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Original Article

Therapeutic effect of bone marrow mesenchymal stem cells in a rat model of carbon tetrachloride induced liver fibrosis

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ABSTRACT

Background: Liver fibrosis is a major medical problem with high mortality and morbidity rates where the formation of regenerative nodules and cirrhosis leads to loss of liver function and may result in the development of hepatocellular carcinoma. bone marrow mesenchymal stem cells (BM-MSCs) have drawn attention as a novel approach for treatment of liver fibrosis. This study aimed to evaluate the therapeutic effect of BM-MSCs on the liver structure in carbon tetrachloride (CCl_4) induced liver fibrosis in male rats relative to resveratrol and *Silybum marianum* as standard drugs derived from herbal plants.

Methods: Fifty adult male albino rats (Sprague Dawley strain; 180–220 g mean body weight) were purchased from the Laboratory Animal Unit in the Nile Center of Experimental Research, Mansoura, Egypt. Liver function were determined, isolation and preparation of BM- MSCs and detection of cell-surface markers by flow cytometry.

Results: Animals exposed to CCl_4 developed liver injury characterized by significant increase of liver enzymes, malondialdehyde (MDA), tumor necrosis factor alpha (TNFα), and CYP450, inhibition of antioxidant enzymes, and decreased albumin. Treatment with stem cells enhanced liver state more effectively than resveratrol and *S. marianum*. It significantly decreased AST, ALT, ALP, MDA, TNF-α, and CYP450 and increased albumin, SOD, GSH, GST, and CAT. Histopathological study and atomic force microscope results confirmed the therapeutic effects of MSCs.

Conclusions: BM-MSCs could restore liver structure and function in CCL_4 induced liver fibrosis rat model, ameliorating the toxicity of CCl_4 and improving liver function tests.

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At a glance of commentary

Scientific background on the subject

Liver fibrosis is the final stage of all chronic hepatic disease. No effective therapy is currently available for Liver fibrosis except liver transplantation. Hence, we investigated the effect of bone marrow-derived mesenchymal stem cells (BM-MSCs) in a rat model of carbon tetrachloride (CCL_4) induced liver fibrosis.

What this study adds to the field

Our study showed that BM-MSC treatment inhibited fibrosis formation and the progression of CCL_4-induced liver fibrosis. Therefore, BM-MSC therapy leads to the improvement of hepatic fibrosis and may provide a new strategy for anti-fibrosis therapy in the future.

The liver is a vital organ that plays a key role in metabolic activities and detoxification of exogenous and endogenous substances [1]. Liver fibrosis is induced by exposure to toxic chemicals or injury. It is characterized by the accumulation of collagen and extracellular matrix proteins leading to dysfunction and fibrosis [2]. The most effective therapy for advanced hepatic cirrhosis is liver transplantation, but this is limited because of organ donor shortage, surgical complications, immunological suppression, and high cost. Thus, new therapies are needed for cell-based therapy, such as cell transplantation can be a potential alternative to liver transplantation [3].

Stem cells have the ability to implant in the target tissues and secrete many factors that could change or improve the function of damaged tissue [4]. Stem cells are advantageous due to their high capacity for self-renewal, multipotent differentiation, and low immunogenicity [5]. The properties of Bone marrow mesenchymal stem cells (BM-MSCs) suggest a potential use in liver fibrosis treatment and regeneration of hepatocytes [6]. BM-MSCs demonstrated anti-fibrotic effects and stimulated regeneration of injured liver in animal models of liver fibrosis [7]. They have the ability to secrete multiple bioactive molecules that are capable of stimulating recovery of hepatic injured cells and inhibiting inflammation [8]. An in vitro study has shown that BM-MSCs induce apoptosis and suppress collagen synthesis in hepatic cells. BM-MSCs were found to be capable of differentiating into hepatic stellate cells (HSCs) in vitro, and the differentiated cells gain hepatocyte-like morphologies and express hepatocyte specific markers [9]. Hence, activated HSCs represent an attractive target for antifibrotic therapy. Several in vitro studies have demonstrated the ability of MSCs to modulate HSC activation indirectly via paracrine mechanisms and directly through cell–cell contacts [10]. Additionally, in vivo studies have confirmed that BM-MSCs injected through a peripheral vein have antifibrotic effect and anti-inflammatory functions [11]. BM-MSCs alleviated liver damage and improved the liver microenvironment after hepatic ischemia/reperfusion injury [12]. Bone marrow-derived stem cells include mesenchymal stem cells (MSCs) and hematopoietic stem cells, MSCs are multipotent adult stem cells present in bone marrow [13].

Resveratrol (3, 4′, 5-trihydroxy-trans-stilbene) is a natural polyphenolic flavonoid found in many plant species, such as grapes, nuts, and berries. It has been suggested to have antioxidant, anti-inflammatory, antiplatelet aggregation and anti-neoplastic activities [14]. Resveratrol was shown to have antioxidant and anti-inflammatory effects and also to reduce hepatotoxicity [15].

Silybum marianum has been used for decades for treatment of diseases of the liver, spleen and gall bladder [16]. *S. marianum* is reported to have antioxidant, anti-inflammatory, anticarcinogenic, and growth modulatory effects [17]. The aim of this study was to evaluate the therapeutic effect of BM-MSCs on the liver structure in carbon tetrachloride (CCL4) induced liver fibrosis in rats, and to assess this effect in comparison to *S. marianum* and resveratrol as standard drugs.

Materials and methods

Experimental animals

Fifty adult male albino rats (Sprague Dawley strain; 180–220 g mean body weight) were purchased from the Laboratory Animal Unit in the Nile Center of Experimental Research, Mansoura, Egypt. They were housed in plastic cages and were given food and water ad libitum throughout the period of the experiment. The animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Research Ethics Committee of Faculty of Pharmacy, Suez Canal University (Code: 201603PHDA1).

Isolation and preparation of BM- MSCs

Isolation of BM- MSCs was done as previously described [18]. The six -week old male albino rats were anesthetized by inhalation of halothane. Skin was sterilized by application of 70% ethyl alcohol and cut. The femur and tibia were dissected and immersed in 70% ethyl alcohol for 1–2 min. The bones were then washed in a petri dish containing phosphate buffer saline (PBS) (1X, ph = 7.1) (Hyclone, USA). Bone marrow was extracted under laminar air flow (Unilab biological safety cabinet class II, China). The ends of the bones were cut with sterile scissors and bone marrow was harvested by flushing the bones with Dulbecco's modified Eagles medium (DMEM) (Lonza, Belgium). The marrow plugs were cultured in 20 ml complete medium (CM) consisting of DMEM (Lonza, Belgium) supplemented with 10% (v/v) FBS (Lonza, Belgium) and 1% (w/v) penicillin-streptomycin (Lonza, USA). The cultures were incubated at 37 °C in 5% humidified CO_2 incubator (Shellac, USA) for 7–10 days. When the cultures approached 80–90% confluence, cells were washed twice with phosphate buffer saline (PBS) and digested with 0.25% trypsin-EDTA (Lonza, Belgium) for approximately 5 min at 37 °C. After centrifugation at 2400 rpm for 20 min, cells were suspended with CM and incubated in a culture Falcon flask.

Detection of cell-surface markers by flow cytometry

The third generation of cells was taken and digested by 2.5 g/L trypsin to prepare 1×10^6/mL single cell suspension. BM-MSCs were stained with antibodies conjugated with fluorescein

isothiocyanate (FITC) or phycoerythrin (PE). CD45-FITC (positive markers of BM-MSCs) and CD105-PE (negative markers of BM-MSCs) antibody (BioLegend Company, USA) were used as the control isotypes. CD45-FITC and CD105-PE antibody were added and mixed with BM-MSCs. The solution was incubated at room temperature for 15 min. After two washes with PBS, the fluorescence intensity was determined by flow cytometry.

Experimental groups

Total 50 rats were divided randomly into 5 groups, each composed of 10 male rats:

Group (1): Normal group fed a basal diet, given saline twice a week for 8 weeks.

Group (2): Liver fibrosis (positive control) group, received the intra peritoneal injection of CCl_4 (0.5 mg/kg twice a week for 8 weeks). CCl_4 was prepared as a 50% (v/v) solution in olive oil. CCl_4 was chosen in this study to induce liver fibrosis because the CCL_4 induced liver fibrosis model is the most closely resembling that of human [19].

Group (3): Resveratrol group, received resveratrol dissolved in water orally (30 mg/kg/) + CCl_4 (0.5 mg/kg twice a week for 8 weeks) [20]. Resveratrol Extra® was purchased from Pure Encapsulations Inc., Sudbury, MA, USA.

Group (4): *S. marianum* group, received *S. marianum* extract orally (200 mg/kg daily) + CCl_4 (0.5 mg/kg twice a week for 8 weeks). Seeds of *S. marianum* were obtained from National Research Center, Dokki, Giza, Egypt. Extraction was performed as described before [21].

Group (5): BM-MSCs group, Received BM-MSCs one month after starting CCl_4 injection (2×10^6 cells/rat).

BM-MSCs were administered by intravenous injection through the penile vein. The first dose consisted of 10^6 cells/rat in 0.2 ml DMEM and after one week rats received the second dose of cells (10^6 cells/rat) in 0.2 ml DMEM.

At the end of the experimental period, rats were fasted overnight and sacrificed under halothane anesthesia. Blood was collected by cardiac puncture and centrifuged at 8600×*g* for 20min 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.

Liver function tests

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed using Spin react diagnostic kits (Spinreact, Gerona, Spain). Serum alkaline phosphatase (ALP) was assayed using Spectrum diagnostics (Obour city, Cairo, Egypt). Serum albumin was assayed using Diamond diagnostics kit (Heliopolis, Cairo, Egypt).

Determination of antioxidant enzymes and tumor necrosis factor alpha (TNF-α) levels

Liver tissue was homogenized in ice-cold PBS, pH 7.2. Antioxidant enzymes, glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GSH), and lipid peroxides (expressed as malondialdehyde (MDA), were determined as described (Bio diagnostics Co. Giza, Egypt). TNF-α was determined in the liver using ELISA kits (Sun red Biological Technology CO. Shanghai, China).

Expression of cytochrome P450

Expression of cytochrome P450 (CYP450) was determined by semi-quantitative PCR. Total RNA was extracted from liver tissue of all groups by Direct-Zol RNA miniprep (Zymod Research CORP, USA). RNA concentration was measured spectrophotometrically (Nanodrop 2000, Thermo Scientific, USA.). Reverse transcription was done using 1 μg of total RNA and a cDNA kit (high-capacity cDNA archive kit, USA). Two μl of the cDNA sample was mixed with 25 pico mole of each primer and 12.5 μl of Taq PCR (master mix kit, QIAGEN Inc, Valencia, CA, USA). Distilled water was added to a volume of 25 μl, and the resulting mixture was subjected to PCR amplification (BioRad Thermal Cycler, USA).

The primers were designed at NCBI international site as following: primers CYP450; (Rattus norvegicus cytochrome P450, family 1, subfamily a, polypeptide 1 (Cyp1a1), mRNA) Forward: 5′-CTTCCTCACAGCCAAAGCAG -3′ and reverse: 5′-GAATGTGGTGACGGCCAAGA -3′ (NM_012540.2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Forward: 5′-TGCCACTCAGAAGACTGTGG-3′ and reverse: 5′-GGATGCAGGGATGATGTTCT -3′ (NM_017008.4) was used as an internal control. The cycling parameters were as follows: The PCR cycle consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The amplicon sizes for CYP450 and GAPDH were 100 and 85 bp, respectively. The resulting products were electrophoresed in a 1.5% agarose gel to detect gene bands. The signal intensity of the bands was measured using BioRad Gel Documentation model (universal hood2, USA) software and the changes in expression were normalized to GAPDH control.

Histopathological examination

Haematoxylin and Eosin (H&E)

Liver tissues were collected and fixed in 4% paraformaldehyde (Merck Millipore, Germany). Paraffin liver sections were deparaffinized by xylene, dehydrated using alcohol, washed, and stained in hematoxylin (Merck Millipore, Germany) for 5 min, washed quickly, and then differentiated by 1% acid alcohol for 30 s and washing for 10 min. Slides were stained with eosin solution (Merck Millipore, Germany) for 2–3 min, then washed and mounted. Photographs were acquired using a digital image-capture system (Olympus CX40; Olympus, Tokyo, Japan).

Assessment of fibrosis

The grade of hepatic fibrosis was assessed in individual biopsy specimens in a blinded manner using Metavir scoring system [22]. The fibrosis score is assessed on a five-point

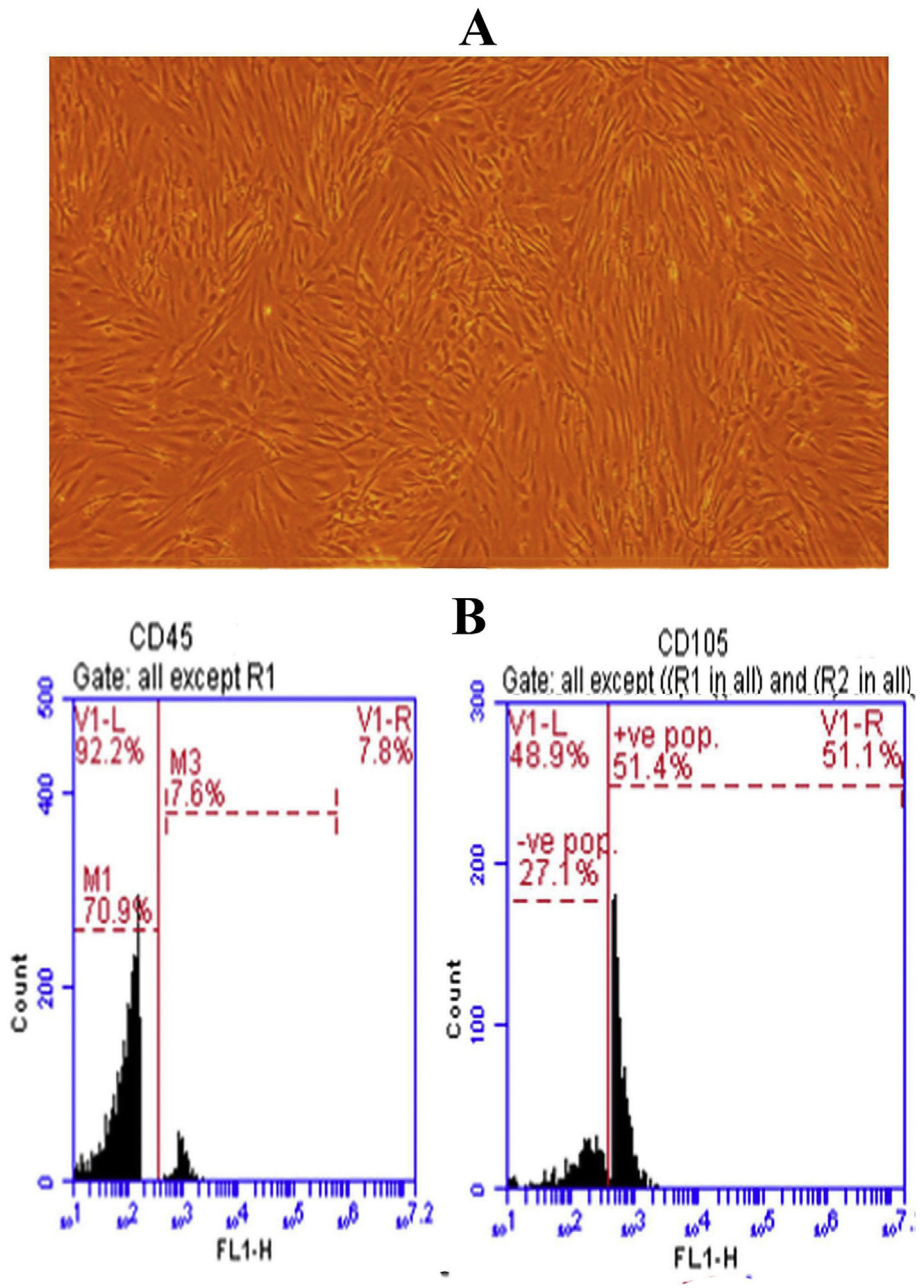

Fig. 1 (**A**) Morphology of BM-MSCs, nine days after isolation (X 100) showing spindle shape fibroblasts-like cells. **(B)** Flow cytometry charts for cells stained with CD105 and CD45 antibodies. Abbreviation: BM-MSCs: bone marrow-mesenchymal stem cells.

scale (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis).

Masson trichrome (MTC)

Liver section was deparaffinized, dehydrated, and washed. Slides were stained with Weigert's iron hematoxylin for 5 min and washed. Slides were stained with Biebrich scarlet acid fuchsin solution for 5 min and washed. Slides were differentiated in 1% phosphomolybdic-phosphotungstic acid solution for 5 min, then transferred to aniline blue solution, and stained for 5 min. Sections were differentiated in 1% acetic acid solution for 1 min, washed, dehydrated, and mounted with mounting medium. All sections were randomly evaluated with blindfold manner. Images were captured by a digital camera connected to a CX31 light microscope (Olympus, Japan).

MTC (× 200 magnification) was examined in ten separate fields of its stained sections (at different time periods of the experiment). The mean MTC percentage area per examined field was calculated as the average of the pooled readings from these fields in each specimen.

Table 1 Serum levels of ALT, AST, ALP and albumin in all the study groups.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Albumin (g/dL)
Normal	40.0 ± 4.4	142.1 ± 4.7	55.53 ± 13.53	6.79 ± 0.49
Positive control	86.7 ± 5.7[a]	196.9 ± 23.8[a]	123.38 ± 5.78[a]	4.46 ± 0.37[a]
resveratrol	53.4 ± 9.2[a,b]	161.3 ± 7.5[a,b]	75.75 ± 10.96[a,b]	5.00 ± 0.57[a,b]
Silybum marianum	52.3 ± 6.5[a,b]	153.0 ± 8.3[a,b,c]	85.42 ± 10.89[a,b]	5.44 ± 0.65[a,b]
BM-MSCs	44.2 ± 4.2[b,c,d]	145.9 ± 7.3[b,c,d]	62.72 ± 6.58[b,c,d]	6.42 ± 0.64[b,c,d]

Data are presented as mean ± SD. Comparisons were performed by ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$.
Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BM-MSCs: Bone marrow Mesenchymal stem cells.
[a] Significantly different from normal group.
[b] Significantly different from positive control group.
[c] Significantly different from resveratrol group.
[d] Significantly different from *Silybum marianum* group.

Atomic force microscopy (AFM)
AFM was conducted at the Nanotechnology center, Mansoura University, Mansoura, Egypt. Stiffness and roughness of the liver tissue were assessed as previously described [23].

Statistical analysis

Parameters from different groups were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. All values are presented as mean ± SD. Differences were considered significant at $p < 0.05$. Data were analyzed using SPSS/PC program (version 17; SPSS, Chicago, IL, USA).

Results

Characterization of BM-MSCs

BM-MSCs were identified by their adhesiveness and fusiform like shape [Fig. 1A]. To characterize the isolated cells, flow cytometry was performed for cell-surface markers. BM-MSCs were consistently positive for CD105, expressed in more than 54.3% of the cells, and negative for hematopoietic surface markers CD45, expressed in less than 7.9% of the cells [Fig. 1B].

Biochemical parameters

Serum AST, ALT and ALP were higher in positive control group compared to the normal group ($p < 0.05$). Serum levels of these enzymes were less in both resveratrol and *S. marianum* treated groups [Table 1]. The group treated with BM-MSCs had lower serum AST, ALT and ALP levels compared to the positive control group ($p < 0.001$) and the resveratrol and *S. marianum* treated groups ($p < 0.05$). There was no significant difference between the levels of these enzymes in the BM-MSCs treated group and the normal group ($p > 0.05$) [Table 1]. There was a significant reduction of serum albumin in positive control group compared to the normal group ($p < 0.001$). Serum albumin levels were increased in groups treated with resveratrol and *S. marianum*, but the group treated with BM-MSCs showed significantly higher serum albumin levels compared to the two other treated groups ($p < 0.001$) with no significant difference between its levels in BM-MSCs treated group and the normal group [Table 1].

SOD, CAT, GSH, and GST were less in positive control group relative to the normal group ($p < 0.05$). They were increased upon treatment with resveratrol or *S. marianum* in comparison with positive control group [Table 2]. In the BM-MSCs treated group; there was a marked increase in all these antioxidant enzymes compared with positive control group ($p < 0.05$). Their levels in the BM-MSCs group were close to the normal levels [Table 2]. MDA and TNF-α levels increased significantly in the positive control group when compared with the normal group and significantly decreased in all treated groups ($p < 0.05$), with their levels in the BM-MSCs treated group significantly lower than resveratrol or Silybum marianum treated groups ($p < 0.05$) and not different from the levels of MDA and TNF-α in the normal healthy group ($p > 0.05$) [Table 2].

Semi-quantitative RT-PCR of cytochrome P450

To determine the effects of BM-MSC on the activity of CYP450 enzyme, the expression of CYP450 was analyzed using semi-quantitative RT-PCR in the liver tissues [Fig. 2A]. Expression of CYP450 was significantly increased in the positive control group compared to the normal group ($p < 0.05$). CYP450 expression levels in the BM-MSC treated group were significantly decreased compared to the positive control, resveratrol and *S. marianum* groups ($p < 0.05$). Moreover, the results revealed that there was no significant difference between resveratrol treatment and *S. marianum* treatment [Fig. 2B].

Histopathological examination

Haematoxylin and Eosin staining
The hepatic cell damage was assessed by histological examination of liver tissues using H&E stain and METAVIR scoring system [Fig. 3]. Histopathological study of liver sections of normal group revealed the normal liver structure scoring fibrosis (F0). Non-inflammatory cells in the periportal areas were observed [Fig. 3A]. Liver of the positive control group showed inflammation with congested blood vessels and necrosis of hepatocytes (*F 3*) [Fig. 3B]. The liver of Resveratrol treated rats showed mild fibroblastic proliferation radiating from the portal area (*F 1*) [Fig. 3C]. Histopathological

Table 2 Levels of SOD, GST, CAT, GSH, MDA and TNF-α in all the study groups.

Groups	SOD (U/g tissue)	GST (U/g tissue)	CAT (U/g tissue)	GSH (mg/g tissue)	MDA (m mol/g)	TNF-α (U/g tissue)
Normal	1269.2 ± 33.2	4.52 ± 0.39	602.8 ± 139.2	17.17 ± 4.43	15.96 ± 1.49	463.9 ± 30.6
Positive control	920.5 ± 52.3[a]	3.46 ± 0.47[a]	306.8 ± 65.0[a]	6.01 ± 0.71[a]	28.42 ± 6.05[a]	839.7 ± 59.0[a]
resveratrol	1166.8 ± 113.3[a,b]	4.06 ± 0.92[b]	535.8 ± 39.6[b,d]	15.40 ± 2.84[b]	21.99 ± 1.08[a,b]	677.2 ± 15.5[a,b,d]
Silybum marianum	1184.6 ± 129.5[a,b]	4.14 ± 0.70[c,a,b]	405.9 ± 28.6[a,b,c]	13.77 ± 2.41[a,b]	23.40 ± 3.67[a,b]	620.5 ± 17.7[a,b,c]
BM-MSCs	1246.6 ± 83.9[b]	4.50 ± 0.69[b]	625.9 ± 42.08[b,c,d]	16.35 ± 3.43[b,d]	17.35 ± 1.75[b,c,d]	490.6 ± 46.3[b,c,d]

Data are presented as mean ± SD. Comparisons were performed by ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$.
Abbreviations: BM-MSCs: Bone marrow Mesenchymal stem cells; CAT: catalase; GSH: glutathione reduced; GST: glutathione −S- transferase; SOD: superoxide dismutase; TNF-α: Tumor necrosis factor alpha.
[a] Significantly different from normal group.
[b] Significantly different from positive control group.
[c] Significantly different from resveratrol group.
[d] Significantly different from *Silybum marianum* treated group.

examination of the livers of *S. marianum* group showed mild intralobular fibroblastic proliferation (*F 1*) [Fig. 3D]. Microscopical examination of liver sections of rats treated with the BM-MSCs showed normal tissue and decrease of the severity of histopathological changes induced by CCl_4 (*F 0*) [Fig. 3E]. The degree of fibrosis was scored according to the METAVIR scoring system. The BM-MSC treated group showed significant improvement in hepatic fibrosis compared to positive control, *S. marianum* and resveratrol groups ($p < 0.05$). The level of fibrosis in BM-MSCs treated group is not significantly different from the normal group [Table 3].

Masson trichrome staining (MTC)

Liver tissues of the normal group showed no collagen proliferation as recorded by MTC stain [Fig. 4A]. In contrast, collagen was detected surrounding the central vein and extended to the portal area in the liver tissue from the positive control group. Collagen also divided the hepatic parenchyma into labels [Fig. 4B]. Normal tissue and no collagen proliferation recorded in the BM-MSCs group [Fig. 4E]. In addition, the analysis results of histopathological fibrosis confirmed that cirrhosis was markedly reduced by BM-MSCs treatment, compared to the positive control group ($p < 0.001$), where treatment with *S. marianum* as a standard therapy did not show an impressive reduction in fibrosis, but moderately reduced inflammatory activity [Fig. 4F].

Atomic force microscope (AFM) results

AFM was used for cell imaging and observing changes in surface morphology and ultra-structure of cells in all studied groups. AFM image of the liver in the normal group showed roughness and stiffness within the normal range [Fig. 5A]. CCl_4 administration resulted in significantly increased roughness [Fig. 5B]. Liver in resveratrol and *S. marianum* treated groups showed decreased roughness and stiffness [Fig. 5C & D]. The cell surface appeared normal with significantly decreased roughness and stiffness in BM-MSCs treated rats [Fig. 5E]. Also, the analysis of AFM results confirmed that stiffness was significantly reduced by BM-MSCs treatment compared to positive control, *S. marianum* and resveratrol groups ($p < 0.05$) [Fig. 6A]. On the other hand, the roughness in BM-MSCs treated group was significantly reduced compared to positive control group ($p < 0.05$) [Fig. 6B].

Discussion

In this study, we investigated the effect of BM-MSCs on hepatic fibrosis and its ability to restore the liver structure in an experimental CCl_4 model of liver fibrosis. *S. marianum* and resveratrol were used in this study as reference drugs. The therapeutic potentials of *S. marianum* in the treatment of liver fibrosis were reported and were attributed to its antioxidant and anti-fibrotic activity [24]. The effect of BM-MSCs was investigated in a different model of fibrotic liver in comparison to Silymarin as a standard drug [25]. Another study investigated the effect of MSCs combined with resveratrol in treatment of partially hepatectomized rats [26]. Resveratrol has an important therapeutic effect in the treatment of liver fibrosis and protects liver cells from damage and hence causes liver regeneration by stimulating protein biosynthesis [27].

Several studies have reported that stem cells secrete cytokines and growth factors to promote regeneration, inhibit inflammation, and reduce the generation of extracellular matrix (ECM) and degradation of the intrahepatic excess in the ECM [28,29].

Liver fibrosis is characterized by excess accumulation of collagens and other ECM proteins and by the clinical impairment of liver function [30]. CCl_4 can generate free radicals resulting in hepatic fibrosis, liver dysfunction, a significant increase in serum parameters of liver function and a decrease in liver synthetic capacity [31].

The ideal strategy to treat liver fibrosis is to generate new hepatocytes to replace damaged cells without causing excessive ECM deposition [32]. Studies have shown that BM-MSCs have the lasting ability to generate hepatocytes and bile duct cells in the repair process after liver injury when there is severe liver damage [33]. It has been investigated that BM-MSCs transplantation can restore normal function of liver in acute and chronic liver disease [34].

In the present study, BM-MSCs were identified by their morphology, adherence and fusiform shape. CD105 gene expression as a positive surface marker of BM- MSCs and CD45 as a negative surface marker were also detected [35]. The potential therapeutic benefit of BM- MSCs can only be achieved through their homing efficiency to the target site. The

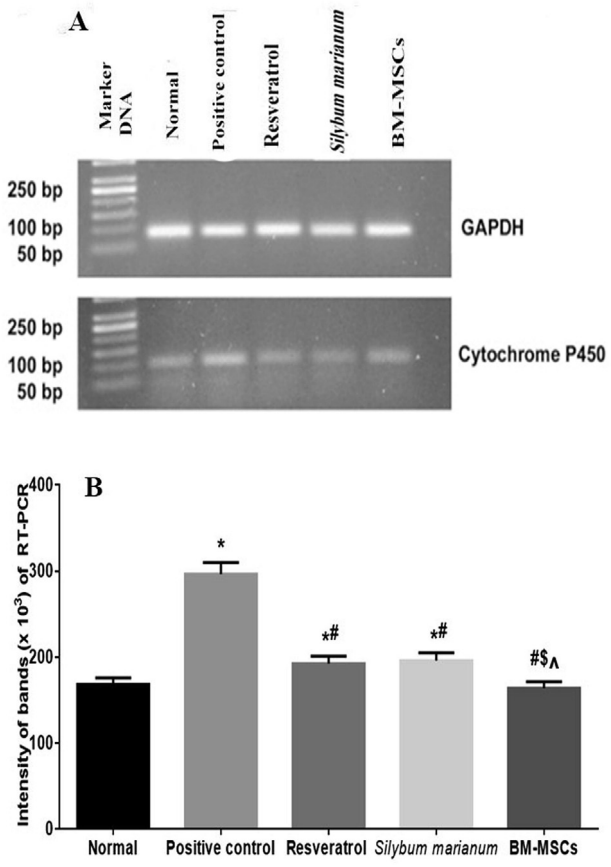

Fig. 2 Gene expression of CYP450 levels. **(A)** Reverse Transcription – Polymerase Chain Reactions (RT-PCR) of CYP450 gene (semi-quantitative) using agarose gel electrophoresis by BioRad Gel Documentation model (Universal hood2, USA). **(B)** The relative expression percentages of different groups to negative control (100%) by measuring the intensity of bands. Data are presented as mean ± SD. Comparisons were performed by ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$. *Significantly different from normal group. #Significantly different from positive control group. $Significantly different from resveratrol group. ^Significantly different from *Silybum marianum* treated group.

homing of BM- MSCs to the hepatic cell was detected by PKH26 dye which was used to label BM- MSCs [36].

Liver enzymes, ALT, and AST are useful serum markers for liver inflammation and necrosis in liver tissue [37,38]. In this study, AST, ALT and ALP levels of BM-MSC-treated groups improved more efficiently than those of resveratrol and *S. marianum* groups. Similarly, bilirubin and albumin levels changed positively in BM-MSC-treated groups. Previous findings showed that transplanted BM-MSCs could restore the serum albumin level and significantly suppressed transaminase activity and liver fibrosis in injured liver of rats [7].

The levels of SOD, GSH, GST, and CAT in CCl_4 induced liver fibrosis group were reduced. Similar results were found by Wang et al. [39]. These results may be due to destruction of hepatic cells and failure of liver detoxification by antioxidant enzymes and subsequent increased production of reactive oxygen species ROS [40]. In the present study, injection of BM-MSCs to rats ameliorated the toxic effects induced by CCl4 via increasing SOD, GSH, GST, and CAT and subsequently reducing oxidative stress. BM-MSCs were suggested to scavenge the reactive oxygen species (ROS) that trigger a cascade of events resulting in hepatic fibrosis [39].

The present study demonstrated that, after injection of BM-MSCs, the level of MDA in liver tissues decreased significantly relative to resveratrol and *S. marianum* treated groups. Similar results were reported by Sayyed et al. [41]. BM-MSCs secrete numerous factors, such as nitric oxide and prostaglandin E2 [42], which enhance antioxidant defenses, inhibit oxidation factors, thereby exerting anti-inflammatory effects and reduce necrosis of hepatocytes [43]. These key functions of BM-MSCs may be responsible for the liver marker recovery after transplantation.

Several studies have shown that serum levels of TNF-α are significantly elevated in liver diseases [44–46]. In the present study Serum levels of TNF-α were significantly decreased in BM-MSCs group. These results are in agreement with Lee et al. [47] who found that serum level of TNF-α decreased significantly after BM-MSCs treatment due to deactivation of macrophages and hepatic stellate cells.

On the other hand, induction of liver damage by CCl_4 caused significant increase in the level of CYP450. Cytochrome P450 1A1 (CYP1A1) is considered as an important marker for liver fibrosis induced by carbon tetrachloride as CCl_4 is metabolised by CYP1A1 to the trichloromethyl radicals (CCl_3* and/or CCl_3OO). This radical can bind to cellular molecules (e.g. nucleic acid, protein, lipid). So, it is obvious that the reductive metabolism of CCl_4 to reactive intermediates by CYP1A1 enzymes is an essential prerequisite for its toxicity [48,49]. The effect of CCl_4 on the level of CYP450 was also reported by Recknagel et al. [50]. The expression levels of CYP450 can be elevated by a process induction of CCl_4 to generate free radicals that trigger a cascade of events resulting in hepatic fibrosis, liver dysfunction and a decrease in liver synthetic capacity [7,51]. The results of the present study demonstrated that CYP450 expression was restored to the normal level after treatment with BM-MSCs. This may be due to the ability of BM-MSCs to differentiate to functional hepatocytes at the level of gene expression [50].

The gold standard tool for diagnosis of liver fibrosis is a histopathological examination that demonstrates the presence of fibrosis with other changes as described in previous studies [52]. In the current study, these changes and fibrosis area disappeared after treatment with BM-MSCs compared to other treated groups by decreasing fibrosis at the end stage through regenerative properties. These results match with Fang et al. [53]. BM-MSCs can differentiate into functional hepatic cells and then produce a series of growth factors and cytokines to ameliorate liver injury [54].

In this study, we employed the METAVIR scoring system to evaluate inflammation and estimate the stage of fibrosis. According to this scoring system, histological improvement was observed in hepatic fibrosis after BM-MSCs treatment. Through histological H&E and MTC staining, we showed that

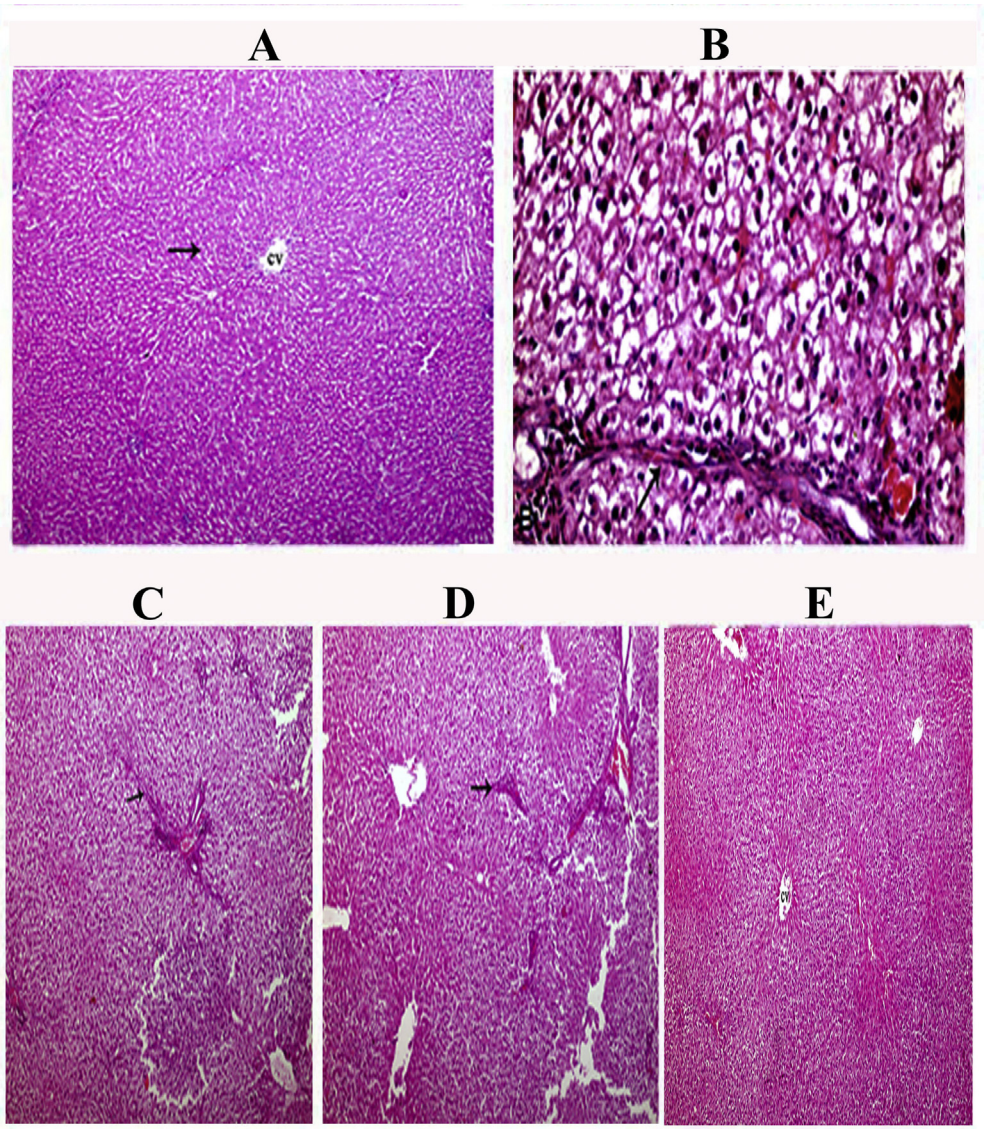

Fig. 3 Photomicrographs of liver tissue stained with Haematoxylin and Eosin. **(A)** Normal group showing normal portal area structures (arrow) and most of hepatocytes were within normal limits (*F0*). **(B)** Positive control group showing intralobular fibroblastic proliferation forming thin porto-portal bridging fibrosis (*F3*). **(C)** Resveratrol group showing mild fibroblastic proliferation (*F1*). **(D)** *Silybum marianum* group showing intralobular fibroblastic proliferation (*F1*). **(E)** BM-MSCs group showing mild degeneration of hepatocytes with normal central vein (CV) (*F0*). (H&E, 100x).

BM-MSC administration resulted in significant improvement of hepatic fibrosis compared to positive control group. BM-MSCs ameliorated liver fibrosis by down-regulating the profibrotic genes and up-regulating anti-fibrotic hepatic genes [55]. Our results are in agreement with previous reports [56,57]. Based on these results, BM-MSC transplantation is effective for the treatment of liver fibrosis and restores normal liver structure, which corresponds to previous studies [18,58].

Table 3 Comparison of fibrosis among different studied groups.

Fibrosis score	Normal (10)	Positive control (10)	Resveratrol (10)	*Silybum marianum* (10)	BM-MSCs (10)
F0	8	0	0	0	2
F1	2	1	6	5	7
F2	0	2	4	5	1
F3	0	7	0	0	0

The fibrosis score is assessed on a five-point scale using Metavir scoring system (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis). Abbreviation: BM-MSCs: Bone marrow Mesenchymal stem cells.

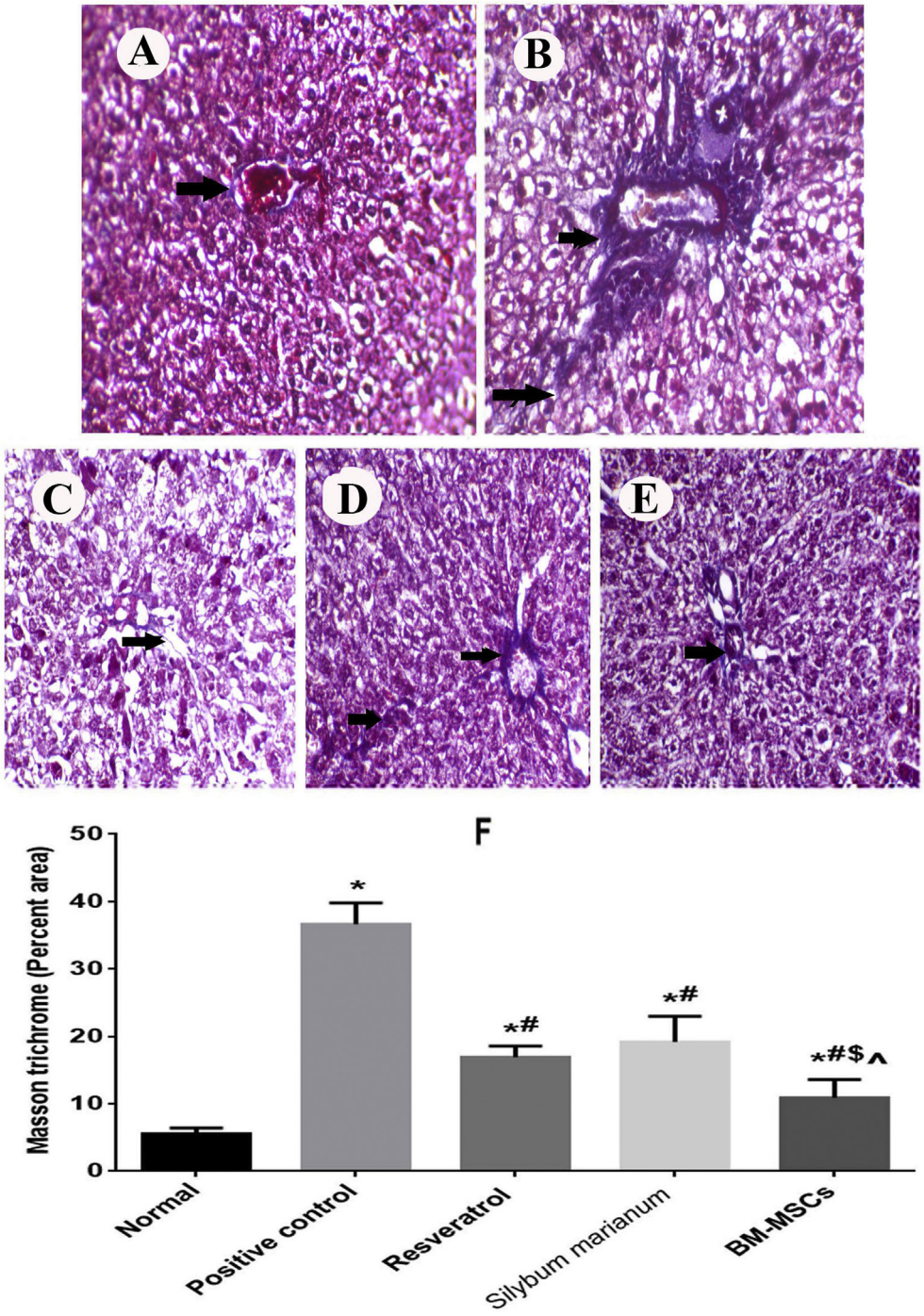

Fig. 4 Photomicrographs of liver tissue stained with Masson trichrome. **(A)** Normal group showing minute sheet of periportal collagen fibers, **(B)** Positive control group showing marked periportal collagen fibers and also intra-lobular collagen fibers deposition. **(C)** Resveratrol group showing mild periportal collagen fibers deposition. **(D)** *Silybum marianum* group showing mild degree of collagen fibers. **(E)** BM-MSCs group showing mild periportal collagen fibers deposition. **(F)** The percentage of the collagen content and fibrosis area values are expressed as mean ± SD for ten rats in each group. (MTC-X200). Data are presented as mean ± SD. Comparisons were performed by ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$. *Significantly different from normal group. $^{\#}$Significantly different from positive control group. $^{\$}$Significantly different from resveratrol group. $^{\wedge}$Significantly different from *Silybum marianum* treated group.

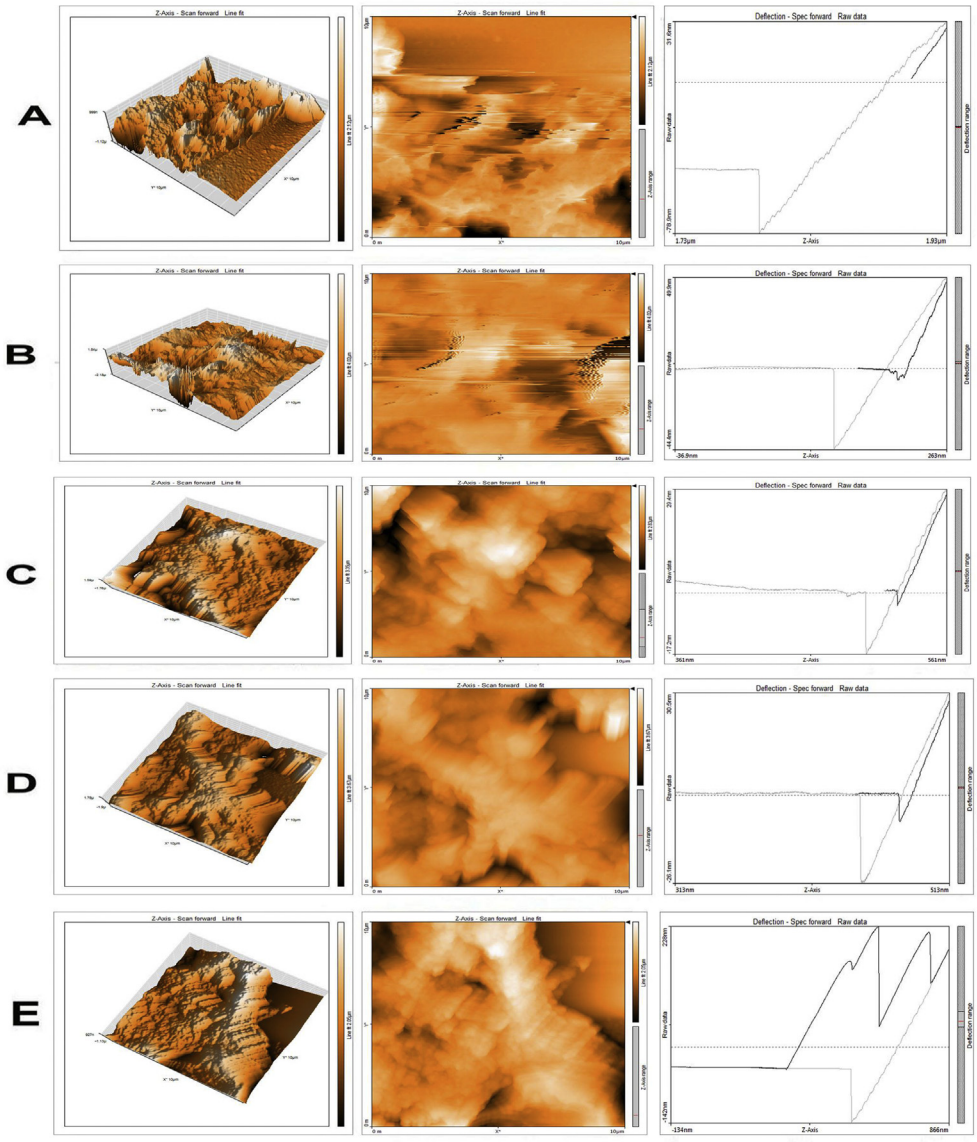

Fig. 5 Atomic force microscope (AFM) image of liver: **(A)** force distance curve of normal group where roughness (sq. = 339 nm), height 31.742Nn, width 42.97 nm and stiffness 0.73 N/m. **(B)** force distance curve of Positive control group where roughness (sq. = 590.62 nm), height 47.4Nn, width 39.84 nm and stiffness 1.1 N/m. **(C)** force distance curve of resveratrol group where roughness (sq. = 474.09 nm), height 29.26Nn, width 35.94 nm and stiffness 0.81 N/m. **(D)** force distance curve of *Silybum marianum* group where roughness (sq. = 508.04 nm), height 30.17Nn, width 35.55 nm and stiffness 0.84 N/m. **(E)** force distance curve of BM-MSCs group where roughness (sq. = 345.97 nm), height 128Nn, width 166 nm and stiffness 0.77 N/m.

AFM is a powerful tool for nano-scale imaging of cells and an important diagnostic instrument [59]. AFM determines the surface roughness and stiffness of organs with qualitative and quantitative information on cell membranes [60]. In the current study, the stiffness and roughness of the liver tissues returned to the normal level after treatment with BM-MSCs with significant decrease when compared with positive control group and other treated groups. This may be explained by the anti-fibrotic activities of BM-MSCs in liver and increased regeneration of the tissue and restore the normal surface of the liver tissues [61–63]. The regenerative capabilities and resolution of hepatic fibrosis of both resveratrol and *S. marianum* were less than that of BM-MSCs. Histopathological observations and AFM provided supportive evidence for the biochemical and molecular analyses.

BM-MSCs exert their beneficial effect either by undergoing differentiation in the liver tissue or by a paracrine mechanism via release of cytokines/chemokines that help to reduce the inflammation, fibrosis and oxidative stress associated with the disease [64]. BM-MSCs stimulate hepatic regeneration in liver injury through a number of cellular mechanisms including generation of de novo hepatocytes through transdifferentiation and/or cell fusion, paracrine stimulation of endothelial differentiation and vasculogenesis, antifibrogenic modulation of the stromal microenvironment [12], and secretion of hepatotrophic growth

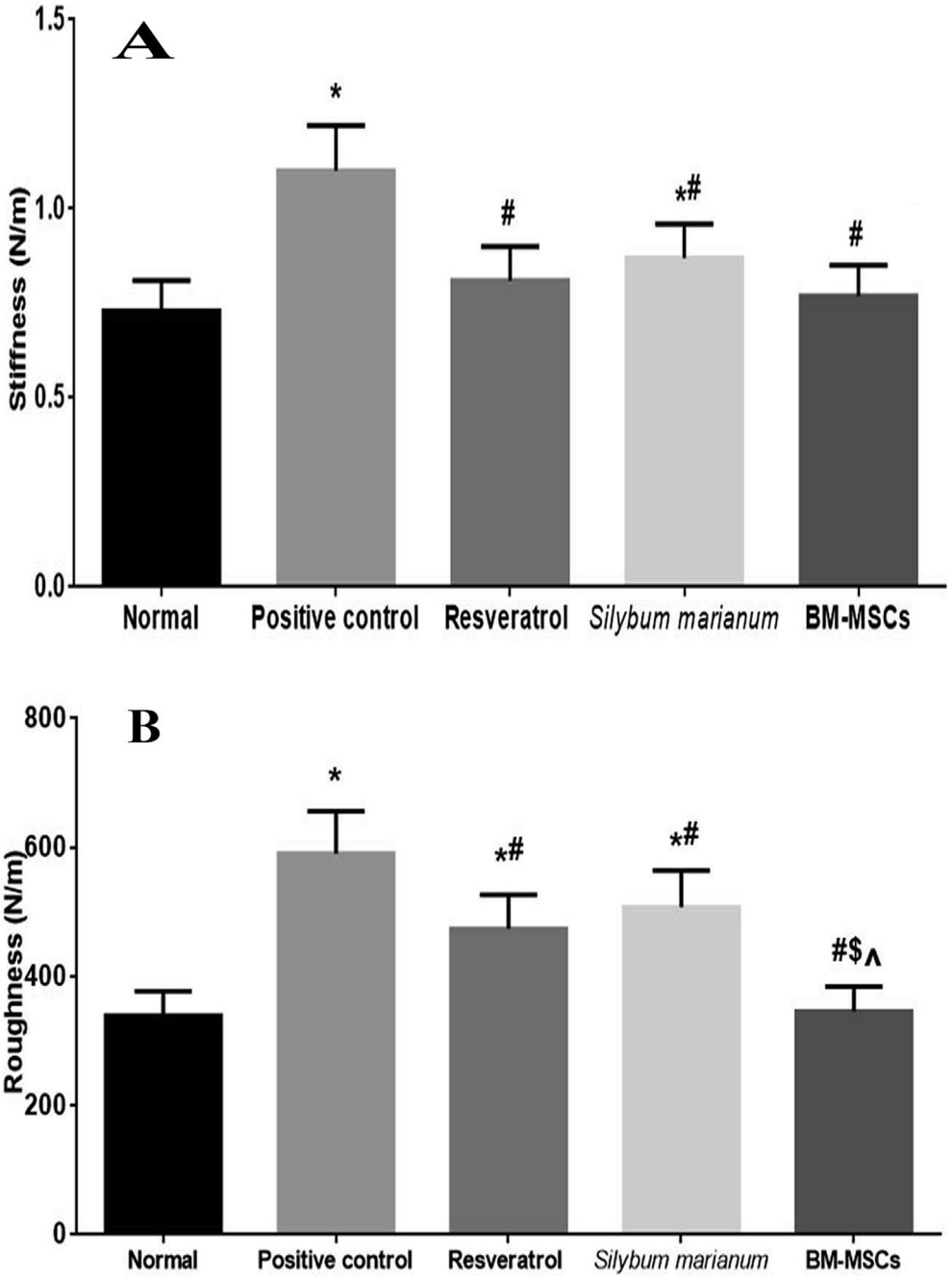

Fig. 6 Atomic force microscope (AFM) relation between different studied groups. **(A)** Comparison of stiffness among different studied groups. **(B)** Comparison of roughness among different studied groups. Data are presented as mean ± SD. Comparisons were performed by ANOVA followed by Bonferroni's post-hoc test for multiple comparisons. Differences were considered significant at $p < 0.05$. *Significantly different from normal group. #Significantly different from positive control group. $Significantly different from resveratrol group. ^Significantly different from *Silybum marianum* group.

factors which are multipotent endogenous repair factors secreted primarily by hepatic mesenchymal cells [65]. These factors act to promote cell survival, suppress chronic inflammation and resolve fibrosis by inhibiting extracellular matrix (ECM) production and deposition, inducing myofibroblasts apoptosis [66].

From the previously discussed results, BM-MSCs could restore the liver structure and function and markedly decreased the induced liver fibrosis in the experimental model. The regenerative capabilities and resolution of hepatic fibrosis of BM-MSCs were significantly more efficient than that of both resveratrol and *S. marianum*. Histopathological

observations and AFM provided supportive evidence for the biochemical and molecular analyses. Stem cell therapy may offer hope to patients waiting for liver transplantation.

Conflicts of Interest

The authors declare no conflicts of interests.

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