Original Research

Mesenchymal stem cells relieve fibrosis of *Schistosoma japonicum*-induced mouse liver injury

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Abstract

Mesenchymal stem cells (MSCs) have gained popularity for their potential as seed cells to treat various human diseases, including pathogenic infections. *Schistosoma japonicum* (*S. japonicum*) infection is characterized by formation of parasite egg granulomas and host liver fibrosis. MSCs have been proposed as useful treatments of *S. japonicum* infection, but the efficacy and underlying mechanisms remain unknown. Herein, we report that MSCs were able to ameliorate *S. japonicum*-induced liver injury *in vivo* and this effect was enhanced by combining MSCs with conventional drug praziquantel (PZQ). Kunming strains of mice were infected with *S. japonicum* and treated with vehicle, MSCs, PZQ or PZQ + MSCs. MSC treatment not only prolonged the survival time of infected mice but reduced egg granuloma diameter and decreased the concentrations of serum transforming growth factor-β1 and hyaluronic acid. MSC treatment also inhibited collagen deposition and reduced the expression of collagen type 3, α-smooth muscle actin and vimentin in infected mouse liver tissues. Collectively, our findings suggest that MSC treatment represents a novel therapeutic approach for *S. japonicum*-induced liver injury and fibrosis.

Keywords: mesenchymal stem cell, *Schistosoma japonicum*, praziquantel, fibrosis

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Introduction

Mesenchymal stem cell (MSC)-based cell therapy has recently emerged as an effective approach to treat a variety of diseases, including lung fibrosis,[1] liver injury,[2,3] kidney injury,[4,5] bacterial infection[6,7] and myocardial infarction.[8] MSCs are multipotent and are capable of differentiating into cardiomyocytes[9,10] and hepatocyte-like cells[11–13] *in vitro* and *in vivo*. Under the influence of some growth factors or cytokines, MSCs can also differentiate into functional hepatocytes, which have been demonstrated as effective in regenerating liver structure and promoting recovery of liver function.[14] In addition, some studies have suggested that MSCs can secrete interleukin (IL)-10,[15] hepatic growth factor and nerve growth factor[16] to induce activated hepatic stellate cell (HSC) apoptosis to amend fibrosis. Thus, MSCs have already shown enormous potential for treating acute and chronic injuries, as well as fibrosis, by facilitating tissue reconstitution.

Schistosomiasis is a widespread parasitic infective disease that remains endemic in many countries. The main pathogenic agent of schistosomiasis is the *Schistosoma japonicum* species of blood fluke. If left untreated, or in a severe form, *S. japonicum* infection can lead to life-threatening liver complications, including hepatic pipe stem fibrosis, 'tortoise back' fibrosis and portal hypertension. The most commonly used therapeutic drug is praziquantel (PZQ), which has demonstrated efficacy in both prevention of *S. japonicum* infection and substantial reduction of worm burden in infected patients.[17] However, the hepatic fibrosis caused by schistosomiasis is not eliminated by PZQ and has remained a clinical challenge. Considering the previous successes of MSCs in infectious disease therapy and their evidenced antifibrotic effects, it is worthwhile to explore the potential of MSCs for treating *S. japonicum* infection and investigate the effects of MSCs on the underlying mechanisms of schistosomiasis.

In this study, we transplanted rat bone marrow-derived MSCs into a mouse model of *S. japonicum*-induced liver fibrosis and found that MSCs could inhibit *S. japonicum*-induced mouse liver fibrosis and prolong the survival time of infected mice. Our findings indicate that

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MSC treatment may represent a novel and useful approach for ameliorating *S. japonicum*-induced liver injury and fibrosis.

Materials and methods

Parasites and animals

Cercarie of *S. japonicum* were harvested from infected *Oncomelania hupensis* snails. A total of 102 female Kunming mice, 6–8 weeks old and weighing 24 ± 1 g, were used for infection experiments. All procedures involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Jiangsu University. Seventy mice were divided into five groups randomly ($n = 14$ each). Group 1 was used as the uninfected controls. In groups 2–5, each mouse was infected through the abdominal skin with seven cercarie once a week for three consecutive weeks (for a total of 21 cercarie) to induce liver fibrosis. At the ninth week after infection, mice in groups 2–5 were injected with either 0.3 mL of phosphate-buffered saline (PBS; intravenous), 0.3 mL of MSCs (5×10^5 cells suspended in PBS; intravenous), PZQ (300 mg/kg; intragastric) or PZQ combined with MSCs. PZQ was purchased from Nanjing Pharmaceutical Factory Co, Ltd (Jiangsu, China). Mice were sacrificed by anesthesia overdose at two or four weeks after MSC transplantation. Another 32 mice were randomly divided into four groups (groups 6–9) for survival analysis. Mice in group 6 were used as the uninfected control group ($n = 8$) and mice in groups 7–9 were infected with 10 cercarie once a week for three consecutive weeks (for a total of 30 cercarie). Mice in group 7 served as infected controls without any treatment; mice in group 8 were injected once a week, for two consecutive weeks with PBS (0.3 mL; intravenous) and mice in group 9 were injected with MSCs (5×10^5 cells suspended in 0.3 mL PBS; intravenous).

MSC isolation and culture

MSCs were isolated from male Sprague-Dawley rats, as described previously.[18] The cells in the femoral bone were flushed out with PBS, collected by centrifugation at 1000 rpm for 10 min, and cultured in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The medium was changed at 48 h after cells adhered to the culture dishes. When 80% confluence was reached, the cells were trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA, USA) and passed into new dishes for further expansion.

Histology and immunohistochemistry

The right lobes of the livers were harvested and immediately fixed in 4% paraformadehyde. The mice liver tissues in different groups were embedded in paraffin and sliced into 4-μm sections, dewaxed and rehydrated, then stained with hematoxylin and eosin and subjected to Masson's trichrome staining. For immunohistochemical analyses, the slices were heated in citrate buffer to recover antigens, incubated in 3% H_2O_2 for five minutes to block endogenous peroxidase, then blocked with 5% bovine serum albumin for 20 min. Tissue sections were then incubated with monoclonal primary antibodies against vimentin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or E-cadherin (1:200; Santa Cruz Biotechnology) at 37°C for 80 min, followed by washing and incubation with horseradish peroxidase (HRP)-labeled biotin–streptavidin goat anti-mouse secondary antibodies (1:2000; KangChen Bio-tech, Shanghai, China) at 37°C for 20 min. Slices were visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin for microscopic examination.

Mean egg granuloma diameter

We made pathological slices of liver samples harvested from each mouse, and the diameters of the 20 largest egg granulomas in each group were measured and calculated as described by Bartley *et al*.[19] Only granulomas that appeared circular in form were considered.

Liver index and spleen index

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

Enzyme-linked immunosorbent assay and chemiluminescence

Mice serum samples were collected and analyzed to determine levels of transforming growth factor-β1 (TGF-β1) and hyaluronic acid (HA). TGF-β1 was detected by enzyme-linked immunosorbent assay (ExCellBio, Shanghai, China) and HA (Tigsun Diagnostics, Beijing, China) by chemiluminescence assays, according to the manufacturers' protocols.

Realtime polymerase chain reaction

Total RNA was extracted from mice livers using the Trizol reagent (Invitrogen). The cDNAs were synthesized using a reverse transcription kit (Invitrogen). The expression of collagen 3 mRNA was detected by realtime polymerase chain reaction (PCR), according to the manufacturer's instructions. The reaction mixture contained 10 μL 2 × SYBR Green Mix (Qiagen, Hilden, Germany), 0.5 μL 10 μmol/L of each primer, 0.1 μL *Taq* DNA polymerase and 1 μL cDNA. The thermal profile for PCR was as follows: 94°C for five minutes, then 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and 85°C for eight seconds, followed by a final extension at 72°C for 10 min. The relative expression of collagen 3 mRNA was normalized to β-actin (internal control) using the $^{\Delta\Delta}$Ct method.[20] The primer sequences were as follows: collagen 3: forward, 5′ CTGGTCAGCCTGGAGATAAG 3′, reverse, 5′ ACCAGGAC TACCACGTTCAC 3′ and β-actin: forward, 5′ CACGAAA CTACCTTCAACTCC 3′, reverse, 5′ CATACTCCTGCTT GCTGATC-3′.

Western blotting

Liver tissues were lysed in standard RIPA buffer. Protein concentration was measured and equal proteins were loaded and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene fluoride membrane and blocked by incubation with 5% non-fat milk for one hour. After incubation with the primary antibodies overnight at 4°C and secondary HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:2000; Kangchen Bio-tech), respectively, the proteins were detected with enhanced chemiluminescent substrate (ECL; Millipore, Billerica, MA, USA). The dilutions of the primary antibodies were as follows: mouse polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000; Kangchen Bio-tech), anti-vimentin (1:1000; Santa Cruz Biotechnology) and anti-α-smooth muscle actin (α-SMA, 1:200; Chemicon, Temecula, CA, USA). The expression of α-SMA, vimentin and GAPDH of mice in each group were examined. Each reaction was performed in triplicate. The difference was expressed according to gray density image analysis of α-SMA/GAPDH and vimentin/GAPDH.

Statistical analysis

Differences were analyzed between the following treatment groups: PBS group versus MSC group and PZQ combined with PBS group versus PZQ combined with MSC group. Data are presented as means $\pm$ standard deviation (SD). Statistical analysis was performed by analysis of variance or log-rank (Mantel–Cox) test using Prism software (GraphPad, San Diego, CA, USA). Statistical P values less than 0.05 were considered significant.

Results

MSCs prolong survival time and decrease the liver and spleen indexes of *S. japonicum*-infected mice

To evaluate the effect of MSCs on the survival of *S. japonicum*-infected mice, a survival study was performed. As shown in Figure 1a, death rates for mice in the infected control group and the PBS group were recorded after one week of treatment, while death rates for mice in the MSC group were recorded after the fourth week of treatment. At eight weeks after treatment, we found that the percentage of survival in the MSC group was 62.5% versus 12.5% in the PBS group ($P < 0.05$), suggesting that MSCs prolong the survival of infected mice.

To investigate the effect of MSCs on the recovery of hepatic fibrosis, the liver index and spleen index were measured.

The PBS group presented with infection-induced enlargement of the liver and spleen (Figures 1b left and d left), but these features were not apparent in the MSC group (Figures 1b right and d right). The liver index and spleen index are shown in Figures 1c and e, respectively. The liver index in the PZQ + MSC group was 0.057 ± 0.009, lower than 0.070 ± 0.010 in the PZQ + PBS group after two weeks of treatment. Likewise, the liver index in the MSC group was 0.066 ± 0.008, lower than 0.075 ± 0.011 in the PBS group after four weeks of treatment (Figure 1c) ($P < 0.05$). As shown in Figure 1e, the spleen index was 0.017 ± 0.002 in the MSC group, lower than 0.026 ± 0.006 in the PBS group ($P < 0.01$); likewise, the spleen index in the PZQ + MSC group was 0.012 ± 0.002, lower than 0.017 ± 0.001 in the PZQ + PBS group after two weeks of treatment ($P < 0.01$). Moreover, the spleen index was 0.024 ± 0.005 in the MSC group, much lower than 0.033 ± 0.004 in the PBS group ($P < 0.01$), and 0.007 ± 0.002 in the PZQ + MSC group, lower than 0.011 ± 0.002 in the PZQ + PBS group after four weeks of treatment ($P < 0.05$). These data indicate that MSC treatment decreases the liver and spleen indexes of *S. japonicum*-infected mice.

MSCs decrease liver injury of infected mice

We further observed liver histological changes after four weeks of MSC treatment. Comparison with the PBS-treated mice indicated that MSC treatment alleviated liver injury. Likewise, comparison with PZQ + PBS indicated that PZQ + MSCs could relieve liver injury by decreasing infiltration of inflammatory cells, reducing collagen deposition and fibrosis formation, and diminishing the diameter of granulomas (Figure 2a). As shown in Figure 2b, the mean egg granuloma diameter was 505.20 ± 122.32 μm in the MSC group, significantly smaller than that in the PBS-treated group (786.45 ± 93.55 μm, $P < 0.01$). In addition, the diameter in the PZQ + MSC group (316.00 ± 71.48 μm) was significantly smaller than that in the PZQ + PBS group (371.15 ± 77.84 μm, $P < 0.05$). These results demonstrate that MSCs can alleviate liver injury.

MSCs reduce serum HA and TGF-β1 levels of infected mice

HA and TGF-β1 are non-invasive markers for fibrosis, so we then examined the serum levels of HA and TGF-β1 of infected mice. The results presented in Figure 3a revealed that the serum HA was significantly lower in the MSC group (319.43 ± 21.36 μg/mL) than in the PBS group (388.25 ± 45.68 μg/mL) after two weeks of treatment ($P < 0.05$). In addition, the HA concentration in the MSC group (186.00 ± 30.14 μg/mL) was significantly lower than in the PBS group (333.44 ± 43.58 μg/mL) after four weeks of treatment ($P < 0.01$). A statistically significant difference in HA concentrations was also observed between the MSC groups after two and four weeks of treatment. As shown in Figure 3b, serum TGF-β1 was significantly lower in the MSC group ($[5.06 \pm 1.38] \times 10^4$ pg/mL) than in the PBS group ($[8.72 \pm 2.44] \times 10^4$ pg/mL) after two weeks of treatment ($P < 0.05$), and was lower in the MSC group ($[4.69 \pm 2.03] \times 10^4$ pg/mL) than in the PBS group ($[11.52 \pm 3.62] \times 10^4$ pg/mL) after four weeks of treatment ($P < 0.05$). Our results suggest that MSC administration significantly decreases HA and TGF-β1 concentrations in infected mice.

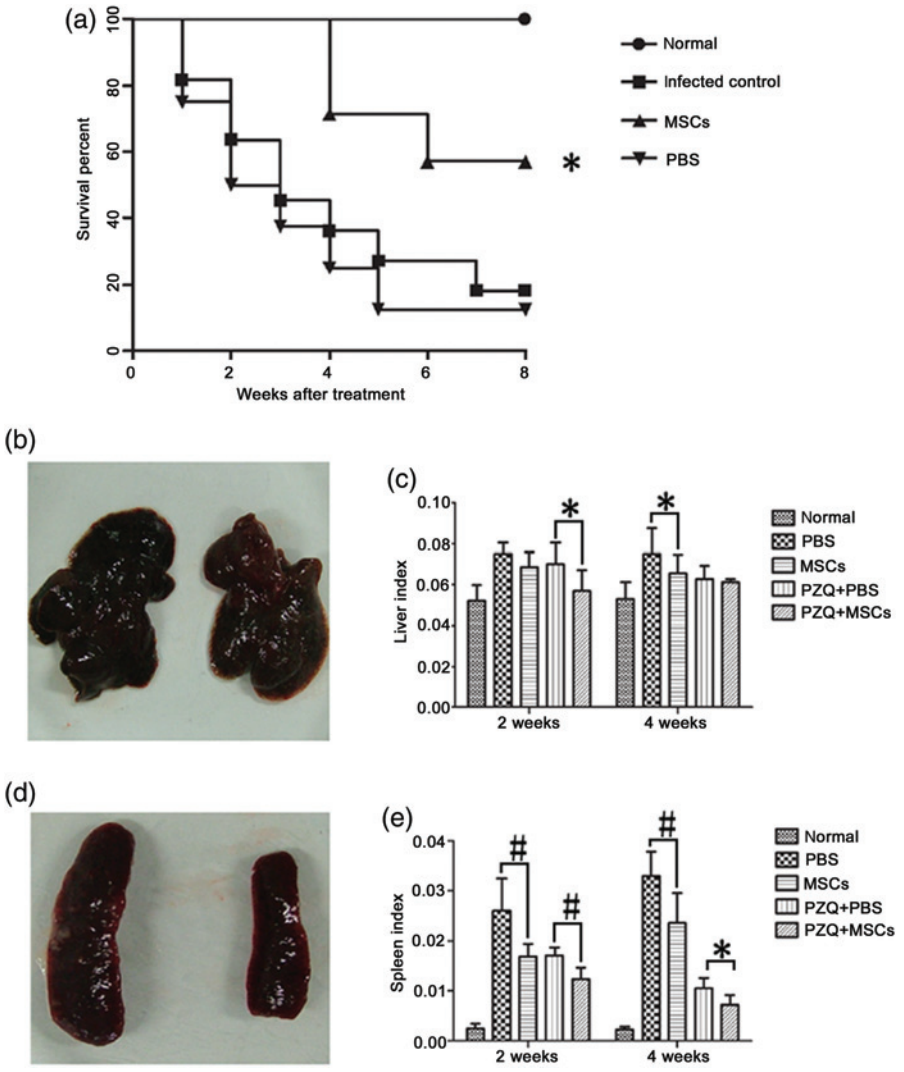

Figure 1 MSCs prolong the survival of *S. japonicum*-infected mice and decrease the liver and spleen indexes. (a) The survival curves of mice in each group ($n = 8$). $^{*}P < 0.05$, compared with the PBS group and infected control. (b) The appearance of infected liver from mice treated with PBS (left) or MSCs (right). (c) Liver index at two and four weeks after treatment ($n = 6$). $^{*}P < 0.05$. (d) The appearance of infected spleens of mice treated with PBS (left) or MSCs (right). (e) Spleen index at two and four weeks after treatment ($n = 6$). $^{*}P < 0.05$, $^{\#}P < 0.01$. MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PZQ, praziquantel. (A color version of this figure is available in the online journal)

MSCs inhibit collagen deposition in the livers of infected mice

Next, we assessed the deposition of collagen by Masson's trichrome staining and realtime PCR. In the data images, collagen in the liver tissue sections is presented as light green (Figure 4a). Compared with the PBS group, collagen deposition in the MSC groups was decreased. The collagen deposition in the PZQ + MSC group was also less than in the PZQ + PBS group. Realtime PCR results supported this qualitative observation by measuring the mRNA expression of collagen type 3 in liver tissues. Specifically, collagen 3 mRNA in the MSC group was significantly lower (1.82 ± 0.84) than in the PBS group (3.56 ± 1.07, $P < 0.05$); likewise, the level was significantly lower in the PZQ + MSC group (1.06 ± 0.34) than in the PZQ + PBS group (2.51 ± 0.84, $P < 0.01$) (Figure 4b). These results indicate that collagen deposition is inhibited in MSC-treated mice.

MSCs promote mesenchymal–epithelial transition

To explore the potential mechanism of MSCs on liver fibrosis, we detected the expression of mesenchymal–epithelial transition (MET)-associated proteins including vimentin, E-cadherin and α-SMA. Immunohistochemical staining results showed that there were less vimentin-positive cells in the MSC group than in the PBS group; also, these cells were decreased in the PZQ + MSC group as compared with the PZQ + PBS group (Figure 5a). In contrast to vimentin, there were more E-cadherin-positive cells detected in the MSC group than in the PBS group, and these cells were also increased in the PZQ + MSC group as compared with the PZQ + PBS group (Figure 5b). Western blotting results of α-SMA, vimentin and GAPDH are shown in Figure 5c. The gray density image analysis indicated that the ratio of α-SMA/GAPDH was 0.37 ± 0.09 in the MSC group, which was significantly lower than in the PBS group (0.56 ± 0.34, $P < 0.05$); additionally, the ratio was

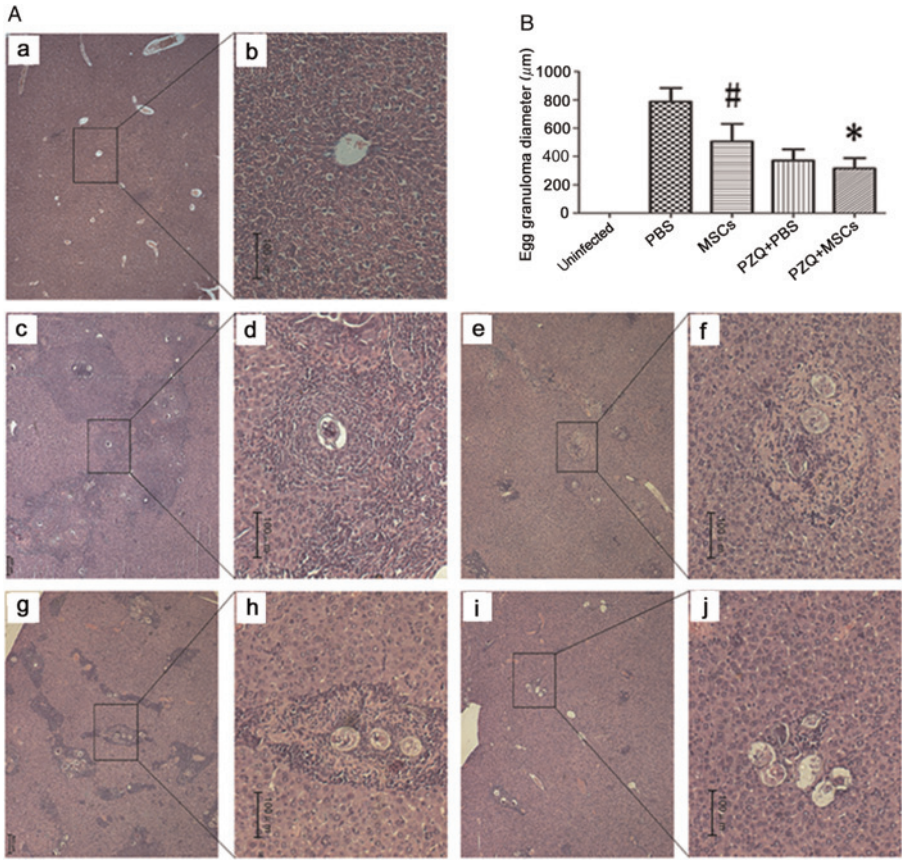

Figure 2 MSCs promote the relief of injured livers after four weeks of treatment. (A) MSCs promoted the alleviation of liver injury. Uninfected control (a, b); PBS (c, d); MSCs (e, f); PZQ + PBS (g, h); PZQ + MSCs (i, j). Scale bars: 0.1 mm. (B) Mean egg granuloma diameter in each group ($n = 20$). $^{\#}P < 0.01$ versus PBS group; $^{*}P < 0.05$ versus PZQ + PBS group. MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PZQ, praziquantel. (A color version of this figure is available in the online journal)

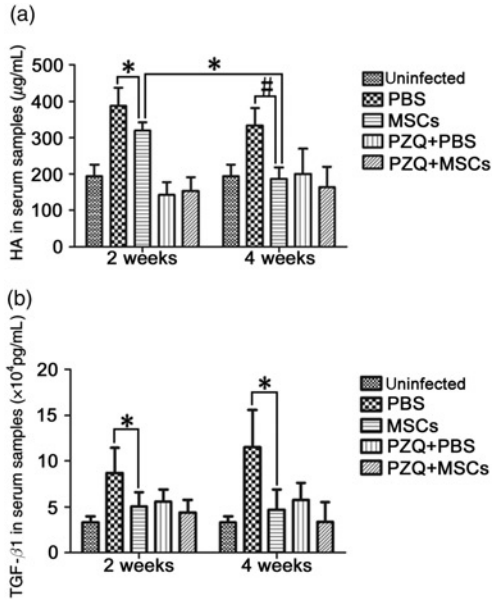

Figure 3 MSCs decrease the serum HA and TGF-β1 concentrations. (a). Serum HA concentration in each group at two and four weeks after treatment ($n = 6$). $^{*}P < 0.05$, $^{\#}P < 0.01$. (b). Serum TGF-β1 concentration in each group at two and four weeks after treatment ($n = 6$). $^{*}P < 0.05$. MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PZQ, praziquantel; TGF-β1, transforming growth factor-β1; HA, hyaluronic acid

significantly lower in the PZQ + MSC group (0.20 ± 0.02) than in the PZQ + PBS group (0.42 ± 0.07, $P < 0.05$) (Figure 5d). The ratio of vimentin/GAPDH was also lower in the MSC group (0.56 ± 0.08) than in the PBS group (0.89 ± 0.04, $P < 0.01$) (Figure 5e). Together, these data suggest that MET is induced after MSC treatment in the liver of *S. japonicum*-infected mice.

Discussion

In the present study, we have evaluated the efficacy of MSCs in ameliorating *S. japonicum*-induced liver injury, and demonstrated that MSC treatment not only alleviated liver fibrosis but also prolonged the survival time of infected mice. MSCs have emerged as promising candidates for cell therapy and have been successfully applied to treat various diseases. A previous study of *Schistosoma mansoni*-infected mice found bone marrow cells localized in the liver tissues.[21] Studies from others[22] have also demonstrated that injured tissues can secrete chemotactic factors to recruit MSCs and the numbers of MSCs homing to the liver have been shown to be independent of the route of MSC infusion. Another study revealed that repeated administration of MSCs can improve their protective effect.[15] All of these findings, together with evidence that MSCs from a certain organ can differentiate into other tissue-type

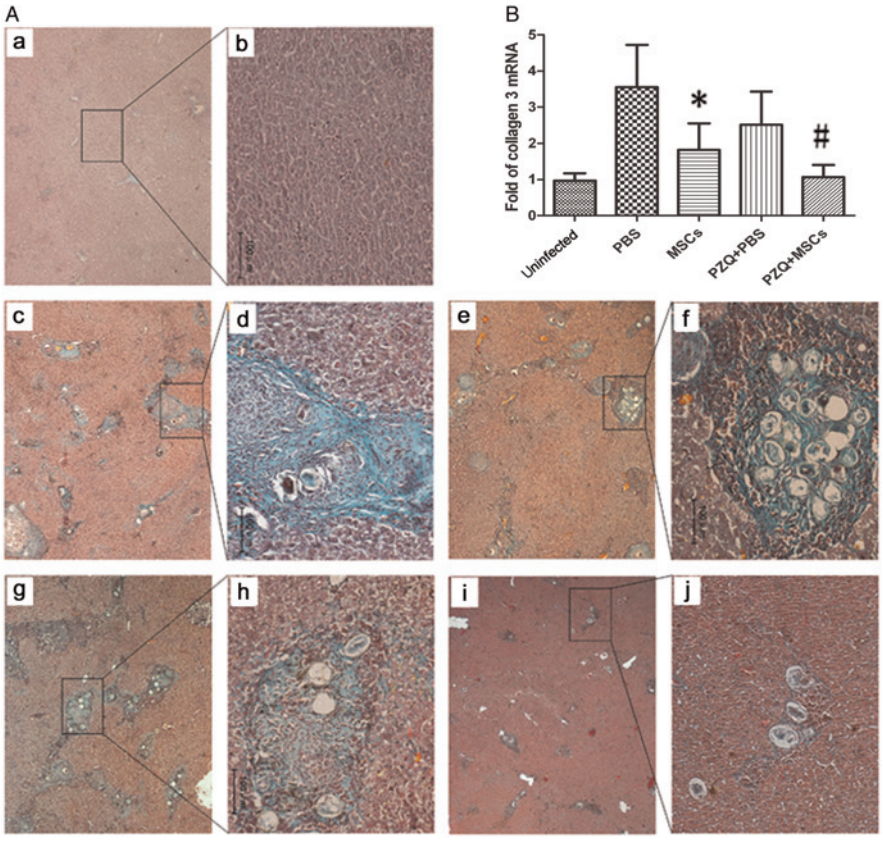

Figure 4 MSCs decrease collagen deposition in livers after four weeks of treatment. (A) Collagen deposition in the livers of mice. Uninfected control (a, b); PBS (c, d); MSCs (e, f); PZQ + PBS (g, h); PZQ + MSCs (i, j). Scale bars: 0.1 mm. (B) The expression of collagen type 3 mRNA in liver tissues ($n = 6$). $^{*}P < 0.05$ versus PBS group; $^{\#}P < 0.01$ versus PZQ + PBS group. MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PZQ, praziquantel. (A color version of this figure is available in the online journal)

cells, especially that pretreating with injured liver tissue can enhance the differentiation of MSCs into hepatocyte-like cells,[13] support the notion that MSCs may be beneficial for therapy to recover organ structure and/or function. We have previously demonstrated that transplantation of MSCs could efficiently promote repair of injured liver and kidney by transdifferentiation and paracrine mechanisms.[3,5] In this study, we investigated the effects of MSCs on periportal liver fibrosis by repeated and direct exposure to *S. japonicum*.[23] Intravenous repeat injections of MSCs into the *S. japonicum*-infected mice markedly ameliorated liver injury, as indicated by the recovery of liver and spleen indexes and decreased collagen deposition, indicating that MSC treatment may be an effective clinical approach to treat *S. japonicum*-induced liver injury.

PZQ, an effective anthelminthic drug, is routinely used throughout the world to treat schistosomiasis in human patients and in experimental animal models. In our study, PZQ was administered to *S. japonicum*-infected mice as a single-agent therapy *via* the intragastric route or as a dual therapy in combination with MSCs in order to determine if MSCs could enhance the beneficial effects of PZQ therapy. Indeed, combining PZQ with MSCs greatly improved the efficacy of PZQ for repairing *S. japonicum*-induced hepatic fibrosis, suggesting that MSCs can be utilized as an effective adjuvant for treating liver fibrosis caused by *Schistosomiasis*.

In order to investigate the mechanisms used by MSCs to inhibit *S. japonicum*-induced liver fibrosis, we determined the MET-associated markers, namely vimentin, E-cadherin and α-SMA. It has been previously observed that schistosomiasis is accompanied by egg-derived antigen (soluble egg antigen, SEA) which stimulates host immunomodulatory factors, including inflammatory cells (such as macrophages, lymphocytes and eosinocytes) and cytokines (including TGF-β1, interferon-γ, tumor necrosis factor-α, ILs).[24] Some previous reports have shown that the critical basis of liver fibrosis is the accumulation of myofibroblasts, which may result from the activation and proliferation of HSCs or from the resident epithelial cells, including hepatocytes and bile duct cells, via epithelial–mesenchymal transition (EMT).[25–27] Meanwhile, the fibroblasts and myofibroblasts themselves can secrete TGF-β1 to promote EMT through the TGF-β1/Smad signaling pathway, subsequently leading to fibrosis.[28] Thus, inhibition of TGF-β1 or the TGF-β1/Smad signaling pathway has antifibrotic effects.[29,30]

MSCs have been reported to inhibit inflammatory cell infiltration and cytokine secretion.[31,32] Cho *et al.*[33] reported that active HSCs can switch their phenotype to myofibroblast-like cells and, in that manner, contribute to liver fibrosis. It has been shown that E-cadherin can antagonize TGF-β1-induced HSC activation. Therefore, the alleviation of liver fibrosis in *S. japonicum*-infected

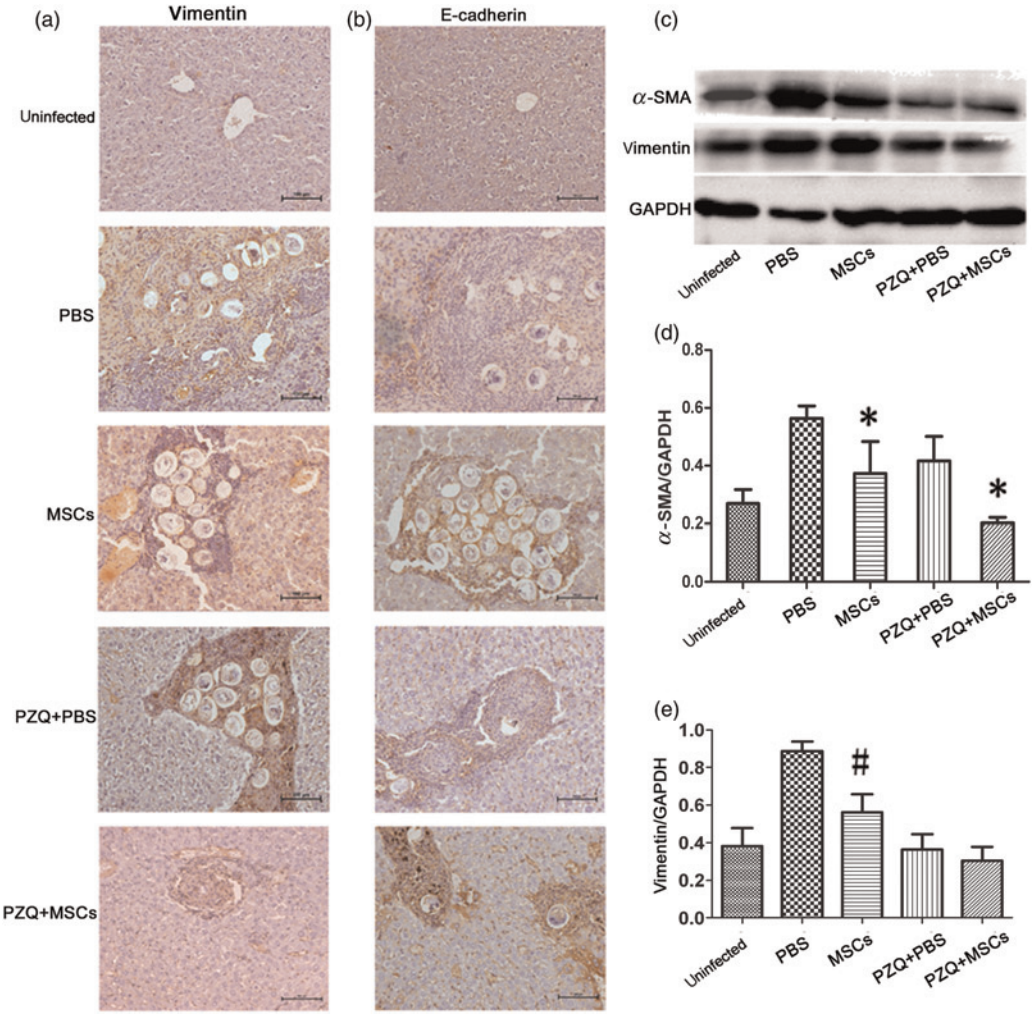

Figure 5 Expression of vimentin, E-cadherin and α-SMA in the liver tissues of mice after four weeks of treatment. (a). Immunohistochemical staining of vimentin. (b). Immunohistochemical staining of E-cadherin. (c). Western blotting results of α-SMA, vimentin and GAPDH. (d). Gray density image analysis of α-SMA ($n = 6$). $^{*}P < 0.05$, MSC group versus PBS group; PZQ + MSC group versus PZQ + PBS group. (e) Gray density image analysis of vimentin ($n = 6$). $^{\#}P < 0.01$ versus PBS group. MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PZQ, praziquantel; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (A color version of this figure is available in the online journal)

mice could be, at least partially, explained by the inactivation of HSCs by MSCs, which would be consistent with previously reported findings that indicated MSCs can inhibit the activation of HSCs and induce apoptosis to relieve fibrosis.[34] α-SMA, the major marker of HSC activation and proliferation, has also been examined in our study. We observed a reduction of α-SMA in mice treated with either MSCs alone or PZQ alone; combined treatment of PZQ with MSCs caused a significantly higher degree of inhibition of α-SMA expression, indicating that MSCs can inhibit liver fibrosis by suppressing HSC activation and reversing the EMT, at least partially.

Collectively, our data from this study has demonstrated that administering MSCs to *S. japonicum*-induced liver fibrotic mice can promote the recovery of injured tissues, reduce egg granuloma diameter and inhibit collagen deposition, as well as prolong the survival time. Our findings suggest that MSC treatment represents a novel and useful therapeutic approach for *S. japonicum*-induced liver injury and fibrosis.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. HX, XZ, FM, MW and HX conducted the experiments; HQ and WZ supplied critical reagents; HX and YY wrote the manuscript; and WX designed and supervised the experiments and revised the manuscript.

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