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Transplantation With Autologous Bone Marrow-Derived Mesenchymal Stem Cells for Alcoholic Cirrhosis: Phase 2 Trial

Ki Tae Suk,[1*] Jung-Hwan Yoon,[2*] Moon Young Kim,[3] Chang Wook Kim,[4] Ja Kyung Kim,[5] Hana Park,[6] Seong Gyu Hwang,[6] Dong Joon Kim,[1] Byung Seok Lee,[7] Sae Hwan Lee,[8] Hong Soo Kim,[8] Jae Young Jang,[9] Chang-Hyeong Lee,[10] Byung Seok Kim,[10] Yoon Ok Jang,[3] Mee Yon Cho,[11] Eun Sun Jung,[12] Yong Man Kim,[13] Si Hyun Bae,[14] and Soon Koo Baik[3]

Bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation has been suggested as an effective therapy for liver cirrhosis. The efficacy and safety of autologous BM-MSC transplantation in the treatment of alcoholic cirrhosis were investigated. Seventy-two patients with baseline biopsy-proven alcoholic cirrhosis who had been alcohol-abstinent for more than 6 months underwent a multicenter, randomized, open-label, phase 2 trial. Patients were randomly assigned to three groups: one control group and two autologous BM-MSC groups that underwent either one-time or two-time hepatic arterial injections of 5×10^7 BM-MSCs 30 days after BM aspiration. A follow-up biopsy was performed 6 months after enrollment, and adverse events were monitored for 12 months. The primary endpoint was improvement in fibrosis quantification based on picrosirius red staining. The secondary endpoints included liver function tests, Child-Pugh score, and Model for End-stage Liver Disease score. Outcomes were analyzed by per-protocol analysis. In terms of fibrosis quantification (before versus after), the one-time and two-time BM-MSC groups were associated with 25% (19.5 ± 9.5% versus 14.5 ± 7.1%) and 37% (21.1 ± 8.9% versus 13.2 ± 6.7%) reductions in the proportion of collagen, respectively ($P < 0.001$). In the intergroup comparison, two-time BM-MSC transplantation in comparison with one-time BM-MSC transplantation was not associated with improved results in fibrosis quantification ($P > 0.05$). The Child-Pugh scores of both BM-MSC groups (one-time 7.6 ± 1.0 versus 6.3 ± 1.3 and two-time 7.8 ± 1.2 versus 6.8 ± 1.6) were also significantly improved following BM-MSC transplantation ($P < 0.05$). The proportion of patients with adverse events did not differ among the three groups. *Conclusion:* Autologous BM-MSC transplantation safely improved histologic fibrosis and liver function in patients with alcoholic cirrhosis. (HEPATOLOGY 2016; 00:000–000)

Liver cirrhosis is an advanced stage of liver fibrosis which presents with regenerative nodules surrounded by fibrous bands that develop in response to chronic liver injury.[1-3] Currently, the most effective therapy for advanced liver cirrhosis is liver transplantation.[4] However, this procedure is associated with several limitations, including the lack of donors, surgical complications, immunosuppression

Abbreviations: AC, alcoholic cirrhosis; AE, adverse event; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BM, bone marrow; BM-MSC, BM-derived MSC; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GGT, gamma-glutamyl transpeptidase; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; MSC, mesenchymal stem cell; PE, phycoerythrin.

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**These authors contributed equally to this work.*

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following transplantation, and high medical cost.[5,6] New therapeutic approaches such as bioartificial liver, stem cell therapy, and regenerative medicine are being investigated in an attempt to improve the prognosis of patients with liver cirrhosis.[7-9]

There has been increasing interest in the transplantation of bone marrow (BM) cells, hematopoietic stem cells, and mesenchymal stem cells (MSCs) as potential treatments for liver cirrhosis.[10-12] Even though the precise therapeutic mechanisms of stem cell treatments have not yet been elucidated, BM and hematopoietic stem cells have been shown to accelerate liver regeneration, reduce hepatic fibrosis, and restore liver function *in vivo*.[13-15] MSCs, more specifically, have the potential for self-renewal and differentiation into multiple cell lineages including the hepatocytes lineage, and more attention should be paid to the clinical applications of MSCs.[16,17] Some clinical studies have demonstrated the efficacy and feasibility of BM-derived MSC (BM-MSC) therapy in patients with chronic liver diseases, mostly viral cirrhosis.[18,19]

Although alcohol is one of the common causes of liver cirrhosis worldwide and alcoholic cirrhosis (AC) is responsible for a high rate of mortality,[20,21] limited resources have been invested into research on AC. Moreover, there are very few studies that explore the use of BM-MSCs in patients with AC. In our previous clinical trial involving 11 patients with AC, autologous BM-MSC transplantation induced a histological and quantitative improvement in hepatic fibrosis.[11] Based on the promising results of that pilot study, the efficacy and safety of autologous BM-MSC transplantation in the treatment of patients with AC were assessed in this phase 2 trial.

Materials and Methods

PATIENTS AND TRIAL DESIGN

From January 2013 to November 2015, a randomized open-label trial was prospectively conducted in 12 university-affiliated hospitals in Korea (clinicaltrials.gov; NCT01875081). Patients with biopsy-proven AC were recruited, and all patients discontinued alcohol intake for at least 6 months prior to participating in this study. The diagnosis of AC was made through imaging studies, history of alcohol intake, and pathologic examination of liver biopsy.[20] It was hypothesized that 6 months of abstinence from alcohol would reduce active inflammation and permit stable liver histology, effectively minimizing selection bias.[22] The eligibility criteria included Child-Pugh score B or C and age between 20 and 70 years. Patients meeting the following criteria were excluded: age >70 years; cirrhosis related to hepatitis A, B, C, and E viruses; human immunodeficiency virus infection; Child-Pugh class A; Model for End-Stage Liver Disease (MELD) >20; treating physician's decision not to enroll due to severe clinical condition of the

ARTICLE INFORMATION:

From the [1]Department of Internal Medicine, Hallym University College of Medicine, Chuncheon, South Korea; [2]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, South Korea; [3]Department of Internal Medicine, Wonju Severance Christian Hospital, Yonsei University, Wonju College of Medicine, Wonju, South Korea; [4]Department of Internal Medicine, Uijeongbu St Mary's Hospital College of Medicine, The Catholic University, Uijeongbu, South Korea; [5]Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University Health System, Yonsei University College of Medicine, Seoul, South Korea; [6]Department of Internal Medicine, Bundang CHA Medical Center, CHA University, Seongnam, South Korea; [7]Department of Internal Medicine, Chungnam National University College of Medicine, Daejeon, South Korea; [8]Department of Internal Medicine, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, Cheonan, South Korea; [9]Department of Internal Medicine, Soonchunhyang University College of Medicine, Seoul, South Korea; [10]Department of Internal Medicine, College of Medicine & Hospital, Catholic University of Daegu, Daegu, South Korea; [11]Department of Pathology, Wonju Severance Christian Hospital, Yonsei University, Wonju College of Medicine, Wonju, South Korea; [12]Department of Pathology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University, Seoul, South Korea; [13]Phamicell Co., Ltd., Sungnam, South Korea; [14]Department of Internal Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University, Seoul, South Korea.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Soon Koo Baik, M.D., Ph.D.
Department of Internal Medicine
Yonsei University Wonju College of Medicine
20 Ilsan-ro
Wonju 26426, South Korea
Tel: +82-33-741-1223
E-mail: baiksk@yonsei.ac.kr

or

Si Hyun Bae, M.D., Ph.D.
Division of Hepatology, Department of Internal Medicine
College of Medicine, The Catholic University of Korea
222 Banpo-daero
Seocho-gu, Seoul 137-041, South Korea
E-mail: baesh@catholic.ac.kr

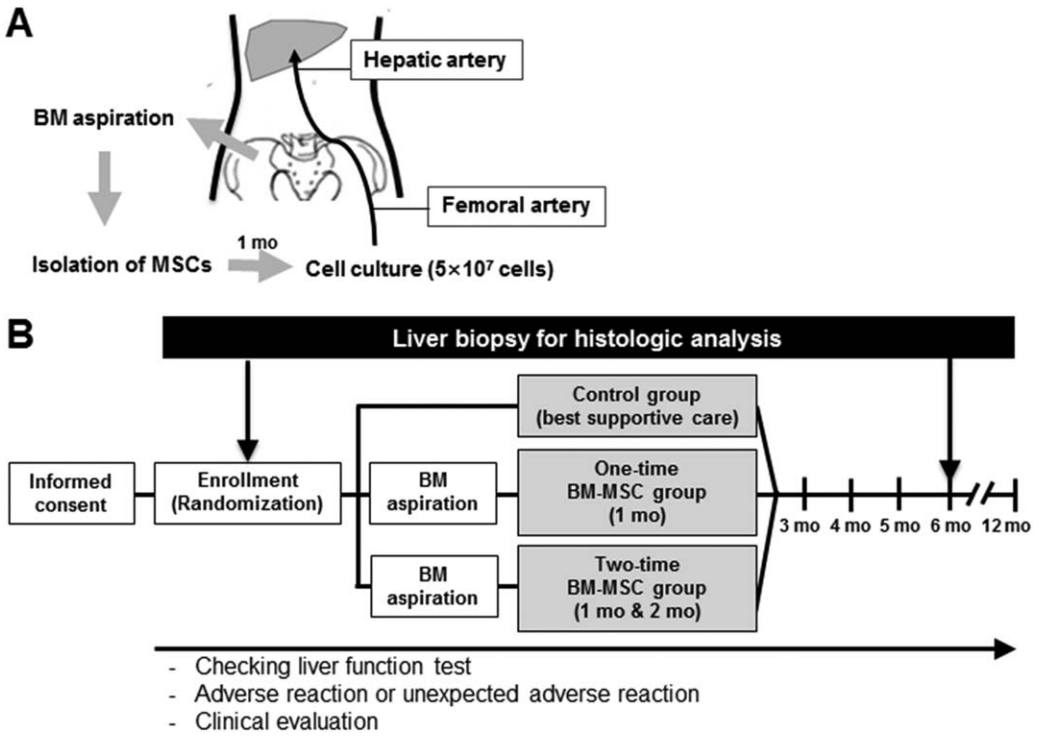


FIG. 1. Study design. (A) MSC isolation, amplification, and injection process. (B) Overall process of trial.

patient; inability to undergo hepatic angiography; sepsis; history of high-dose steroid or antibiotics; presence of liver tumor; or history of other cancer or pregnancy.

The institutional review board of all participating hospitals and the Korea Food and Drug Administration approved the protocol (clinical trial no. 27), and written informed consent to participate in this study was received from all participating patients. The study protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Following enrollment, BM aspiration for the isolation of BM-MSCs was performed in the two BM-MSC groups. Isolated BM-MSCs were cultured for 1 month and injected into the liver through the hepatic artery (Fig. 1A). All eligible participants were assigned randomly, in a 1:1:1 ratio, to the control group (no transplantation), the one-time BM-MSC group (hepatic arterial injection of 5×10^7 autologous BM-MSCs 1 month after BM aspiration), and the two-time BM-MSC group (hepatic arterial injection of 5×10^7 autologous BM-MSCs 1 and 2 months after BM aspiration). Random assignment was performed in SAS through the proc plan procedure with the stratified block randomization method, with patients processed on the basis of Child-Pugh scores B and C (Fig. 1B).

Baseline evaluations included family and alcohol history, abdominal ultrasound and computed tomographic scan, X-ray, electrocardiography, complete blood count, electrolytes, liver function test, viral markers, and Child-Pugh score. The MELD score was calculated as described.[23] Blood analysis was performed using standard methodologies. Serum biochemical parameters included aspartate transferase (AST), alanine transferase (ALT), albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), blood urea nitrogen, creatinine, international normalized ratio (INR), α-fetoprotein, carcinoembryonic antigen, prothrombin time, blood glucose, triglycerides, and total cholesterol. The levels of hepatitis A, B, C, E, and other virus markers were evaluated. Antinuclear antibody, antimitochondrial antibody, and anti-smooth muscle antibody tests were also performed.

BM ASPIRATION, ISOLATION, AND CELL CULTURE OF BM-MSCs

All manufacturing and product-testing procedures for the generation of clinical-grade autologous MSCs

(Livercellgram; Pharmicell Co., Ltd., Seongnam, South Korea) were performed in a manner consistent with good manufacturing practice and Ministry of Food and Drug Safety regulatory guidelines. Approximately 10-20 mL of BM was aspirated from the posterior iliac crest of patients under local anesthesia. BM mononuclear cells were isolated by density-gradient centrifugation (Histopaque-1077; Sigma-Aldrich, St. Louis, MO). Mononuclear cells were plated in 75-cm^2 flasks (Falcon, Franklin Lakes, NJ) with low-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco) and 20 g/mL gentamicin (Gibco) and cultured at 37°C in a 5% CO_2 atmosphere. After 5-7 days, nonadherent cells were removed by replacing the medium, and adherent cells were cultured for another 2-3 days. Colonized cells were detached with a trypsin/ethylene diamine tetraacetic acid solution (Gibco) and replated in 175-cm^2 flasks. When the cultures approached 70%-80% confluence, the cells were serially subcultured up to passage 4 or 5 for injection. For the second injection in the two-time BM-MSC group, some passage-1 cells were harvested during cell culture and cryopreserved in 10% dimethyl sulfoxide (Sigma-Aldrich) and 90% FBS. These cells were thawed according to the injection schedule and subcultured up to passage 4 or 5.

VIABILITY, IMMUNOPHENOTYPE, AND DIFFERENTIAL POTENTIAL OF LIVERCELLGRAM

The viability of the BM-MSCs (Livercellgram) was assessed by trypan blue exclusion assay.

The immunophenotypes of Livercellgram (CD14, CD34, CD45, CD73, and CD105) were analyzed using the final harvested cells on the day of injection. For immunophenotype analysis, MSCs were stained with the following antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): anti-CD14-FITC, anti-CD34-FITC, anti-CD45-FITC, anti-CD73-PE, and anti-CD105-PE (BD Biosciences, San Jose, CA). Briefly, 5×10^5 cells were resuspended in 0.2 mL of phosphate-buffered saline and incubated with antibodies for 20 minutes at room temperature. FITC-conjugated or PE-conjugated mouse immunoglobulin Gs were used as isotype controls. The intensity of the fluorescence of the cells was evaluated by flow cytometry (Navios; Beckman Coulter, Fullerton, CA).

In addition, the differentiation potential of BM-MSCs to osteoblasts and adipocytes was tested as described.[11] Osteogenic differentiation was determined by plating the cells at 2×10^4 cells/cm^2 in six-well plates and culturing them for 2-3 weeks in osteogenic medium, consisting of low-glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, and 0.2 mM ascorbic acid (Sigma-Aldrich).[24] For adipogenic differentiation, BM-MSCs were plated at 2×10^4 cells/cm^2 in six-well plates and cultured for 1 week. Differentiation was subsequently induced with an adipogenic medium consisting of 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/mL insulin, and 100 μM indomethacin in high-glucose Dulbecco's modified Eagle's medium for an additional 3 weeks. Differentiated cells were fixed in 4% paraformaldehyde for 10 minutes and stained with fresh oil red-O solution (Sigma-Aldrich) for the detection of lipid droplets.[11]

AUTOLOGOUS BM-MSC INJECTION

On the day of the injection, MSCs were harvested using trypsin/ethylene diamine tetraacetic acid, washed twice with phosphate-buffered saline and once with Plasma Solution A Inj (Multiple Electrolytes Injection, Type 1, USP; CJ HealthCare, Seoul, Korea) and resuspended at a final concentration of 5×10^6 cells/mL in 10 mL of Plasma Solution A Inj (total 5×10^7 cells).

For each injection, 5×10^7 cells in 10 mL of Plasma Solution A Inj were injected at day 30 ($\pm$7) for the one-time BM-MSC group and at days 30 ($\pm$7) and 60 ($\pm$7) for the two-time BM-MSC group. Continuous monitoring was performed in addition to the injection procedure, and any hemodynamic changes were recorded.

LIVER BIOPSY AND HISTOLOGY

Paired liver biopsies were performed at baseline and 6 months after enrollment. Liver biopsy specimens $\geq$15 mm in length and $\geq$1.2 mm in width were used for this study. Five-micrometer-thick sections of paraffin-embedded liver biopsy samples were prepared and stained with hematoxylin and eosin, Masson's trichrome, and picrosirius red.

Picrosirius red staining was performed for the quantification of the total amount of collagen. Paraffin-embedded liver biopsy specimens were deparaffinized,

rehydrated with distilled water, and stained with a picrosirius red staining kit (Polysciences, Warrington, PA) according to the manufacturer's instruction. The entire biopsy core was analyzed for fibrosis quantification. The amount of collagen was estimated from the collagen proportionate area, expressed as the percentage of the total area that was positive for picrosirius red stain on microscopy (BX51; Olympus, Tokyo, Japan) using a computerized image-analysis system (IMT i-solution, Vancouver, BC, Canada). Fibrosis quantification was performed by one operator (Y.O.J.) who was blinded to the histological semiquantitative and clinical data. In addition, the fibrosis grade was evaluated with the Laennec fibrosis scoring system by two liver pathologists who were blinded to the clinical data of the patients (Supporting Table S1).[25] To estimate the chance-adjusted agreement, the kappa value was calculated for the interobserver agreement ($\kappa = 0.947$). When the two pathologists disagreed, they reached a consensus on the fibrosis score by discussion.

ENDPOINTS AND FOLLOW-UP

The primary outcome was improvement in fibrosis quantification based on picrosirius red staining. The secondary endpoints included liver function test, Child-Pugh score, and MELD score.

After enrollment, patients were followed up according to the study design (Fig. 1B). All patients were treated in accordance with the treatment guideline for AC, which indicates supportive therapy, nutrition therapy, thiamine, hepatotonics, and nonselective beta-blockers.[2]

All patients who received autologous BM-MSC injections were included in the safety evaluation. Adverse events (AEs) were monitored every week by a telephone call to the patients and their family and during the patients' monthly visits to the hospital. AEs were classified according to the Common Terminology Criteria for AEs, 3.0, and assessed from the time at which patients provided written informed consent to the end of the study or dropout. Multiple occurrences of specific events were counted once per patient, and severe AEs were summarized. Any evidence of alcohol consumption would result in the subject being withdrawn from the study.

STATISTICAL ANALYSIS

Sample size for this study was determined on the basis of the primary endpoint of histologic improvement. For the phase 2 clinical trial with a randomization ratio of 1:1:1 among the three groups, a significance level of 0.05, standard deviation of 0.7, and power value of 90% were selected based on a previous report.[11] When the rate of potential loss to follow-up was established at 20%, it was found that a total of 72 patients were needed to complete this study.

As this study required double liver biopsy and consequent paired analysis to compare the effects of BM-MSCs, the outcomes were analyzed by per-protocol analysis; i.e., only those patients who remained in the protocol and thus had two biopsies were analyzed. Quantitative data were expressed as mean ± standard deviation unless otherwise stated. Comparisons were made using analysis of variance, Kruskal-Wallis test, general linear model analysis (repeated regression), paired sample t test, or independent-sample t test for continuous variables. The chi-squared test or Fisher's exact test was used for the comparison of groups. Data were analyzed with statistical software (SPSS, version 13.0; SPSS, Inc., Chicago, IL) and GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). For all tests, $P < 0.05$ was considered significant.

Results

PATIENTS

During the study period, 81 patients were screened. A total of 72 eligible participants were assigned randomly to the three groups. Among these patients, 17 were excluded after randomization and 55 (18 in the control group, 18 in the one-time autologous BM-MSC group, and 19 in the two-time autologous BM-MSC group) completed the study with follow-up liver biopsy (Fig. 2). Of the 17 excluded patients, four were excluded due to alcohol. Two patients in the control group spontaneously admitted alcohol drinking and withdrew informed consent. Two patients in the two-time BM-MSC group drank alcohol after randomization and were excluded from further study. Following the second liver biopsy, five patients were excluded because of follow-up loss (n = 3) and dropout (n = 2). Therefore, data from 16 patients in the control group, 17 in the one-time autologous BM-MSC group, and 17 in the two-time autologous BM-MSC group were considered for the evaluation of secondary endpoints. Sixty-eight of the randomized patients were included in the safety analysis population. None of the

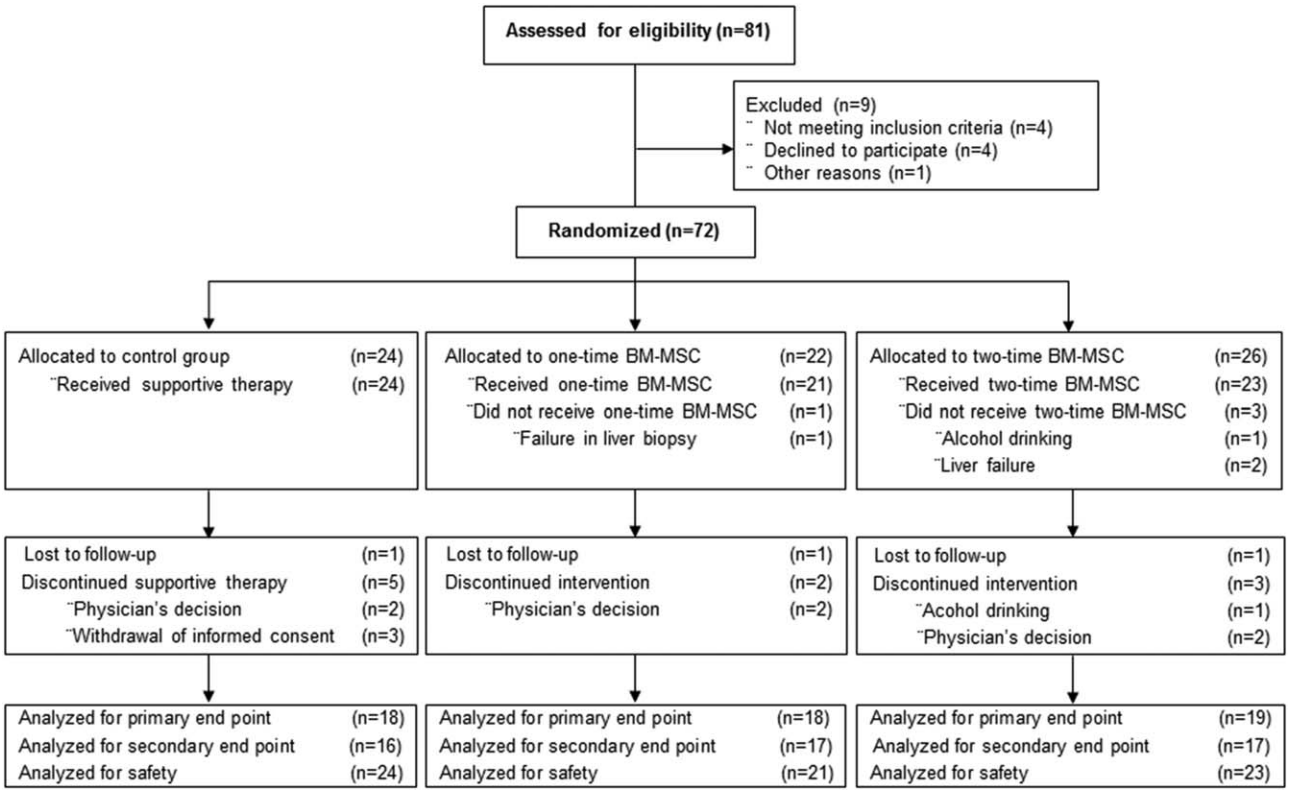


FIG. 2. CONSORT diagram.

differences in baseline characteristics between the three study groups were statistically significant (Table 1).

VIABILITY, IMMUNOPHENOTYPE, AND DIFFERENTIATION POTENTIAL OF BM-MSCs

The mean viability percentage of the BM-MSCs (Livercellgram) as assessed by trypan blue exclusion assay was 88.2 ± 6.1% for the one-time BM-MSC group and 87.5 ± 6.3% (first injection) and 86.2 ± 6.8% (second injection) for the two-time BM-MSC group (Fig. 3A; Supporting Tables S2-S4). The criteria for the clinical use of MSCs were viability >70% and the absence of microbial contamination (bacteria, fungus, viruses, or mycoplasma) when tested 3-4 days before administration.

In the immunophenotyping evaluation, flow-cytometric analysis demonstrated that both groups of BM-MSCs were positive for the MSC markers CD73 and CD105 (>85% of cells positive) and negative for hematopoietic cell markers such as CD14, CD34, and CD45 (<3% of cells positive) (Fig. 3A; Supporting Tables S2-S4).[11]

At the end of the second passage, BM-MSCs had successfully differentiated into osteoblasts and adipocytes as evidenced by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium staining for alkaline phosphatase activity and oil red-O staining for lipid droplets (Fig. 3B).

PRIMARY OUTCOME

In the fibrosis quantification (before versus after), one-time and two-time BM-MSC groups were associated with 25% (19.49 ± 9.48% versus 14.51 ± 7.05%) and 37% (21.05 ± 8.94% versus 13.22 ± 6.70%) reductions in the collagen proportion area ($P < 0.001$) following BM-MSC therapy, respectively. The control group was not associated with any significant changes in fibrosis quantification (17.50 ± 9.51% versus 17.46 ± 9.04%) (Fig. 4 and Table 2). In the intergroup comparison, significant differences between the control and BM-MSC groups were observed in terms of fibrosis quantification (Fig. 4B). However, no significant

TABLE 1. Baseline Characteristics of Patients

Variables	Control Group (n = 18)	One-Time BM-MSC Group (n = 18)	Two-Time BM-MSC Group (n = 19)	P
Male (n, %)	17 (94)	15 (83)	17 (89)	NS
Age*	53.7 (8.2)	53.1 (8.7)	54.4 (7.9)	NS
Functional analysis				
Child-Pugh score*	8.1 (1.3)	7.6 (1.0)	7.8 (1.2)	NS
MELD score*	7.1 (4.2)	4.5 (3.4)	4.5 (3.9)	NS
Histologic analysis				
Laennec score*	5.3 (1.2)	5.3 (0.7)	5.7 (0.5)	NS
Picrosirius red staining* (%)	17.5 (9.5)	19.5 (9.5)	21.1 (8.9)	NS
Biochemical analysis				
AST* (IU/L)	43 (12)	48 (38)	40 (20)	NS
ALT* (IU/L)	29 (13)	29 (19)	21 (10)	NS
Albumin* (g/dL)	3.4 (0.6)	3.7 (0.7)	3.6 (0.8)	NS
Bilirubin* (mg/dL)	2.8 (2.4)	1.7 (1.1)	1.7 (1.4)	NS
ALP* (IU/L)	126 (62)	120 (51)	135 (82)	NS
GGT* (IU/L)	71 (74)	50 (32)	83 (76)	NS
INR	1.39 (0.24)	1.26 (0.16)	1.27 (0.20)	NS

*Continuous variables are expressed as mean values (standard deviation).
Abbreviation: NS, not significant

differences could be detected between the one-time BM-MSC group and the two-time BM-MSC group ($P = 0.329$).

These results were further confirmed by the Laennec fibrosis score (Supporting Fig. S1). According to the Laennec fibrosis score, histological improvements were observed in 27% of the control group, 61% of the one-time BM-MSC group, and 37% of the two-time BM-MSCs group.

SECONDARY OUTCOMES

Child-Pugh scores, which evaluate overall liver function, significantly improved in the one-time BM-MSC group (7.6 ± 1.0 versus 6.3 ± 1.3; $P = 0.035$) and the two-time BM-MSC group (7.8 ± 1.2 versus 6.8 ± 1.6; $P = 0.003$) (Fig. 5A and Table 2). The control group was not associated with changes in Child-Pugh score (8.1 ± 1.3 versus 7.4 ± 1.5; $P > 0.05$). There was a significant difference in the change of Child-Pugh scores between the control group and the one-time BM-MSC group ($P = 0.014$).

In contrast, no significant changes could be detected in the MELD scores of the control group (7.1 ± 4.2 versus 6.0 ± 3.4), the one-time BM-MSC group (4.5 ± 3.4 versus 4.2 ± 2.5), and the two-time BM-MSC group (4.5 ± 3.9 versus 5.4 ± 3.7) ($P > 0.05$) (Fig. 5B and Table 2).

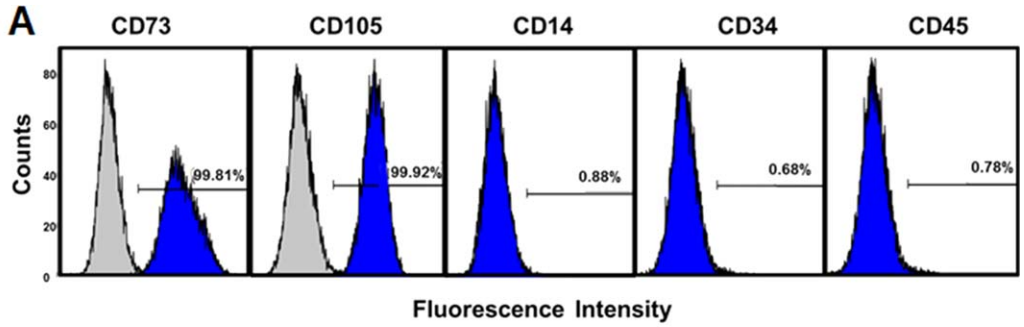
Changes in other relevant laboratory parameters (AST, ALT, albumin, bilirubin, ALP, GGT, and INR) following BM-MSC therapies are presented in Table 3. In the one-time BM-MSC group, improvements in AST, ALT, albumin, bilirubin, GGT, and INR did not reach statistical significance. The level of ALP was significantly decreased in the one-time BM-MSC group and the two-time BM-MSC group.

SAFETY

Of the total sample of 72 patients, 24 in the control group and 44 who received autologous BM-MSC transplantation were monitored regularly for any sign of possible AEs related to stem cell therapy such as fever, hypersensitivity reactions, and acute rejection fever. No difference in the incidence of AEs (total and serious) was observed among the three groups. One instance of therapy-related AE (fever) was observed in the two-time BM-MSC group. Finally, no evidence of development of BM-MSC-related tumors was found during the follow-up period (Table 4).

Discussion

Based on previous *in vivo* and *in vitro* studies using MSCs, MSC transplantation has been identified as a promising approach for liver regeneration and associated with several advantages including early acquisition, strong capability for differentiation, low immunogenicity, and immunomodulatory properties.[26-28] However, some issues remain to be explored, including the optimal timing of injection, the optimal type of transfused MSCs, the most effective number of MSCs



Study group		Viability[b]	CD73[b]	CD105[b]	CD14[b]	CD34[b]	CD45[b]
One-time BM-MSC group (n=18)	First[a]	88.2 (6.1)	98.78 (1.96)	97.99 (4.05)	0.88 (0.37)	0.77 (0.23)	0.71 (0.29)
Two-time BM-MSC group (n=19)	First[a]	87.5 (6.3)	99.23 (1.84)	98.48 (3.06)	0.78 (0.25)	0.78 (0.11)	0.79 (0.18)
	Second[a]	86.2 (6.8)	99.23 (1.22)	98.74 (2.50)	0.82 (0.34)	0.72 (0.20)	0.64 (0.21)

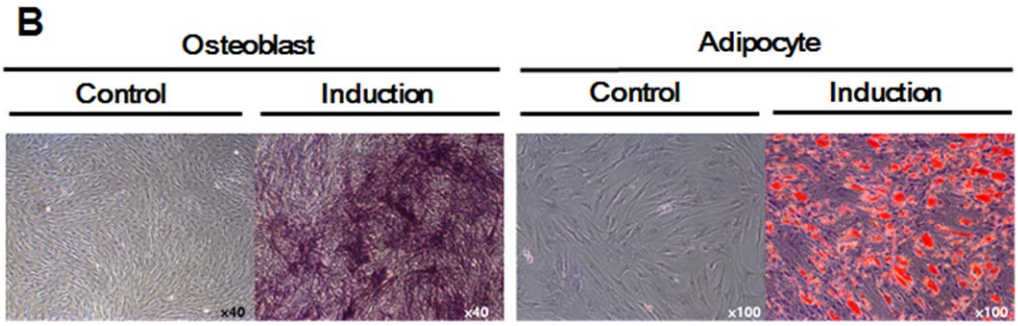


FIG. 3. Immunophenotypes and differentiation potential of the MSCs. (A) Expression of cell-surface antigens (CD73, CD105, CD14, CD34, and CD45) was evaluated by flow cytometry. Representative histograms for the positive populations (above) and mean values of each antigen with standard deviations (below). (B) (Left) BM-MSCs stained positively for endogenous ALP activity, indicating osteogenic differentiation in osteogenic medium, or stained negatively in control medium. (Right) BM-MSCs stained positively for lipid droplets, indicating adipogenic differentiation in adipogenic medium, or stained negatively in control medium (×200). [a]Continuous variables are expressed as mean values (standard deviation). [b]Percentage.

injected, and the best route of administration. Other concerns include the fibrosis-inducing and tumor-promoting capacity of MSCs.[29] The current study, a randomized phase 2 trial following a previous study, demonstrated the efficacy and safety of autologous BM-MSC transplantation (one-time or two-time hepatic arterial injection of 5×10^7 BM-MSCs) for patients with AC.

Several previous studies using MSCs were limited in that virally infected patients were enrolled, which may have resulted in selection bias caused by heterogeneity of antiviral therapy and differences in viral load or immune-stage among patients.[18,30,31] Moreover, current antiviral therapies are effective at treating hepatic fibrosis in patients with virus-related cirrhosis. If patients remain alcohol-abstinent before and during the study period, AC is a suitable target disease for which the efficacy and feasibility of autologous BM-MSC can be evaluated without the aforementioned sources of bias. Given this perspective, the present study has significance because it suggests a novel strategy for the treatment of AC.

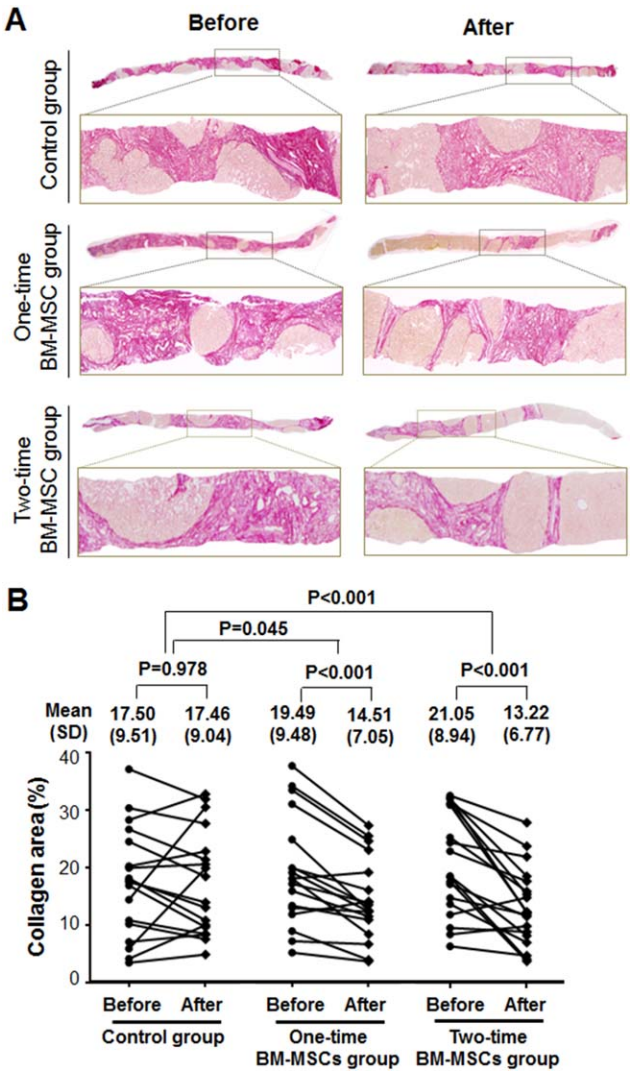


FIG. 4. Quantitative histological analysis of collagen area. Histological analysis was performed by picrosirius red staining and evaluated with an image analysis program. (A,B) Picrosirius red staining (×12.5 [above] and ×40 [below]) of a section from a liver biopsy specimen showed a change in the collagen proportion (stained red) before and after transplantation. Data are mean and standard deviation. Abbreviation: SD, standard deviation.

In the present trial, two BM-MSC groups with one-time or two-time injections were associated with 25% and 37% reductions in the collagen proportionate area, respectively. This finding is consistent with our previous study which demonstrated that autologous MSC (5×10^7 cells) transplantation through the hepatic artery induced histologic improvement in 6 of 11 patients (54.5%).[11] Possible mechanisms that underlie fibrosis regression-causing properties of MSCs include hepatocyte-like cell differentiation and the antifibrotic activities of MSCs.[32,33] In addition, MSCs express trophic factors such as growth factors, cytokines, and chemokines, which are associated not only with anti-inflammatory, apoptotic, and antifibrosis activities but also with angiogenesis and cell regeneration.[34] However, the present study did not reveal improved efficacy of two-time BM-MSC transplantation compared with one-time BM-MSC transplantation. With regard to the time after alcohol consumption, improvements were clearly BM-MSC transplant-related and not time-related because we enrolled patients who discontinued alcohol intake for at least 6 months prior to participating in the study. In summary, BM-MSC therapy leads to a histologic improvement in patients with AC, and one-time injection of BM-MSCs appears to be sufficient for the induction of regression of fibrosis.

The Child-Pugh score was significantly improved in the BM-MSC groups following the treatment (Fig. 5A). This finding was consistent with previous studies that similarly demonstrated improvements in the Child-Pugh score.[11,35] Although the changes in the level of albumin and bilirubin were statistically insignificant, a mild improvement in these values along with consequent clinical effect on ascites and encephalopathy may have affected the observed reduction of Child-Pugh score. Under the assumption that the Child-Pugh score includes clinical indices such as ascites and hepatic encephalopathy, it may be claimed that BM-MSC transplantation in patients with AC was demonstrated to efficiently induce histological and clinical improvements. Taken together, BM-MSC transplantation can be expected to become a new treatment for liver fibrosis in the near future.

The MELD uses the patient's serum bilirubin, serum creatinine, and INR to predict survival in end-stage liver disease; the score does not factor in the clinical sign.[23,36] Our result was not in accordance with those of previous studies which have reported an improved MELD score following MSC transplantation.[18,35,37-39] Compared to previous studies of patients with high MELD scores (mean score 9-10), our trial included patients with relatively low MELD scores (5.2 ± 3.4). This suggests that previous reports included more severe patients with virus-related cirrhosis instead of AC. Because this study recruited stable AC patients with relatively low MELD scores due to alcohol abstinence over 6 months, it can be surmised that the improvement in MELD score was not dramatic enough to show statistical significance.

TABLE 2. Primary and Secondary Outcomes

Variable	Control Group		One-Time BM-MSC Group		Two-Time BM-MSC Group	
	Before	After	Before	After	Before	After
Picrosirius red staining (%)*						
PPA	17.5 (9.5)	17.5 (9.0)	19.5 (9.5)	14.5 (7.1)†	21.1 (8.9)	13.2 (6.8)†
ITT	18.7 (9.8)	17.5 (8.8)	18.7 (9.6)	14.5 (6.9)†	19.6 (9.4)	13.2 (6.5)†
Child-Pugh score*						
PPA	8.1 (1.3)	7.4 (1.5)	7.6 (1.0)	6.3 (1.3)†	7.8 (1.2)	6.8 (1.6)†
ITT	8.1 (1.3)	7.4 (1.5)	7.7 (0.9)	6.3 (1.3)†	7.9 (1.2)	6.8 (1.6)†
MELD score*						
PPA	7.1 (4.2)	6.0 (3.4)	4.5 (3.4)	4.2 (2.5)	4.5 (3.9)	5.4 (3.7)
ITT	7.2 (4.1)	6.0 (3.4)	4.9 (4.2)	4.2 (2.5)	5.8 (4.0)	5.4 (3.7)

*Continuous variables are expressed as mean values (standard deviation).
†$P < 0.05$ comparing data between after and before.
Abbreviations: ITT, intention-to-treat; PPA, per-protocol analysis.

Recently, clinical trials using MSCs have demonstrated that MSC transplantation improves biochemical indices in patients with liver failure.[18,30,31,37] In this study, the level of ALP was associated with a significant reduction. Improving trends were observed for other serologic markers. As mentioned previously, the stable conditions of patients with minimal abnormality in biochemical indices might be the cause of insignificance in the improvements of AST, ALT, bilirubin, and GGT in our study.

Although our previous preliminary trial with 11 patients led to some severe AEs including gastroenteritis, splenic infarction, and cerebral infarction, these AEs were not related to BM-MSC transplantation and did not occur in this phase 2 study. Only one patient was affected by fever following BM-MSC transplantation; this sign disappeared after admission. Serious AEs were not observed in this study, and AEs did not stop the study. Further, when considering that there were no differences in the incidence of AEs among the three groups, it can be concluded that autologous BM-MSC treatment is safe and well tolerated in patients with AC.

When conducting a clinical study in AC patients, a major concern for clinicians is the risk of bleeding following an invasive procedure such as liver biopsy and BM aspiration. One patient presented with biopsy-related hemoperitoneum before enrolling in the study. This incident was attributed to a technical problem. For patients with prolonged INR or severe ascites, transjugular liver biopsy or delayed liver biopsy might be recommended. The quality of the BM-MSCs is another important issue for the evaluation of efficacy. The high quality of the BM-MSCs used in this study was unambiguously demonstrated by flow cytometry; the cells were found to be positive for MSC markers (found in >85% of the cells) and negative for hematopoietic cell markers (found in <3% of the cells).

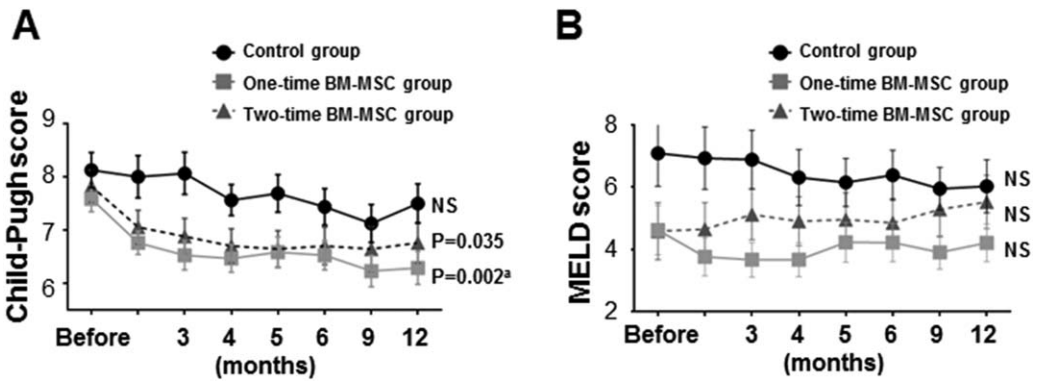


FIG. 5. Serial changes in Child-Pugh score (A) and MELD score (B). [a]$P < 0.05$ comparing data between the control group and the one-time BM-MSC group in general linear model analysis (repeated regression).

TABLE 3. Changes in Liver Function Tests

	Control Group (n = 16)		One-Time BM-MSC Group (n = 17)		Two-Time BM-MSC Group (n = 17)	
Variable	Before	After	Before	After	Before	After
AST* (IU/L)	43 (12)	42 (19)	48 (38)	39 (16)	40 (20)	44 (31)
ALT* (IU/L)	29 (13)	27 (17)	29 (19)	24 (10)	21 (10)	23 (13)
Albumin* (g/dL)	3.4 (0.6)	3.5 (0.7)	3.7 (0.7)	3.8 (0.6)	3.6 (0.8)	3.6 (0.7)
Bilirubin* (mg/dL)	2.8 (2.4)	1.9 (1.0)	1.7 (1.1)	1.6 (1.0)	1.7 (1.4)	2.2 (1.8)
ALP* (IU/L)	126 (62)	123 (59)	120 (51)	100 (33)†	135 (82)	114 (48)†
GGT* (IU/L)	71 (74)	75 (59)	50 (32)	64 (57)	83 (76)	81 (66)
INR*	1.39 (0.24)	1.30 (0.15)	1.26 (0.16)	1.24 (0.12)	1.27 (0.21)	1.27 (0.16)

*Continuous variables are expressed as mean values (standard deviation).
†$P < 0.05$ comparing data between after and before.

Although the efficacy and safety of autologous BM-MSCs were conclusively determined, one limitation of this study is that the precise mechanism for fibrosis reduction through BM-MSC transplantation was not elucidated. The hypothesis that MSCs transdifferentiate into hepatocytes remains to be confirmed in a further study; <1% of the total liver mass was observed to be made up of transdifferentiated MSCs in an animal transplantation model.[40] Although tracing injected BM-MSCs in the body is difficult, such a technique would be necessary to understand the mechanism through which BM-MSCs bring about fibrosis reduction and functional improvement. Considering the poor prognosis of AC patients and the limited treatment options for AC, further research on the therapeutic roles of BM-MSCs is highly merited. A phase 3 study assessing the efficacy, effectiveness, and safety of BM-MSCs is now under preparation in Korea. It can be expected that the precise mechanisms underlying the application of BM-MSCs in humans will be elucidated through further study.

TABLE 4. Adverse Events

	Control Group (n = 24)		One-Time BM-MSC Group (n = 21)		Two-Time BM-MSC Group (n = 23)		
Variable, n (%)	Total AE	Serious AE	Total AE	Serious AE	Total AE	Serious AE	*P**
Abdominal pain	3 (13)		2 (10)		5 (22)	2 (9)	NS
Anemia					2 (9)		NS
Bruise or epistaxis	1 (4)				4 (17)	2 (9)	NS
Chest tightness					1 (4)		NS
Constipation					2 (9)		NS
Dysuria			1 (5)		1 (4)		NS
Edema or ascites	2 (8)		2 (10)	1 (5)	4 (17)		NS
Elevated liver enzyme	1 (4)				1 (4)	1 (4)	NS
Fever			1 (5)		3 (13)	1 (4)†	NS
General weakness	3 (13)		3 (15)	1 (5)	2 (9)		NS
HCC	3 (13)	1 (4)					NS
Headache			1 (5)				NS
Hematoma					2 (9)		NS
Hepatic encephalopathy	3 (13)	3 (13)					NS
Infection	2 (8)		4 (20)		5 (22)	1 (4)	NS
Insomnia	1 (4)		3 (15)		1 (4)		NS
Itching sensation					1 (4)		NS
Muscle cramp			4 (20)				NS
PUD	1 (4)		2 (10)		1 (4)		NS
SBP			2 (10)	1 (5)	1 (4)		NS
Skin rash			1 (5)		1 (4)		NS
Variceal bleeding	4 (17)	4 (17)	3 (15)	1 (5)	5 (22)	3 (13)	NS
Total	24	8	30	5	43	9	

**P* value for the difference among control, one-time BM-MSC, and two-time B-MSC groups.
†BM-MSC transplantation-related AE.
Abbreviations: HCC, hepatocellular carcinoma; NS, not significant; PUD, peptic ulcer disease; SBP, spontaneous bacterial peritonitis.

In summary, autologous BM-MSC transplantation in patients with AC was found to be associated with histologic reduction of hepatic fibrosis, followed by functional and clinical improvement. Consequently, BM-MSC treatment may serve as a potential supplementary therapeutic tool to improve liver function in patients with AC. The findings of this study underscore the importance of future phase 3 studies to investigate the effectiveness of autologous BM-MSCs in improving the prognosis and survival of AC patients.

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Author names in bold designate shared co-first authorship.

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