Bone marrow-derived mesenchymal stem cells effectively regenerate fibrotic liver in bile duct ligation rat model

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Abstract

Mesenchymal stem cells (MSCs) have attracted lots of attention for the treatment of acute liver failure and end-stage liver diseases. This study aimed at investigating the fundamental mechanism by which bone marrow-derived MSCs (BM-MSCs) induce liver regeneration of fibrotic liver in rats. Rats underwent bile duct ligation (BDL) surgery and four weeks later they were treated with either BM-MSCs (3×10^6 cells /rat, once, tail vein injection) or silymarin (100 mg/kg, daily, orally) for four weeks. Liver function tests and hepatic oxidative stress were determined. Hepatic injury and fibrosis were assessed by H and E, Sirius red staining and immunohistochemical expression of α -smooth muscle actin (α -SMA). Hepatocyte growth factor (HGF) and the gene expression of cytokeratin-19 (CK-19) and matrix metalloproteinase-2 (MMP-2) in liver tissue were determined. BDL induced cholestatic liver injury characterized by elevated ALT and AST activities, bilirubin and decreased albumin. The architecture damage was staged as Metavir score: F3, A3. Fibrosis increased around proliferating bile duct as indicated by sirius red staining and α -SMA immunostaining. Fibrogenesis was favored over fibrolysis and confirmed by decreased HGF with increased expression of CK-19, but decreased MMP-2 expression. BM-MSCs treatment restored deteriorated liver functions and restored the histological changes, resolved fibrosis by improving liver regenerative capabilities ($P < 0.001$), increases in HGF and MMP-2 mRNA and downregulating CK-19 mRNA. Silymarin, however, induced similar but less prominent effects compared to BM-MSCs. In conclusion, liver regenerative capabilities can be stimulated by BM-MSCs via augmentation of HGF that subsequently up-regulate MMP-2 mRNA while downregulating CK-19 mRNA.

Keywords: Bone marrow-derived mesenchymal stem cells, hepatic regeneration, bile duct ligation, cytokeratin-19, hepatocyte growth factor, matrix metalloproteinase-2

Experimental Biology and Medicine 2016; **241**: 581–591. DOI: 10.1177/1535370215627219

Introduction

Liver regeneration is one of the defining features of liver to maintain a constant size despite liver mass loss by injury as trauma or partial hepatectomy.¹ Generally, minor and transient liver injury usually results in mitotic division of surviving mature hepatocytes and bile duct cells causing replacement of dead cells and reconstruction of normal hepatic structure and function.^{2,3} Although, the mechanism of liver regeneration is still unclear, many molecular and cellular factors were involved. These include hepatocyte growth factor (HGF) which is a multipotent endogenous repair factor secreted primarily by hepatic mesenchymal

cells promoting cells survival,⁴ suppressing chronic inflammation and resolving fibrosis by inhibiting extracellular matrix (ECM) production and deposition, inducing myofibroblasts apoptosis.^{5,6} In ECM, matrix metalloproteinases (MMPs) are the major family of calcium-dependent enzymes that degrade collagenous and non-collagenous ECM substrates. To ensure the tight regulation of the turnover and constant remodeling of ECM, MMPs are regulated at several levels.⁷ Enhancing their activity along with decreasing ECM production is fundamental in fibrolysis⁸ and hence regeneration.

The self-regeneration process in the liver lasts until it reaches a level to which most of the original mass is

recovered.⁹ On the other hand, worse and repeated insults cause engraftment of hepatic progenitor cells as a final attempt to restore liver homeostasis.^{2,3} Indeed, it has been shown that hepatic self-regeneration is compromised in severe and chronic liver injury.¹⁰ Therefore, Therapeutic approaches/interventions to stimulate liver regenerative capabilities are highly requested as most of patients are clinically presented with chronic liver injuries. Cell-based therapies using mesenchymal stem cells (MSCs) have attracted lots of attention for the treatment of acute liver failure and end-stage liver diseases because of their self-renewal capability and differentiation potential.¹¹ Bone marrow-derived MSCs (BM-MSCs) have shown promising benefits for hepatic fibrosis in experimental and clinical studies.^{12–14} MSCs demonstrated anti-fibrotic effects^{13–15} and stimulated regeneration^{16,17} of injured liver in animal models of liver fibrosis. Even under critical circumstances as lethal irradiation, fulminant hepatic failure, or 90% hepatectomy, the use of BM-MSCs has been demonstrated to produce spectacular therapeutic effects.¹¹ The exact mechanism of liver regeneration by MSCs is not fully understood. We used silymarin in our study as a reference drug to compare the beneficial effects achieved by BM-MSCs as silymarin, a flavonolignan mixture of milk thistle (*Silybum marianum*), is the most well-researched plant in the treatment of liver disease.¹⁸ It has been found that these substances exert hepatoprotective, antioxidant, antiinflammatory, anti-fibrotic properties, and anti viral activities. In addition, they stimulate protein biosynthesis and liver regeneration, increase lactation and possess immuno-modulation activity.¹⁹ The present study therefore aimed to investigate the effect of BM-MSCs on cholestasis rat model and outlined the fundamental mechanism by which BM-MSCs induce liver regeneration in this rat model.

Materials and methods

Animals

Male Wistar rats (250 ± 30 g; $n = 40$), purchased from the faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, were kept in a temperature- and humidity-controlled environment in a 12 h light-dark cycle. Rats were allowed free access to drinking water and standard rodent chow. Animal experiments received approval from the Ethical Committee of the Faculty of Pharmacy, Zagazig University, Egypt (No. P11/2/2013).

Preparation of BM-MSCs

The femurs and tibias of six-week-old male albino Wistar rats were carefully dissected away from attached soft tissue. The ends of the bones were cut, and the bone marrow was aseptically flushed with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL). Nucleated cells were isolated with a density gradient (Ficoll/Paque [Pharmacia]) and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). All cells were incubated at 37°C, in an atmosphere of 5% humidified CO₂ for 12–14 days. When cells grew to 80%

confluency, they were harvested with 0.25% trypsin and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask (Falcon). The resulting cultures were referred to as first-passage cultures.²⁰

Identification of BM-MSCs

Cells were identified as being MSCs by their morphology, adherence, surface markers and their power to differentiate into osteocytes, chondrocytes, and adipocytes. Differentiation into osteocytes was achieved by adding 1–1000 nmol/L dexamethasone (DEX), 0.25 mmol/L ascorbic acid, and 1–10 mmol/L beta glycerophosphate to the medium. Differentiation of MSCs into osteoblasts was confirmed by morphological changes, Alzarin red staining of differentiated osteoblasts. Differentiation into chondrocyte was achieved by adding 500 ng/mL, bone morphogenetic protein-2 (BMP-2; R&D Systems, USA) and 10 ng/mL transforming growth factor b3 (TGFb3) (Peprotech, London) for three weeks. *In vitro* differentiation into chondrocytes was confirmed by morphological changes, Alcian blue staining of differentiated chondrocytes. Induction media for adipogenic differentiation contained 0.5 µmol/L 3-isobutyl-1-methylxanthine, 200 µmol/L indomethacin, 10 µmol/L insulin, and 1 µmol/L DEX. Differentiation of MSCs into adipocyte was confirmed by morphological changes, oil red staining of differentiated adipocyte. MSCs were also identified by surface marker CD90 (+ve), CD105 (+ve) and CD73 (+ve) using flow cytometry. The detection of cluster of differentiation 29 (CD29) gene expression as a surface marker of rat MSCs was also performed by RT-PCR.²¹

RT-PCR detection of CD29 gene expression

Total RNA was extracted from cells using RNeasy Purification Reagent (Qiagen, Valencia, CA), and then a sample (1 µg) was reverse transcribed with M-MLV (Moloney–Murine Leukemia virus) reverse transcriptase (RT) for 30 min at 42°C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers (UniGene Rn.25733) forward: 5'-AATGTTTCAGTGCAGAGC-3' and reverse: 5'-TTGGGATGATGTCGGGAC-3'. PCR was performed for 35 cycles, with each cycle consisting of denaturation at 95°C for 30 s, annealing at 55°C to 63°C for 30 s, and elongation at 72°C for 1 min, with an additional 10-min incubation at 72°C after completion of the last cycle. To exclude the possibility of contaminating genomic DNA, PCRs were also run without RT. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.²¹

Labeling of BM-MSCs with PKH26

BM-MSCs were labeled with PKH26 (Sigma-Aldrich, Saint Louis, MO). Cells were first centrifuged, washed twice in serum-free medium and were pelleted and suspended in dye solution. Cells were injected intravenously into rat tail vein. After one month, liver tissues were

examined with a fluorescence microscope to detect and trace the cells.²¹

Drugs

Silymarin (Legalon® 140 mg) was purchased from Chemical Industries Development (CID; Giza, Egypt) under license of MADAUS, Cologne, Germany.

Experimental design

Induction of liver fibrosis by BDL operation. Surgical procedures of bile duct ligation (BDL) were performed under ketamine hydrochloride (50 mg/kg) and diazepam (3 mg/kg) anesthesia. Double ligation at the common bile duct and complete cutting at midpoint were conducted under aseptic conditions. Sham-operated rats received an identical laparotomy and isolation of the common bile duct without ligation.²²

Experimental groups. Four weeks following BDL or sham operation, BDL rats were randomly divided into three groups ($n=10$ each). BDL group: rats were injected with 0.2 mL PBS. BM-MSCs group: rats were injected (via tail vein) with 0.2 mL PBS containing 3×10^6 BM-MSCs per rat. Silymarin group: rats were given silymarin (100 mg/kg) orally daily for four constitutive weeks. Sham group: four weeks after sham operation, rats ($n=10$) were injected with 0.2 mL phosphate-buffered saline (PBS). At the end of treatment, blood was collected and centrifuged ($3000 \times g$, 4°C , 20 min) to separate serum for the measurement of liver function tests. All rats were euthanized, livers were harvested and were divided into two parts; one part was immediately flash frozen in liquid nitrogen and kept at -80°C for further biochemical analyses and the other part was kept in 10% formalin for histopathological examination.

Biochemical analyses

Liver function tests. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total bilirubin (TB) were assayed using Spinreact diagnostic kits (Spinreact, Gerona, Spain).

Hepatic lipid peroxides and reduced glutathione (GSH)

Liver tissue was homogenized in ice-cold PBS, pH 7.2. Lipid peroxides, expressed as malondialdehyde (MDA), and GSH were determined in the resultant 10% homogenate using Biodiagnostics kits (Biodiagnostics Co., Giza, Egypt).

Hepatocytes growth factor

HGF was measured in liver tissue after cell lysis using Quantikine® ELISA kit (R&D Systems Inc, USA).

CK-19 and MMP-2 gene expression analysis using real-time PCR. Total RNA was extracted from liver samples using SV Total RNA isolation system (Promega, Madison, WI), reverse transcribed into cDNA and amplified by PCR using RT-PCR kit (Stratagene, USA). Reactions

were performed in a 50 μL final volume (25 μL SYBR Green Mix (2x), 0.5 μL cDNA, 2 μL primer pair mix (5 pmol/ μL each primer), 22.5 μL H_2O). PCR reaction was: 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle) and 95°C for 15 s, 60°C for 30 s and 72°C for 30 s (40 cycles) then 72°C for 10 min (1 cycle). Relative gene expression was calculated using $2^{-\Delta\Delta\text{Ct}}$ method.

PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) using RNA sequences from GenBank. The sequences of forward and reverse primers were as follows: cytokeratin-19 (CK-19) (forward: 5'-ACCATTTGAGAACTCCAGGATTGTC-3' and reverse: 5'-AGACG GAACAGGCTCTGCGCATGAG-3'), MMP-2 (forward: 5'-GAGATCTGCAAACAGGACAT-3' and reverse: 5'-GGTTCTCCAGCTTCAGGTA A-3') and β -actin (forward: 5'-ATCATGTTTGAGACCTTCAACACC-3' and reverse: 5'-TAGCTCTTCTCCAGGGAGG-3').

Histopathological examination

Liver slices fixed in 10% formalin were embedded in paraffin, were cut into 5 μm sections and stained with either H&E or Sirius red stains. Photographs were acquired using a digital image-capture system (Olympus CX40; Olympus, Tokyo, Japan). Histopathological scoring of H&E section was done according to the Metavir score, a semiquantitative classifications system consisting of an activity and a fibrosis scores. The fibrosis score is assessed on a five-point scale (0 = no fibrosis, 1 = portal fibrosis without septa, 2 = few septa, 3 = numerous septa without cirrhosis, 4 = cirrhosis). The activity score was graded according to the intensity of necro-inflammatory lesions (A0 = no activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity).²³ The percent of fibrosis were measured in Sirius red-stained sections using image J software and expressed as a percentage of total analyzed areas.

Immunohistochemical analysis

Immunohistochemistry for α -smooth muscle actin (α -SMA) was performed to examine hepatic stellate cells (HSCs) activation. Briefly, liver sections were deparaffinized, hydrated, and heated in citrate buffer for 15 min at 100°C for antigen retrieval. Anti- α -SMA rat monoclonal antibody and a biotin labeled rabbit anti-rat IgG (Abcam, Cambridge, UK) served as primary and secondary antibodies, respectively. The sections were examined microscopically for specific staining, and photographs were acquired using a digital image-capture system (Olympus CX40; Olympus, Tokyo, Japan). Positive cells were counted in 12 random visual fields for each group, and the number was expressed as the integrated optical density value using image J software.

Statistical analyses

All statistical analyses were done using Windows-based SPSS 14.0 (SPSS Inc, Chicago, IL). Results were expressed as mean $\pm$ SD. Statistical difference were sought using Student's *t*-test or one-way ANOVA followed by LSD *post-hoc* test (if more than two sets of data were being compared), taking $P < 0.05$ as statistically significant.

Results

Characterization and homing of BM-MSCs in liver tissue

Isolated and cultured undifferentiated BM-MSCs reached 70–80% confluence on day 14. BM-MSCs were identified by their adhesiveness and fusiform shape (Figure 1a) and their *in vitro* osteogenic, chondrogenic and adipogenic differentiation confirmed by morphological changes and special stains (Figure 1b, c and d), respectively. The immunophenotype of cells isolated from rat bone marrow analyzed by flow cytometry were positive for CD105 (93.9%) and CD90 (88.4%) and CD73 (89.4%) (Figure 1e, f and g), respectively. The gene expression of their surface marker (CD29) was shown in Figure 1(h). BM-MSCs labeled with PKH26 fluorescent dye were detected in the hepatic tissues confirming that these cells homed into the injured liver tissue of recipient rats at 28 days after transplantation (Figure 1j).

Liver function and hepatic oxidative stress

Cholestatic liver injury induced by BDL for eight weeks markedly increased serum ALT and AST activities and TB with concurrent decrement in serum albumin compared with sham operation ($P < 0.001$). These were accompanied with marked depletion of hepatic GSH ($P < 0.001$) and

significant intensification of hepatic MDA level ($P < 0.05$) in BDL-rats as compared with sham-operated rats (Table 1).

Treatment of BDL-rats with either BM-MSCs or silymarin significantly decreased AST ($P < 0.001$), but not ALT activity. The activity of both transaminases was comparable in both BM-MSCs treated and silymarin-treated rats. BM-MSCs and silymarin treatment significantly ($P < 0.001$) lowered TB by 76.8% and 86.2%, respectively. Restoration of plasma albumin was attained only by silymarin treatment ($P < 0.05$). The effects of silymarin were better than those of BM-MSCs on TB and albumin ($P < 0.05$ and $P < 0.01$, respectively). Only BM-MSCs increased hepatic GSH and unexpectedly MDA ($P < 0.05$) compared to BDL rats (Table 1).

Hepatic injury and fibrosis

Eight weeks of BDL for rats suggested hepatic cell damage as assessed by the histological examination of liver tissues using H&E stain. While liver sections from sham-operated rat revealed normal hepatocytes arranged in parallel plates radiating from the central vein (CV) with large bright and vesicular nuclei, no inflammatory cells in the peri-portal areas and normal liver sinusoids (Figure 2a), sections from BDL-rats showed progressive increases in the extent of bile duct proliferation, inflammatory activity, and

Figure 1 BM-MSCs identification and homing into injured liver tissue. Morphological and histological staining of differentiated BM-MSCs into osteoblasts, chondrocytes, and adipocytes; undifferentiated MSCs (a), MSCs differentiated into osteoblasts stained with Alizarin red stain (b), MSCs differentiated into chondrocytes stained with Alcian blue stain and (c), and MSCs differentiated into adipocytes stained with oil red stain (d). Flow cytometry charts for cells stained with CD105 (e), CD90 (f), and CD73 (g) antibodies. UV-transilluminated agarose gel of PCR products of a CD29 gene expression in culture at 261 bp; lane 1 DNA marker, lane 2 BM-MSCs culture (h). Sections taken by fluorescence microscope for injured liver tissue of (i) control rats and (j) recipient rats 28 days following administration of 3×10^6 cells per rat via tail vein revealed homing of BM-MSCs labeled with PKH26 fluorescent dye (red fluorescence). (A color version of this figure is available in the online journal.)

appearance of congested blood vessels (Figure 2b). Treatment with BM-MSCs markedly reduced the extent of cellular infiltration and congestion (Figure 2c). Treatment with silymarin as a standard therapy did not show impressive reduction in the fibrosis, but moderately reduced inflammatory activity (Figure 2d). Metavir scoring for these groups was as follow: sham group (F0, A0), BDL group (F3, A3), MSC group (F1, A1), and silymarin group (F3, A2).

The presence and degree of liver fibrosis were confirmed by Sirius red staining. Sham animals showed the lowest level ($2.2 \pm 0.02\%$), while BDL-rats showed the highest level ($29.7 \pm 0.2\%$) of collagen deposition, as anticipated (Figure 3a and b, respectively). A significant reduction in collagen deposition in BDL-rats was observed after

transplantation of BM-MSCs (Figure 3c) in comparison to BDL. Silymarin alone did not noticeably reduce fibrosis (Figure 3d). These results were further supported by α -SMA immunostaining, where the distribution of α -SMA positive cells was similar to that of collagen deposition in the liver. In sham rats, α -SMA protein was only expressed at the level of the smooth muscle cell layer surrounding blood vessels (Figure 4a). Activated HSCs characterized by increased expression of α -SMA in the liver of BDL-operated rats. Upon the induction of hepatic fibrosis by BDL, α -SMA protein expression is only observed in the fibrotic areas surrounding proliferating bile ducts (Figure 4b). The expression of α -SMA was significantly reduced by BM-MSCs treatment (Figure 4c) and to a less extent by silymarin (Figure 4d).

Table 1 Effect of BM-MSCs and silymarin on liver function and hepatic oxidative stress in BDL rats.

	Sham	BDL	BDL + BM-MSCs	BDL + silymarin
Serum ALT (U/L)	12.6 ± 1.1	30 ± 3.15^c	28.6 ± 5.5	27 ± 4.8
Serum AST (U/L)	73.1 ± 4.5	156.5 ± 15.2^c	$110.8 \pm 6.3^{***}$	$113.2 \pm 5.3^{***}$
Serum TB (mg/dL)	0.35 ± 0.08	4.5 ± 0.4^c	$1.04 \pm 0.4^{b***@}$	$0.62 \pm 0.2^{***}$
Serum albumin (g/dL)	3.5 ± 0.14	2.9 ± 0.22^c	$2.8 \pm 0.53^{@@}$	$3.3 \pm 0.21^*$
Hepatic GSSH (mg/g tissue)	43.9 ± 9.4	29.5 ± 5.4^c	$37.8 \pm 4.6^*$	33.3 ± 5.7
Hepatic MDA (nmol/g tissue)	29 ± 2.5	37.7 ± 5.1^a	$48.1 \pm 11.5^*$	43.3 ± 6.6

Rats subjected to BDL were treated on week 4 following operation with either BM-MSCs (3×10^6 cells, once) or silymarin (100 mg/kg body weight/daily) for 4 weeks. All results were expressed as mean $\pm$ SD; $n = 10$ each. $^aP < 0.05$, $^bP < 0.01$, and $^cP < 0.001$ compared with sham, $^*P < 0.05$, and $^{***}P < 0.001$ compared with BDL rats, $^@P < 0.05$, and $^{@@}P < 0.01$, compared with silymarin-treated rats (BDL + silymarin).

Figure 2 Sections of liver tissue from sham-operated rats (a), rats with BDL (b), BDL rats treated with BM-MSCs (c), and BDL rats treated with silymarin (d) stained with hematoxyline and eosin. Liver sections from sham rats showed normal hepatocytes arranged in parallel plates radiating from the central vein (CV) with large bright and vesicular nuclei. No inflammatory cells were seen in the peri-portal areas and the liver sinusoids were rather normal. BDL showed progressive increase in the level of bile duct proliferation (arrows) and inflammatory activity (arrow heads). Congested blood vessels were also noticed (asterisk). BM-MSCs markedly reduced the extent of cellular infiltration and congestion. Silymarin did not show dramatic reduction in fibrosis, but moderately reduced inflammation. Scale bar, 20 μ mol/L. (A color version of this figure is available in the online journal.)

Figure 3 Sections of liver tissue from sham-operated rats (a), rats with BDL (b), BDL rats treated with BM-MSCs (c), and BDL rats treated with silymarin (d) stained with Sirius red (collagen fibers are stained red). Representative photographs of liver sections were taken from three different rats from each group. (e) Sirius red-stained areas were measured using image J software and expressed as a percentage of total analyzed area. Results are means $\pm$ SD from 12 consecutive fields; $^{\circ}P < 0.001$ as compared to sham group, $^{***}P < 0.001$ as compared to BDL group and $^{@@@}P < 0.001$ as compared to silymarin group. (A color version of this figure is available in the online journal.)

Liver regenerative capacity

Hepatic injury was associated with a notable (37%) decrease in hepatic HGF, upregulation of *CK-19* mRNA and downregulation of *MMP-2* mRNA as seen in BDL-rats compared to sham-operated rats ($P < 0.001$). Liver regeneration was confirmed in BDL rats treated with BM-MSC as pointed out by significant increase in hepatic HGF level ($P < 0.001$), remarkable down regulation for *CK-19* gene expression by 57% and upregulation of *MMP-2* mRNA by 93% if compared to untreated BDL control rats ($P < 0.001$). Hepatic regeneration induced by BM-MSCs treatment was more prominent than that induced by silymarin as indicated by the significant differences between the treated groups on HGF ($P < 0.01$), *MMP-2* mRNA ($P < 0.05$), and *CK-19* mRNA ($P < 0.001$) (Figure 5). Table 2 shows how the selected regeneration parameters were strongly and significantly correlated with liver function

tests performed. Liver enzymes ALT and AST and TB were negatively correlated with serum HGF and hepatic *MMP-2* mRNA ($P < 0.001$) and positively with hepatic *CK-19* mRNA ($P < 0.05$, 0.001 and 0.001, respectively). Moreover, serum HGF and hepatic *MMP-2* mRNA were negatively correlated with the fibrosis parameters; sirius red and α -SMA staining, and hepatic hydroxyproline content (hydroxyproline data are not part of the current manuscript) at $P < 0.001$. On the other hand, hepatic *CK-19* mRNA was positively correlated with fibrosis parameters ($P < 0.001$).

Discussion

In this study, we investigated the effect of BM-MSCs on hepatic fibrosis in BDL rat model and outlined the fundamental mechanism of liver regeneration by BM-MSCs

Figure 4 Expression of α -SMA in liver tissue from sham-operated rats (a), rats with BDL (b), BDL rats treated with BM-MSCs (c), and BDL rats treated with silymarin (d). In sham rats, α -SMA protein is only expressed at the level of the smooth muscle cell layer surrounding blood vessels (blue arrows). Activated HSCs characterized by the expression of α -SMA increased in the liver of BDL-operated rats and was only observed in the fibrotic areas surrounding proliferating bile ducts (red arrows). BM-MSCs significantly reduced the expression of α -SMA. Silymarin did not markedly decrease α -SMA. Scale bar, 20 μ m. Representative photographs of liver sections were taken from three different rats from each group. (e) Positive cells were counted to record the expression (%). Results are expressed as mean $\pm$ SD. $^cP < 0.001$ as compared to sham group, $^{***}P < 0.001$ as compared to BDL group, and $^{@@@}P < 0.001$ as compared to silymarin group. (A color version of this figure is available in the online journal.)

treatment. The key findings of our study are (i) i.v. administration of BM-MSCs at (3×10^6 cells into BDL rats via tail vein) recovered liver function, architectural changes and resolved fibrous damage and (ii) resolution of liver fibrosis by BM-MSCs was attained by increasing the liver regeneration mediated via increasing HGF, subsequent upregulation of MMP-2 mRNA and downregulating CK-19 mRNA.

Liver possess a high regenerative capacity regulated by continuous remodeling process which is affected by the equilibrium between fibrogenesis and fibrolysis.²⁴ From clinical perspective, chronic diseases including liver fibrosis are not diagnosed until the condition has reached an advanced stage. Therefore, studying therapeutic drugs using established fibrosis model rather than preventive drugs are imperative. BDL, a commonly used rat model of liver fibrosis, stimulates the proliferation of biliary

epithelial cells and oval cells resulting in proliferating bile ductules and an accompanying portal inflammation, fibrosis and a secondary biliary cirrhosis, similar to that of humans.^{25,26} Unlike other liver fibrosis models, spontaneous recovery from fibrosis due to the high self-regeneration capacity of liver tissue is not seen in BDL model.^{27,28} We have recently reported that a well-established hepatic fibrosis (Metavir score: F2, A2) arise after four weeks of BDL surgery²⁹ and this represents a suitable point to start anti-fibrotic treatment. Liver dysfunction was evident in our BDL control rats as indicated by significant increases in serum TB, ALT, and AST along with decrease in serum albumin level, in accordance with previous reports.^{27,29} The detergent action of accumulated bile acids is partly responsible for the plasma membrane damage leading to further oxidative stress (increased MDA and depleted GSH) and

Figure 5 Effect of BM-MSCs and silymarin treatment on (a) HGF (pg/mL) and the gene expression of (b) *MMP-2* and (c) *CK-19* in liver tissue of BDL rats. Results were expressed as mean $\pm$ SD, $n=6$ for each group. ^c $P < 0.001$ and ^b $P < 0.01$ as compared to sham group, ^{**} $P < 0.01$ and ^{***} $P < 0.001$ as compared to BDL group and [@] $P < 0.05$, ^{@@} $P < 0.01$ and ^{@@@} $P < 0.001$ as compared to silymarin group. (A color version of this figure is available in the online journal.)

consequently extensive oxidative damage. These results are in consistence with previous reports.^{30,31}

The changes brought by BDL in our model favored fibrogenesis; namely, the decrease in HGF and *MMP-2* mRNA and increased *CK-19* mRNA. Hepatic fibrosis was confirmed by high fibrosis score in Sirius red-stained liver sections from BDL rats as well as increased positive area for α -SMA till the endpoint of our experiment. The decrement in HGF was responsible in part for the reduced self-proliferative capabilities of the liver and consequently misbalances between collagen deposition and degradation in

ECM due to the down regulation of *MMP-2* mRNA.³² The increased expression of *CK-19* mRNA revealed activation of HSCs, which represent a hallmark in the process of fibrosis as previously reported.³³

Treating BDL rats with BM-MSCs was evaluated after four weeks of BM-MSCs transplantation to make sure homing took place as previously reported.³⁴ We detected PKH26-labeled BM-MSCs in the injured liver tissue of recipient rats at 28 days after transplantation. In these rats, restoration of liver functions and hepatic GSH suggested a possible therapeutic role of BM-MSCs in cholestatic liver injury involving an antioxidant effect. This finding is consistent with previous studies which used mouse model of severe hepatic and renal damage.^{35,36} The unexpected increase in lipid peroxidation could be related to the proliferation process during liver regeneration as reported beforehand.^{37,38} Lipid peroxides can act like modulators of cell division influencing the initiation and cessation of mitosis in the regenerating liver,³⁷ might also activate transcriptional factors to serve as signal transducers between cytoplasm and nucleus and could influence the activity of ornithine decarboxylase, which is deeply involved in polyamine synthesis, essential in liver regeneration.³⁹

Hepatic cell proliferation and hence liver regeneration was attained by treating BDL-rats with BM-MSCs as evident by increased hepatic HGF and *MMP-2* mRNA and decreased *CK19* mRNA. HGF is among cytokines secreted by MSCs.^{40,41} It was considered as a humoral hepatotrophic and hepatoprotective factor that enhances liver regeneration.⁴² It was recently reported to be responsible for trans-differentiation of MSCs into hepatic lineage and was also involved in enhancement of MSCs potential for the reduction of liver fibrosis.⁴³ HGF also up-regulates the expression of urokinase-type plasminogen activator and MMPs. The latter are also a critical requirement in the normal hepatic regenerative process. As MMPs may facilitate hepatic regeneration directly via their effect on ECM remodeling, a process that is integral to tissue regeneration and/or indirectly, through their effects on growth of new capillaries from pre-existing vessels (angiogenesis) by facilitating endothelial migration through the degraded ECM. Also, MMPs release pro-endothelial growth factors from the ECM.^{32,44} In addition, the reduction of liver fibrosis (function of MMPs) itself is associated with improved hepatic regeneration.⁴⁵ This was further confirmed by the strong positive correlation between hepatic HGF level and *MMP-2* mRNA ($r=0.819$, $P < 0.0001$).

Down regulation of *CK-19* gene expression following infusion of BM-MSCs into BDL rats suggested the ability of BM-MSCs to differentiate into mature hepatocyte-like cells. *CK-19* is excreted from BM-MSCs only during the early stage of MSC-to-hepatocyte differentiation, but not in the late stage of MSCs differentiation. The mRNA levels of immature hepatocyte markers in MSCs (AFP, Nestin, and *CK19*) decreased as differentiation progressed.⁴⁶ This may explain the significant down-regulation of *CK-19* gene expression in BM-MSCs-treated groups if compared with untreated BDL rats. Homing of BM-MSCs into injured liver tissue and subsequent improvements in liver functions and architecture and the resolution of

Table 2 Correlation between selected regeneration parameters and liver function tests and the extent of liver fibrosis

	Correlated parameters	R	P
Serum ALT (U/L)	Serum HGF	-0.663	0.000
	Hepatic MMP-2 mRNA	-0.754	0.000
	Hepatic CK-19 mRNA	0.425	0.03
Serum AST (U/L)	Serum HGF	-0.842	0.000
	Hepatic MMP-2 mRNA	-0.842	0.000
	Hepatic CK-19 mRNA	0.721	0.000
Serum total bilirubin (mg/dL)	Serum HGF	-0.679	0.000
	Hepatic MMP-2 mRNA	-0.714	0.000
	Hepatic CK-19 mRNA	0.679	0.000
Fibrosis expression (OD-Sirus red stain)	Serum HGF	-0.820	0.000
	Hepatic MMP-2 mRNA	-0.830	0.000
	Hepatic CK-19 mRNA	0.890	0.000
α -SMA expression	Serum HGF	-0.848	0.000
	Hepatic MMP-2 mRNA	-0.851	0.000
	Hepatic CK-19 mRNA	0.898	0.000

hepatic fibrosis were strongly correlated with selected regeneration parameters. This would reveal that homed BM-MSCs may be in part responsible for the process of liver regeneration.

The therapeutic potentials of silymarin in cholestatic liver fibrosis was extensively reported^{29,47} and was attributed to its antioxidant and anti-fibrotic activity matching to our current report. Besides, silymarin has an important therapeutic implication in repairing the damaged hepatocytes and restoring the normal liver functions⁴⁸ by stimulating protein biosynthesis namely the synthesis of ribosomal RNA and hence liver regeneration.^{18,49} In our study, although silymarin was able to exert beneficial effect improving liver function tests in BDL rats and induced better effects on TB and albumin, its regenerative capabilities were less than that of BM-MSCs. Histopathological observations provided supportive evidence for the biochemical and molecular analyses.

Conclusions

Taken together, our results suggest increased liver regenerative capabilities by BM-MSCs attributed to increased HGF, subsequent upregulation of MMP-2 mRNA and downregulation of CK-19 mRNA.

Authors' contribution: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; SEE and HEM suggested the research idea, AMHG, NNY, LAR, and MAS conducted the experiments and recorded the results. AMHG and NNY performed statistical analysis, collected data from literatures and wrote the manuscript. SEE and HEM supervised the experimental work and revised the results and the manuscript. All authors revised the manuscript prior to submission.

ACKNOWLEDGMENTS

The authors acknowledge the support given by Faculty of Pharmacy, Zagazig University for using Molecular Biology laboratory and animal unit facilities.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

No. P11/2/2013.

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(Received May 7, 2015, Accepted December 21, 2015)