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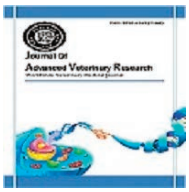

Antifibrogenic Effect of Mesenchymal Stem Cells against Thioacetamide-Induced Liver Fibrosis in Rats

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ABSTRACT

Liver fibrosis is one of the most prevalent health problem in the world and resulting in high morbidity and mortality. Therefore, the antifibrogenic potential of mesenchymal stem cell in liver fibrosis induced by thioacetamide (TAA) and some of its underlying mechanisms was investigated. A total of 40 male albino rats were randomly divided into 4 groups 10 rats per every group as Group 1; normal control group, Group 2; Control group for only a single dose of mesenchymal stem cells (MSCs, $3x10^6$ cell/ml), Group 3; TAA-treated group (200mg TAA /kg body weight I/P three times a week for 6 weeks) and Group 4; rats injected with TAA for six weeks then injected intravenous with a single dose of MSCs ($3x10^6$ cell/ml) per rat at tail vein for another eight weeks. MSCs improved liver biomarker via decreasing serum level of ALT and AST in comparison to fibrotic group with significant increase in the level of albumin and total protein and improved oxidative status of the hepatic tissue. TAA is successfully induced liver fibrosis that was assessed histopathologically by Crossman's trichrome staining and immunostaining of α-smooth muscle actin (α-SMA) and hepatocyte growth factor (HGF). MSCs successfully improved pathological alterations in hepatic tissues induced by TAA as well as it could suppress α-SMA and increase the level of HGF in immunostained sections. Finally, MSCs have therapeutic effect on experimentally induced liver fibrosis using TAA via its regenerative capacity and anti-fibrotic effect. Therefore, the obtained results recommend that, MSCs could be used as a complementary treatment in hepatic fibrosis.

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Introduction

Liver is a primary organ involved in metabolism of food and drugs as well as it perform various vital functions such as remove damaged red blood cells from the blood in coordination with spleen, produces bile, clotting factors, stores vitamins, minerals, protein, fats and glucose from diet. Moreover, filteration of toxic substances from the body, like alcohol, chemotherapeutic drugs, antibiotics and toxicants that is considered the most important task of the liver (Waugh and Grant, 2001). However, hepatic damage may occur if accumulation of toxins is faster than the liver metabolizing ability (Bigoniya *et al.*, 2009). Liver injury can be categorized based on the histologic lesion produced such as inflammatory, necrotic, cholestatic and cytotoxic (Cullen, 2005).

Cirrhosis is the end result of liver fibrosis, that remain as the main causes of morbidity and mortality all over the world with increasing economic impacts (Sánchez-Valle *et al.*, 2012). As a hepatotoxic agent, thioacetamide (TAA) was first reported by Fitzhugh and Nelson (1948). TAA is an organosulfer compound that used as fungicid and as a stabilizer of motor fuel as well as in paper industries (Singh *et al.*, 2012). Centrilobular hepatic necrosis with a subsequent regenerative response was produced in animals by only one dose of TAA (Mangipudy *et al.*, 1995). Moreover, liver cirrhosis and hepatocarcinoma could also be induced by chronic administration of TAA (Natarajan *et al.*, 2006).

Mesenchymal stem cells (MSCs) could be readily obtained from BM aspirates and developed in vitro (Le Blanc *et al.* 2004). Several studies have reported that bone marrow-derived mesenchymal stem cells (BM-MSCs) could be differentiated into hepatocyte-like cells, restoring liver function after hepatic cell injury (Houlihan and Newsome, 2008). However, there are several factors including low cell yields from BM causing the use of BM-MSCs is limited (Huttmann *et al.*, 2003).

Till now, an efficient anti-fibrotic drugs are not developed athough the great progress in the field of recent medicine. The modern treatment of hepatic fibrosis is confined to the withdrawal of the harmful agents and liver transplantation in the late stages (Kim *et al.*, 2014; Liedtke *et al*, 2013). Thus, the search for new medicines is still ongoing.

Stem cell therapy holds pronounced potential for the restoration of injured tissues. Therefore, the present work was conducted to evaluate the role of mesenchymal stem cells in treatment of liver fibrosis induced by TAA in association with studying the effect of MSCs treatment on sequential hepatic

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alteration and blood parameters during regeneration of hepatic fibrosis.

Materials and methods

Stem cell isolation

MSCs was isolated from the hind limbs above the hip and below the ankle of 6 weeks-old male rats following the protocol of Abdel Aziz *et al.* (2007). To obtain the required concentration of cells (3x10^6 cells/mL), the cells were counted by using a flow cytometer)Amnis® CellStream® Flow Cytometer, Catalog Number: CS-100196, Luminex Corporation(as described by Abdel Aziz *et al.* (2007). The cells were suspended in phosphate buffer solution (PBS).

Animals and Experimental Design

Forty male albino rats weighting 150-175g were kept at constant environmental and nutritional condition throughout the period of the experiment. The animals were exposed to good ventilation, humidity and to a 12-hr light/dark cycle. Constant supplies of standard pellet diet, fresh and clean drinking water were supplied ad-libitum in accordance to the study protocol appropriated by the ethical committee of the faculty of veterinary medicine, Benha University, for animal care and experimentation. Animals were left for seven days before beginning of the experiment for adaptation. After that, 20 rats were received physiological saline 1ml I/P for 6 weeks, after that, this group was subdivided into two groups as follow; Group 1; control group (10 rats) that still receive physiological saline 1ml I/P for another 8 weeks. Group 2; mesenchymal stem cells group (10 rats) treated with a single dose of 3x10^6 BM-MSCs per rat by intravenous infusion at tail vein for 8 weeks. The liver fibrosis was induced in the remaining rats (20 rats) by intraperitoneal (I/P) injection of TAA at a dose of 200 mg TAA /kg body weight three times a week for 6 weeks (Kasahara, 1977). Then these rats were subdivided into two groups 10 rats per each, Group 3; TAA-treated group (fibrosis model, 10 rats). In the therapeutic study, rats of group 4 received single dose of 3x10^6 BM-MSCs per rat by intravenous infusion at tail vein (Lee *et al.* 2009) for another 8 weeks after TAA discontinuation. The experiment design was summarized in Table 1.

Sampling

Body weight of rats was firstly recorded. Then, rats were anesthetized with ethyl ether for collection of blood samples from the retro-orbital venous plexus located at the medial canthus of the eye at the end of the experiment (14 weeks). the serum for biochemical analysis was separated by centrifugation of blood samples at 4000 rpm/10 min. After that, all rats were sacrificed and the whole liver tissue were directly removed, washed in ice-cooled isotonic saline, blotted dry and weighed specimen from liver of all groups were used for histopathological and immuno-histochemical examinations, the remaining part was preserved at −20°C for further examinations.

Biochemical Analysis

For evaluation of hepatotoxicity, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were estimated following the protocol of Reitman and Frankel (1957), alkaline phosphatase (ALP) activity according to Tietz *et al.* (1983). Meanwhile, serum total protein and Albumin levels were evaluated according to Weichselbaum (1964) and Doumas *et al.* (1997), respectively. Subsequently, globulin and A/G ratio were also calculated.

Oxidative Stress Biomarkers

10% homogenates from liver samples was prepared by homogenization in 0.1 M cold phosphate buffer saline (pH 7.4) using a Teflon pestle. Then, the homogenates were centrifuged at 14,000× g for 15 min at 4 °C to get the supernatant, which was used to assay of L-malondialdehyde (L-MDA) (Placer *et al.*, 1966) and nitric oxide concentration (NO) (Miranda *et al.*, 2001), using commercial kits (Biodiagnostic, Cairo, Egypt).

Determination of liver α-smooth muscle actin (α-SMA) concentration

Liver tissue homogenate used for measurement of the concentrations of α-smooth muscle actin (α-SMA) by using Rat α-Smooth Muscle Actin (α-SMA) ELISA kits (Cusabio Biotech®) Catalog Number: (CSB-E14027r) according to the manufacturer's instruction.

Histopathological Examination

Liver specimens were taken from different parts of the liver. The specimens were preserved in 10% neutral buffered formalin for histopathological examination. The fixed tissues were processed routinely, embedded in paraffin, sectioned, de-paraffinized and rehydrated according to the technique described by (Bancroft and Gamble (2008). The extent of degree of liver fibrosis was evaluated by assessing the histopathological changes in the liver sections stained with hematoxylin and eosin (H and E) using standard technique (Bancroft and Gamble, 2008) and Crossman's trichrome stain according to Gomori (1950). For evaluation of fibrosis, a numerical scoring system was applied to assess the grade of fibrosis following the criteria of Ishak system (Ishak *et al.*, 1995), as follow: 0, no fibrosis (normal); 1, fibrous expansion of some portal areas ± short fibrous septa; 2, fibrous expansion of most portal areas ± short fibrous septa; 3, fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging; 4,

Table 1. Experimental design

Groups	No	Type of treatment	Dose and route	Duration	Time of sampling	Type of sampling
Control	10	Physiological saline	1 ml/ rat I/P	14 weeks	After 14 weeks	Serum and liver samples were collected from all groups
MSCs	10	MSCs	3x10^6 cells/ml I/V in tail vein	8 weeks		
TAA	10	TAA	200 mg/kg I/P /3times/a week	6 weeks		
TAA /MScs	10	TAA/ MSCs	200 mg TAA /kg I/P and once MSCs 3x10^6 cells /ml I/V	6 weeks TAA followed by 8 weeks MSCs		

TAA: Thioacetamide; MSCs: Mesenchymal stem cells

fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C); 5, marked bridging (P-P and/or P-C) with occasional nodules (in-complete cirrhosis); 6, cirrhosis.

Immunohistochemical Examination

Sections of the liver in xylene have been deparaffinized and rehydrated in graded alcohol. To block endogenous peroxidase activity, drops of the Hydrogen Peroxide (Thermo Scientific) have been added. The tissues were pretreated for antigenic recovery with a 10 mM citrate buffer, pH 6.0 in a 500 W microwave oven for 10 min. The slides were washed with PBS and blocked for 5 min with ultra V Scientific blocking solution. Sections were incubated at 4°C overnight in a humidified chamber with diluted primary monoclonal mouse antibody to α-SMA (1:100) (Santa Cruz Biotech) and diluted 1:50 polyclonal anti- hepatocyte growth factor (anti-HGF) rabbit antibody (Santa Cruz Biotech). The sections were rinsed with PBS again and then incubated for 10 min with a biotinylated anti-mouse goat antibody (Thermo Scientific). The sections have been rinsed with PBS again. Finally, sections were incubated with Streptavidin peroxidase (Thermo Scientific). Slides were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 10 min to visualize the reaction. The slides were counter stained with haematoxylin, then dehydrated and mounted. Primary antibodies for negative controls were omitted and replaced by PBS. To record the immunopositive (dark brown) reaction, the immune-stained sections were examined.

Statistical Analysis

Statistical analysis was carried out by one way ANOVA followed by Tukey test using SPSS V. 19 software (SPSS, Chicago, IL, USA) (Howitt and Cramer 2011). The results were expressed as mean and stander error (± SE). Differences at P<0.05 were considered statistically significant.

Results

Body weight, liver weight and Liver index in rats

Effect of treatment with MSCs on body weight, liver weight and liver index in TAA induced hepatic fibrosis in rats and their control were presented in Table 2. A significant (P < 0.05) increase in liver weight and Liver index with significant (P < 0.05) decrease in the body weight were demonstrated in TAA treated rats compared to control normal group. Meanwhile, treatment of TAA injected rats with MSCs resulted in significant (P < 0.05) decrease in liver weight and liver index with significant (P < 0.05) increase in body weight. Interestingly, injection of MSCs to normal rats resulted in insignificant change in body weight, liver weight and liver index in comparison to normal control group.

Table 2. effect of treatment with MSCs on body weight, liver weight and Liver index in TAA- induced hepatotoxicity in rats and their control.

	Body weight (gm)	Liver weight (gm)	Liver index
Control	230±2.1[a]	5.34± 0.25[a]	2.32
TAA	192.33±10.6[b]	7.48±0.70[b]	3.99
MSCs	232.33±2.0[a]	5.48±0.56[a]	2.36
TAA+MSCs	212±19.98[a]	5.93± 0.45[a]	2.79

Data are presented as (Mean ± SEM. Values with different superscripts in the same column are significantly different (ρ<0.05). Liver index = (liver weight/body weight×100). TAA: Thioacetamide; MSCs: Mesenchymal stem cells

Liver function parameters

The administration of TAA induced extensive liver injury that demonstrated by significant (P < 0.05) increase in ALT, AST and ALP activities in comparison to control group in association with significant (P < 0.05) decrease in total protein, albumin, globulin and A/G ratio. On the other hand, MSCs control group did not induce significant changes in the activities of all previous biochemical parameters (ALT, AST, ALP, total protein, albumin, globulin and A/G ratio) in comparison with control group. Meanwhile, significant (P < 0.05) improvement in serum level of ALT, AST, ALP, total protein, albumin, globulin and A/G ratio was detected in TAA/MSCs treated group in comparison to TAA-treated group (Table 3).

Antioxidant Effect of MSCs against TAA-Induced oxidative liver damage

TAA was able to induce oxidative liver injury as significant elevation in the level of L-MDA to 166% and in NO concentration to 195% in comparison to control group. Meanwhile, Treatment with MSCs in TAA-induced hepatotoxicity in rats resulted in significant reduction in liver L-MDA (70%) and NO (63.5%) concentrations in comparison to TAA treated group. Interestingly, injection of MSCs to normal rats produced no significant change in liver L-MDA and NO concentration when compared with normal control group (Table 4).

Table 3. Serum biochemical changes in different treated groups compared to their corresponding controls, 14 weeks after treatment with MSCs.

	Control	TAA	MSCs	TAA/MSCs
ALT (U/L)	30.73±0.43[c]	161.52±2.16 [a]	29.24±1.02 [c]	67.24±3.90 [b]
AST (U/L)	45.14±0.13[c]	205.66±1.27 [a]	47.20±0.81 [c]	115.73±1.29 [b]
ALP (U/L)	85.07±7.66[c]	165.42±4.22 [a]	83.84±9.24 [c]	104.38±1.66 [b]
Total protein (g/dl)	5.94±0. 12 [a]	4.83±0.01 [c]	5.71±0.11 [a]	5.43±0.01 [b]
Albumin (g/dl)	3.62±0.08 [a]	2.41±0.01 [c]	3.59±0.05 [a]	3.28±0.02 [b]
Globulin (g/dl)	2.44±0.03 [a]	1.90±0.01 [c]	2.42±0.05 [a]	2.37±0.01 [b]
A/G ratio	1.48±0.01 [b]	1.26±0.01 [c]	1.48±0.01 [b]	1.38±0.01 [a]

Data represent mean values ± SEM (n = 10).
Values having different superscripts within same row are significantly different (P<0.05). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; A/G: Albumin/globulin; TAA: Thioacetamide; MSCs: Mesenchymal stem cells

Table 4. Effects of MSCs treatment on oxidative stress biomarkers in TAA fibrotic liver.

	Control	TAA	MSCs	TAA+MSCs
MDA (nmol/g. liver)	71.7±0.70[a]	118.75±0.86[c]	70.67±0.56[a]	83.52±0.86[b]
NO (µmol/g. liver)	35.36±0.85[a]	69.00±0.73[c]	36.50±0.76[a]	43.83±0.31[b]

Values are mean ± SEM (n =10). The presence of different superscripts means significant differences (P<0.05) between groups in the same row.
MDA: Malondialdehyde; NO: Nitric oxide; TAA: Thioacetamide; MSCs: Mesenchymal stem cells.

Effect on alpha-smooth muscle actin (α-SMA) in liver homogenate

TAA administration resulted in significant (P < 0.05) increase in hepatic α-SMA level (302%) in comparison to that of the normal control group. On the other side, the use of MSCs dose (3x10^6 cells/ mL I.V) for 8 weeks as a treatment for liver fibrosis producing significant (P < 0.05) suppression of α-SMA levels (46.5%) in hepatic tissue homogenate as compared to TAA group (Table 5).

Pathological assessment

Microscopic examination

Examination of hepatic tissue obtained from control rats showed normal histological structure of hepatic tissue. The hepatic tissue preserved its normal hepatic lobular structure with central veins and hepatic cords. Interestingly, no histological differences were demonstrated between control group and MSCs treated group. Meanwhile, the liver of rats injected with TAA showed extensive pathological alterations as severe congestion of the central veins and hepatic sinusoids, marked congestion and thrombosis of the portal blood vessels with mild vasculitis represented by proliferation of the lining endothelium with perivascular edema as well as perivascular mononuclear leukocytic infiltration (Fig. 1a) was detected. Furthermore, marked proliferation of the biliary epithelium with formation of irregular tubules and newly formed bile ductules. Portal to portal fibrous bridge (Fig. 1b) was also observed. The hepatocytes displayed severe vacuolar and hydropic degeneration characterized by pale vacuolated cytoplasm as well as severe swelling with ballooning degeneration and reticulation of cytoplasm with occasionally fatty change that appeared more pronounced and affected large areas of hepatic parenchyma (Fig. 1c). The hepatocytes characterized by clear fat vacuoles in their cytoplasm, pushed the nucleus to one side giving signet ring appearance. Megalocytosis with severe enlargement of nucleus and nucleolus and the presence of two or more nuclei in the hepatic cell (Fig. 1d) was noticed. Additionally, focal areas of necrosis and apoptotic changes of hepatocytes were also observed in the hepatic parenchyma (Fig. 1e). Fibrosis with formation of fibrous bridges connecting between the portal areas resulting in distinct nodular feature

Table 5. Treatment effect of MSCs on liver α-SMA concentration in TAA-induced liver fibrosis in rats.

	Control	TAA	MSCs	TAA+MSCs
α-SMA concentration (ng/g.tissue)	0.42 ± 0.007 ^{a}	1.27 ± 0.006 ^{b}	0.44 ± 0.002 ^{a}	0.59 ± 0.016 ^{c}

Data are presented as Mean ± SEM). Values with different superscripts in the same row are significantly different (P<0.05).
α-SMA: α-smooth muscle actin; TAA: Thioacetamide; MSCs: Mesenchymal stem cells.

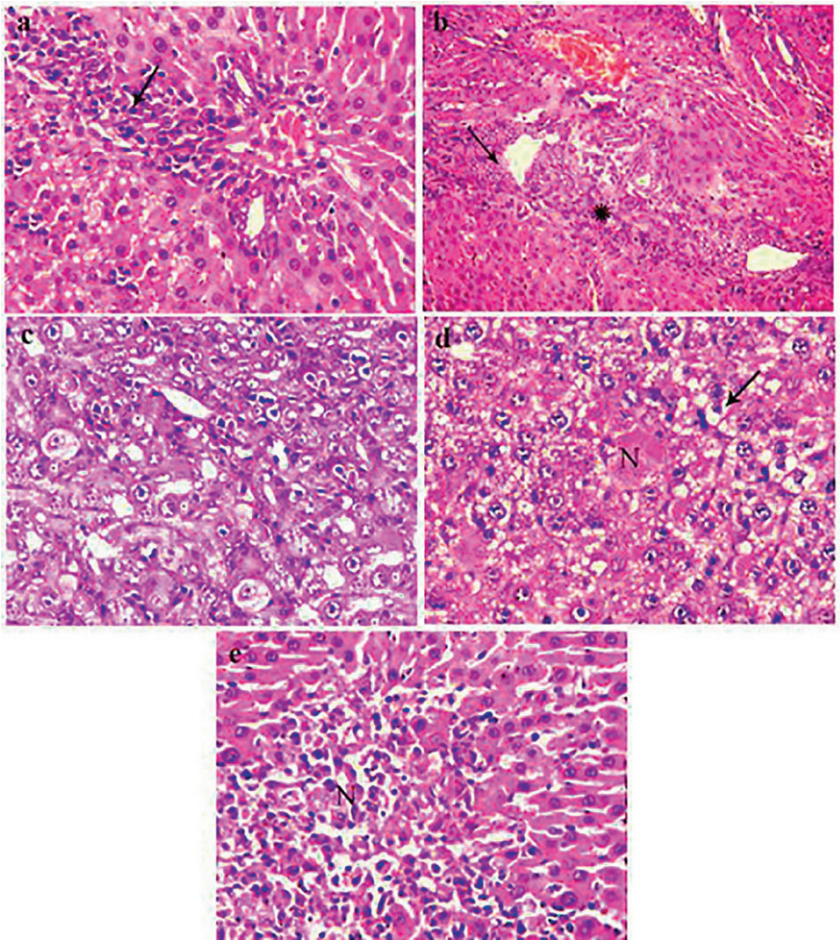

Fig. 1. H&E stained section of liver obtained from rats received 200 mg/kg b.wt TAA three times (a) week for six weeks and sacrificed eight weeks post TAA discontinuation, showing. a: Congestion of portal blood vessels with mononuclear leukocytic cellular infiltration (arrow, x400), (b) Hyperplasia of the lining epithelium of bile duct (arrow) with portal to portal fibrous bridge (asterisk, x100), (c) Hepatocytes showed extensive balloning degeneration and reticulation, (x200), (d) Diffuse hydropic degeneration of hepatocytes (arrow) with focal area of coagulative necrosis in the hepatic parenchyma (N, x200), (e) Marked areas of coagulative necrosis in the hepatic parenchyma (N) characterized by shrunken hepatocytes with hyper-eosinophilic cytoplasm and pyknotic nuclei admixed with mononuclear leukocytic infiltration (x 400).

with pseudo-lobulation (Fig. 2 a,b), which separate the hepatic lobules from each other at which fibrous septa appeared complete, thick and contained proteinaceous eosinophilic edematous substance, congested blood capillaries, mononuclear leukocytic cellular infiltrations with newly formed bile ductules were observed (Fig. 2c,d). Fibrous bridging from the central vein to adjacent portal areas was detected (Fig. 2e). Severe fibrosis of hepatic tissue was also detected by positive Crossman's trichrome stain (Fig. 2f).

Concerning liver obtained from rats (group 4) received therapy by single dose (3x10^6 cells/ mL I.V) of MSCs for another 8-weeks post induction of liver fibrosis revealed good restoration of the hepatic parenchymal cells, as mild congestion of the central vein and hepatic sinusoid. Interestingly, the portal area nearly showing normal histological structures except few mononuclear leukocytic cellular infiltration (Fig. 3a). Additionally, mild degenerative changes in the hepatocytes with activation of Von-Kuepfer cells (Fig. 3b) as well as the hepatocytes have small intracytoplasmic clear vacuoles was demonstrated (Fig. 3c). However, marked decrease in the intensity and spreading of fibrous tissue proliferation that appeared as thin strands of the fibrous connective tissue in between hepatic lobules in most examined sections, as ensured also by Crossman's trichrome stain (Fig. 3d,e). In the meantime, focal areas of regenerated hepatocytes which mostly seen in all examined sections. This area appears in the form of vesiculated nucleus and more eosinophilic cytoplasm (Fig. 3f). Some examined sections showed complete regeneration of hepatic lobules. The histopathological grading of hepatic fibrosis in various studied groups is summarized in Table 6.

Immunohistochemical analysis of α-SMA and HGF

Normal positive reaction of α-SMA around the central and portal veins was observed in liver sections obtained from the negative control group and MSCs treated group (Fig. 4a,b) where normal myofibroblasts exist. In contrary, thioacetamide

Table 6. Effect of MSCs on the pathological grading of TAA-induced liver fibrosis in rats in different treatment groups.

		Pathological grading of hepatic fibrosis						
Group	Number	0	I	II	III	IV	V	VI
Control	10	10	0	0	0	0	0	0
TAA	10	0	0	0	1	3	6	0
MSCs	10	10	0	0	0	0	0	0
TAA+MSCs	10	0	6	3	1	0	0	0

TAA: Thioacetamide; MSCs: Mesenchymal stem cells.

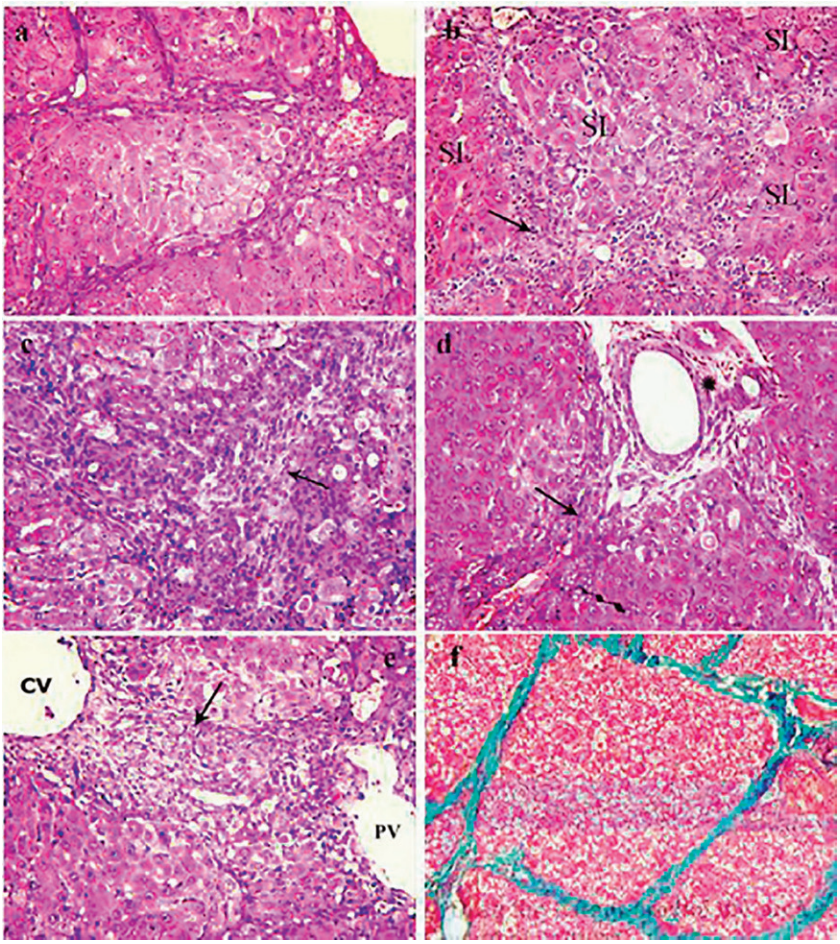

Fig. 2. H&E stained section of liver obtained from rats received TAA (200 mg/kg b.wt) three times a week for six weeks and sacrificed eight weeks post TAA discontinuation, showing, (a) Pseudo-lobulation with formation of unusual hepatic lobules, with extensive apoptosis (x200), (b) Strands of fibrous connective tissue infiltrated by mononuclear inflammatory cells (arrow) and replacing the hepatic parenchyma forming pseudo-lobulation (SL), with presence of apoptotic bodies in hepatic cells (x200), (c) The proliferated fibrous connective tissue strands containing proteinaceous eosinophilic edematous substance (arrow, x200), (d) Peri-ductual with peri-portal connective tissue proliferation, with the connective tissue septa of the pseudo-lobulation containing mononuclear cells (arrow) and newly formed bile ductules (zigzag arrow, x200), (e) Fibrous bridging (arrow) from the central vein (CV) to adjacent portal areas (PV, x200), (f) Extensive collagen deposition with multiple pseudolobular formation (Crossman's trichrome, x200).

injected rats showed marked immunopositive response of α-SMA along the portal to portal fibrous stands, extending among the parenchyma, in the peri-sinusoidal space and surrounding hepatocytes (Fig. 4c). There was a marked expression of α-SMA in the portal triads along the proliferated fibrous connective tissue particularly peri-canalecular around the proliferated bile duct and the newly formed bile ductules (Fig. 4d). Interestingly, hepatic sections obtained from rats' administrated thioacetamide and MSCs revealed scattered mild positivity of α-SMA in the hepatic parenchyma (Fig. 4e).

The positive immunohistochemical reaction for HGF was appeared intra-cytoplasmic as the cytoplasm has been colored dark brown. The HGF expression in the hepatocytes of hepatic tissue obtained from rats treated with TAA is considered weak in comparison to the hepatic tissue of rats treated with both TAA plus MSCs. In this group (TAA plus MSCs) HGF expression is markedly extensive in comparison to other groups. Figure 5 showed the results of IHC for evaluation of HGF expression in different groups.

Discussion

Fibrosis of liver is a complicated dynamic response generated by different kinds of harmful agents, including chemical toxicity, drug, viral or alcoholic (Liedtke *et al*., 2013). In the meantime, an alternative way for advanced hepatic fibrotic patients is liver transplantation. Consequently, there is an urgent necessity to find new approaches for anti-fibrotic treatment. Recently, several anti-fibrotic drugs with well-established safety profiles were suggested (Lee *et al*., 2015). Additionally, the mesenchymal stem cells found to serve as a potentially relevant therapeutic agent for the treatment of hepatic diseases. Accordingly, the present research investigated the potential hepato-therapeutic effects of MSCs in liver fibrosis experimentally induced in rat model using thioacetamide (Wallace *et al*., 2015).

In the current study, the use of TAA resulting in high serum ALT, AST and ALP with significant decreases in the levels of albumin, globulin, total protein and A/G ratio that are considered important indicators of hepatic injury and toxicity. The elevation of ALT, AST and ALP could be attributed to either destruction of hepatocytes or changes in the cell membrane permeability resulting in their outflow from injured hepatocytes into the blood (Kaplowitz, 2001). Meanwhile, The reduction in the concentration of total protein and albumin in hepatotoxic disorder could be attributed to disturbances in the carbohydrate, lipid and protein metabolisms or disturbed protein biosynthesis in the cirrhotic liver (Low *et al*., 2004). These biochemical results confirmed the obtained histopathological changes in liver of rats injected with TAA. Necrosis of hepatic cells and inability of liver to perform its metabolic and excretory function may be due to free radicals and toxic metabolites produced from TAA metabolism (Ahmed *et al*., 2012). Interestingly, BM-MSCs infusions by tail vein resulting in marked improvement in these biochemical parameters that could be due to the ability of MSCs to protect hepatocytes from necrosis and apoptosis as confirmed by the results of

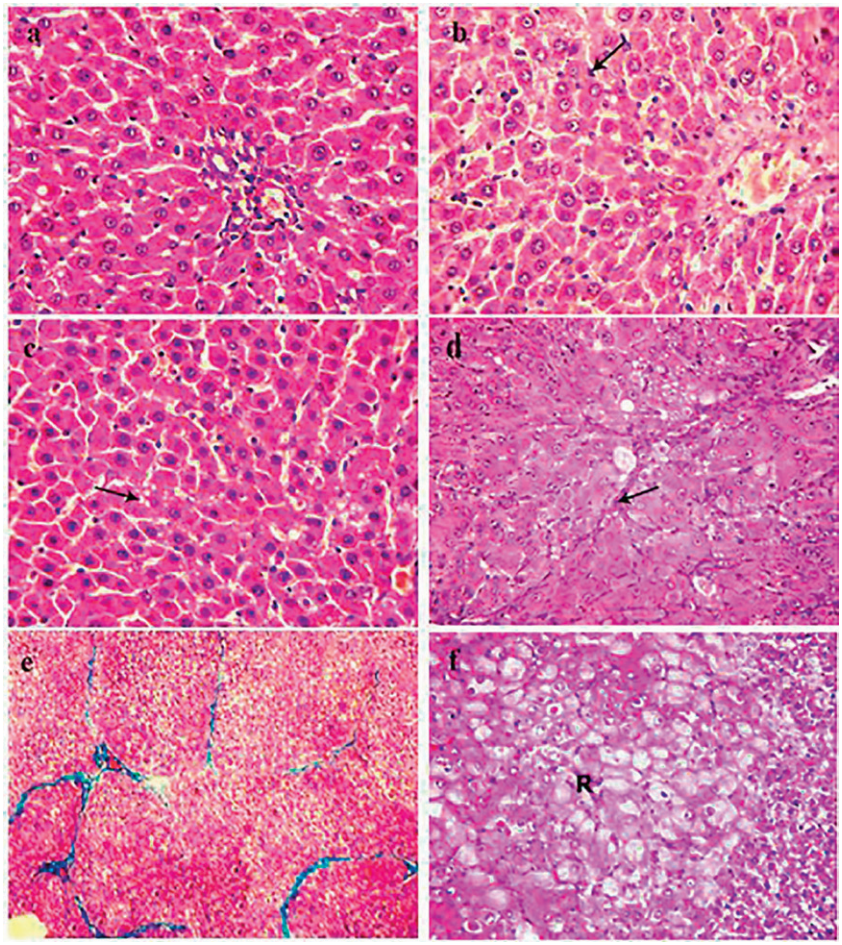

Fig. 3. H&E stained section of liver obtained from rats, received TAA (200 mg/kg b.wt) three times a week for six weeks then treated with single dose (3x106 cells/ mL I.V) of MSCs and sacrificed after 8 weeks post treatment, showing, (a) nearly normal histological structures of the portal area except few mononuclear leukocytic cellular infiltration (x200), (b) Mild degenerative changes in the hepatocytes with activation of Von-kupfer cells (arrow, x200), (c) The hepatocytes have small intracytoplasmic clear vacuoles (arrow, x200), (d) remnant of fibrous connective tissue proliferation in between the hepatic lobules (arrow, x200), (e) Thin fibrous strands in between the hepatic lobules (Crossman's trichrome, x200), (f) Focal regenerated area of hepatocytes in the hepatic parenchyma (R, x 200).

histopathological examination in association with its ability to reduce ROS damage induced by TAA. These findings are matched with Qiao *et al.* (2011), who reported that treatment with MSCs in fibrotic liver resulting in improvement in serum protein activity and an increase in serum albumin levels. This could be attributed to MSCs was able to promote partial recovery of liver function and suppression of liver inflammation.

In the current work, induction of liver fibrosis via TAA injection resulted in obvious oxidative stress in the hepatic tissue, this was demonstrated by a substantial rise in lipid peroxidation content such as MDA and NO. According to the obtained results there is a beneficial correlation between the NO level and the hepatic fibrosis rate that completely agree with Sagor *et al.* (2015). The elevation in the level of MDA and

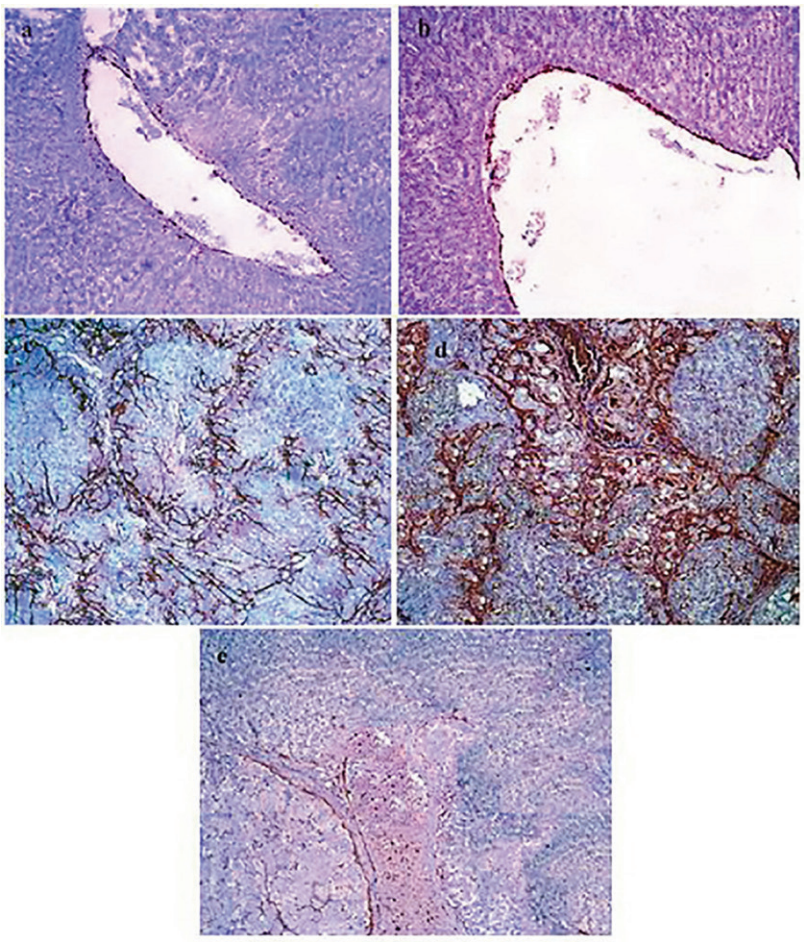

Fig. 4. Micrograph of hepatic tissue obtained from different groups with immunohistochemistry staining for α-SMA, (a) The negative control group showing normal positive staining reaction of α-SMA in the smooth musculature of the blood vessels (×100), (b) MSCs treated group showing normal expression of α-SMA positive staining in the smooth musculature of the blood vessels (×100), (c&d) TAA treated group showing strong immunopostive reaction of α-SMA (×100), (e) TAA plus MSCs group showing marked reduction in the positive immunoreaction of α-SMA (×100).

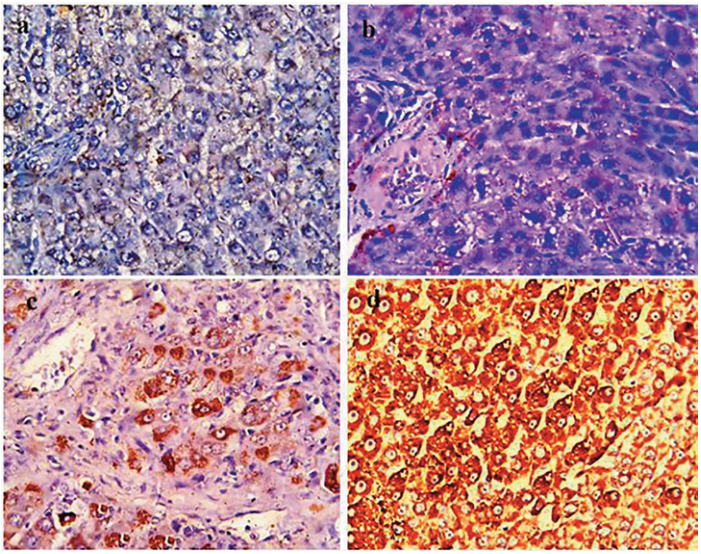

Fig. 5. immunohistochemistry staining for HGF in the hepatic tissue obtained from different groups, (a) The negative control group, showing weak immunopostive reaction (×400), (b) MSCs treated group (II) showing very weak immunopostive reaction, (c) TAA treated group (III) showing immunopostive staining in few number of cells (×400), (d) TAA plus MSCs treated group (IV) showing strong immunopostive reaction (×400).

NO occur as a consequence of tissue injury, hepatocyte necrosis, activation of the inflammatory cells including macrophages. Furthermore, hepatic stellate cell (HSCs) activation into a myofibroblast phenotype that allows great amounts of collagen to be expressed and deposited, in association with the release of fibrogenic mediators and failure of the antioxidant defense mechanism were also induced (Tacke and Weiskirchen, 2012).

The microscopical finding in fibrotic group releved severe degree of hydropic degeneration and fatty changes. Moreover, focal areas of necrosis and apoptic changes with mononuclear leucocytic infiltration were also observed in the hepatic parenchyma. These results were in compelete agreement with the results of Rui *et al*. (2014). These findings may be attributed to production of reactive metabolites such as sulfoxide thioacetamide (TASO) and Thioacetamide-S, S dioxide ($TASO_2$) resulting from the comprehensive metabolism of microsome cytochrome P450 (CYP2E1), which contributes to the toxic effects of TAA (Hajovsky *et al*., 2012). These toxic metabolities induced oxidative stress in the hepatic cells which leads to alterations in cell permeability, increase intracellular concentration of Ca^{++}, increase in nuclear volume, enlargement of nucleoli and also inhibition of mitochondrial activity, caused necrosis of hepatocytesand significantly affected hepatic cells situated in the perivenous acinus area as previously mentioned by Bigoniya *et al*. (2009). Meanwhile, moderate degree of degenerative changes in hepatocytes were observed in treated rats with MSCs in the form of vacuolar and hydropic degeneration. These results completely agreed with the results of Choi *et al*. (2013), who validated the ability of MSCs within 1 to 3 weeks to migrate to the injured liver area and to differentiate to functional hepatocytes. Localization of differentiated cells derived from transplanted MSCs was time-dependent changed from around centri-lobular hepatic veins to parenchyma, which could clarify the obtained results that MSCs may be able to induce complete recovery of hepatic cells with long duration. Furthermore, the obtained results revealed that the intraperitoneal injection of TAA induced hepatic fibrosis within 6 weeks as demonstrated by Crossman trichrome stain as well as increased the expression of α-SMA. These findings could be due to the activation of myofibroblasts as a common hallmark of fibrosis, which leads to the destruction of the architecture and gradual decrease in organ function as a result of production of excessive amounts of the extracellular matrix. Consequently, the measurement of α-SMA expression is therefore commonly used to determine myofibroblast existence and activity. Additionally, it has been found that, α-SMA levels increase in the early stage of fibroblast differentiation process (Corallo *et al*., 2014). However, the strong immune-positive reaction of α-SMA expression in liver sections following the administration of TAA in the present study was matched with the findings of Abd-Elgawad *et al*. (2016).

In the meantime, the hepatic tissue appeared nearly with normal histological structure hepatic lobules with central veins and arrangement of hepatic cords in MSCs treated rats post induction of hepatic fibrosis except only thin strands of fibrous connective tissue in between the hepatic lobules associated with mild degenerative changes in hepatocytes. The obtained results may be attributed to MSCs can exert anti-fibrotic effect in fibrotic liver via its capacity to decrease hepatic stellate cell proliferation and synthesis of collagen Type I via secreting IL-10. Additionally, MSCs could promote hepatic stellate cell apoptosis through the secretion of HGF and nerve growth factor (NGF) lead to a significant decrease in collagen deposition and proliferation (Mansour *et al*., 2015) as demonstrated by clear reduction in a hepatic α-SMA expression in MSCs treated rats post induction of hepatic fibrosis in the current study in comparison to TAA group.

Hyperplasia of the biliary epithelium with newly formed bile ductules, besides, inter acinar mononuclear leukocytic infiltration, these findings agreed with the results of Ling *et al*. (2013). Interestingly, post MSCs treatment in fibrotic rats, the bile duct showed mild degree of hyperplasia of biliary epithelium with fewer numbers of leukocytes. Multiple focal areas of regeneration of hepatic parenchyma was seen scattered in the hepatic tissue obtained from rats treated with TAA and MSCs. These results agreed with the findings of Hwang *et al*. (2012), who indicated that the administrated MSCs, was firstly transdifferentiated into hepatic oval cells and subsequently into hepatocyte-like cells as well as it stimulate the regeneration of endogenous parenchymal cells, and enhance fibrous matrix degradation. This method decreased inflammation, repared damaged hepatocytes, and resolved fibrosis, that leading to an general enhancement of hepatic function (Christ and Dollinger, 2011).

Conclusion

Following to the obtained results in the current study, it could be concluded that, MSCs have therapeutic effect on hepatic toxicity induced by TAA, consequently, it could be used successfully for the treatment of hepatic fibrosis and cirrhosis but its effect is time dependent.

Conflict of interest

The authors declare that there are no conflicts of interest.

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