Original Paper

Mesenchymal Stem Cells with Enhanced *Bcl-2* Expression Promote Liver Recovery in a Rat Model of Hepatic Cirrhosis

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Key Words

Mesenchymal Stem Cells • *Bcl-2* gene • Hepatic cirrhosis • CCl₄ treated rats

Abstract

Background/Aims: Mesenchymal stem cell (MSC) transplantation has emerged as an option for the treatment of chronic hepatic cirrhosis, while its therapeutic efficacy could be improved. The *bcl-2* gene is anti-apoptotic and can help cell survival and proliferation. Therefore, we explored whether transplanted MSCs with enhanced *bcl-2* expression may be beneficial in the treatment of experimental cirrhosis in rats. **Methods:** MSCs were isolated from rat bone marrow, expanded *in vitro* and transfected with adeno-associated virus (AAV) engineered the *bcl-2* gene (AAV-*bcl-2*). Rats with cirrhosis induced by carbon tetrachloride (CCl₄) were treated with AAV-*bcl-2* infected BMSCs-AAV-*bcl-2*, with the cells traced *in vivo* post transplantation. Liver pathology and function were evaluated 7, 14, 21, and 28 days post transplantation, respectively. **Results:** On day 7 post transplantation, the infused AAV-*bcl-2* had integrated into the hepatocyte-like cells (HLCs) that expressed albumin (ALB), Cytokeratin 18 (CK18), and hepatocytes nuclear factor 4a (HNF4a). On day 28 post transplantation, rats in the cirrhosis + BMSCs-AAV-*bcl-2* group showed the most dense HLCs, highest mRNA and protein levels of ALB, CK18, and HNF4a, compared to the other groups. Their liver function recovered most rapidly in 4 week observation, while histological sign of cirrhosis remained at the end of this period. **Conclusion:** BMSCs over expressing *bcl-2* gene showed better survival, and enhanced the differentiation into hepatocytes-like cells, and appeared to promote the recovery of liver function in rats with experimental cirrhosis.

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Introduction

Cirrhosis is the end stage of chronic liver disease and characterized by distortion of hepatic cytoarchitecture, formation of regenerative nodules, aberrant angiogenesis and shunts, leading to the loss of liver function with increased probability of hepatocellular

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carcinoma [1-3]. Currently, orthotopic liver transplantation (OLT) remains the most effective treatment for cirrhosis, which, however, is limited in clinical practice because of donor shortage, surgical complications, immunological rejection and high medical costs [4].

Thus, alternative options have been considered for the treatment of cirrhosis in the past decades, with stem cell transplantation appeared promising. In 2000, Theise et al. reported the presence of Y chromosome-positive hepatocytes in autopsied women who had received therapeutic bone marrow transplantation from male donors, suggesting that bone marrow cells may be useful for stem cell therapy [5, 6]. Indeed, bone marrow-derived mesenchymal stem cells (BMSCs) have been shown to differentiate into hepatocyte-like cells (HLCs) *in vitro* and *in vivo* [7-10]. MSCs have advantages including easy acquisition, strong proliferative capacities and *ex vivo* expansion. Moreover, they have immune-modulatory properties, are able to migrate into damaged tissues, and secrete trophic factors such as growth factors and cytokines, which promote the regeneration of injured liver tissue [11-13].

Despite the major advancements mentioned above for MSCs cell therapy, several critical issues and concerns remain. Several lines of evidence suggest that MSCs might promote tumor growth *in vivo* [14, 15], although some other studies report some anti-tumorigenic effects of MSCs [16-19]. The surviving of engrafted human MSCs may be low in normal and acutely injured livers [18]. Thus, researchers have applied genetic modification of MSCs to improve cell survival, in order to counteract liver fibrosis [20], improve the therapeutic effect [21], or attenuate hepatocellular carcinoma [22].

In the present study, we used adeno-associated virus (AAV) as a vector for overexpressing the *bcl-2* gene in BMSCs. Compared to other virus vectors, adeno-associated virus vector (AVV) has a number of advantages as a gene delivery system, including the lack of any associated disease with a wild type virus [23, 24], possible integration of the gene into the host genome [25, 26], and ability to transduce non-dividing cells [27]. Here *bcl-2* was chosen as it is an apoptosis repressor gene. Overexpression of *bcl-2* has been shown to protect neuronal cells from death induced by a variety of stimuli, such as trophic factor deprivation, free radical generation and glutamate excitotoxicity [28-31]. Moreover, *bcl-2* may play a key role in the progression of liver fibrosis as activated human hepatic stellate cells are resistant to most pro-apoptotic stimuli due to *bcl-2* overexpression [32-34]. Using histological, biochemical and serological measurements, we demonstrate here that administration of BMSCs with *bcl-2* overexpression provides enhanced therapeutic effects in a rat model of liver cirrhosis.

Materials and Methods

Animals and experimental design

Male Sprague-Dawley rats, at one-month of age in the beginning of the experiment, were purchased from the animal facility of the Second Affiliated Hospital of Harbin Medical University. Animals were housed at constant temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$), with a 12/12 hr light/dark illumination cycle and free accesses to food and water. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals set up by the Harbin Medical University Administrative Panel on Laboratory Animal Care.

Carbon tetrachloride (CCl_4) is commonly used to induce experimental liver cirrhosis in rodents, which recapitulates the histological, biochemical, cellular and molecular changes associated with human liver fibrosis [35]. Rats were separated into two groups, the experimental ($n = 85$) and control ($n = 5$) groups, with the former treated with CCl_4 (i.p., 50% in sterilized saline) at 0.5 ml/100 g once a day in 3 successive days. Control rats were injected same volume of sterilized saline. Five CCl_4 treated and the control rats were examined at the end of experiment to evaluate the establishment of the model. The remaining 80 rats in the experimental group were randomly divided into the following four sub-groups (each with 20 rats): Cirrhosis + Saline (0.3 ml), Cirrhosis + AVV-*bcl-2* (diluted in 0.3 ml saline, containing 2×10^{11} virus particles), Cirrhosis + BMSCs (10^7 cells/ml, diluted in 0.3 ml saline), and Cirrhosis + BMSCs-AVV-*bcl-2* (10^7 cells/ml, diluted in 0.3 ml saline, containing $1-4 \times 10^{11}$ virus particles, averaged around 2×10^{11}), to assess the effect of cell therapy at day 7, 14, 21, or 28, respectively, after cell transplant.

BMSCs isolation and expansion

BMSCs were obtained from 4-week old rats. Bone marrow was acquired by flushing the femoral mid-shafts with a syringe. After three centrifugations, cells were seeded (5×10^5 cell/ml) in a 25 cm² culture flask with DMEM/F12 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL) and penicillin (10,000 unit, pH 7.4). Cultures were maintained at 37 °C with 5% CO₂ and the medium was changed every 3 days to remove non-adherent cells. When the cells reached 80% confluence, BMSCs were digested with 0.25% trypsin and 0.1% EDTA and subsequently passaged.

BMSCs from the second passage (P2) were used for phenotyping analysis. The cells were incubated for 30 min with FITC or PE-labeled monoclonal antibodies against CD29, CD34, CD44, and CD45 (BD Bioscience, San Jose, CA) and analyzed with flow cytometer (BD FACScalibur, San Jose, CA).

Recombinant AVV and infection of BMSCs

A recombinant AVV expressing human *bcl-2* gene (NM_000633) was purchased from Genechem Biotech Inc. (Shanghai, China). The recombinant AAV virions were purified by the company. Also, the ability of this recombinant construct to express *bcl-2* was assessed by the provider.

The third-passage BMSCs were plated at a density of 10^5 cells per well in six-well culture plates. Twenty-four hours later, AAV-*bcl-2* (MOI=1) virion was added and incubated for 4 hr to infect the cells. The cultures were then washed with 10 mM phosphate-buffered saline (PBS, pH = 7.4) and the culture medium replaced. Parallel control cultures of BMSCs (AAV-BMSCs) were trans-infected with blank vector. Effective trans-infection was confirmed under a microscope (Olympus, Tokyo, Japan) which identified all BMSCs-AAV-*bcl-2* cells in red color due to the expression of the cherry report gene. Cell cultures for transplantation were checked for viability by trypan blue and viability was >90%.

Histological and immunohistochemical analyses

Paraffin-embedded liver tissues were cut into 5 mm-thick sections, which were stained with hematoxylin and eosin. Fibrosis was evaluated using the Laennec fibrosis scoring system [8]. According to this system, a liver specimen may be scored by any number from 0 to 6, with 0 indicating no definite fibrosis, 1 minimal fibrosis, 2 mild fibrosis, 3 moderate fibrosis, 4 mild but definite cirrhosis, 5 moderate cirrhosis, and 6 severe cirrhosis. For immunohistochemical analysis, liver sections were incubated with primary antibody to albumin (ALB, sc-46293, 1:200; Santa Cruz, Dallas, TX), CK18 (ab133263, 1:200; Abcam, Cambridge, UK), or HNF4α (ab41898, 1:100; Abcam, Cambridge, UK) at 4°C overnight, then washed and incubated with the biotinylated secondary antibody at 22°C for 30 min. After several rinses with buffered saline, the sections were incubated with the streptavidin-HRP conjugate solution at 22°C for an additional 30 min. Finally, the sections were developed with a solution of 0.03% DAB and 0.03% hydrogen peroxide in Tris/HCl-buffered saline (0.05 M, pH=7.6). The images were acquired using a Nikon microscope (Japan) under a high magnification (40 ×) and positively labeled cells were counted.

Analysis of colocalization of transplanted BMSCs with hepatocytic markers

Immunofluorescence was used to identify the transplanted BMSCs and track their potential hepatocyte differentiation. At first, sections were incubated in the blocking solution with 3% (w/v) bovine serum albumin (BSA), 0.1% Triton X-100, and 0.05% Tween-20. Next, the sections were incubated with goat anti-ALB (Santa Cruz; 1:100), rabbit anti-CK18 (Abcam, 1:200), or mouse anti-HNF4α (Abcam, 1:100) at 4°C overnight. After rinses, the sections were incubated with a mixture of FITC- and TRITC-conjugated secondary antibodies for 2 hr at 4°C. The nuclei were stained with Hoechst. Fluorescent images were obtained and analyzed with a Leica fluorescence microscope (Germany).

Biochemical analysis

Blood samples were collected at the time points when rats were sacrificed. They were kept at room temperature for 2 hr and then at 4°C overnight. At first, blood samples were centrifuged at 3000 rpm for 10 min for serum separation. All biochemical parameters were measured with a PUZS-300 automatic biochemical analyzer in the Second Affiliated Hospital of Harbin Medical University. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBL), and ALB levels were measured to evaluate liver function.

Reverse transcription-polymerase chain reaction (RT-PCR)

At the time the animals were killed, liver tissues were collected with total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA purity and concentration were determined using a spectrophotometer (Ultrospec 2100 pro UV/Visible, Amersham Bioscience, Freiburg, Germany), and cDNA was synthesized from total RNA (1 µg) using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster city, CA) with oligo-dT. For RT-PCR, amplification was performed to measure the mRNA levels of ALB, CK18, and HNF4α using sequence-specific primers (All primers were purchased from Invitrogen). The mRNA levels of the target genes were acquired from the values of the threshold cycle, with β-actin mRNA as a control.

Western blot assay

Liver tissues were homogenized using an extraction buffer containing proteinase inhibitors (Jianglai Biotech, Shanghai, China). The lysates were then centrifuged at 12,000 rpm for 10 min at 4°C, and the protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Twenty microgram of proteins was electrophoresed on a 5% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skin milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hr at room temperature. Then the membranes were incubated with primary antibodies to anti-ALB (Santa Cruz; 1:200), anti-CK18 (Abcam; 1:200), or anti-HNF4α (Abcam; 1:200) at 4°C overnight, and incubated with the horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Specific protein bands were developed using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ). The membranes were also probed with β-actin antibody as loading control.

Statistical analysis

Data were expressed as means ± standard deviations (SD). Statistical analysis was performed using the Kruskal-Wallis nonparametric analysis, with posthoc tests between individual groups. Statistical analysis was performed using SPSS software (version 20.0, Chicago, IL). P values <0.05 was considered significant for all tests.

Results

Verification of BMSC antigenic phenotype and expression of reporter gene

The BMSCs obtained by bone marrow aspiration and expanded *in vitro* were small and in micro-sphere shape in the first 24 hrs (Fig. 1A), and increased in number and size in the following 2-3 days (Fig. 1B). The cells of third generation appeared similar to fibroblasts in the spindle-shaped fusiform morphology and expressed the cherry report gene appearing in red under the fluorescent microscope (Fig. 1C). The flow cytometric analysis showed that >97% of the expanded BMSCs expressed CD44 (Fig. 1D) and CD29 (Fig. 1E), two typical mesenchymal markers. Moreover, absence of contaminating hematopoietic cells in the BMSCs population was verified by the lack of surface antigens CD45 (Fig. 1F) and CD34 (Fig. 1G) characteristic of hematopoietic progenitor cells.

Verification of CCl₄ induced hepatic cirrhosis and functional impairment

CCl₄-treated rats surviving one month showed pathological changes of moderate or severe cirrhosis, as evidenced by broad septa and minute nodules (Fig. 2B). In contrast, controls received saline lacked any of these histological changes (Fig. 2A). Higher levels of ALT (Fig. 2C) and AST (Fig. 2D), lower levels of ALB (Fig. 2E), and higher levels of TBL (Fig. 2F) were found in the sera of CCl₄-treated rats relative to controls, indicating live function impairments in the former.

Colocalization of *bcl-2* gene-modified BMSCs with hepatocyte-like cell markers

To explore a possibility that the infused *bcl-2* gene-modified BMSCs might differentiate into hepatocyte-like cells in the damaged liver, we performed immunofluorescent staining to

Fig. 1. Morphology and antigenic phenotyping of the BMSCs. The BMSCs are small and in spherical shape at the first 24 hr culture (A). They increase in number and size in the following 2-3 days of culture (B). The cells of third generation appear similar to fibroblasts in the spindle/fusiform shape and express the cherry report gene in red (C). The flow cytometric analysis shows that >97% of the expanded BMSCs expressed CD44 (D) and CD29 (E), but lack of CD45 (F) and CD34 (G).

Fig. 2. Carbon tetrachloride induced liver cirrhosis and functional impairments. The liver tissue from normal controls (A) and CCl_4 -treated rats (B) were histologically stained. Biochemical analyses show higher levels of ALT (C) and AST (D), lower levels of ALB (E), and higher levels of TBL (F) in serum of CCl_4 -treated rats compared to normal controls. Data are expressed as mean $\pm$ SD; $n = 5$ for each group, ** $p < 0.01$ versus control group.

determine colocalization of *bcl-2* protein with a set of hepatocyte antigen markers, HNF4a, ALB, and CK18, in liver tissue 7 days after the infusion of AVV or *bcl-2* gene-modified BMSCs. *Bcl-2* positive cells co-expressed HNF4a (Fig. 3A-D), CK18 (Fig. 3E-H), and ALB (Fig. 3I-L). Moreover, the amounts of double labeled were significantly higher in the BMSCs-AVV-*bcl-2* group compared to the AVV-*bcl-2* group (Fig. 3M). In immunohistochemical analysis, ALB-, CK18-, and HNF4a-positive cells appeared mostly frequent in liver tissue of the rats in BMSCs-AVV-*bcl-2* group among the 4 groups (Fig. 4A-M). Quantitative analysis indicated that the Cirrhosis+saline group had the fewest ALB-, CK18-, and HNF4a-positive cells, while the Cirrhosis + BMSCs-AVV-*bcl-2* group had the most, and the other two groups had middle amounts of cells, with significant statistical difference (Fig. 4M). In parallel with the above immunohistological data, RT-PCR measurements of the corresponding mRNAs (Fig. 5) and Western blot (Fig. 6) analyses of corresponding proteins showed similar trends of differences among the groups.

Bcl-2-modified BMSCs attenuated serological and histological signs of cirrhosis

The recovery processes of liver function and histology in the animal groups were examined with on days 7, 14, 21, or 28 after infusions of saline, AVV-*bcl-2*, BMSCs, or BMSCs+AVV-*bcl-2*. Serum ALB levels progressively increased along the four-week recovery period in all 4 groups, of which the fastest recovery happened in the BMSCs-AVV-*bcl-2* group (Table 1). On day 28, serum ALB in this group was comparable to that in the normal healthy

Fig. 3. Colocalization of *bcl-2*-modified BMSCs with hepatocyte markers. Immunofluorescence was performed on liver sections with antibodies to *bcl-2*, HNF4a, ALB, and CK18 7 days after the infusion of AVV vector or *bcl-2* gene-modified BMSCs. Liver cells express *bcl-2* (red, A, E, I), HNF4a (green, B), CK18 (green, F), and ALB (green, J). Co-localization of *bcl-2* and the latter three proteins are indicated in images D, H, and L. The histogram (M) shows the statistical comparison (nonparametric test) between the BMSCs-AVV-*bcl-2* and AVV-*bcl-2* groups in terms of percentages of the double-stained cells over *bcl-2* positive cells. Data are expressed as mean $\pm$ SD; n = 5 for each group, *p < 0.05, **p < 0.01 versus AVV-*bcl-2* group.

Fig. 4. Treatment of *bcl-2* gene-modified BMSCs enhanced expression of hepatic cellular markers. Immunohistochemical analysis was performed on liver sections with antibodies to ALB, CK18, and HNF4a 28 days after the infusion of saline, AVV-*bcl-2*, BMSCs, or BMSCs-AVV-*bcl-2*. Representative images A-D show the ALB positive cells in liver sections from the above groups, respectively, E-H CK18 positive cells, I-L HNF4a positive cells. The histogram (M) shows the statistical comparison among the four groups. Data are expressed as mean $\pm$ SD; n = 5 for each group, *p < 0.05, **p < 0.01 versus cirrhosis + saline group. ++p < 0.01 versus cirrhosis + AVV-*bcl-2* group.

rats (46 vs 50). Serum ALT levels progressively decreased along the four-week period in all 4 groups, of which the fastest recovery occurred in the BMSCs-AVV-*bcl-2* group (Table 2). On day 28, the serum ALT in this group was close to that in normal healthy rats (58 vs 51). Similarly, levels of serum AST (Table 3) and TBL (Table 4) tended to progressively decrease

Fig. 5. Treatment of *bcl-2* gene-modified BMSCs enhanced mRNA levels of hepatic markers. RT-PCR analysis was performed with liver tissue of the cirrhosis + saline, cirrhosis + AVV-*bcl-2*, cirrhosis + BMSCs, and cirrhosis + BMSCs-AVV-*bcl-2* groups. The levels of mRNAs of ALB, CK18, and HNF4a (A, B and C) show statistically significant difference among the four groups. Data are expressed as mean $\pm$ SD; n = 5 for each group, *p < 0.05, **p < 0.01 versus cirrhosis + saline group. ++p < 0.01 versus cirrhosis + AVV-*bcl-2* group.

Fig. 6. Treatment of *bcl-2* gene-modified BMSCs elevated protein levels of hepatic markers. Western blot analysis was performed with liver tissue of the cirrhosis + saline, cirrhosis + AVV-*bcl-2*, cirrhosis + BMSCs, and cirrhosis + BMSCs-AVV-*bcl-2* groups to compare protein levels of ALB, CK18, and HNF4a among these groups. A shows representative western blot images. The histogram (B) shows the statistical comparison among the four groups. Data are expressed as mean $\pm$ SD; n = 5 for each group, *p < 0.05, **p < 0.01 versus cirrhosis + saline group. ++p < 0.01 versus cirrhosis + AVV-*bcl-2* group.

along the 4 week period in all groups, with the fastest recovery in the BMSCs-AVV-*bcl-2* group.

We further assessed the extent of cirrhosis histologically among the 4 groups by the Laennec scores. At the end of four week period post CCl₄ induction, the recovery of liver cirrhosis was not significant in the cirrhosis + saline group relative to normal control. However, in the other three groups, histological recovery was noted, with the fastest seen in the BMSCs-AVV-*bcl-2* group, although its recovery was not completely comparable to healthy controls (Table 5).

Discussion

MSCs are defined as plate-adhering, fibroblast-like cells possessing the capability of self-renewal and differentiation into multiple mesenchymal cell lineages [36]. MSCs are also characterized to express surface markers, such as CD105, CD73 and CD90, but lack of

Table 1. The comparisons of recovery processes of serum ALB levels (g/L) after liver cirrhosis. Note: Data are expressed as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to week 1 in the same group. + $p < 0.05$, compared to the cirrhosis+saline group at the same time point

	Cirrhosis+ saline	Cirrhosis+ AVV-bcl-2	Cirrhosis+ BMSCs	Cirrhosis+ BMSCs-AVV- bcl-2
Week 1	23.2 (4.3)	20.1 (3.3)	24.3 (3.2)	19.4 (3.2)
Week 2	24.9 (3.4)	29.3 (3.4)*	27.3 (4.3)	30.1 (2.9)*,+
Week 3	30.4 (3.9)*	34.4 (2.9)*	33.2 (3.9)*	37.1 (4.3)**,+
Week 4	35.3 (2.1)*	39.1 (5.4)**	40.3 (5.0)**	45.5 (3.4)**,+

Table 2. The comparisons of recovery processes of serum ALT levels (IU/L) after liver cirrhosis in rats of the four groups. Note: Data are expressed as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to week 1 in the same group. + $p < 0.05$, compared to the cirrhosis+saline group at the same time point

	Cirrhosis+ saline	Cirrhosis+ AVV-bcl-2	Cirrhosis+ BMSCs	Cirrhosis+ BMSCs-AVV- bcl-2
Week 1	1521.3 (161.0)	1582.1 (178.9)	1609.3 (167.2)	1643.3 (158.9)
Week 2	1129.8 (103.2)	789.3 (128.3)	892.7 (128.1)	469.4 (67.2)**,+
Week 3	421.6 (39.1)*	253.8 (31.1)*,+	369.8 (23.0)*	89.9 (10.1)**,+
Week 4	102.7 (12.3)**	70.6 (7.1)**,+	67.5 (5.8)**,+	58.1 (4.2)**,+

Table 3. The comparisons of recovery processes of serum AST levels (IU/L) after liver cirrhosis in rats of the four groups. Note: Data are expressed as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to week 1 in the same group. + $p < 0.05$, compared to the cirrhosis+saline group at the same time point

	Cirrhosis+ saline	Cirrhosis+ AVV-bcl-2	Cirrhosis+ BMSCs	Cirrhosis+ BMSCs-AVV- bcl-2
Week 1	1651.3 (198.7)	1492.8 (188.9)	1659.3 (132.9)	1634.3 (198.9)
Week 2	1099.8 (103.2)	809.3 (89.3)*	792.7 (102.9)*	520.4 (67.2)*,+
Week 3	397.6 (29.0)*	253.8 (42.1)*	289.3 (23.0)**	109.3 (10.1)**,+
Week 4	117.4 (10.9)**	87.6 (8.7)**	64.5 (6.3)**	38.1 (4.6)**,+

Table 4. The comparisons of recovery processes of serum TBL levels (umol/L) after liver cirrhosis in rats of the four groups. Note: Data are expressed as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to week 1 in the same group. + $p < 0.05$, compared to the cirrhosis+saline group at the same time point

	Cirrhosis+ saline	Cirrhosis+ AVV-bcl-2	Cirrhosis+ BMSCs	Cirrhosis+ BMSCs-AVV- bcl-2
Week 1	238.4 (21.0)	249.2 (19.4)	251.9 (20.1)	229.8 (23.0)
Week 2	194.2 (12.3)	159.3 (14.3)*	172.3 (15.9)	121.3 (17.2)*,+
Week 3	110.2 (7.3)*	83.4 (7.4)*	72.1 (8.3)*	51.4 (9.2)**,+
Week 4	23.0 (4.5)**	19.2 (3.8)**	20.0 (2.5)**	14.2 (3.2)**,+

Table 5. The comparisons of recovery processes of liver cirrhosis (Laennec scores) in rats of the four groups. Note: Data are expressed as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to week 1 in the same group. + $p < 0.05$, ++ $p < 0.01$, compared to the cirrhosis+saline group at the same time point

	Cirrhosis+ saline	Cirrhosis+ AVV-bcl-2	Cirrhosis+ BMSCs	Cirrhosis+ BMSCs-AVV- bcl-2
Week 1	5.74 (0.11)	5.54 (0.13)	5.49 (0.13)	5.42 (0.15)
Week 2	5.71 (0.15)	5.39 (0.14)	5.22 (0.14)+	5.03 (0.12)*,+
Week 3	5.65 (0.12)	5.16 (0.11)*,+	4.91 (0.17)*,+	4.58 (0.20)*,+
Week 4	5.52 (0.17)	4.88 (0.15)*,+	4.48 (0.15)*,+	4.03 (0.17)**,+

the expression of CD45, CD14 and CD34 [37]. In accordance with these criteria, the BMSCs applied in the present study appear similar to fibroblasts in the spindle-shaped fusiform morphology and can expand well *in vitro*. They express CD44 and CD29, two typical mesenchymal markers, but not CD45 and CD34.

Experimental cirrhosis was induced in rats by intraperitoneal injection of CCl_4 using established methods [38-40]. It should be noted that CCl_4 was given at a relatively high dosage, allowing an induction of liver cirrhosis in a short period. This might account for the severe liver dysfunction and cirrhosis in the CCl_4 -treated rats in the present study, relative to previous reports [38-40].

Rodent and human MSCs have been shown to differentiate into HLCs possessing the functions of hepatocytes [10, 41, 42], which express hepatocyte markers such as AAT, AFP, albumin (ALB), CK18, and HNF4a [43-45]. In accordance, the infused BMSCs in the present study expressed ALB, CK18, and HNF4a. Interestingly, in the present study liver cells in rats infused with only AVV-*bcl-2* vectors also expressed these markers, indicating a possibility that some of the surviving hepatic cells in the injured liver might be infected by the infused vectors *in vivo*. Importantly, there were more liver cells that expressed *bcl-2* together with ALB, CK18 and HNF4a in the rats infused with BMSCs-AVV-*bcl-2* relative to animals infused with AVV-*bcl-2* vectors only. Among the four animal groups, the cirrhosis + BMSCs-AVV-*bcl-2* group had the most, the cirrhosis + saline group the fewest cells showing colocalization, while the other two groups had numbers in-between. Thus, it appears that the *bcl-2* protein may facilitate a differentiation of BMSCs into functional hepatocytes in injured rat liver. This interpretation was supported by the findings of the highest mRNA and protein levels of hepatic markers in the cirrhosis + BMSCs-AVV-*bcl-2* group.

Several *in vivo* studies have been performed to evaluate the therapeutic potential of MSCs in the context of liver fibrosis and liver function. Jang et al. [8] reported that BMSCs attenuated hepatic fibrosis in thioacetamide-treated rats and improved liver function, as evidenced by the normalized levels of serum ALB, ALT, AST, and TBL. In the present study, we also found that rats in the cirrhosis + saline group showed some automatic serological recovery, however, the recovery in the other 3 groups was significantly promoted, with the fastest recovering processes seen in the cirrhosis + BMSCs-AVV-*bcl-2* group. Together, infusions of AVV-*bcl-2* and BMSCs can accelerate liver recovery following CCl_4 injury, and there is a synergistic effect between these two treatments.

A number of mechanisms such as cell transdifferentiation, immunomodulation, anti-fibrosis and trophic actions are proposed for the therapeutic effect of MSCs in liver fibrosis/cirrhosis. Although we have not specifically addressed these individual mechanisms, our data can provide some heuristic clues. First, AVV-*bcl-2* can infect hepatocytes in the injured liver, which might exert some anti-apoptotic effect. Reports have shown that *bcl-2* can prevent doxorubicin induced apoptosis of human liver cancer cells [46], while staurosporine induced hepatic apoptosis is associated with down-regulation of *bcl-2* and *bcl-XL* [47]. Second, *bcl-2* protein expressed in the BMSCs-AVV-*bcl-2* complex might help infused BMSCs develop towards liver cells. This interpretation appears in line with other report also showing that enhanced *bcl-2* expression in grafted neuroepithelial cells promotes the differentiation of these stem cells into enteric neurons [48].

In summary, we demonstrate here that genetic modification with the anti-apoptotic *bcl-2* gene increases cell survival, and potentially enhances the differentiation of infused BMSCs into HLCs. Such modified BMSCs also promote the recovery of liver function and lessen cirrhotic histology in CCl_4 -treated rats. The data suggest that transplantation of gene-engineered BMSCs may serve an approach for the treatment of cirrhosis.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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