Effect of Silymarin and/or Bone Marrow-Derived Mesenchymal Stem Cells on Carbon Tetrachloride-Induced Hepatotoxicity in Rats

Tariq I Almundarij1*, Abdel Kader A Zaki1,2, Yousef M. Alharbi1, Saleh M. Albarak1, Tamim S Alqarawi2 and Faten A M Abo-Aziza3*

1Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, Saudi Arabia
2Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
3Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, Cairo, Egypt

*Corresponding Authors: Faten A M Abo-Aziza (faten.aboaaziza@gmail.com)
Tariq I Almundarij (tmdrj@qu.edu.sa)

ABSTRACT
Hepatic fibrosis has been diagnosed in more than 10% of the world population and is still with limited treatments. This work explored whether silymarin and bone marrow-derived mesenchymal stem cells (BM-MSCs) combination can remodel the hepatotoxicity induced by carbon tetrachloride (CCl4) in rats. Fifty rats were equally distributed into five groups: healthy control, CCl4, hepatotoxicity rat model, CCl4 + silymarin treated, CCl4 + BM-MSCs treated. Serum liver function tests, hepatic tissue oxidative enzymes and cytokines were assessed. Akt and P-Akt proteins expression was estimated by Western blot. Livers were examined histologically using two types of staining. The cultured BM-MSCs positively expressed CD73, CD105, and CD29 and negatively expressed CD34 Akt proteins phosphorylation.

INTRODUCTION
Liver disease is a broad term that covers all the potential problems that inhibit the liver from performing its designated functions. The main responsibility of the liver is to detoxify the majority of drugs and toxins and get rid of them outside the body. However, some of these toxins can cause liver injury, which is termed hepatotoxicity. Furthermore, the general liver complaint in many countries is the fatty liver disease, which is the accumulation of excess fat in the liver cells [1]. Nonalcoholic Fatty Liver Disease (NAFLD) is considered one of the most primary causes of cirrhosis. Fibrosis of the liver is a huge problem that leads to organ dysfunction [2]. Hepatic fibrosis has been diagnosed in more than 10% of the world population and is still a main common health disease with limited treatment options and a variety of underlying etiologies [3]. Fibrosis is typically accompanied by the proliferation of hepatic parenchymal cells in the early stage as well as by interchanging mediators and cells. A number of issues arise when the liver is diagnosed with fibrosis, which follows the pathological process of chronic disease that begins with the damage and ends with the healing process, matrix protein deposition, and remodeling. It is accompanied by loss of both endothelium and parenchyma by macrophage stimulation and inflammatory cues [4]. Hepatic stellate cells (HSCs) have been documented to induce and form fibrosis through a membranous signals cascade as PI3K/Akt/mTOR [5]. These signals are responsible for cell cycles and differentiation [3]. Prevalence of 7 – 10% NAFLD has been reported in Saudi Arabia [6]. The main risk factors that are very popular in Saudi Arabia are diabetes mellitus, hyperlipidemia as well as obesity, with some records suggesting 23.7%, 54% and 35.5% prevalence respectively [7]. The most recent WHO data released in 2017 recorded 1,995 liver disease deaths in Saudi Arabia which is 2.05% of total deaths. The age depending mortality rate was 13.33 from each 100,000 population and Saudi Arabia was ranked as #99 in the world for liver disease [8]. Scientists face several problems when dealing with the abovementioned damage to liver tissue, notwithstanding the myriad of the used therapy to prohibit complications and slope the progression rate. Although liver transplantation is widely considered an effective solution; Its implementation is limited due to the high costs, donor’s scarcity and rejection of the transplanted organ [9]. Liver cells transplantation can play an alternative technique to ameliorate liver regeneration. Nonetheless, there is a deficiency in superior in vitro primary hepatocytes. They are difficult to propagate and their hepatic characteristics are easily lost [10]. Significant evidence shown in past studies suggests that treatment by silymarin can improve acute and chronic hepatic diseases [11]. Silymarin is a natural complex derived from the common plant (Silybum marianum). The activity of silymarin includes the enhancement of hepatic glutathione and stimulation of RNA polymerase I activity, in addition to its contribution to

Keywords: Bone marrow-derived mesenchymal stem cells, Carbon tetrachloride, Hepatotoxicity, Rat, Silymarin

Correspondence:
Faten A M Abo-Aziza
Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, Cairo, Egypt
Email: faten.aboaaziza@gmail.com
Tariq I Almundarij
Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, Saudi Arabia
Email: tmdrj@qu.edu.sa
the hepatic antioxidant protection [12]. The hepatoprotective and antioxidant activity of silymarin inhibits the free radicals produced from the metabolism of toxic materials such as CCl₄ and ethanol [12]. In the case of liver fibrosis, silymarin has an anti-fibrotic effect during signal transduction and also inhibits protein kinases. In addition, it may interact with intracellular signaling pathways [13].

There is a need for additional support to control liver damage. In recent years, mesenchymal stem cells (MSCs) have been studied extensively. Their dual characteristics of continuous own-renewal and the ability to differentiate into a variety of mature tissue types favor their use in regenerative medicine [15]. These MSCs are adult stem cells found in numerous tissues, including bone marrow and the umbilical cord, that have the ability to give rise to diverse cell types in the laboratory, such as cartilage, bone, fat, tendon, ligament, and muscle cells. Numerous studies have established the positive effects of MSCs on the regeneration of damaged tissue, reduction of inflammation and oxidative stress, and modulation of immune reactions [15]. These MSCs can be separated from numerous tissues (e.g., adipose tissue, bone marrow, amniotic fluid, menstrual blood, the umbilical cord tissue and blood) and purified. Thus, because of the shortage of sources for the transplantation of liver or hepatocyte, mesenchymal stromal cell therapy is being seen as an effective novel approach for the repair of liver damage [1].

In the current study, a CCl₄-induced hepatotoxicity rat model was created because of the close similarity to liver fibrosis and the umbilical cord tissue and blood) and purified. Thus, because of the shortage of sources for the transplantation of liver or hepatocyte, mesenchymal stromal cell therapy is being seen as an effective novel approach for the repair of liver damage [1]. In the current study, a CCl₄-induced hepatotoxicity rat model was created because of the close similarity to liver fibrosis and the umbilical cord tissue and blood) and purified. Thus, because of the shortage of sources for the transplantation of liver or hepatocyte, mesenchymal stromal cell therapy is being seen as an effective novel approach for the repair of liver damage [1].

**Materials and Methods**

**Ethical standard**

The current work was permitted through the Animal Ethics Committee of Qassim University (QU) (No. 3432). The experiments were consistent with the guides recognized by the International Animal Ethics Committee and were carried out according to local laws and regulations.

**Animals**

A total of fifty healthy male Wistar albino rats (150 - 180 g) were transported from the King Saud University laboratory center in Riyadh to suitable housing under hygienic conditions at the Department of Veterinary Medicine of the QU Faculty of Agriculture and Veterinary Medicine in Buraydah, Saudi Arabia. The animals were kept in cages (30 × 35 × 15 cm per 4-5 rats). All animals received a commercial diet formulated to supply all the recommended nutrients [17]. Feed and water were provided ad libitum. Animals care was permitted by the QU Animal Ethics Committee and was monitored throughout the experimental duration.

**CCl₄-induced hepatotoxicity rat model**

The CCl₄-induced rat model is mostly considered as the typical and commonly used of the various liver hepatotoxicity models (4). In the present work, the rat model was implemented via intraperitoneal (i.p.) injection (1.0 mg/kg) of diluted CCl₄ (BDH Chemicals, England) in olive oil (1:1.5, vol/vol) twice a week for 70 days.

**Bone marrow-mesenchymal stem cells (BM-MSCs)**

Five 50-day-old albino Wistar rats were used for isolation of MSCs from BM of the femur and tibia as described previously, with some modification [18]. Briefly, the rats were killed using sodium pentobarbital anesthesia and the bones excised. The BM was harvested by inserting a syringe containing complete conditioned DMEM warmed medium with glucose (1 g/l) + fetal bovine serum (10%) into the bone and extracting the medium with the red marrow, which was collected in Petri dishes (100 mm) containing mL heparin (2000 IU / 0.2 mL). The suspension was centrifuged twice with phosphate buffer saline (PBS) to discard all remaining tissues. After that, alpha minimum essential medium (α-MEM) was added, and the diluted BM was used with similar amount of sodium carbonate buffer solution (0.1%) for lysis of the red blood cells as described previously [19]. The mononuclear cells (MNCs) were isolated and grown as previously mentioned [20]. Briefly, the cells were incubated with 5% CO₂ for 24 h, at 37 °C to adhere, and non-adherent were washed out once the medium was changed. Maintenance of the attached cells was carried out with α-MEM complemented with fetal bovine serum (20%), 55 μM 2-mercaptoethanol, 2 mM L-glutamine, and 2 kinds of antibiotics. Culture dishes were preserved in a humid incubator with 5% CO₂ at 37 °C. The medium was regularly changed every 2 days. When the attached cells of the 1st culture had achieved 80 % confluence, subculture was done and named passage zero. The following passages were numbered accordingly. Adherent cells of the primary cell culture were released by sterile trypsin-EDTA solution (0.25%) at 37 °C after a double wash with PBS. Additional fetal bovine serum (100 μL) was used to trypsin inactivation. The cells were pelleted after centrifugation at low speed followed by division into two parts via the same means. Each part was plated to increase the cell number. All the previous procedures were performed using sterile instruments under aseptic conditions in a laminar flow safety cabinet with filtered air.

**Immunophenotype analysis of BM-MSCs**

Immunophenotype analysis was performed by the detection of cell surface markers using flow cytometry [21]. All major markers for MSCs cell surface as CD45, CD34, CD73, CD29, and CD105 were detected because of the close similarity to liver fibrosis and the umbilical cord tissue and blood) and purified. Thus, because of the shortage of sources for the transplantation of liver or hepatocyte, mesenchymal stromal cell therapy is being seen as an effective novel approach for the repair of liver damage [1].

**Viability % and proliferation capability of BM-MSCs**

Trypan blue staining was used to evaluate the viability of the BM-MSCs with the exclusion of the dead cells [22]. The proliferation of the confluent BM-MSCs (80%) was evaluated using a bromodeoxyuridine (Brd-U) integration assay kit (Invitrogen). Cells were seeded for 2-3 days at a density of 1 × 10⁴ / well on two-well chamber slides (Nunc) and then incubated one day with diluted BrdU solution followed by staining. The positive-stained and total cells number were counted along ten successive images. The proliferation capacity of the BM-MSCs was
measured by way of positive BrdU cells percentage over the total number of nucleated cells [23]. An indirect manner was used for the determination of mitochondrial enzymes activity by applying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were subjected to 1 h incubation in 96-well plates among 0.2 mg MTT / mL medium, at 37 °C to produce formazan following reduction. After that, the solution was discarded, and 0.04 N HCl / 1 mL isopropanol was added to solubilize the formazan. After shaking for 5 min, formazan quantity was then calorimetrically evaluated at 570 nm wavelength [Guan et al., 2011].

Hepatogenic differentiation of BM-MSCs
Induction of hepatogenic differentiated cells was carried out in three steps (conditioning, differentiation, and maturation) according to the method of Ye et al. [24]. The BM-MSCs at the 3rd passage a density of 5 × 10^4 were cultured on collagen type I-coated flasks (Falcon) with a growth medium until confluence. Twenty-four h later, the cells were pre-cultured in a serum free Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco, USA) complemented by 20 ng/mL epidermal growth factor (EGF, ITS-Biosciences, USA) and 10 ng/mL basic fibroblast growth factor (bFGF2, ITS-Biosciences, USA) for two days (conditioning step). For cell differentiation step induction, cells were incubated in IMDM in adding 20 ng/mL hepatocyte growth factor (HGF, ITS-Biosciences, USA), 10 ng/mL bFGF2, and 0.61g/L nicotinamide (Lonza, Switzerland) for seven days. To induce maturation step, the cells were incubated up to 21 days among IMDM complemented with 20 ng/mL HGF, 20 ng/mL oncostatin M (OMS, ITS-Biosciences, USA), 20 ng/mL dexamethasone (Dex, Sigma-Aldrich, USA), and 50 mg/mL insulin transferrin selenium (ITS, Lonza, Switzerland). For steps 2 and 3, the medium was changed twice weekly. The differentiated cells were then assessed morphologically at different time points: at the end of the differentiation step (Day 7), midway (Day 14) and at the termination of maturation step (Day 21).

Injection of BM-MSCs
After xylazine (10 mg/kg) anesthesia, intravenous (i.v.) injection was used as the route of administration in the rats. Prior to injection, xylol was used to wash the tail and to render the four tail veins more prominent. A suspension (5 × 10^6) was prepared and injected slowly into two tail veins. After injection, using cotton balls, a gentle pressure was applied at the site of injection to prevent later draining and leakage of the cell suspensions.

Experimental design
The rats were randomly equally distributed (ten rats / group) as follows: (1) the apparently healthy control group that was i.p. injected with olive oil 2 times / week for 70 days; (2) the CCl4-induced hepatotoxicity rat model group that injected with CCl4 2 times / week for 70 days; (3) the CCl4 + silymarin group received 25 mg/kg silymarin (Sedico Pharmaceutical Co., Cairo, Egypt) via orogastric tube as previously described [Abdel-Salam et al. 2012] once a day (2 mg/kilograms); (4) the CCl4 + MSC group that on the day 60 and day 70 received a dose of 5 × 10^6 BM-MSCs through the tail veins; (5) the CCL4 + BM-MSC + silymarin group treated via the same regimen as groups 2.3, and 4. On Day 80, animals were sacrificed, blood samples and livers were collected and divided into two parts. One part was immediately stored at -80 °C in liquid nitrogen for homogenization and biochemical analysis, and the other part was stained using Masson’s trichrome (MTC) and hematoxylin and eosin (H&E). The sera were obtained from the blood samples and then deactivated at 56 °C for 30 min for the nonspecific agglutinins removal or inactivate complement proteins, and the samples were stored at -20 °C. Serum amylase, ALT, and ALP concentrations were measured using commercial kits (BioMerieux, USA) and 10 ng/mL basic fibroblast growth factor (bFGF2, ITS-Biosciences, USA) for two days (conditioning step). For cell differentiation step induction, cells were incubated in IMDM in adding 20 ng/mL hepatocyte growth factor (HGF, ITS-Biosciences, USA), 10 ng/mL bFGF2, and 0.61g/L nicotinamide (Lonza, Switzerland) for seven days. To induce maturation step, the cells were incubated up to 21 days among IMDM complemented with 20 ng/mL HGF, 20 ng/mL oncostatin M (OMS, ITS-Biosciences, USA), 20 ng/mL dexamethasone (Dex, Sigma-Aldrich, USA), and 50 mg/mL insulin transferrin selenium (ITS, Lonza, Switzerland). For steps 2 and 3, the medium was changed twice weekly. The differentiated cells were then assessed morphologically at different time points: at the end of the differentiation step (Day 7), midway (Day 14) and at the termination of maturation step (Day 21).

Liver function parameters
Following instructions in the enclosed pamphlets, commercially available kits (BioMerieux, SA) were used to calorimetrically estimate the levels of the serum protein profile (total protein and albumin) and liver-associated enzymes involving alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and the hepatocellular markers of total bilirubin (T. Bil) and direct bilirubin (D. Bil). The globulin level was also calculated.

Antioxidant activity
Specimens of the dissected livers were weighed and homogenized in PBS (pH 7.4) to give a 20% (w/v) homogenate, followed by sonication at low speed [25]. Ten min. centrifugation was done at 10,000 r.p.m and 4 °C and the supernatants were taken for malondialdehyde (MDA) determination to present the hepatic lipid peroxide formation. Further dilution of the supernatants to 2% was carried out with PBS to determine hepatic glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) enzymes activities using SPECTRUM kits (BioMerieux Ltd, UK). Absorbencies were measured spectrophotometrically at 450 nm, 340 nm, 560 nm, and 520 nm, respectively.

Cytokine profiles
ELISA kits (Assaypro, USA) were used to perform the assay of the levels of cytokines (TNF-α, IL-4, IL-10, and IL-17) in the collected sera, and the color change was measured by microplate ELISA reader (Dynatic product, USA) at 450 nm according to the manufacturer’s instructions.

Western blot analysis
Three equal pieces of liver tissue were dissected from each group and homogenized separately over ices in lysis buffer for 30 min, followed by a cooling centrifugation for 10 min at 12,000 r.p.m to clarify the lysate. The supernatants were then collected and kept at -80 °C to be used. Protein concentrations were commercially determined by available kits (BioMerieux, SA). Identical portions were subsequently run or electrophoresed on 10% SDS-polyacrylamide gel. Proteins were then separated and transferred to nitrocellulose membranes following by blocking using 5% skim milk in 1 × Tris-buffered saline/Tween 20 (TBST) for 2 h and incubated overnight with primary antibodies against Akt (1:1000) and p-Akt (1:2000) (Sigma-Aldrich, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (PharMingen). β-actin was applied on the same membrane as the loading control and then bands immunoactivity were visualized using chemiluminescence. The intensities of the bands were analyzed quantitatively by means of IMAGEJ software and relatively normalized and calculated to the correlated control β-actin [26].

Histopathological studies
Immediately after the experiment ended, the livers were preserved in neutral buffered formalin solution (10%). The fixed specimens were treated via the conservative paraffin-embedding technique, ethanol dehydration, xylene cleaning, and melted paraffin wax embedding inside an incubator adjusted at 60 °C. Paraaffin blocks were obtained to get sections of 4 µm thick followed by H & E staining. Another section from the same paraaffin block was stained with Masson’s trichrome staining (MTC) to
confirm and evaluate the fibrosis degree, and also to detect any hidden minor proliferative fibrous reaction that might have failed to be taken up by the H & E inspection [27]. All sections were examined microscopically and photographed.

**Statistical analysis**

The results were calculated and statistically analyzed via the Windows SPSS version 19 software. One-way analysis of variance (ANOVA) was used to identify differences between groups. In the status of significant differences, the Student-Newman-Kuels test was performed. All data were recorded on an individual basis. Duncan’s New Multiple Range test was applied to determine significant differences ($P <0.05$ and $P <0.01$). Data were stated as means ± standard error (SE).

**RESULTS**

The BM-MSCs were isolated from the rat BM. The cells were cultured to adhere on five culture dishes. The cells were regularly observed via inverted microscope and they continued to multiply to reach 80% confluence after 14 days (Figure 1A). Serial passages were performed, and the viability and cell proliferation were assayed at the 3rd passage, with Trypan blue examination showing cell viability of 95.54 ±3.22%. Proliferation was evaluated by BrdU incorporation assay. The percentage of BrdU positive cells with brown-stained nuclei was 75.12 (Figure 1C). The proliferation was indicated by the OD of the formazan measurement in cells at 80% confluence. The released formazan was 0.111 ±0.08. Phenotypic analysis showed that the cultured BM-MSCs positively expressed CD73 (91.13%), CD105 (89.51%), and CD29 (88.75%), while they negatively expressed CD34 and CD45 antibody staining. The untreated control sample exhibited 0.21% +ve population and 99.79% –ve population (Figure 2).

![Figure 1: Isolation and proliferation of BM-MSCs (40×).](image)

The photomicrograph of cultured BM-MSCs showed their spindle and stellate-shaped appearance with 80% confluence (A). The cell proliferation assay was evaluated via BrdU incorporation of the BM-MSCs for 24 h. BM-MSCs before BrdU staining with no stained nuclei (B). The BrdU positive cells significantly increased upon reaching the required confluence and the brown-stained nuclei can be seen (C). BM-MSCs: bone marrow-mesenchymal stem cells, BrdU: bromodeoxyuridine.
Cultured BM-MSCs positively expressed CD73 (91.13%), CD105 (89.51%), and CD29 (88.75%), while they negatively expressed CD34 and CD45 antibody staining. The untreated sample resulted in 0.21% +ve population and 99.79% -ve population. The morphology of the BM-MSCs changed from spindle-shaped to oblate-shaped after differentiation to hepatocyte-like cells (Figure 3). Firstly, the majority of cells exhibited a fibroplastic appearance, with a few cells being round. The cells were changed from their original cylindrical shape to semi-spindle and spherical-shaped cells on day 7. The majority of cells had a noticeable round appearance with a few cells displaying faint nuclei. From day 14 this cell morphology often changed to more compact oblateness. On day 21 of maturation, polygonal honeycomb-like overloaded cells with hexagonal borders and distinct single or double nuclei were observed and very little intercellular space was seen.
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(A) Day 2, (B) Day 3, (C) Day 7, (D) Day 14, (E) Day 21. (A) On day 2, the morphology of the BM-MSCs had changed from spindle to semi-spindle shape (arrowheads). (B) On day 3, the majority of cells exhibited a fibroplastic appearance (white arrowheads) and a few showed a round appearance (yellow arrowheads). (C) On day 7, the majority of cells were round or spherical-shaped (white arrowheads) with a few cells displaying faint nuclei (yellow arrowheads). (D) By day 14, all cells exhibited a fibroplastic appearance (white arrowheads) and with a few cells displaying faint nuclei (arrowheads). (E) On day 21, the honeycomb-like overloaded cells can be seen, displaying oblateness with hexagonal borders and having a distinct single nucleus (white arrowheads) or double nuclei (yellow arrowheads) with very little intercellular space. BM-MSCs: bone marrow-mesenchymal stem cells.

Evaluation of the hepatic enzymes ALP, AST, and ALT in the sera of the CCl4-induced hepatotoxicity rat model and the effects of silymarin or BM-MSCs or both were used to evaluate the hepatic status (Table 1). Hepatotoxicity led to alteration in some liver function parameters. Compared with the apparently healthy group, in the CCl4 hepatotoxicity model, there were significant increases in ALT (P <0.01), AST (P <0.01), ALP (P<0.01), T. Bili (P<0.05), and D. Bili (P<0.05) with a significant decline in albumin (P<0.05) and unchanged total protein or globulin levels. The results revealed that albumin was significantly elevated, and ALT, AST, T. Bili and D. Bili were significantly l (P<0.05) in the silymarin group. The i.v. injection of BM-MSCs significantly restored the activities of ALT, AST, ALP and the T. Bili and D. Bili levels (P<0.05), relative to group of CCl4 hepatotoxicity rat model. On the other hand, the BM-MSCs + silymarin significantly ameliorated all the biochemical indices and restored them to normal levels by significantly increasing the total protein, globulin and albumin (P<0.05) as well as significantly reducing the levels of ALT (P<0.05), AST (P<0.01), ALP (P<0.05), T. Bili (P<0.05), and D. Bili (P<0.05).

Table 1: Effect of silymarin and/or BM-MSCs on bio-indices and liver enzyme activities in serum of the CCl4-induced hepatotoxicity rat model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U%)</th>
<th>T. Protein (g%)</th>
<th>Albumin (g%)</th>
<th>Globulin (g%)</th>
<th>T. Bili mg/dl</th>
<th>D. Bili mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>82.12 ±3.51</td>
<td>128.76±4.99</td>
<td>117.55 ±5.78</td>
<td>7.83 ±1.22</td>
<td>4.55 ±1.09</td>
<td>3.12 ±0.98</td>
<td>0.45 ±0.02</td>
<td>0.23 ±0.04</td>
</tr>
<tr>
<td>CCl4</td>
<td>114.65**±9.54</td>
<td>183.65+7.08</td>
<td>163.63**±8.54</td>
<td>6.63 ±1.11</td>
<td>3.01*±0.55</td>
<td>3.31 ±0.88</td>
<td>0.87 ±0.04</td>
<td>0.43 ±0.03</td>
</tr>
<tr>
<td>CCl4+Silymarin</td>
<td>95.43+4.86</td>
<td>131.53±3.95</td>
<td>148.73±5.82</td>
<td>7.56 ±1.22</td>
<td>3.99±0.07</td>
<td>3.43 ±0.23</td>
<td>0.54±0.04</td>
<td>0.33±0.08</td>
</tr>
<tr>
<td>CCl4+BM-MSCs</td>
<td>85.65+3.65</td>
<td>133.43±4.11</td>
<td>129.43±5.34</td>
<td>8.83±0.88</td>
<td>4.97±0.53</td>
<td>4.52±0.51</td>
<td>0.51±0.03</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>CCl4+Silymarin+BM-MSCs</td>
<td>82.54+6.54</td>
<td>129.65±3.54</td>
<td>121.32±7.54</td>
<td>8.94±0.93</td>
<td>4.65±0.76</td>
<td>5.76±0.33</td>
<td>0.55±0.10</td>
<td>0.21±0.04</td>
</tr>
</tbody>
</table>

Means in the same column with * and** are differ significantly from the healthy control at P<0.05 and P<0.01, respectively.

Means in the same column, a and b are differ significantly from CCl4 at P<0.05 and P<0.01, respectively.

Figure 4: Livers, gross and histological views (400×)
(A) Healthy control, (B) CCl₄-induced hepatotoxicity model, (C) CCl₄+silymarin treated, (D) CCl₄+BM-MSCs treated, (E) CCl₄+silymarin+BM-MSCs treated. (Scale bar: 40 μm). The gross view of a liver from the healthy control group in Figure 4 (A) displays a normal appearance and size. The histological view shows the consistent structure of the liver as a solid organ possessing plates of hepatocytes (h) radiating from the central vein (cv). The stained section shows a typical portal area (p) and slit-like sinusoids (s) lined by endothelial cells (arrowhead). In Figure 4(B), hepatomegaly and a dark, severely congested hepatic surface with fibrotic patches, rough surfaces, and a hard texture are seen in the gross view of the liver of the CCl₄-induced hepatotoxicity rat model. Histologically, the liver displays abnormal architecture, as seen in the engorgement of the central vein with blood and in the hepatocytes with swollen vacuolated cytoplasm and pyknotic nuclei with collagen fiber (c) deposited between them. Mononuclear cellular infiltration (mn) and erythrocytes are also seen. The liver from the CCl₄ + silymarin group (Figure 4C) is seen anatomically as enlarged and congested with a few fibrotic nodules. Histologically, the liver shows a moderate amount of collagen fiber (c) between the hepatocytes and the obliterated central vein. The liver of the CCl₄+BM-MSCs group (Figure 4D) macroscopically exhibits a normal size with a dark, congested surface and disappearance of the fibrotic nodules. Histologically, this liver exhibits a dilated central vein with a few collagen fibers between the hepatocytes. Some hepatocytes display a dark-colored nucleus and acidophilic cytoplasm. The liver from the CCl₄ + silymarin + BM-MSCs group shown in Figure (4E) has been restored to a nearly normal appearance, hepatic surface, and size, and the fibrotic nodules and congestion have disappeared. Histologically, the restored architecture of the hepatic lobules can be observed. The hepatocytes are arranged in cords radiating from the central vein and appear with eosinophilic granular cytoplasm and rounded vesicular nuclei. BM-MSCs: bone marrow-mesenchymal stem cells, CCl₄: carbon tetrachloride.

The antioxidant activities in hepatic tissue were affected in the CCl₄-induced fibrosis rat model relative to the group of healthy control (Table 2). A significantly increased activity of MDA (P < 0.01) and a significantly decreased activities of CAT (P < 0.01), SOD, and GSH-Px (P < 0.05) were noticed in the CCl₄-induced fibrosis rat model relative to the normal group. Silymarin administration or BM-MSCs transplantation separately significantly decreased MDA (P < 0.01) and increased CAT and SOD (P < 0.01). However, compared to the CCl₄-induced fibrosis rat model, the BM-MSCs + silymarin therapy significantly restored hepatic tissue antioxidant activities by decreasing MDA (P < 0.05) and markedly increasing CAT and GSH-Px (P < 0.05) activities without changing SOD activity.

Table 2: Effect of silymarin and/or BM-MSCs on hepatic tissue antioxidant enzyme activities of the CCl₄-induced hepatotoxicity rat model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MDA (mol/mg tissue)</th>
<th>CAT (nmol/g tissue)</th>
<th>SOD (U/g tissue)</th>
<th>GPx (U/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy control</td>
<td>33.45 ± 1.65</td>
<td>38.23 ± 3.54</td>
<td>9.33 ± 2.65</td>
<td>71.87 ± 3.76</td>
</tr>
<tr>
<td>CCl₄</td>
<td>47.43* ± 2.56</td>
<td>19.44** ± 1.76</td>
<td>5.98* ± 1.22</td>
<td>43.65* ± 3.65</td>
</tr>
<tr>
<td>CCl₄+Silymarin</td>
<td>25.54* ± 2.22</td>
<td>32.96* ± 3.76</td>
<td>8.54* ± 1.88</td>
<td>48.46 ± 4.12</td>
</tr>
<tr>
<td>CCl₄+BM-MSCs</td>
<td>23.20* ± 2.05</td>
<td>35.16* ± 2.51</td>
<td>8.99* ± 1.32</td>
<td>52.54 ± 4.33</td>
</tr>
<tr>
<td>CCl₄+Silymarin+BM-MSCs</td>
<td>34.65* ± 3.88</td>
<td>29.54* ± 2.93</td>
<td>7.65 ± 2.87</td>
<td>70.54* ± 3.16</td>
</tr>
</tbody>
</table>

Means in the same column with * and ** are differ significantly from the healthy control at P < 0.05 and P < 0.01, respectively. Means in the same column, a and b are differ significantly from CCl₄ at P < 0.05 and P < 0.01, respectively.

TNF-α, IL-4, IL-10, and IL-17 serum levels in all the studied groups of rats are presented in Table 3. The CCl₄-induced hepatotoxicity was associated with an elevated level of pro-inflammatory cytokine; TNF-α (P < 0.05) and a decrease in anti-inflammatory cytokines; IL-4, IL-10, and IL-17 (P < 0.05) relating to the normal healthy rats. Otherwise, the level of IL-17 raised significantly (P < 0.05) and TNF-α significantly reduced in the rats treated with silymarin (P < 0.05). Treatment with BM-MSCs or both silymarin and BM-MSCs restored the cytokine levels, as confirmed by a significant reduction in TNF-α serum level and a significant rise in the IL-4, IL-10, and IL-17 levels (P < 0.05).

Table 3: Effect of silymarin and/or BM-MSCs on serum cytokine concentration (ng/mL) of the CCl₄-induced hepatotoxicity rat model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TNF-α (ng/ml)</th>
<th>IL-4 (ng/ml)</th>
<th>IL-10 (ng/ml)</th>
<th>IL-17 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy control</td>
<td>9.87 ± 1.94</td>
<td>11.87 ± 1.22</td>
<td>9.66 ± 1.67</td>
<td>14.65 ± 1.32</td>
</tr>
<tr>
<td>CCl₄</td>
<td>16.43* ± 1.04</td>
<td>7.96* ± 1.11</td>
<td>5.65* ± 1.54</td>
<td>9.54* ± 1.02</td>
</tr>
<tr>
<td>CCl₄+Silymarin</td>
<td>10.76* ± 1.44</td>
<td>11.99* ± 2.08</td>
<td>7.31* ± 1.54</td>
<td>13.90* ± 1.22</td>
</tr>
<tr>
<td>CCl₄+BM-MSCs</td>
<td>10.54* ± 1.45</td>
<td>11.55* ± 1.33</td>
<td>9.31* ± 1.33</td>
<td>14.08* ± 1.32</td>
</tr>
<tr>
<td>CCl₄+Silymarin+BM-MSCs</td>
<td>10.43* ± 1.55</td>
<td>10.95* ± 1.35</td>
<td>10.11* ± 1.54</td>
<td>15.91* ± 1.29</td>
</tr>
</tbody>
</table>
Means in the same column with * and ** are differ significantly from the healthy control at $P<0.05$ and $P<0.01$, respectively. Means in the same column, * and # are differ significantly from CCl$_4$ at $P<0.05$ and $P<0.01$, respectively.

The expression of Akt and p-Akt proteins was evaluated by Western blot. Bands of Akt were expressed in the liver protein of all experimental groups (Figure 5). Bands of p-Akt were more visible in the CCl$_4$-induced hepatotoxicity and CCl$_4$ + silymarin groups than in the other groups. Densitometric measurements were performed of the Akt and p-Akt band intensities using IMAGEJ software with β-actin as the control. The highest p-Akt band intensity was found in the CCl$_4$-induced hepatotoxicity group, which indicated Akt phosphorylation (Figure 6).

**DISCUSSION**

The topic of liver damage control is continually being discussed, with the hope of finding the ideal method for amelioration. Although cell therapy was established for liver damage management, there is disagreement regarding the outcome. Incompetence in the treatment of fibrosis has been reported by some authors [28]. Various factors, such as timing and dose, can interfere with the results. Previously, BM-MSC transplantation has been shown to result in maintenance of fibrogenesis via the stimulation of HSC and myofibroblasts [29]. Similar results were obtained in a cirrhotic liver mouse model [30] and immunocompromised mice [31] treated with MSCs. The homing of stem cells to the liver and differentiating to myofibroblasts has been previously reported [32]. Therefore, in the present study, BM-MSCs were transplanted alone or in combination with silymarin to a CCl$_4$-induced hepatotoxicity rat model. The isolated BM-MSCs can be provoked for hepatocyte-like cells differentiation by some growth factors, as previously discussed [33]. The properties of the BM-MSCs isolated in the present work via phenotyping to viability and proliferation are similar to those of the cells in a previous study [34].

Various laboratory models have been used in the past. The CCl$_4$-induced hepatotoxicity rat model was chosen due to its similarity to human hepatotoxicity [Jiménez et al., 1992]. The solvent CCl$_4$ is widely used in the chemical industry and its hepatic and renal toxic activity is widely known. As a potential environmental contaminant, it provides a means for volatile chemicals contacting and absorption. In the present study, the CCl$_4$-induced hepatotoxicity rat model showed a significant decrease in albumin levels accompanied by a significant rise in ALT, AST, and ALP, compared to the apparently healthy group. The significant decrease in albumin may have been due to decreased synthesis by the dysfunctional liver or to dehydration resulting from diarrhea caused by the CCl$_4$ treatment [35]. Previous studies have reported CCl$_4$-induced steatosis, fibrosis, cirrhosis [36], and eventually cell death [37] and lipid peroxidation [4]. Normally, liver transaminases are found and synthesized inside the cell [AST in the mitochondria and ALT inside the cytoplasm]. The toxicity has led to leakage of enzymes into the blood [27] and CCl$_4$ treatment resulted in an increase of serum ALP with high levels of direct and indirect Bil [38]. These parameters indicated cholestasis and pathological variation of biliary flux [39].

In the present study, we used two well-known management applications: silymarin as a standard hepatoprotective drug and BM-MSCs as a cell-based therapy. The oral injection dose and duration of the silymarin treatment were parallel to those of Abdel Salam et al. [40]. However, Abenavoli et al. [41] used silymarin...
for a short time of five days and at a low dose of 50 mg/kg, while Onalan et al. [42] used 300 mg/kg/day for seven days. The results revealed a significant elevation in albumin and a significant reduction in ALT, AST, T. Bili and D. Bili in the silymarin group. The i.v. injection of BM-MSCs significantly restored the activities of ALT, AST, ALP, T. Bili and D. Bili levels compared to the CCl₄ hepatotoxicity rat model group. On the other hand, the BM-MSCs + silymarin significantly ameliorated all the biochemical indices and restored them to normal levels by significantly increasing the total protein, globulin, and albumin as well as significantly reducing the ALT, AST, ALP, T. Bili, and D. Bili the hepatoprotective effect of silymarin has been discussed earlier [43]. Silymarin has the ability to suppress enzyme leakage and to maintain the integrity of the plasma membrane, hence, preventing liver damage. The potential of silymarin to treat fibrotic rat liver has been previously reported [44] and was attributed to the anti-fibrotic activity of the silymarin. In addition, it has been used to repair damaged hepatocytes [45]. The mechanism was previously attributed to the elevation of protein bioynthesis by the ribosomal RNA [46] and was confirmed by liver function tests [Mohamed et al., 2016]. Concerning BM-MSC treatment and hepatic damage, Zhang et al. [1] reported that the cell-based therapy was begun as a new and effective strategy to repair liver damage. The obtained data were in parallel with the work of Leibacher et al. [15], who demonstrated the beneficial effects of MSCs on the regeneration of damaged tissue, reduction of inflammation and oxidative stress, and modulation of immune reactions.

The antioxidant activities in hepatic tissue were affected in the CCl₄-induced hepatotoxicity rat model relative to the healthy control group. MDA activity significantly increased along with a significant decline in CAT, SOD, and GPx. The data were in parallel with previously recorded findings [47]. Individual separate administration of silymarin or BM-MSCs significantly reduced MDA and increased CAT and SOD. However, compared to the CCl₄-induced hepatotoxicity rat model, the BM-MSCs + silymarin therapy significantly restored hepatic tissue antioxidant activities by decreasing MDA and markedly increasing CAT and GPx activities without changing SOD activity. These antioxidant activities and immunomodulation features were suggested in an earlier study [48]. It is a common knowledge that oxidative stress is caused by the exhaustion of antioxidant enzyme activity in response to reactive oxygen species [ROS]. An elevated MDA is commonly used as a biomarker of lipid peroxidation [49]. The positive support of silymarin in the oxidant/antioxidant balance has been investigated previously [25] and results confirmed that silymarin defensive effects may be at least partially concerning to its antioxidant activity [11, 50].

CCl₄ induced hepatotoxicity was associated with increased pro-inflammatory cytokines; TNF-α and reduced anti-inflammatory cytokines; IL-4, IL-10 and IL-17 relative to the healthy control group. Furthermore, a significant decrease in the level of IL-17 and significant decrease of TNF-α were reported in rats treated with silymarin. BM-MSCs therapy or both silymarin and BM-MSCs combination restored the cytokines levels as indicated by a significantly reduced TNF-α serum level and significantly raised IL-4, IL-10 and IL-17 levels. This data was parallel to the previously recorded [51]. It was known the involvement of IFN-γ in the pathogenesis of liver fibrosis [52]. On the contrary, IL-4, IL-10 and IL-17 were previously considered as anti-fibrotic cytokines and their elevation indicated shifting cytokines from anti-inflammatory to pro-fibrolytic condition [52]. Several experimental investigations have explored that IL-17 signaling of fibrogenesis and the production of pro-fibrotic cytokine in liver [Meng et al., 2012]. Treatment with BM-MSCs increased serum level of IL-10 and decreased Th17 which reduced HSC activation and collagen deposition [53]. Upon histopathological examination, the liver slides stained by H & E and MTC revealed a regular structure in the healthy control group. Those of the CCl₄-treated group displayed abnormal architecture, including engorgement of the central vein with blood, swollen vacuolated cytoplasm and pylonotic nuclei in the hepatocytes, and collagen fiber deposition between the cells. Mononuclear cellular infiltration and erythrocytes were also observed. The CCl₄ + silymarin-treated group showed moderate collagen fiber deposition and an obliterated central vein. The CCl₄ + BM-MSCs-treated group showed a dilated central vein with a small amount of collagen between the hepatocytes. The CCl₄ + silymarin + BM-MSCs-treated group exhibited remodeling of hepatic lobules construction. The hepatocytes were aligned in cords radiating from the central vein and displayed eosinophil granular cytoplasm and rounded vesicular nuclei. The Akt and p-Akt expression was evaluated in the present study via Western blot. Bands of p-Akt were much more prominent in the CCl₄-induced hepatotoxicity group than in the other groups, indicating a higher phosphorylation of the Akt signal accompanied by higher collagen levels in the liver tissue. Densitometric measurement of the band intensities was performed for Akt and p-Akt using IMAGEJ software with β-actin as the control. The p-Akt band intensity was higher in CCl₄ group than the various other groups. This result was in parallel with the work of Wang et al. [26]. The collagen that was formed was believed to have originated in the hepatic stellate cells [54]. These data further supported the findings of earlier biochemical work [55].

These data suggest that the BM-MSCs with silymarin had a synergistic effect via an antioxidant defense mechanism in attenuating or alleviating oxidative stress in the CCl₄-induced hepatotoxicity rat model. The combination succeeded in enhancing immunomodulation and histopathological features. The findings of the current investigation prove the usefulness of BM-MSCs and their synergism with silymarin in providing a significant hepatoprotective effect against hepatotoxicity induced by CCl₄.

CONCLUSION

In light of the results of the present study, treatment using a combination of BM-MSCs and silymarin could protect the liver from hepatotoxicity by inhibiting collagen deposition and improving liver enzyme and antioxidant activity. Furthermore, the results were supported by the Akt/p-Akt signaling cascade via down-regulation of Akt phosphorylation.

AUTHOR CONTRIBUTIONS

TIA and AAZ collaborated in the study design. AAZ and FAMA planned the experiments. FAMA isolated BM-MSCs and completed their proliferation, characterization and hepaticogenic differentiation. TSA, YMA and SMA shared in BM-MSCs characterization. FAMA, YMA and SMA carried out the biochemical, histological and immunological studies. AAZ and FAMA interpreted the results, made the write up and statistically analyzed the data and made
illustrations. All authors have read and approved the final manuscript.

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POTENTIAL CONFLICT OF INTEREST
The data and research results are honest, and the authors have no conflicting financial interest.

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