cells were then injected twice, at weeks 4 and 8, through the hepatic artery. One patient was withdrawn because of ingestion of alcohol. Finally, 11 patients completed the follow-up biopsy and laboratory tests at 12 weeks after the second injection. The primary outcome was improvement in the patients' histological features. *Results:* According to the Laennec fibrosis system, histological improvement was observed in 6 of 11 patients (54.5%). The Child-Pugh score improved in ten patients (90.9%) and the levels of transforming growth factor-β1, type 1 collagen and α-smooth muscle actin significantly decreased (as assessed by real-time reverse transcriptase polymerase chain reaction) after BM-MSCs therapy ($P < 0.05$). No significant complications or side effects were observed during this study. *Conclusions:* Bone marrow-derived mesenchymal stem cells therapy in alcoholic cirrhosis induces a histological and quantitative improvement of hepatic fibrosis.

Cirrhosis, the end stage of progressive hepatic fibrosis, is characterized by distortion of the hepatic architecture and the formation of regenerative nodules, angiogenesis and shunt formation, leading to the loss of liver function and the development of hepatocellular carcinoma (1–5). The main causes of cirrhosis are chronic alcohol abuse, and hepatitis B and C viruses. Alcoholic cirrhosis (AC), one of the major medical complications of alcohol abuse, is a major cause of chronic liver disease worldwide (6–8).

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The most effective therapy for advanced cirrhosis is currently liver transplant. However, this procedure has several limitations, including lack of donors, surgical complications, immunological suppression and high medical cost (9). Alternative approaches that circumvent the use of the whole organ, such transplantation of cells of various origins, have recently been accepted (10, 11). For example, stem cell transplantation has been suggested as an effective alternate therapy for hepatic disease (12, 13).

Several previous studies using animal models of liver diseases have demonstrated that bone marrow (BM) cell transplantation may accelerate the liver regeneration process, reduce hepatic fibrosis and improve liver function and the survival rate (14–17). The prospects for stem cell transplantation as a therapy for hepatic disease, as determined by initial translational pilot studies testing the direct hepatic administration of BM-derived stem cells, have been encouraging and have suggested enhanced liver regeneration prior to partial hepatectomy, showing improved liver function in advanced chronic liver disease (18–24). Among the stem cells, mesenchymal stem cells (MSCs) in particular have practical advantages in regenerative medicine because of their high capability for self-renewal and differentiation without ethical or tumorigenic problems. A phase I–II trial of transplantation with autologous bone marrow-derived mesenchymal stem cells (BM-MSCs) has been conducted, and it now seems both feasible and safe to use MSCs to treat patients with decompensated cirrhosis (13). In experimental models, BM-MSCs are capable of differentiating into hepatocytes and exhibit antifibrotic effects. However, there have been no reports regarding the effect of autologous BM-MSCs therapy on humans with AC.

We therefore investigated the antifibrotic effect of BM-MSCs on alcoholic cirrhosis as a phase II clinical study.

Patients and methods

Patients

This study was conducted prospectively as an open-label trial involving one centre in Korea. The Institutional Review Board of the hospital and the Korea food and drug administration (KFDA) approved the protocol (KFDA clinical trial no. 687), and written informed consent to participate in this study was received from all of the participating patients. The registered number of Clinicaltrial. gov is NCT01741090. This study was conducted following the principles of the Declaration of Helsinki (revised in Edinburgh 2000).

Patients between 37 and 60 years of age with biopsy-proven AC who visited the Yonsei University Wonju College of Medicine, Wonju Christian Hospital between May 2010 and Jan 2011 and discontinued alcohol intake for at least 6 months prior to participating in this study (to exclude the influence of inflammation by recent alcohol consumption) were considered eligible for the study (25, 26). We presumed that 6 months of abstinence from alcohol might further reduce the active inflammation and allow stabilization of the liver histology in most of our participants and play an essential role in eliminating the bias that may occur with severe inflammation resulting from recent consumption of alcohol.

Patients who did not provide informed consent were excluded from this study. In addition, patients younger than 20 or older than 65 years, with hepatitis B or C virus-related cirrhosis, severe liver failure (serum bilirubin >85 μmol/L), hepatic encephalopathy, hepatorenal syndrome, recurrent gastrointestinal bleeding, spontaneous bacterial peritonitis, presence of liver tumour or history of other cancer and pregnant or lactating were also excluded from this study.

Radiological imaging studies including ultrasonography and computed tomography scan, and laboratory studies were conducted on each patient to determine the Child-Pugh score and the model for end-stage liver disease score. Other causes of liver disease were excluded by performing serological tests for hepatitis B and C infection, and autoantibodies.

According to these criteria, 12 consecutive patients were initially considered for this study. One patient who had continued to consume alcohol (continuous alcohol intake of more than 60 g/week) was withdrawn from this study. Thus, 11 patients were ultimately enrolled. The general characteristics of the patients are listed in Table 1.

BM aspiration, isolation of MSCs and cell culture

All manufacturing and product testing procedures for the generation of clinical-grade autologous MSCs were performed under good manufacturing practice conditions (FCB-Pharmicell Co., Ltd., Sungnam, Korea). Approximately, 10–20 ml of BM was aspirated from the posterior iliac crest of patients under local anaesthesia. BM mononuclear cells were isolated by density-gradient centrifugation (Histopaque-1077, Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cells ($2–3 \times 10^5$ cells/cm^2) were plated in a $75cm^2$ flask (Falcon, Franklin Lakes, NJ, USA) with low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco), and cultured at 37 °C in a 5% CO_2 atmosphere. After 5 days, non-adherent cells were removed by replacing the medium. When the cultures approached 80% confluence, the cells were harvested by treatment with a trypsin/EDTA solution (Gibco) and replated at a density of $4–5 \times 10^3$ cells/cm^2 in $175cm^2$ flasks. Cells for injection were serially subcultured up to passages four or five. During culture, some passage 2 cells were harvested and cryopreserved in 10% dimethyl sulfoxide (Sigma-Aldrich) and 90% foetal bovine serum (FBS) for the second injection.

Table 1. General characteristics of the patients

	Pre-stem cell therapy (Total n = 11)	Post-stem cell therapy (Total n = 11)	P
Age (years)/ sex (males: females)	50 ± 8 (37–60)/10:1	50 ± 8 (37–60)/10:1	
Aetiology (alcohol)	11 (100%)	11 (100%)	
Albumin (g/dl)	3.5 ± 0.6 (2.6–4.5)	3.9 ± 0.5 (2.9–4.5)	0.016
AST (U/L)	49 ± 20 (22–82)	43 ± 19 (23–79)	0.103
ALT (U/L)	16 ± 7 (6–28)	15 ± 7 (9–32)	0.843
γ-GT (NL<60 U/L)	54 ± 12	53 ± 11	0.471
Total bilirubin (mg/dl)	1.3 ± 0.9 (0.2–3.6)	1.1 ± 0.7 (0.4–2.9)	0.163
Prothrombin time (INR)	1.2 ± 0.1 (1.0–1.4)	1.1 ± 0.1 (1.0–1.3)	0.050
Platelet count (/mm^3)	119 546 ± 66 872 (51 000–246 000)	112 364 ± 34 989 (73 000–179 000)	0.642
Creatinine (mg/dl)	0.7 ± 0.2 (0.5–1.0)	0.6 ± 0.2 (0.4–0.9)	0.311
Child-Pugh score	7.1 ± 0.9 (6–9)	5.4 ± 0.7 (5–7)	0.000
MELD score	9.2 ± 2.8 (6–15)	8.3 ± 2.4 (6–13)	0.005
Histology			0.020
F4A	1 (9.1%)	4 (36.4%)	
F4B	3 (27.3%)	4 (36.4%)	
F4C	7 (63.6%)	3 (27.3%)	-

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, gamma glutamyl transferase; MELD, model for end-stage liver disease.

Immunophenotypes and differentiation assays of BM-MSCs

The immunophenotypes of the BM-MSCs (CD14, CD34, CD45, CD73 and CD105) were analyzed on the day of injection, and their differentiation potentials were determined (osteogenesis and adipogenesis; Fig. 1). For immunophenotype analysis, BM-MSCs were stained with antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD14-FITC, CD34-FITC, CD45-FITC, CD73-PE and CD105-PE (BD Biosciences, San Jose, CA, USA). Briefly, 5×10^5 cells were resuspended in 0.2 mL phosphate-buffered saline (PBS) and incubated with FITC- or PE-conjugated antibodies for 20 min at room temperature. FITC- or PE-conjugated mouse IgGs were used as the control isotype at the same concentration as the specific primary antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (Epics XL; Beckman Coulter, Miami, FL, USA).

The osteogenic differentiation was determined by first plating the cells at 2×10^4 cells/cm^2 in six-well plates and then leaving them in the following osteogenic medium for 2–3 weeks: low-glucose DMEM medium supplemented with 10% FBS, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone and 0.2 mM ascorbic acid (Sigma-Aldrich) (27). The osteogenic differentiation was quantified from the release of p-nitrophenol from p-nitrophenyl phosphate by the enzyme alkaline phosphatase (28).

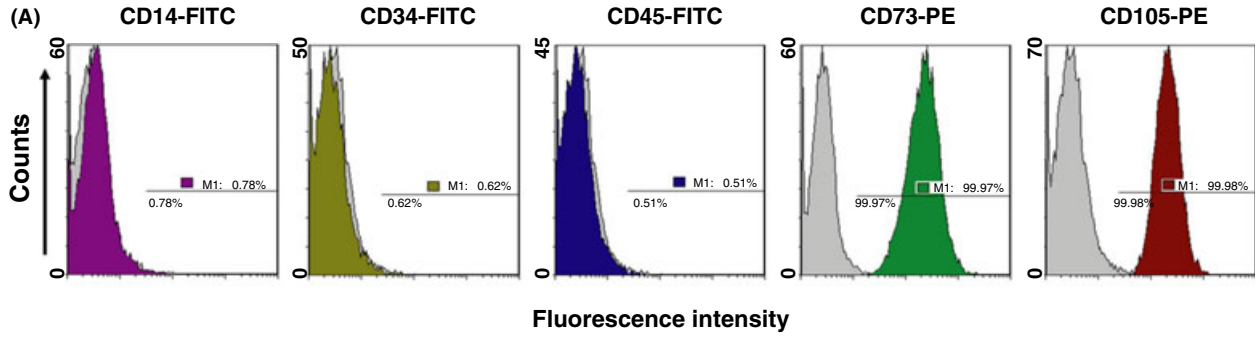
For adipogenic differentiation, BM-MSCs were plated at 2×10^4 cells/cm^2 in six-well plates, cultured for 1 week and then differentiation was induced with an adipogenic medium (10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin and 100 μM indomethacin in high-glucose DMEM) for an additional 3 weeks. The differentiated cells were fixed in 4% paraformaldehyde for 10 min and stained with fresh Oil Red-O solution (Sigma-Aldrich) to show lipid droplets (Fig. 1C).

Autologous BM-MSCs injections

On the day of the injection, MSCs were harvested using trypsin/EDTA, washed twice with PBS and once with saline solution and then resuspended at a final concentration of 5×10^6 cells/ml in 10 ml of saline solution (i.e., 5×10^7 cells). Criteria regarding the clinical use of MSCs included viability greater than 80%, absence of microbial contamination (bacteria, fungus, virus or mycoplasma) when tested 3–4 days before administration, expression of CD73 and CD105 by more than 90% of the cells, and absence of CD14, CD34 and CD45 in less than 3% of the cells, as assessed by flow cytometry. Each time the 5×10^7 cells in 10 ml of saline were injected at weeks 4 and 8 via the right hepatic artery by a coaxial angiographic catheter (Boston Scientific, Natick, MA, USA). Injections of BM-MSCs were performed using coaxial angiographic catheters (Boston Scientific) placed in the hepatic artery. Continuous monitoring was performed via leukapheresis as well as the injection procedure, and any hemodynamic changes were recorded.

Follow-up

After enrollment, patients were followed up according to the study design. The patients did not receive any other medications during the study. Alcohol side effects were monitored every week by phone call in the patients and their family and during their monthly visits to the hospital. Clinical and biochemical tests for the assessment of the side effects present were also performed at each hospital visit. Any evidence of drinking alcohol would result in the subject being withdrawn from the study. A follow-up liver biopsy was performed 12 weeks after the second therapeutic BM-MSCs injection.



(B)

	CD14	CD34	CD45	CD73	CD105
First injection (mean±SD)	1.0±0.3	0.9±0.3	0.8±0.3	99.6±0.6	98.8±2.0
Second injection (mean±SD)	1.0±0.2	0.7±0.2	0.8±0.3	98.9±1.6	98.6±1.3

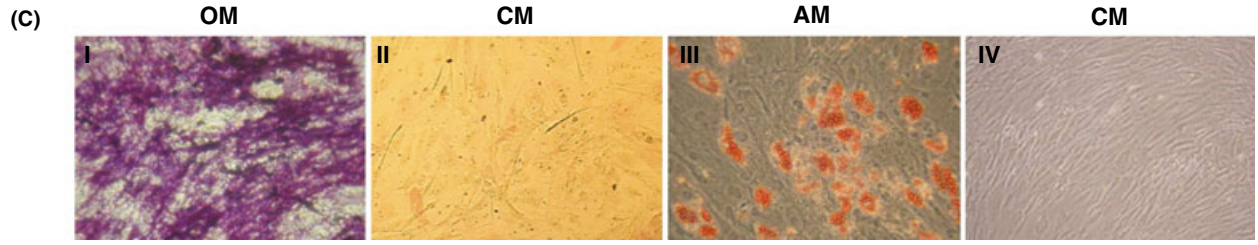


Fig. 1. Immunophenotypes and differentiation potentials of the BM-MSCs. (A) The expressions of cell-surface antigens (CD14, CD34, CD45, CD73, and CD105) were evaluated by flow cytometry. (B) Representative histograms for the positive populations represent averages for 11 patients with the indicated standard deviations. (C) BM-MSCs stained positively for endogenous alkaline phosphatase activity, indicating osteogenic differentiation within an osteogenic medium (OM; I), or stained negatively in control medium (CM; II). BM-MSCs stained positively for lipid droplets, indicating adipogenic differentiation within adipogenic medium (AM; III), or stained negatively in CM (IV) (×200).

Primary and secondary outcomes

The primary outcome was the improvement of histological findings, which required improvement of at least 1 point on the Laennec fibrosis scoring system. The secondary outcome included changes in the Child-Pugh score, direct markers related to hepatic fibrosis and the development of side effects.

Fibrosis assessment

Histomorphological and immunohistochemical analysis

Paired liver biopsies were performed at baseline and 12 weeks after the second therapeutic BM-MSCs injection. Five micrometer thick sections of paraffin-embedded liver biopsy samples were prepared and stained with hematoxylin and eosin (H&E), Masson's trichrome (MTC), α-smooth muscle actin (α-SMA) and Picrosirius Red.

Fibrosis was evaluated using both the Laennec fibrosis scoring system (Table S1, Fig. 2) and the METAVIR fibrosis scoring system by two liver pathologists were blinded to the clinical data of the patients (29, 30). To estimate the chance adjusted agreement, the kappa value was calculated for inter-observer agreement equalling the value of 0.87. When the two pathologists disagreed, they discussed and reached a consensus on the fibrosis score.

Liver biopsy specimens that were ≥15 mm long and ≥1.2 mm wide were required for the study. In fragmented biopsies, the total length was estimated by adding the maximum dimensions of each individual fragment. In using the Laennec system, the thickness of the predominant type of septae in each specimen was chosen and the smallest nodule was selected for scoring. The Laennec fibrosis scoring system was used because this system divides cirrhosis into three subclasses, allowing for a more detailed estimation of fibrosis after intervention (29). In addition, to estimate any treatment-induced change in liver fibrosis, the fibrosis area was quantified as a percentage of the total area that was positive for MTC stain in the digital photomicrographs using a computerized image-analysis system (Analysis

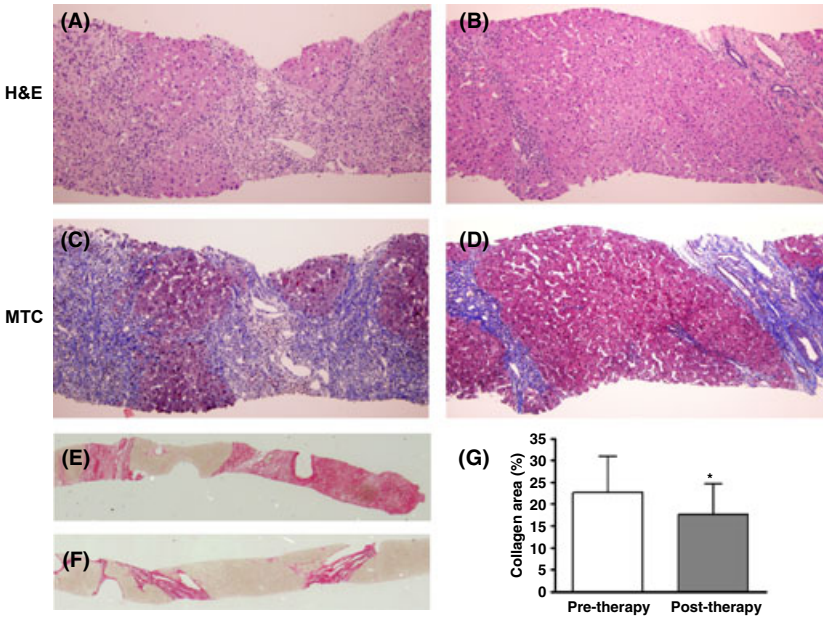


Fig. 2. Histological and Immunohistochemical Analysis. Histological analysis was evaluated by (A, B) H&E and (C, D) MTC staining (×100). BM-MSCs therapy induced an improvement of cirrhosis from (A, C) F4C pre-therapy to (B, D) F4B post-therapy according to the Laennec fibrosis scoring system. Picrosirius Red staining of a section from a liver biopsy specimen showed a change in collagen proportion stained as red from (E) pre-therapy to (F) post-therapy (×40). (G) The relative area of collagen stained by Picrosirius Red was analyzed with an image-analysis programme. The percentage of collagen proportionate area decreased from 22.6 ± 8.4% (pre-therapy) to 17.7 ± 6.9% after BM-MSCs therapy. Data are mean and SD values, $*P < 0.001$.

3.0, Soft Imaging System, Münster, Germany). To quantify the fibrosis area, microscopic areas were selected randomly at an original magnification of ×100. For immunohistochemical analysis, tissue sections were incubated with primary antibody against α-SMA (diluted 1:800, Neomarkers, Fremont, CA, USA) for 90 min at room temperature after washing with buffer. Tissue sections were incubated with the chromogen 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA, USA) for 5–7 min. Prior to mounting, the sections were counterstained with hematoxylin and then dehydrated. An UltraVision LP Large Volume Detection System (Lab Vision, Runcorn, UK) was used as the detection system. Morphological analysis of immunopositive cells was also performed with a computerized image-analysis system.

Picrosirius Red staining was performed to quantify the total amount of collagen. Five micrometer thick sections of paraffin-embedded liver biopsy specimens were deparaffinized and rehydrated with distilled water and stained with a Picrosirius Red staining kit (Polysciences, Warrington, PA, USA) according to the manufacturer's instructions. In addition, the amount of collagen (the main component of fibrous tissue) was estimated from the collagen proportionate area, expressed as the percentage of the total area that was positive for Picrosirius Red stain on microscopy (Olympus BX51, Tokyo, Japan) using a computerized image-analysis system (IMT i solution, Vancouver, Canada). While measuring the collagen proportionate area, we eliminated image artefacts and structural collagen in large portal tracts and blood vessel walls (31).

Gene expressions of transforming growth factor β1, type 1 collagen and α-smooth muscle actin

Total RNA was isolated from paired liver biopsy samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA purity and concentration were determined using a spectrophotometer (Ultrospec 2100 pro UV/Visible, Amersham Bioscience, Freiburg, Germany). cDNAs were then synthesized using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers. For polymerase chain reaction (PCR) amplification, sequence-specific oligonucleotide primers for the genes of interest were designed based on human specific sequences in the GenBank database (Table S2). Quantitative real-time PCR was carried out using SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) and performed in an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Data were analyzed using SDS2.2.2 software (Applied Biosystems). The cycle threshold (Ct) values of the target genes were normalized to the Ct values of the endogenous control (glyceraldehyde-3-phosphate dehydrogenase). Relative changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

Table 2. Changes in clinical histological markers induced by BM-MSCs therapy (Pre, before BM-MSCs therapy; Post, after BM-MSCs therapy)

	Child-Pugh score		MELD score		Histology	
Patient no.	Pre	Post	Pre	Post	Pre	Post
1	7	7	8	7	F4C	F4B
2	6	5	8	6	F4C	F4A
3	7	5	11	10	F4B	F4A
4	6	5	8	8	F4A	F4A
5	8	6	13	11	F4C	F4C
6	7	5	8	8	F4B	F4A
7	9	7	15	13	F4C	F4C
8	8	6	10	10	F4C	F4B
9	7	5	7	6	F4B	F4B
10	6	5	7	6	F4C	F4B
11	7	5	6	6	F4C	F4C

BM-MSCs, bone marrow-derived mesenchymal stem cells; MELD, model for end-stage liver disease

Table 3. Image analysis of Picrosirius Red staining for collagen proportionate area

	Laennec fibrosis histology			
Patient no.	Pre	Post	Pre* (n = 11)	Post* (n = 11)
1	F4C	F4B	24.1	12.7
2	F4C	F4A	33.7	21.6
3	F4B	F4A	26.7	21.8
4	F4A	F4A	4.9	4.8
5	F4C	F4C	30.1	26.8
6	F4B	F4A	16.5	11.8
7	F4C	F4C	32.6	28.4
8	F4C	F4B	19.6	16.5
9	F4B	F4B	16.1	14.3
10	F4C	F4B	21.2	15.6
11	F4C	F4C	23.3	20.3
Mean			22.6	17.7
SD			8.4	6.9

*Percentage of the collagen area

Statistical analysis

The outcomes were analyzed by per-protocol (PP) analysis. Comparisons were made using Fisher's exact test, Mann–Whitney *U*-test and Wilcoxon signed-rank test. Results are expressed as mean ± SD values. The level of statistical significance was set at $P < 0.05$. A medical statistician (E.H.C.) supported the study design and analysis of the data.

Results

The characteristics of the patients in this study are listed in Table 1. All patients had paired biopsy-proven cirrhosis, and the cohort had a median age of 50 years (range 37–60 years). There was a male to female ratio of 10:1. The immunophenotypes for CD14, CD34, CD45, CD73 and CD105 cells were determined and osteogenic or adipogenic differentiation was induced on the day of autologous BM-MSCs injection (Fig. 1). In both the first and second injected cell populations, CD73 or CD105 (which are positive markers of BM-MSCs) was expressed in more than 98% of the cells, and CD14, CD34, or CD45 (which are known to be negative markers of BM-MSCs) was expressed in less than 1% of the cells (Fig. 1B). BM-MSCs from all patients could be differentiated into osteocytes and adipocytes (Fig. 1C).

Primary outcome

Histological and immunohistochemical analysis

The Laennec fibrosis scoring system revealed detailed individual changes within the cirrhotic tissue (F4A–F4C; Table S1). Histological analysis was evaluated by H&E and MTC staining (Fig. 2). According to the Laennec fibrosis system, histological improvements were observed in six patients (54.5%) following BM-MSCs therapy (Table 2).

These results were further confirmed by immunohistochemical staining revealing α-SMA expression and Picrosirius Red staining (Fig. 2). The relative expression of the collagen proportionate area stained by Picrosirius Red was analyzed using an image-analysis programme. The percentage of collagen proportionate area decreased significantly from 22.6 ± 8.4% to 17.7 ± 6.9% following BM-MSCs therapy ($P < 0.001$) (Table 3) (Fig. 2G).

Secondary outcome

Changes in laboratory data after BM-MSCs therapy

The Child-Pugh score were used to evaluate overall liver function; scores improved in 10 of the 11 patients (90.9%), from 7.1 ± 0.9 (pre-therapy) to 5.4 ± 0.7 (post-therapy; $P < 0.001$; Tables 1 and 2). Changes in the value of the other relevant laboratory parameters [albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and creatinine according to BM-MSCs therapy are listed in Table 1. MELD scores decreased from 9.2 ± 2.8 to 8.3 ± 2.4 following BM-MSCs therapy ($P < 0.005$; Tables 1 and 2).

Gene expression associated with fibrosis

After stem cell administration, the relative expressions ($2^{-\Delta\Delta Ct}$ values) of transforming growth factor β1 (TGF-β1), type 1 collagen (collagen-1) and α-smooth muscle actin (α-SMA) in real-time PCR significantly decreased from 3.8 ± 1.4 to 2.6 ± 1.2 ($P = 0.013$), from 4.5 ± 2.6 to 3.1 ± 2.8 ($P = 0.021$), and from 4.3 ± 1.9 to 2.3 ± 1.6 ($P = 0.007$) respectively (Fig. 3).

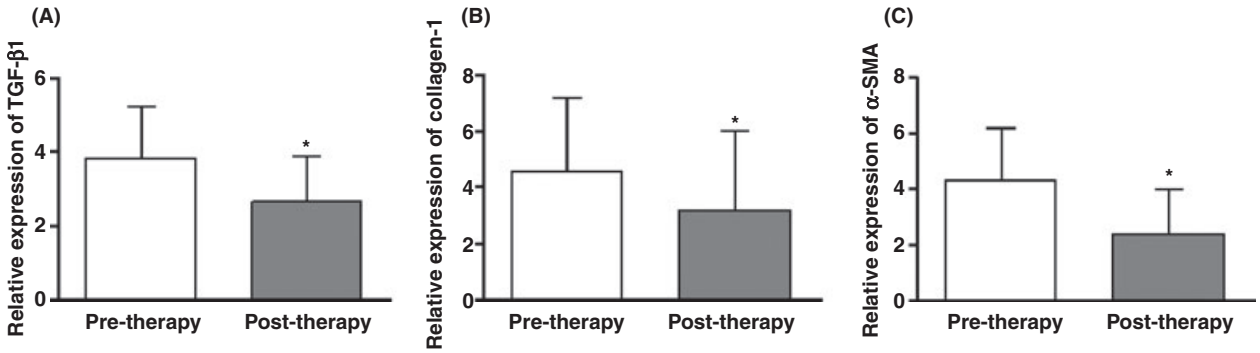


Fig. 3. Gene expression analysis associated with fibrosis. After administration of BM-MSCs therapy, the relative expressions ($2^{-\Delta\Delta Ct}$ values) of (A) TGF-β1, (B) collagen-1, and (C) α-SMA by real-time PCR decreased significantly from 3.8 ± 1.4 to 2.6±1.2 (*P = 0.013), from 4.5 ± 2.6 to 3.1 ± 2.8 (*P = 0.021), and from 4.3 ± 1.9 to 2.3 ± 1.6 (*P = 0.007) respectively. Data are mean and SD values.

Safety

All 11 patients tolerated the therapy. They were monitored regularly for any sign of possible side effects of the stem cell therapy such as fever, hypersensitivity reaction and acute rejection fever. There was no evidence of stem cell therapy related tumour developing during the follow-up period.

Discussion

At the overt cirrhotic stage, end stage liver disease often necessitates liver transplantation (32). However, it has several limitations, such as shortage of organ donors, high medical cost, a widening donor recipient gap and life long dependence on immunological suppression. Hence, stem cell transplantation has been suggested as an effective alternate therapy for hepatic disease (10, 12, 13).

Stem cell therapies have shown promising benefits for hepatic fibrosis in experimental and clinical studies (13, 14, 19, 22). BM comprises two main populations of stem cells, hematopoietic stem cells and MSCs, of which the latter have been considered as alternative cell sources for liver or hepatocyte transplantation (24). In liver damage, MSCs are able to differentiate into hepatocytes, stimulate the regeneration of endogenous parenchymal cells, migrate to damaged sites and enhance fibrous matrix degradation (antifibrotic effects). Furthermore, several clinical studies have demonstrated favourable effects such as BM-MSCs improving the liver function in patients with hepatitis B or C virus-related cirrhosis (22, 33). However, no previous study has examined the effect of autologous BM-MCs on hepatic fibrosis in patients with alcoholic cirrhosis.

We aimed to determine the safety and antifibrosis effect of MSCs on alcohol-related hepatic fibrosis in humans. After autologous BM-MSCs injection, histological improvements were observed in 6 of the 11 patients (54.5%). The Child-Pugh score improved in ten patients (90.9%), and the expressions of TGF-β1, collagen-1 and α-SMA were significantly decreased ($P < 0.05$). Importantly, no significant complications or side effects were observed during this study. These results indicate that BM-MSCs therapy has potential as an antifibrotic treatment in cirrhosis and a bridging therapy for liver transplantation in advanced cirrhosis with hepatic insufficiency. Current antiviral therapies can improve the hepatic fibrosis in patients with B or C virus-related cirrhosis, but the only treatment for alcoholic cirrhosis is alcohol abstinence. Therefore, our results suggest a novel strategy for the treatment of alcoholic cirrhosis.

There is a clear histological variability of severity within cirrhosis classified as F4 by the METAVIR system. Cirrhosis is currently considered to be potentially reversible if the cause of the injury is removed. The lack of subclassification within cirrhosis can be problematic when assessing the antifibrotic effect of agents such as antiviral drugs. For instance, even though antifibrotic therapy leads to the improvement of hepatic fibrosis from F4C to F4A in the Laennec system, the lack of change under the conventional METAVIR system will lead to the false conclusion that treatment is ineffective. Hence, further histological subclassification of cirrhosis is required (4, 29). In this study, we applied the new Laennec fibrosis scoring system to provide a more detailed classification of F4 cirrhosis.

The quality of interventional studies of alcoholic liver disease patients is crucially dependent on monitoring the alcohol intake (6, 7). In this study, we did our best to monitor the subjects' alcohol re-intake and alcohol abstinence, using serological liver function tests including serum AST/ALT ratio and γ-glutamyl transpeptidase, and mean corpus volume in complete blood counts. Furthermore, alcohol intake was monitored every week by phone calls and in monthly face to face interviews with patients and their family members. This was effective in preventing alcohol re-intake in most of the participants because of the formation of a good rapport.

This study was subject to some limitations. Firstly, this pilot study was designed and performed as an open-label single arm study without control. Indeed, lack of a control group is certainly a major limitation of this study. However, repeating biopsies in control group would be ethically difficult if no active treatment is being offered. Nevertheless, future prospective controlled studies would be necessary to validate the beneficial effect of MSCs on hepatic fibrosis shown in this study. Although being an open-label study, the pathologist was blinded to the clinical data and the kind of therapy used, which enabled us to be confident about the accuracy of our histological data.

Secondly, like all biopsy studies there were potential limitations regarding liver biopsy sampling, and in particular sampling error. In this study, our criteria of excluding biopsy specimens smaller than 15 mm should have minimized the magnitude of any sampling error. In addition, we incorporated other methods to assess hepatic fibrosis in this study to improve the validity of our morphometric analysis and measurement of TGF-β1, collagen-1 and α-SMA by quantitative real-time PCR of the biopsied liver tissues (31).

The final limitation of our study was that we did not track the injected MSCs or their localization in patient's body. Although tracing injected stem cells in the body is a complicated procedure and the interpretation of tracing studies has recently created considerable controversy, it is very important to understand the way stem cells act to improve liver function and liver volume.

This is the first study to determine the effect of BM-MSCs on hepatic fibrosis in patients with AC. The obtained results support the approval of this class of agents as a therapy for hepatic fibrosis. In conclusion, BM-MSCs therapy leads to the histological improvement of hepatic fibrosis, and so further prospective controlled studies are needed to be it accepted as a new strategy for antifibrosis therapy of AC.

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Conflicts of Interest. None to declare.

The statistical analysis of the entire data pertaining to efficacy (specifically primary and major secondary efficacy endpoints) and safety (specifically serious adverse events as defined in the federal guidelines) have been independently confirmed by a biostatistician who is not employed by a corporate entity.

The corresponding author had full access to all of the data and takes full responsibility for the veracity of the data and analysis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Laennec scoring system for staging fibrosis in liver biopsy specimens.

Table S2. Primer sequences for quantitative PCR.