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ORIGINAL ARTICLE

Amelioration of Bilharzial Liver Fibrosis Using Mesenchymal Stem Cells

[1]Nadia S. Elnahas, [2]Sahar M. Selim, [1]Amany A. Rady[*], [2]Dalia A. Shafey, [2]Hebat Allah M. S. Sallam, [3]Doha M. Taie

[1]Department of Medical Parasitology, Faculty of Medicine, Menoufia University, Shebin El-Kom ,Menoufia, Egypt
[2]Department of Clinical and Molecular Parasitology, National Liver Institute, Menoufia University, Shebin El-Kom ,Menoufia, Egypt
[3]Department of Pathology, National Liver Institute, Menoufia University, Shebin El-Kom, Menoufia, Egypt

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Corresponding Author:
Amany Ahmed Rady
Assistant Professor of Medical Parasitology
Parasitology Department
Faculty of Medicine Menoufia University, Egypt
Tel: 00201093959050
dramanyrady@yahoo.com

ABSTRACT

Background: *Schistosomiasis mansoni causes fibrosis that progress to cirrhosis and liver cell failure. Mesenchymal stem cells (MSCs) based therapy has promising effect in treatment of liver diseases.* ***Objectives:*** *Studying the therapeutic potential of stem cell injection on liver fibrosis in Schistosoma (S.) mansoni infected mice.* ***Methods:*** *A) Pilot study was carried out to ensure homing of MSCs in the fibrosed liver of S. mansoni infected mice. B) Experimental study: S. mansoni infected mice groups (untreated, PZQ treated, MSCs treated and PZQ+ MSCs treated) and non-infected control mice were studied to evaluate the effect of treatment regarding the parasitological parameters and by assessment of serum albumin level and liver enzyme, alanine tranferase (ALT). Stained liver sections by hematoxylin and eosin (H &E), Masson trichrome (MT) and immunohistochemical for transforming growth factor beta-1 (TGF-β1) were also examined.* ***Results:*** *Serum albumin level was significantly improved in PZQ and PZQ +MSCs treated groups. Serum ALT level was significantly reduced in PZQ and MSCs treated groups. High significant reduction in granuloma size was found in MSCs treated group and granuloma number significantly reduced in PZQ +MSCs treated group .TGF-β1 expression encountered significant reduction in PZQ treated group and non-significant reduction in other infected treated groups.* ***Conclusion:*** *Peripherally injected MSCs can migrate and settle in the injured liver of S. mansoni infected mice leading to significant reduction in granuloma size, ameliorate hepatic fibrosis and hepatic functions improvement.*

INTRODUCTION

Schistosomiasis is an infectious disease of poverty [1]. It is a widely spread chronic helminthic infection and remains endemic in many countries [2]affecting more than 200 million people worldwide .It is found mainly in tropical regions and more debilitating than malaria [3].

Bone marrow derived mesenchymal stem cells (BM-MSCs) are unique multipotent cells, capable of *in vitro* and *in vivo* differentiation into other cell types like functional hepatocytes. So, they are proposed as effective treatment of many diseases [4]. MSCs have been demonstrated an effectiveness in regenerating liver structure and enhancing recovery of liver function [5]. In addition, some studies reported that MSCs can induce apoptosis of activated hepatic stellate cell (HSC) which is responsible for fibrosis [6, 7].

The present study aimed to investigate homing of peripherally injected BM-MSCs in fibrosed livers of *S. mansoni* infected mice and their effectiveness in the regeneration of liver cells. Also, antifibrotic therapeutic potential of BM-MSCs either alone or in combination with anti-*Schistosome* drug "praziquantel" was assessed.

METHODOLOGY

Animals and ethics statement

Swiss albino female mice aging 6-8 weeks, weighing 20±2 grams were purchased from Schistosome Biological Supply Program (SBSP) Unit at Theodor Bilharz Research Institute (TBRI) (Giza, Egypt), caged in groups and maintained under standard conditions. All animal protocols were conducted in accordance with the valid international guidelines for animal experimentation and approved by the TBRI's animal research committee.

S. mansoni infection

An Egyptian strain of *S. mansoni* cercariae were provided by SBSP \TBRI. Cercariae were shed from laboratory-bred, infected *Biomphalaria alexandrina* according to the method described by Pellegrino et al. [8]. Mice were infected with 60-80 cercaria suspended in 0.2 ml solution by subcutaneous injection [9].

Pilot study

It was carried out on 5 mice. Initially, each mouse was infected with 60-70 *S. mansoni* cercaria .Then, 250 microns of Labeled MSCs with red fluorochrome PKH26 (Sigma– Aldrich Co., USA) suspended in PBS was injected to tail vein of mice 8 weeks post infection (p.i.) Mice were euthanized at 12th week p.i. and the livers were harvested and examined under fluorescence microscope (Leica DM 5500 B, Germany) (magnification 200x).

Study design

Mice were randomly divided into five groups, 10 per each. Group I served as non- infected control. Group II; *S. mansoni* infected control. Group III; infected and treated with praziquantel (500 mg/kg at two divided doses) oral administration for 2 successive days[10] .Group IV infected and treated with BM-MSCs by a dose of 1x10^{6}cells. Group V infected and treated with both MSCs and praziquantel as previous groups. All treatment regimens started 8th week p.i.

Drug

Praziquantel (Distocide, 600 mg tablets) was obtained from E.I.P.I.Co. Cairo, Egypt. Fresh drug suspension in Cremophore-EL was prepared and given orally to animals by stainless steel canula.

Treatment with BM-MSCs

MSCs isolation and culture

Mesenchymal stem cells isolated from bone marrow were harvested by flushing the tibiae and femurs of 6-week-old male Balb/C mice with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco -BRL, USA). Isolation of nucleated cells with a density gradient by using Ficoll/Paque (Pharmacia) was done. They were resuspended in complete culture medium with 10% penicillin–streptomycin- amphotercin B mixture (Gibco-BRL). Cells were incubated at 37 °C in5% humidified CO_2 for 12–14 days as primary culture or upon formation of large colonies. When large colonies developed (80–90% confluence), cultures were washed twice with PBS. For their detachment, the cells were trypsinized with 0.25% trypsin in 1mM EDTA (Gibco-BRL) for 5 min at 37 °C. [11]. MSCs in culture were characterized by their plastic adhesiveness and spindle shaped fibroblast-like appearance when examined by inverted tissue microscope [12].

PKH26- labeling of stem cells

Using red fluorescent dye PKH26 (Sigma Aldrich Co., USA) according to the manufacture's protocol [13], MSC from the third passage were isolated and washed triple with serum free medium. All procedures were performed at room temperature. Fluorescent microscope was used to examine the liver specimens of mice treated with the labeled cells. (Fig 1A)

Immunophenotyping of BM-MSC

For the immunophenotyping analysis, BM-MSCs were stained with antibodies conjugated with fluorescein isothiocyanate (FITC) forCD37, CD90 andCD105[14] according to the manufacturer's protocol. The fluorescence intensity of the cells was evaluated through flowcytometry (Beckman Coulter, EPICS-XL, USA). (Fig 1B, C, D)

Inoculation of BM-MSCs

A single intravenous inoculums of one million of MSCs suspended in 250µL PBS was injected in the tail vein of each *S .mansoni* infected mice while being hold within specific straightener on the 8th week p.i..

Euthanizing animals

All mice groups were euthanized by decapitation 4 weeks after MSCs transplantation. Their livers were isolated under aseptic condition.

Parasitological Parameters

Worm burden

Mesenteric and portal vasculatures were perfused according to Duvall and DeWitt [15]; the numbers of adult worms were counted and recorded for each mouse. The percent reduction was calculated by comparing the number of worm recovered from each group of mice with their respective controls.

Estimation of liver and spleen indices

The liver and spleen as well as the body of each mouse were weighed. Liver and splenic indices were calculated according to the ratio of liver and splenic weight to body weight respectively.

Histopathological examination of liver sections

Parts from the livers collected from all mice groups were fixed in10% formalin. Paraffin embedded blocks were prepared and sectioned at 4 um thickness. Sections were stained with H&E to study the hepatic morphological changes and MT to assess hepatic fibrosis and examined microscopically at low power of magnification (10x10) using light microscopy.

Measurement of mean egg granuloma size

Stained liver sections from each mouse of all groups were measured for granuloma size. The single-egg granulomas (with intact or degenerated miracidia) were only measured. Measurements of the granuloma size were conducted on non-contiguous granulomas using an ocular micrometer [16].

Mean egg granuloma number

Granuloma count was carried out at low power of magnification (10x10) in five microscopic fields of liver sections under Zeiss light microscope (Oberkochen, Germany). The mean number of granuloma was calculated for each mouse. The mean was calculated for each experimental group from the mean values of the individual mice [17].

Immunohistochemical staining for TGF-β1

Immunohistochemistry was performed by using an avidin biotin complex immunoperoxidase technique [18] according to the manufacturer's protocols. Liver sections with the primary antibody replaced with PBS served as negative controls while colonic cancer sections served as TGF-β1 positive controls. The liver

sections were examined using light microscope. TGF-β1 expression sites were examined intralobulary, in the periportal areas, in hepatocytes and granuloma. The percentage of positively stained cells was estimated per section animal in a semi-quantitative way [19].

Biochemical study of liver function

At 4th week after treatment, blood samples were collected from each mouse and sera were separated by centrifugation at 3000 ×g for 15min at 4∘C. Serum albumin level was determined using commercial kit supplied by Diamond, RA50, Ireland[20]. Alanine aminotransferase (ALT) was measured using Biodiagnostic commercial kits [21].

Statistical analysis:

All values are presented as mean ($\overline{X}$) and standard deviation (SD). Qualitative data were presented in the form of numbers and percentages (%). Also, one way ANOVA (F) test, KrusKall Wallis (KW) test and Post Hoc LSD test were used.

Values of P-value: Non-significant difference if P > 0.05, significant difference if P<0.05*, highly significant difference if P < 0.001 **.

RESULTS

Identification of MSCs:

In culture, MSCs were identified by their spindle shaped fibroblast-like appearance under inverted tissue microscope (Fig 1A).

Flowcytometry immunophenotyping of MSCs:

Flowcytometric analysis showed that MSCs were positive for CD37, CD90 and CD105 (Fig 1 B, C and D).

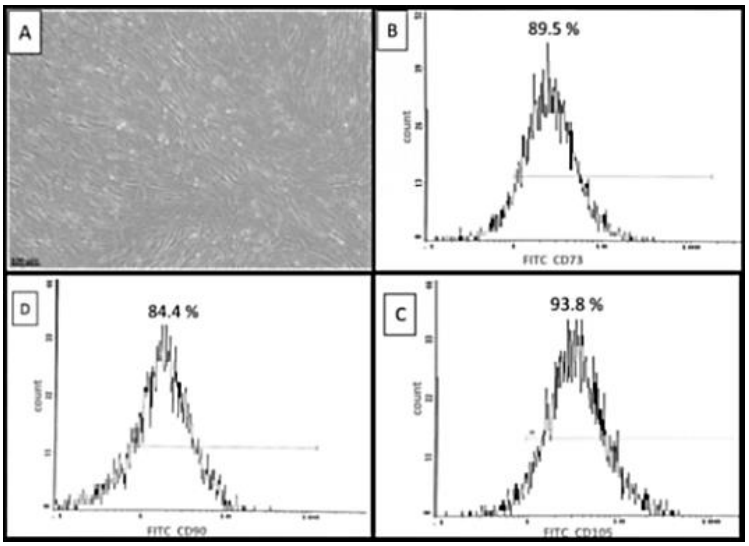

Fig.1: (A) Spindle shaped fibroblast-like MSCs under inverted tissue microscope. (B, C, D) Flowcytometric immunophenotyping of clusters of differentiation of MSCs CD 73+, 90+ and 105 + respectively.

Effect of PZQ, MSC and their combination on mice weight, hepatic and splenic index:

Mean mice weight for each studied group was shown in table (1). High significant increase in mice weight was detected in PZQ and PZQ +MSCs treated groups compared to infected untreated group (p< 0.001), also BM-MSCs treatment caused significant increase in mice weight (p< 0.05).

To investigate the effect of treatment on the recovery of hepatic fibrosis, the liver and splenic indices were measured. In infected control group ,enlargement of liver and spleen were found resulting in an increase in both liver and splenic indices (0.075±0.009 and0.013±0.0036 respectively).Non-significant decrease in liver index was recorded in all treated groups (P>0.05). Splenic index was significantly decreased (p< 0.05) in PZQ (0.0090±0.0014) and PZQ +MSCs (0.009±0.00099) treated groups compared to infected untreated group, while BM-MSCs caused a non-significant decrease in splenic index compared to infected untreated group (P>0.05). It was 0.016±0.0064 (Table 1).

Effect of PZQ, MSC and their combination on worm burden, serum ALT and albumin:

Worm burden encountered a high significant reduction in all treated group compared to infected untreated groups (p< 0.001). In PZQ and PZQ +MSCs treated, the percentage of reduction was 100% and 97.3 % respectively .The total number of worm was reduced significantly in BM-MSCs treated mice with reduction percentage of 33.8 % (p<0.05). (Table, 1).The performance of the liver was monitored by the serum albumin level and liver enzymes. Regarding serum albumin level, PZQ and PZQ +MSCs treatment caused

a high significant increase in comparison to infected untreated group (p<0.001). It was 3.96±0.12 and 3.80±0.24 respectively. Reduction in serum ALT was highly significant in PZQ and BM-MSCs treated groups (p< 0.001). While in PZQ +MSCs treated group, a significant reduction in serum ALT (p< 0.05) was detected. It was 94.2±26.1 and 91.2±37.7respectively (Table 1).

Table 1: Effect of PZQ, BM-MSC and their combination on *S. mansoni*-induced changes in mice weight, hepatic index, splenic index and worm burden Albumin and ALT.

Parameters	Normal	Infected untreated	PZQ treated	BM-MSCs	PZQ + BM-MSCs	P value
Mice weight	32.0±2.29	16.5±2.63	24.4±1.78	19.4±3.09	22.5 ± 2.45	P1<0.001 P2<0.05 P3<0.001
Liver index	0.057±0.006	0.075±0.009	0.074±0.013	0.087±0.023	0.069±0.009	P1>0.05 P2>0.05 P3>0.05
Splenic index	0.0017±0.0009	0.013±0.0036	0.0090±0.0014	0.016±0.0064	0.009±0.00099	P1<0.05 P2>0.05 P3<0.05
Worm burden	0.0±0.0	14.8±1.39	0.0±0.0	9.8±6.78	0.4±0.84	P1<0.001 P2<0.05 P3<0.001
Reduction %			100%	33.8%	97.3%	
ALT	81.4±13.9	184.2±66.5	94.2±26.1	91.2±37.7	114.2±67.5	P1<0.001 P2<0.001 P3<0.05
Albumin	3.92±0.15	3.01±0.39	3.96±0.12	3.46±0.33	3.80±0.24	P1<0.001 P2<0.05 P3<0.001

Effect of PZQ, MSC and their combination on mean hepatic granuloma diameter and number:

The diameter of the hepatic granulomas was reduced significantly as compared to the infected control group in all treated groups. The group of mice treated with BM-MSCs showed the highest reduction followed by PZQ +MSCs treated group and lastly PZQ treated group. The reduction percentages were 66.7%, 20% and 13.3% respectively. These results were highly statistically significant (P<0.001). Regarding the mean granuloma count in treated groups, the PZQ +MSCs treated group showed the highest reduction in number, it was 25.2±7.3 compared to infected control group (32.2±6.5). While in PZQ treated, it was 25.8±12.2, the reduction percentages were 21.7% and 19.8% respectively. These results were highly statistically significant (P<0.001). In the group of mice treated with BM-MSCs, there was high significant increase in granuloma number. It was 36.0±15.9 (Table 2).

Table 2: Effect of PZQ, BM-MSC and their combination on mean hepatic granuloma diameter and number

	Normal	Infected untreated	PZQ treated	BM-MSCs	PZQ + BM-MSCs	KrusKall Wallis	P value
Granuloma number: Mean ±SD	0.0±0.0	32.2±6.5	25.8±12.2	36.0±15.9	25.2±7.3	27.7	**P**<0.001
Reduction %			19.8%		21.7%		
Granuloma size: Mean ±SD	0.0±0.0	0.30±0.07	0.26±0.05	0.1±0.037	0.24±0.084	39.5	**P**<0.001
Reduction %			13.3%	66.7%	20%		

Histopathological examination

Examination of liver sections from the infected control group stained by H&E and Masson's trichrome showed presence of scattered large fibrocellular granulomas with excess deposition of collagen fibers at the periphery and an apparent disturbance of the hepatic lobular architecture (Fig 3B& Fig 4B).

In PZQ treated, small fibrocellular granulomas formed of degenerated ova surrounded by lymphocytes, giant cells, pigmented macrophages and plasma cells with decreased collagen fibers within the granuloma (Fig 3C& Fig 4C).

Marked improvement and regression of the inflammatory granulomatous reactions resulting in small fibrocellular granulomas with few collagen fibers and appearance of scattered small oval shaped cells with deeply stained cytoplasm at the periphery of egg granuloma with restoration of normal hepatic architecture were observed in the group of mice treated with BM-MSCs (Fig 3D& Fig 4D) and PZQ +MSCs treated group (Fig 3E, Fig 4E& Fig 3F).

Liver sections immunohistochemical stained for TGF β1 showed absence of its expression in the control group (Fig 5A). However, expression was detected in the infected control group (Fig 5B) and other treated groups within parenchymal hepatocytes and both portal inflammatory cells and fibroblasts (Fig 5C,D and E) with the highest percentage of expression in the infected control group and lowest expression in group treated by MSC only(Fig 5D).

Effect of praziquantel, BM-MSC and their combination on TGF-β1 expression within granuloma:

The results of the current study revealed significant increase of TGF-β1 percentage of expression within granulomas in infected control (group II)as regards to normal control group (group I). A high significant reduction of TGF-β1 expression within the granulomas was detected in all treated groups. The highest reduction was detected in PZQ treated group followed by PZQ +MSCs treated group and lastly BM-MSCs treated group. The reduction percentages were 52%, 24% and 4% respectively (Table: 3).

Table 3 : TGF-β1 percentage of expression within granulomas in different groups.

	Normal	Infected untreated	PZQ treated	BM-MSCs	PZQ + BM-MSCs	KrusKall Wallis	P value
TGFβ1granuloma %: Mean ±SD	0±0.0	50±24.03	24±10.7	48±20.4	38±20.4	29.9	<0.001
Reduction %			52%	4%	24%		

Results of pilot study:

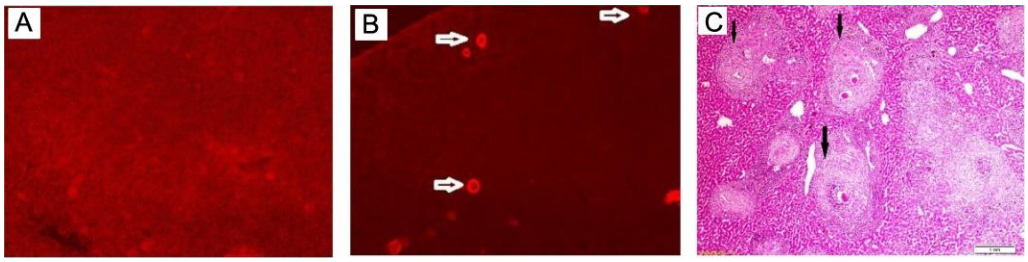

Fig.2: (A, B) .Cryosections showed BM-MSC homing in the infected livers
(A) Iso picture of hepatic tissue with no detection of labeled cells.
(B) Pkh26 labeled BM-MSC (white arrows) using the fluorescence microscope (magnification, 200X).
(C) Bilharzial granulomas in the liver of *S. mansoni* infected mice.

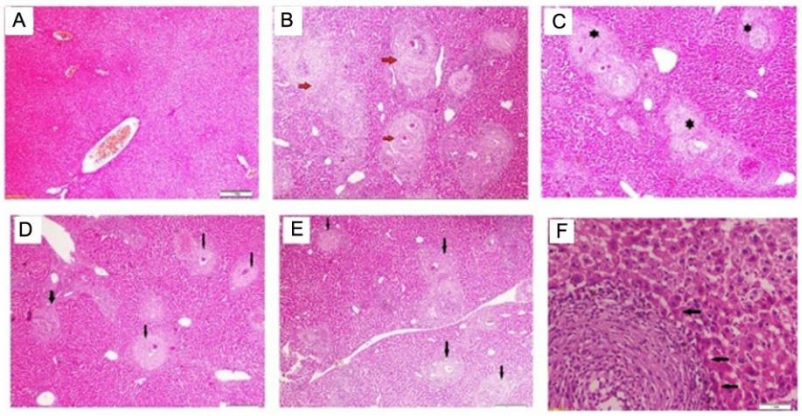

Figure 3

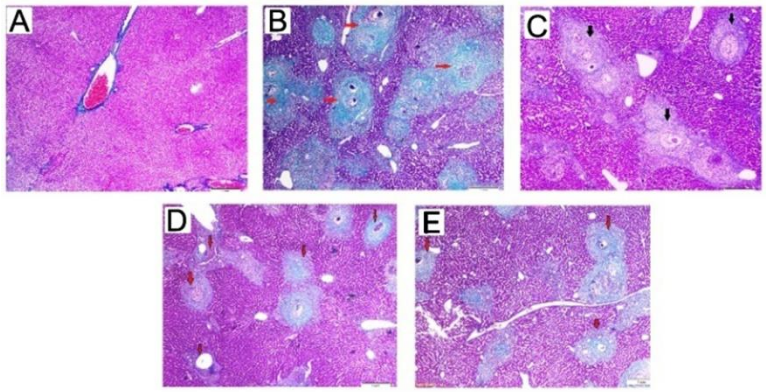

Figure 4

Fig. (3,4): Effect of PZQ, BM-MSC and their combination on histological changes stained by H &E and Masson's trichrome stain. (A) Normal group. (B) Liver section of infected untreated mice. (C) Liver section of infected mice treated by PZQ only. (D) Liver section of infected mice treated by I.V injection of MSC alone. (E) Liver section of infected mice treated by both PZQ and MSC

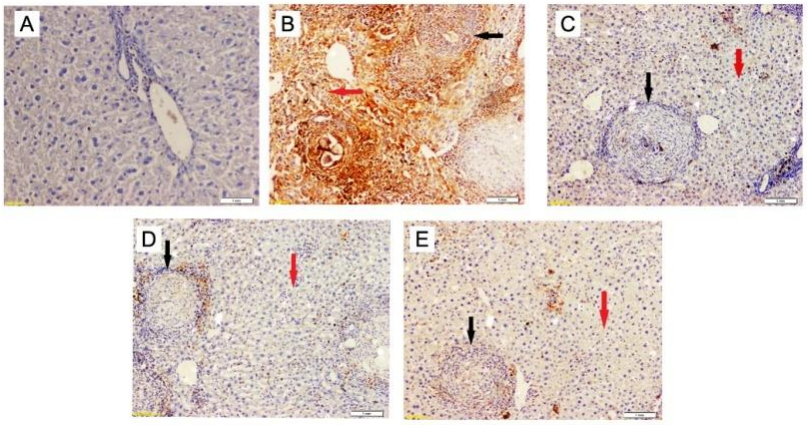

Fig. 5: (A) Normal hepatic tissue showed no cytoplasmic expression of TGF-β1 within hepatocytes. (B) Hepatic tissue of *Schistosoma* infected mice showed strong and diffuse brownish cytoplasmic expression of TGF-β1 within detected granuloma (fibroblasts and inflammatory cells) (black arrows) and within hepatocytes (red arrow).(C) Hepatic tissue of infected mice treated by PZQ only showed weak expression of TGF-β1 .(D) Hepatic tissue of infected mice treated by I.V injection of MSC alone. (E) Hepatic tissue of infected mice treated by both PZQ and MSC. (Immunoperoxidase, 40X). D &E marked decrease of TGF-β1 expression.

DISCUSSION

Treatment and control of schistosomiasis still depend on the available drug, PZQ, and hence seeking for adjunctive therapies is in demand and obligatory, particularly in view of rapid re-infection after treatment and emergence of resistant parasitic strains to PZQ [22].

Currently, several clinical studies showed the significance of stem cell-based therapies for the treatment of a wide range of human diseases[23].

Mesenchymal stem cells (MSCs) have practical advantages because of their high self-renewal capability, their potential for multipotent differentiation, and their low immunogenicity [24].

Fluorescent microscopic examination of liver sections showed that the transplanted labeled MSCs had been settled into the liver. This result is in agreement with Fikry et al. [4].

In the current work, it was found that injection of BM-MSCs alone or in combination with PZQ improved the architecture of the liver and showed a regenerative effect on the injured liver. The regenerative effects of MSCs have been recorded by different authors in many studies [25, 26]. The significant increase in mice weight in all treated groups either with PZQ, MSCs or their combination could indicate an improvement in general condition and increasing the survival of *S. mansoni* infected mice. Our results are in accordance with Xu et al. [2] who evaluated the effect of MSCs on the survival of *S. japonicum* infected mice. They reported an increased survival percentage of MSCs treated group. It was 62.5 % versus 12.5% in infected control group.

Liver and splenic indices were decreased in PZQ and PZQ + MSCs treated groups. Similarly, Xu et al. [2] reported the same results in *S. japonicum* infected mice.

In the current work, total worm burden was significantly reduced in all treated groups. The percentage of reduction was higher in PZQ + MSCs than MSCs treated group. It was 97.3% .This may be due to strong anti-schistosomal activity of PZQ. While in MSCs treated, it was much lowered. It was 33.8% .The noticed difference in the efficacy of MSCs injection on worm burden may be attributed to its minor effect on the vitality as well as the fecundity of the female adult schistosomes. These results are in accordance with El–Mahdi et al [26]**;** they reported non-significant reduction of total worm burden in *S. mansoni* infected mice administered MSCs alone.

There was a significant decrease in the granuloma number and size in PZQ and MSCs + PZQ treated groups. The percentages of reduction were 19.8% and 21.7% in number and 13.3% and 20% in size respectively. However, MSCs treated group, the granuloma numbers were increased with decrease in their size. This may be attributed to the lack of schistosomicidal activity of PZQ on adult worm and minimal effect of MSCs on the fecundity of adult worm. Our results are supported by Xu et al. [2].They documented significant reduction in granulomas size in MSCs + PZQ treated group.

The extent of fibrosis in the present work was determined by density of MT stain and area of fibrosis. The fibrotic area and intensity of MT stain were markedly decreased in MSCs treated group (Fig. 4C).In another study, El–Mahdi et al. [26] estimated the extent of fibrosis by quantitative morphometric analysis of collagen content and reported significant reduction of collagen content after treatment with MSCs . They explained its inhibitory effect on collagen deposition to enhanced fibrotic degradation rather than decrease fibrosis formation. While Fikry et al. [4] explained the antifibrotic effect of BM–MSCs by inactivation of hepatocytes stellate cells (HSCs).

In the current study, histopathological examination of H & E stained sections revealed small fibrocellular granulomas with few collagen fibers and appearance of oval cells in the periphery of granulomas in MSCs and MSCs + PZQ treated groups. The oval cells are small young cells exhibiting small oval shaped nuclei centrally located with deeply stained cytoplasm. Our results are supported by Fikry et al. [4]. They confirmed their results by immunohistochemical staining for CK7.

One of the main mediators involved on fibrosis deposition during hepatic injury is TGF-β1[26].This cytokine stimulates the transition of stellate cells into myofibroblasts, which secrete high amounts of extracellular matrix and inhibit its degradation [27].

The present study showed TGF-β1 expression within hepatocytes and granulomas was significantly decreased in all treated groups when compared with infected control (Table: 3). Positive TGF-β1 expression was seen in hepatocytes (cytoplasmic), fibroblast and inflammatory cells of granulomas. The highest reduction was detected in PZQ treated followed by MSCs + PZQ and lastly MSC treated groups. It was 52%, 24 % and 4%respectively.Similarly; El-Lakkany et al. [19]reported 70.75% reduction of TGF-β1 expression in PZQ treated group 7th week p.i. Also, increased TGF-β gene expression in *S. mansoni* infected livers was reversed significantly after MSC administration. The reduction was 50-60% by quantitative PCR analysis [29].

The results of present study revealed a significant reduction in the serum level of ALT after treatment with PZQ, MSCs and their combination in comparison to infected untreated mice ($p<0.001$).The amelioration of serum level of ALT was more effective in mice group transplanted with MSCs. These results are in accordance with Abdel-Aziz et al. [29].

The present results revealed significant increase in serum level of ALB in all treated groups. In PZQ and PZQ+MSCs treated groups, serum ALB level increased

markedly reaching the normalization level of non-infected control. This may be resulted from anti-fibrotic activity of MSCs and PZQ in addition to the reduction of granuloma size and inflammatory reaction. Also differentiation of MSCs into hepatocytes and restoration of normal liver functions . Our results are supported by Salama et al. [30]. Also, Pulavendran el al. [31] found that the increased serum ALB level was an indicative for recovery of normal liver function by MSCs treatment and explained their results either due to apoptosis prohibition of parenchymal liver cells or inhibition of proliferation and infiltration of inflammatory cells.

CONCLUSION

Our findings in the current study strongly suggest the valuable role of BM-MSCs in liver remodeling following schistosomiasis.BM-MSCs infusion significantly enhanced hepatic regeneration and alleviated hepatic fibrosis. Moreover, combining MSCs with PZQ was better than the administration of each drug alone and can cause significant decrease in formation of collagen fibers and inhibition of subsequent liver fibrosis. However, the mechanism of the antifibrotic effect of MSCs needs more researches to determine the exact mechanism.

Conflicts of interest:

- The authors declare that they have no financial or non- financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher

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