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Healing Effect of Conditioned Media from Bone Marrow-Derived Stem Cells in Thioacetamide-induced Liver Fibrosis of Rat

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Following repeated injury, the liver undergoes tissue remodeling and fibrosis. This study investigated the healing effect of conditioned media (secreta) from rat bone marrow-derived stem cells (BMSCs) in thioacetamide (TA)-induced liver fibrosis rat model. During 2014, thirty male Wistar rats were randomly allocated into two groups of sham control and treatment. The rats of sham control group were subdivided into three subgroups and were sampled on weeks 14, 18 and 20 after fibrosis induction. The rats of treatment group were subdivided into two subgroups and were sampled on weeks 4 and 6 after treatment by conditioned media. Fibrosis was induced by intraperitoneal administration of TA. Animals were valuated histologically and for liver function tests after TA-induced fibrosis and injection of conditioned media. Conditioned media were provided from the supernatant of BMSCs culture flasks. Administration of TA resulted into infiltration of inflammatory cells and deposition of collagen fibers in hepatic tissue. A significant healing was noticed in liver four and six weeks after post-injection of BMSCs conditioned media. Regarding fibrosis and necrosis, a significant reduction appeared 4 and 6 weeks after BMSCs conditioned media therapy. Aspartate aminotransferase (AST) and alanine transaminase (ALT) levels decreased 4 and 6 weeks after BMSCs conditioned media therapy. Regarding alkaline phosphatase (ALP) and albumin levels 4 and 6 weeks after BMSCs conditioned media therapy, a non-significant increase was seen. This study indicated that the BMSCs conditioned media had a positive healing effect in rearrangement of hepatic fibrosis. So it can be an alternative therapeutic choice to ameliorate liver fibrosis.

Key words: Bone marrow, mesenchymal stem cell, conditioned media, liver fibrosis, rat

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INTRODUCTION

Viral hepatitis, alcohol and drug abuse, metabolic and autoimmune diseases and congenital abnormalities were shown to induce injuries in the liver (Mostaghni *et al*., 2011). In the case of hepatic injury, the liver regeneration would fail due to substitution of abundant extracellular matrix, including fibrillar collagen in the hepatocytes. Liver fibrosis happens due to excessive accumulation of extracellular matrix proteins including collagen that occurs in most types of chronic liver diseases. The liver fibrosis is usually insidious and most of the related morbidity and mortality occur after the development of cirrhosis (Davarpanah *et al*., 2009). The traditional view is that cirrhosis is an irreversible disease, but recent evidences indicate that fibrosis is considered as a reversible phenomena (Arthur, 2002). Several strategies have been used to prevent further damages to the liver, to treat complications of fibrosis and to prevent or detect liver cirrhosis and/or cancer in early stages (Farjam *et al*., 2014). Thus, transplantation of the liver is becoming an important option for treating patients with advanced fibrosis (Dehghani *et al*., 2007).

Stem cells were demonstrated to have certain characteristics including self-renewal, pluripotency, proliferation, longevity and differentiation, to be a valuable source for transplantation (Ghobadi *et al*., 2015). The differentiation property into hepatocyte-like cells of adult hematopoietic and non-hematopoietic stem cells have previously been reported (Wang *et al*., 2005). It has been shown that injection of Mesenchymal Stem Cells (MSCs) could improve carbon tetrachloride (CCl_4)-induced liver fibrosis in mice (Fang *et al*., 2004). This may be due to the MSCs effects in minimizing collagen deposition in addition to their capacity to differentiate into hepatocytes (Abdel Aziz *et al*., 2007). Thioacetamide (TA) is an organosulfur compound (C_2H_5NS) with a potent centrilobular hepatotoxicant, undergoing a two-step bioactivation mediated by microsomal CYP2E1 to TA sulfoxide (TASO) and further to TA-S, S-dioxide ($TASO_2$), a reactive metabolite leading to cellular necrosis by covalently binding to liver macromolecules (Chilakapati *et al*., 2005).

There are not many reports on therapeutic effects of conditioned media (secreted bioactive molecules) of MSCs Therefore, the purpose of the present study was to determine whether, intra-peritoneal injection of conditioned media (secreta) of bone marrow-derived stem cells (BMSCs) might prevent or reverse liver fibrosis in a rat model of TA-induced fibrosis.

MATERIALS AND METHODS

Animals: During, 2014, thirty male Wistar rats, weighing 225-250 g were obtained from Laboratory Animal Center of Shiraz University of Medical Sciences (Shiraz, Iran) and were randomly allocated into two groups of sham control and treatment. Sham control group (n = 18) which was induced liver fibrosis without treatment. The rats of sham control group were subdivided into three subgroups and were sampled on weeks 14, 18 and 20 after induction. The rats of treatment group (n = 12) were induced liver fibrosis and was treated by conditioned media (secreta) of BMSCS. The rats of treatment group were subdivided into two subgroups and were sampled on weeks 4 and 6 after treatment by conditioned media. The rats were kept in an air conditioned facility and were subjected to a 12:12 h day light/darkness (light at 7:00 PM) and allowed unlimited access to chow and water at an ambient temperature of 22±2°C and 50% relative humidity.

This study was performed according to the rules of working with laboratory animals of the Ethical Committee of Shiraz University of Medical Sciences (Shiraz, Iran). All blood and tissue sampling were performed under anesthesia and all efforts were made to minimize suffering during the experimental period. Euthanasia was undertaken according to ethical issues of working with laboratory animals of Ethical Committee of Shiraz University of Medical Sciences.

Thioacetamide-induced liver fibrosis: Fibrosis was induced in rats as described previously by intraperitoneal administration of 200 mg kg^{-1} of thioacetamide twice weekly for 14 weeks (Farjam *et al*., 2014). Under ketamin and xylazine anesthesia, the blood samples were taken and tested for liver function tests. Liver tissue samples were provided after sacrificing the animals and the tissue samples were transferred into formalin buffer for histological evaluation.

BMSCs preparation: The BMSCs were prepared from rat bone marrow as described previously (Ai *et al*., 2012). Briefly, femur and tibia were separated from the body and placed in 15 mL falcon tubes containing PBS on ice. After flushing of bone marrow by Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) into a 15 mL falcon, the flushed BM was homogenized with a pipette, then plated in a culture flask containing DMEM, 10% fetal bovine serum (FBS, Bio-sera, UK), 1% penicillin and streptomycin (100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin; Sigma, USA) and 1% L-glutamine (Sigma, USA) and were placed in a CO_2 incubator at 37°C with 5% CO_2 and saturated humidity. The medium was changed every 3 days and cells were subcultured with trypsin/EDTA (Bio-Sera, UK) at 70% confluence. The cells were enumerated at the third passage, stained by trypan blue (Sigma, USA) using a hemocytometer under a light microscope. The BMSCs were morphologically evaluated by inverted microscope (Olympus, USA).

BMSCs osteogenic differentiation: For *in vitro* osteogenic differentiation, cells at 90% confluence were cultivated in DMEM, 15% FBS, 200 µM L-ascorbic acid, 10 mM

glycerolphosphate and 100 nM dexamethasone. The medium was changed twice a week for 3 weeks. After 21 days, osteogenic differentiation was evaluated using alzarin red staining. In brief, BMSCs cultures were fixed with 4% paraformaldehyde for 10 min. Then cells were incubated for 20 min at room temperature in 1% Alizarin red S and 1% ammonium hydroxide. Following incubation, cultures were washed 4 times, 5 min each time with 1 mL dH_2O replacing the water at each 5 min interval and air-dried. Alizarin red S dye binds to calcium ions present in mineralized deposits resulting in a brilliant red staining (all reagents from Sigma-Aldrich, USA).

Characterization by reverse transcription polymerase chain reaction (RT-PCR): The RT-PCR was used to determine the expression of mesenchymal stem cell markers. Briefly, the total RNA was extracted using the column RNA isolation kit (Denazist-Asia, Iran) upon manufacturer's guideline. Then, the total RNA concentration was evaluated by spectrophotometry. From RNA samples, the complementary DNA (cDNA) was provided using AccuPower Cycle Script RT PreMix Kit (Bioneer, Korea) as manufacturer's guideliene. Briefly, for each reaction, 15 µL of total RNA was used to reach a volume of 20 µL with the DEPC water. Twelve thermal cycles was done as follows: 30 sec at 20°C for primer annealing, 4 min at 42°C for cDNA synthesis, 30 sec at 55°C for melting secondary structure and cDNA synthesis and 5 min at 95°C for inactivation.

Then, 1 µL of template (cDNA) was mixed with PCR buffer, H_2O, dNTPs, $MgCl_2$, Taq DNA polymerase and forward and reverse primers (CD45: 450 bp, CD73: 208 bp and marker: 100 bp). The microtubules containing 20 µL of the above mentioned mixture were transferred into thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) and 30 amplification cycles were performed including 30 sec denaturation at 95°C, 30 sec annealing at 64°C and 30 sec extension at 72°C with the 2 min at 95°C for primary denaturation and 5 min at 72°C for final extension. The PCR products were evaluated for presence of defined bands by gel electrophoresis by DNA safe stain in 1.5% agarose gel medium. The bands were visualized using UV radiation by a gel documentation system (UVtec, Cambridge, UK).

Treatment with BMSCs conditioned media: The supernatant of the BMSCs culture flasks lacking presence of any cells was used as conditioned media of BMSCs (1 mL). The conditioned media was intraperitoneally injected after induction of liver fibrosis with thioacetamide and the animals were sacrificed 4 and 6 weeks after injection of BMSCs conditioned media and the tissue samples were transferred into formalin buffer for histological evaluation.

Histological and biochemical evaluations: Liver and blood sampling were done 4 and 6 weeks after BMSCs conditioned media injection. Tissue processing and section preparation were done according to previous studies (Haghighi *et al*., 2013). Briefly, formalin-fixed liver were processed routinely and embedded in paraffin. Blocks were cut at 6 µm and sections were stained with hematoxylin-eosin (H and E) and Masson's trichrome. To assay the function of the liver, serum activities of aspartate aminotransferase (AST), alanine transaminase (ALT) using Randox (UK) kits and alkaline phosphatase (ALP) using Parsazma (Iran) kits were evaluated. Serum albumin was assessed using BioRex (UK).

Statistical analysis: The Kolmogorov-Smirnov test was used to evaluate the normal distribution of serum analysis data. The differences between mean values for control, sham and experimental groups were compared using one way analysis of variances (ANOVA) using SPSS statistical package (version 11.5, Chicago, IL, USA) for Windows. The LSD was used to find significant differences that were considered when $p>0.05$. Moreover, comparison of histological findings between control, sham and experimental groups were compared using Mann-Whitney test with Bonferroni correction to find significant differences at $p<0.01$.

RESULTS

The morphology of BMSCs in different passages under light microscopy was shown in Fig. 1, revealing the spindle shape of cells identical to mesenchymal stem cells. Moreover, after culture of BMSCs in differentiation medium, the cells differentiated toward osteoblasts as verified by positive staining with Alzarin red staining (Fig. 2). The RT-PCR analyses of the BMSCs revealed that these cells were uniformly positive for CD 73 and negative for CD 45 (Fig. 3).

Histological findings of liver after induction of fibrosis by TA and any probable healing changes in liver paranchyma after injection of conditioned media were shown in Fig. 4. Administration of TA resulted into infiltration of inflammatory cells and deposition of collagen fibers in hepatic tissue together with a portal fibrotic bridge. Interstitial and portal hepatitis and liver fibrosis were confirmed histologically. A significant healing effect in hepatic tissue was noticed 4 and 6 weeks after injection of conditioned media verified by both staining methods of H and E and Masson's trichrome with identical findings.

Regarding scoring of fibrosis after administration of TA and conditioned media, it was shown that fibrosis reduced significantly 4 and 6 weeks after conditioned media therapy of BMSCs ($p<0.01$). This difference was also significant when comparing administration of TA without any treatment measure after 4 and 6 weeks with conditioned media treatment group after identical time intervals ($p<0.01$, Fig. 5a).

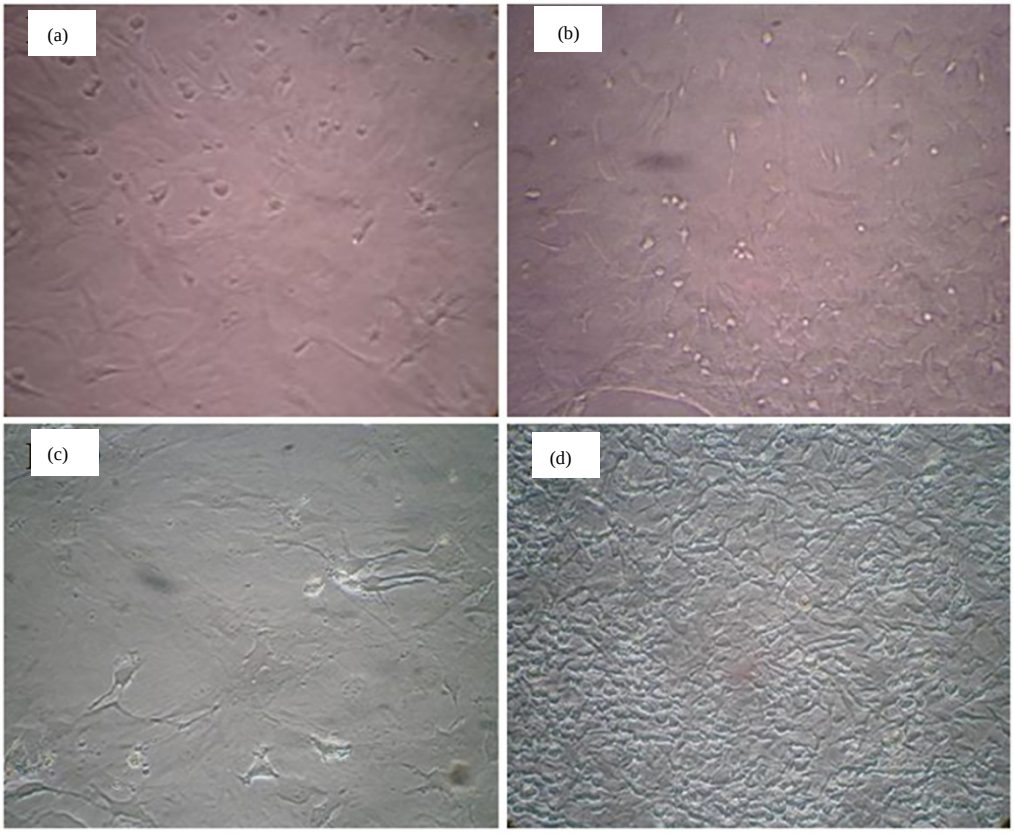

Fig. 1(a-d): Spindle shape morphological characteristics of bone marrow-derived stem cells before using of their conditioned media (secreta) as therapeutic agent on (a): Days 4 (primary culture), (b): Day 7 (the first passage), (c): Day10 and (d): Day 11 (the second passage)

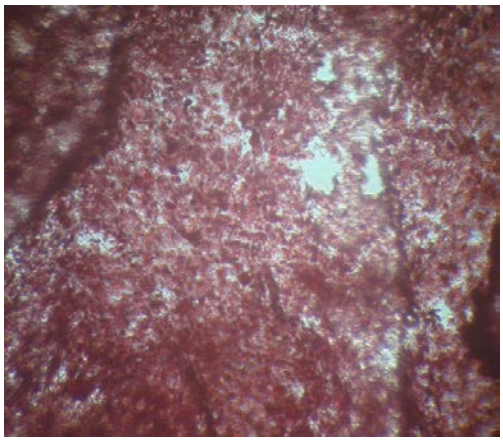

Fig. 2: Cells cultivated in osteogenic medium and were stained with Alizarin red at 21 days after induction (×150)

Regarding scoring of necrosis after administration of TA and conditioned media, it was shown that fibrosis had a significant decreasing trend 4 and 6 weeks after conditioned media therapy of BMSCs ($p<0.05$). This difference was also significant, when comparing the 4 and 6 weeks conditioned media treatment groups ($p<0.05$). The difference was also significant when comparing administration of TA without any treatment measure on day zero and after 4 and 6 weeks with conditioned media treatment group after similar time periods ($p<0.05$, Fig. 5b).

Considering liver function tests, for serum albumin level, an increase was seen in all groups, but the difference was not statistically significant between groups (Fig. 6a). There was a decrease in serum AST level, 4 and 6 weeks after conditioned media therapy of BMSCs when compared with TA-induced fibrosis group without any treatment measure on week 0 and after 4 and 6 weeks and the difference was not statistically significant (Fig. 6b). In relation to serum ALT, 4 and 6 weeks after conditioned media therapy of BMSCs in comparison to TA-induced fibrosis group without any treatment measure on day zero, the decline was statistically significant ($p<0.05$, Fig. 6c). Regarding serum ALP level, an increase was noticed in all groups, but the difference was not statistically significant except between 4 and 6 weeks subgroups of conditioned media group ($p<0.05$, Fig. 6d).

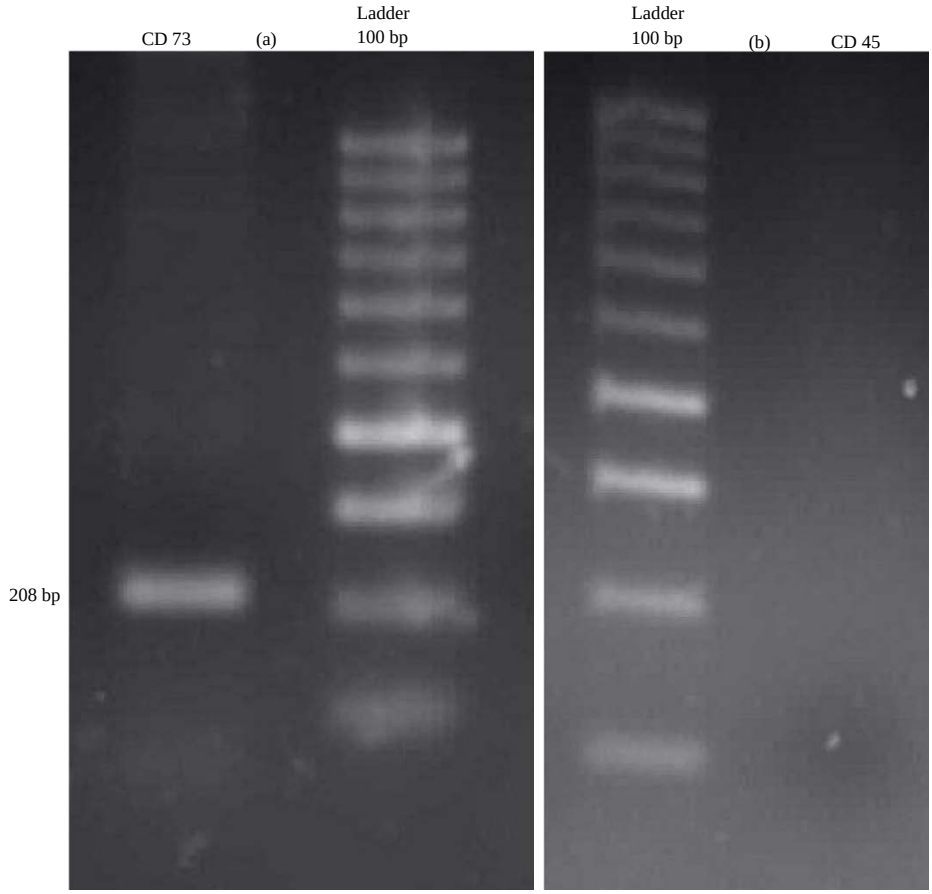

Fig. 3(a-b): Reverse transcription polymerase chain reaction (RT-PCR) analyses of bone marrow-derived stem cells, cells were (a) (a): Uniformly positive for CD 73 and (b): Negative for CD 45

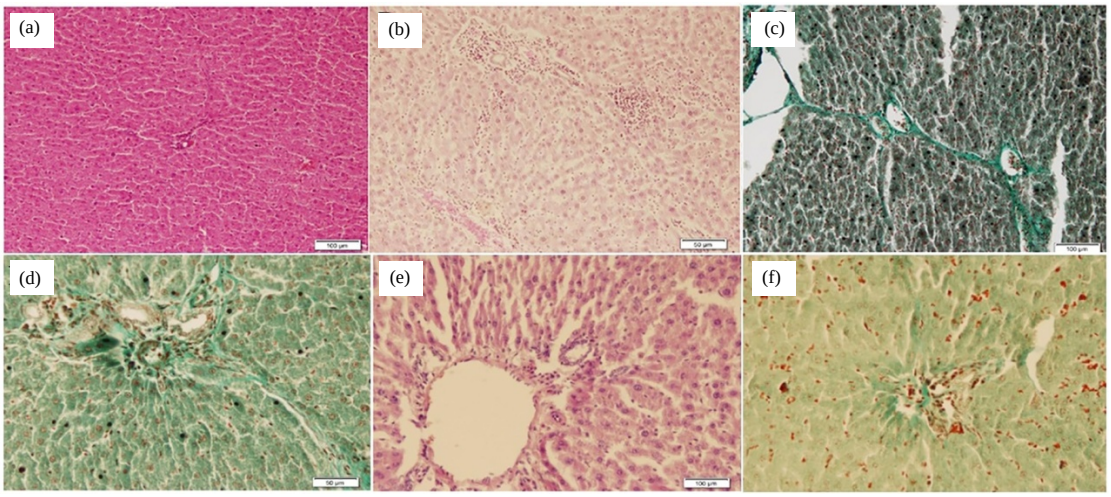

Fig. 4(a-f): Histological changes after administration of thioacetamide and conditioned media (secreta) of bone marrow-derived mesenchymal stem cells (BMSCs) in hepatic tissue, (a): Normal liver, (b): Interstitial hepatitis due to thioacetamide intoxication, (c): Hepatic fibrosis due to thioacetamide intoxication, (d): Hepatic repair after four weeks of therapy with BMSCs, (e): Hepatic repair after six weeks treatment with conditioned media of BMSCs a, b and e hematoxylin-eosin (H and E) staining, c, d and (f): Masson's trichrome staining

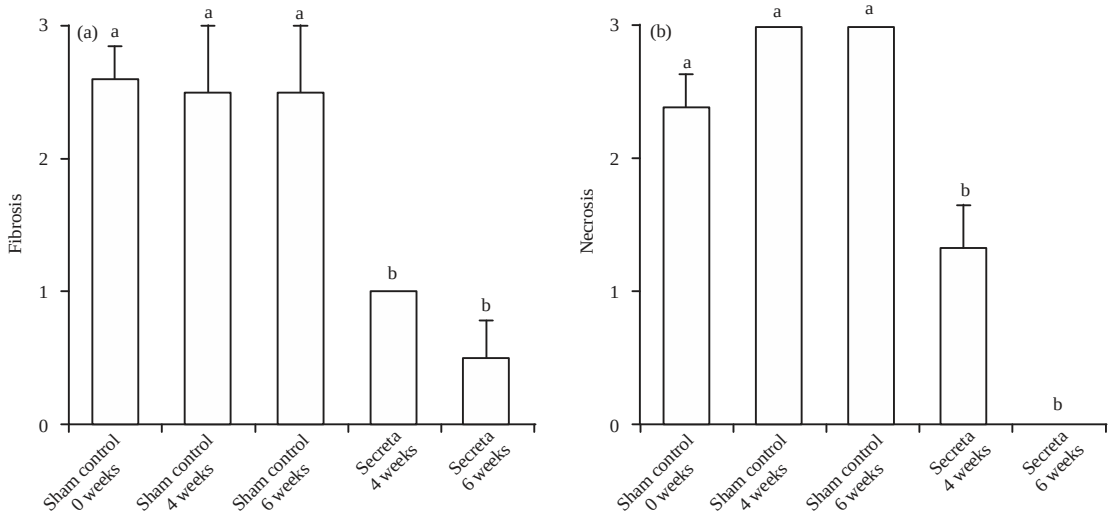

Fig. 5(a-b): Comparison of histological scoring of hepatic tissue between thioacetamide liver fibrosis (sham control) and liver fibrosis induced, which treated with conditioned media (secreta) of bone marrow-derived stem cells (BMSCs), 0, 4 and 6 weeks after induction. Different superscript letters show significant differences between groups ($p<0.01$)

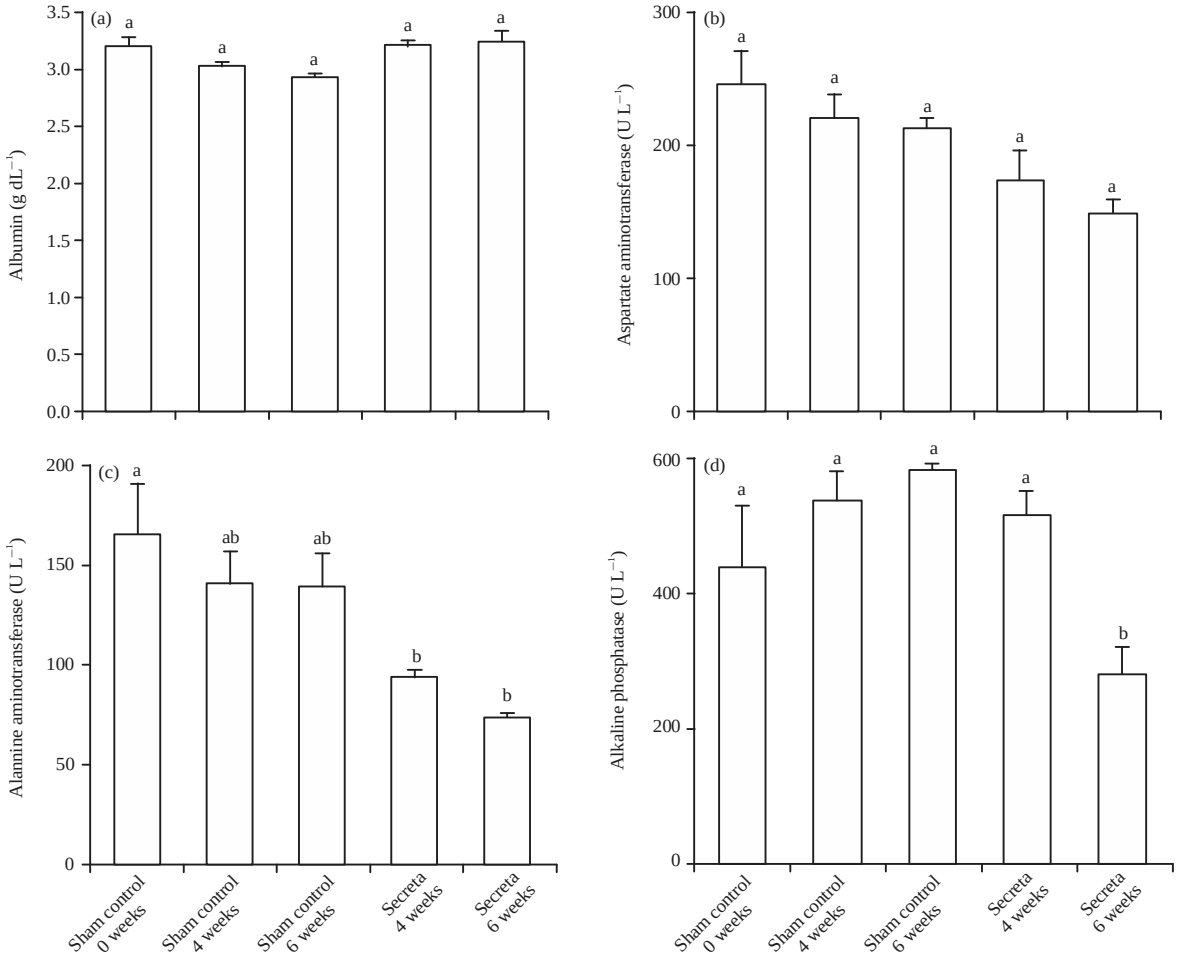

Fig. 6(a-d): Comparison of liver function tests between thioacetamide liver fibrosis (sham control) and liver fibrosis induced which treated with conditioned media (secreta) of bone marrow-derived stem cells (BMSCs), 0, 4 and 6 weeks after induction. Different superscript letters show significant differences between groups ($p<0.05$)

DISCUSSION

Cirrhosis is the end stage of the progressive hepatic fibrosis which is characterized by distortion of the liver architecture and presence of regenerative nodules. Liver transplantation is considered as one of the few available treatment choices for such patients. However, because of severe shortage of organ donors, surgical complications, transplant rejection and the high cost of liver transplantation, there is much interest to reach new treatment modalities for the disease (Nikeghbalian *et al*., 2011).

Many types of cells such as bone marrow and adipose-derived hematopoietic or mesenchymal stem cells, umbilical cord blood cells, adult liver progenitor cells, mature hepatocytes, fetal liver progenitor cells and human embryonic stem cells in both rodents and humans were shown to be able of self-replication, giving rise to daughter hepatocytes and to repopulate liver and to improve liver function. Human embryonic stem cells seem to play an important role in treatment of liver cirrhosis, but there is no clinical application for them in human due to their ethical concerns or difficulties in harvesting or safely and efficiently expanding sufficient quantities. In contrast, mesenchymal stem cells, that can be easily and safely isolated and cultured were used in many clinical trials for treatment of chronic diseases such as liver diseases. Human MSCs can be differentiated into partially functional hepatocyte-like cells and were previously shown to be a potential source for cell therapy in liver disorders. Cell transplantation offers prominent promise for future treatment of cirrhosis and metabolic liver diseases, but still significant technical problems remain that can be overcome through years of intensive research (Malekzadeh *et al*., 2010; Pournasr *et al*., 2011).

Recent studies demonstrated that bone marrow stem cell transplantation can lead to regression of liver fibrosis and seem to be feasible and safe in treatment of decompensated liver cirrhosis too (Mohamadnejad *et al*., 2007). In liver fibrosis, there is excessive accumulation of extracellular matrix, which affects liver function over time and leads to its failure. In a mouse model of liver fibrosis, the effectiveness of human adipose tissue-derived multi-lineage progenitor cells in improving liver fibrosis was shown (Okura *et al*., 2014). Considering BMSCs, it was shown that injection of these cells resulted into improvement in the histological picture of the liver and its enzymatic profile (Ahmed *et al*., 2014).

In the current study, the anti-fibrotic effects of infusion of BMSCs conditioned media in thioacetamide-induced hepatic fibrotic rats were evaluated showing that the conditioned media could enhance healing in liver and improve the liver function tests similar to above mentioned studies. Ayatollahi *et al*. (2014) reported that antioxidant response of BMSCs may be responsible for treatment of their liver fibrosis animal model approach even they used CCl_4 for induction of liver fibrosis. Jang *et al*. (2014) has also studied on the effect of bone marrow-derived mesenchymal stem cells on hepatic fibrosis in a thioacetamide-induced cirrhotic rat model. They showed that BMSCS treatment could attenuate hepatic fibrosis in rats with TAA-induced cirrhosis, while their animal modeling and stem cell source are identical to this study. The increasing trend of liver enzymes after administration of TA could not reach the normal level for ALP and albumin during the 6 weeks period showing the effect of TA still present in liver damaged tissue. Qi *et al*. (2015) reported that stem cell therapy could improve the liver function without any severe procedure-related complications similar to these findings.

In fibrosis, there is a massive loss of hepatocytes and/or an inhibition in the proliferation potential of the mature hepatocytes. In liver damage, an activation of a dormant cell population of resident hepatic progenitor cells happens, while based on the type of liver injury, hepatic progenitor cells produce new hepatocytes and biliary cells to repopulate the liver. The MSCs can be responsible for activation of hepatic progenitor cells and improve damages to the liver (Katoonizadeh *et al*., 2014).

It was previously demonstrated that progenitor cells could synthesize a broad spectrum of growth factors and cytokines affecting intracellular signaling and improve function of the tissue (Hosseinkhani *et al*., 2014). Indeed, as MSCs enter and progress toward an end-stage phenotype, the quantity and array of secreted bioactive factors change as the descendants of MSCs enter new lineage stages (Mohamadnejad *et al*., 2007).

These secreted factors can feed back on the cells themselves and others and govern both their functional status and physiology. Thus, the role of stem cells in differentiation to target tissue and secretion of bioactive molecules to control of other cells differentiations are very important. The trophic effects of MSCs have been documented before showing that MSCs simulated an increased production of Vascular Endothelial Growth Factor (VEGF), resulting into an increase in vascular density and blood flow and a decrease in apoptosis (Tang *et al*., 2004).

There are few reports on the healing effect of conditioned media in tissue injuries. In acute myocardial infarction, the protective and healing effect of the medium of BMSCs have been studied supporting the MSCs' role in turnover dynamics of damaged tissue confirming our results too (Gnecchi *et al*., 2005). In this study, the role of BMSCs conditioned media in improving of liver fibrosis induced by TA was evaluated. Results are in agreement with other previously reports in other tissue using MSCs in treatment of tissue damages such as heart (Gnecchi *et al*., 2005; Tang *et al*., 2004) lung (Ortiz *et al*., 2007) and kidney (Togel *et al*., 2007).

The MSCs can secrete a number of bioactive factors that can provide a microenvironment helping the rearrangement of damaged tissues (Togel *et al*., 2007). The MSCs can produce factors that inhibit scarring (fibrosis) and apoptosis, promote angiogenesis and stimulate host progenitors to

divide and differentiate into functional regenerative units (Mehrabani *et al.*, 2013). In this light, the trophic effects of MSCs may have profound clinical use (Hosseinkhani *et al*., 2014).

CONCLUSION

It can be concluded that the conditioned media had a positive anti-inflammatory and healing effect in rearrangement of hepatic fibrosis. So conditioned media of BMSCs can be an alternative therapeutic choice to ameliorate liver fibrosis, but the mechanisms underlying the effects of BMSCs conditioned media also need more research attention and further studies are necessary to find target molecules which display reliable therapeutic effects.

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