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Regenerative Impact of Adipose Tissue-Originated Stem Cells on Healing of Liver Injuries: Biochemical Assay and Histological Examination

Davood Mehrabani [1,2,3,4], *Samin Arshi* [5*], *Ladan Sadeghi* [5], *Zahra Khodabandeh* [1], *Shahrokh Zare* [1], *Aghdass Rasouli-Nia* [4], *Feridoun Karimi-Busheri* [4]

[1]Stem Cell Technology Research Center, Shiraz University of Medical Science, Shiraz, Iran

[2]Burn and Wound Healing Research Center, Shiraz University of Medical Science, Shiraz, Iran

[3]Comparative and Experimental Medicine Center, Shiraz University of Medical Science, Shiraz, Iran

[4]Department of Oncology, Cross Cancer Institute, Faculty of Medicine, University of Alberta, Edmonton, AB, Canada

[5]Department of Biology, Islamic Azad University, Arsanjan Branch, Arsanjan, Iran

ABSTRACT

Background: The phrase "liver disease" refers to any hepatic ailment that causes tissue damage or alters liver function. As there is reduced organ availability, a search for alternative solutions is necessary. Regenerative medicine by employing stem cell transplantation has opened a new door in hepatic injuries. This study was conducted to determine the healing effect of Adipose Tissue-Derived Stem Cells (ADSCs) in Carbon tetrachloride (CCl_4)-induced liver fibrosis of rats.
Methods: A total of 32 rats were divided into 4 equal groups using randomization. Only 1 mL/kg of distilled water was administered twice weekly for 8 weeks to control group 1. Intraperitoneal (IP) of 1 mL/kg of olive oil was given to control group 2. The Sham group was administered as IP with CCl_4 (1 mL/kg) dissolved in 1 mL of olive oil to create liver injury. The experimental group following liver injury received 2×10^6 ADSCs in the tail vein. A blood sample was provided to asses biochemically and histological evaluation was undertaken 3 and 8 weeks following cell transplantation. ADSCs were characterized before injection to have mesenchymal properties.
Results: ADSCs were confirmed to have mesenchymal properties. Regarding Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), and Alkaline Phosphatase (ALP) in the experimental group, a significant decrease and for albumin, a significant increase in the enzyme was noticed after cell transplantation. Histological assessment revealed the healing effect of ADSCs in hepatic injuries.
Conclusion: Our study revealed that ADSCs could improve liver function and promote liver regeneration. These findings can open a new door to facilitate the treatment of hepatic injuries when AdSC transplantation is targeted.

Keywords: Mesenchymal Stem Cells • Adipose Tissue • Liver Injury • Healing • Rat

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*Correspondence:
Samin Arshi
samin.arshi@gmail.com

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INTRODUCTION

The term "liver disease" refers to any hepatic illness that causes tissue damage or changes in hepatic function. These conditions can be brought on by autoimmune, viral infections, inherited genetic alterations, fat buildup, cancer, or heavy alcohol or drug use. The final

stage of chronic liver disease, liver cirrhosis is characterized by fibrosis, necrosis, and regenerating nodules that alter the liver's normal structure by reducing its functional mass and changing its vascular composition [1]. There is no proven treatment for liver cirrhosis; however, symptoms can be managed with medications like ursodeoxycholic acid and obeticholic acid for primary biliary cholangitis, steroids and immunosuppressive drugs for autoimmune hepatitis, copper chelation for Wilson disease, antiviral drugs for viral hepatitis, and iron chelation and phlebotomy for hemochromatosis [2]. Although the liver has regenerative capabilities, but when chronic damage or extensive fibrosis occurs, tissue regeneration is not possible, and liver transplantation is needed. As there is reduced organ availability, a search for alternative bioengineered solutions to treat or increase life expectancy is necessary. Regenerative medicine by employing transplantation of stem cells has opened a new door as a therapeutic alternative strategy to cure hepatic injuries [3].

Mesenchymal Stem Cells (MSCs) have been extensively utilized in stem cell-based regenerative therapy based on their unique ability of self-renewal and differentiation into several various cell types [4]. Their beneficial properties come from their impact on tissue repair via cellular differentiation and secretion of different trophic factors in the damaged tissue. Kandel et al. demonstrated that Bone Marrow-Derived Stem Cells (BMSCs) could be differentiated into osteoblast cell types [5]. Also, they possess immunomodulation and anti-inflammatory properties targeting them being used in regenerative medicine, especially in hepatic injuries [6,7]. MSCs have been isolated from a variety of tissues, including bone marrow, adipose tissue, endometrial, menstrual blood, Wharton's jelly, amnion, and dental pulp [8-14]. Adipose tissue has become an important source of MSCs called adipose tissue-originated mesenchymal stem cells (ADSCs) and has been extensively used in the treatment of several diseases including liver diseases [15,16]. Several researchers showed that MSCs such as BMSCs and ADSCs could improve liver function and act as a promoting factor for liver regeneration in hepatic injuries due to their immunomodulatory and anti-inflammatory activities that can decrease hepatic inflammation, improve liver function, and decline infection incidences. The injection of MSCs was demonstrated to improve Thioacetamide (TA) and Carbon tetrachloride (CCl_4)-induced liver fibrosis in animal models too [17,18]. So this study was undertaken to evaluate the regenerative impact of ADSCs on the healing of liver injuries to treat liver fibrosis caused by CCl_4 in rat models by biochemical and histological examinations.

MATERIALS AND METHODS

Animals

Thirty-two male Sprague-Dawley rats (200 g ± 20 g) provided by the Laboratory Animal Center of the Shiraz University of Medical Science, Shiraz, Iran were included in this study, while they were caged under 12 hrs light and 12 hrs dark cycle and at temperatures of 20°C-22°C. They had free access to food and water. During experiments, animals were sacrificed based on instructions from the Shiraz University of Medical Sciences Animal Care Committee, Shiraz, Iran, and upon regulations and laws of the Iran Veterinary Organization for laboratory animals. This study was approved by the Arsanjan Azad University Ethics Committee.

CCl_4 dissolved in olive oil (1:1 v/v) was administered intraperitoneally (IP) twice per week for eight weeks to induce hepatic damage. Anesthesia was undertaken using a 10% ketamine and 2% xylazine (Alfasam, Netherlands) mixture and the sacrifice was performed via cervical dislocation. Under anesthesia and sterile conditions, adipose tissue was removed from the abdomen and pelvic regions. They were placed right away in falcon tubes filled with Gibco's Dulbecco's Modified Eagle's Medium (DMEM, Waltham, USA) to be used later for cell culture.

Cell Culture

The isolated adipose tissues were later washed in sterile phosphate buffer saline (PBS, Gibco, Waltham, USA) three times. It was then divided into small pieces with a sterile blade, put into a 15 mL falcon tube with 5 mL of DMEM, and centrifuged at 200 g for 10 min. The supernatant was later removed and the residual precipitate was treated with 1.5 mL of 0.2% collagenase type II (Gibco, USA) at 37°C for 40 min and was finally placed in an incubator with 5% CO_2 at 37°C and saturated humidity. The falcon was removed, and the falcon tube was then filled with 5 mL of DMEM and centrifuged once more at 200 g for 7 min. The supernatant was removed, and the remained pellet of cells was suspended again in 1 mL of culture media with 88% DMEM, 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids (Sigma-Aldrich, USA). Later, they were placed in a culture flask (T25: 25-mL) with 4 mL of culture media and kept at 37°C with saturated humidity in an incubator with 5% CO_2. Media change happened every 3 days to reach an 80% confluent culture and later the cells were subjected to 0.25% (w/v) trypsin-EDTA (Gibco, USA) until passage 3.

Cell Characterization

Characterization of cells was done morphologically by an inverted microscope (Nikon, Tokyo, Japan), and images were captured utilizing a digital camera (Olympus, Tokyo, Japan). Reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique combining reverse transcription of RNA into DNA called complementary DNA or cDNA) and amplification of specific DNA targets using PCR (Applied Biosystems, USA) was also employed to determine the presence of mesenchymal markers (CD73 and CD90) and the absence of hematopoietic markers (CD34 and CD45). They were also assessed for adipogenic and osteogenic induction to verify having mesenchymal properties. RT-PCR indicated the gene expression of CD73 and CD90 as mesenchymal markers. Also, RT-PCR revealed CD34 and CD45 as hematopoietic markers (Table 1).

According to the manufacturer's instructions, the RNA extraction kit (Cinna Gen Inc., Tehran, Iran) analyzed total RNA. Revert AidTM first strand cDNA synthesis kit from Thermo Fisher Scientific, Waltham, USA, was used to create first-strand cDNA. Test PCR runs were conducted using a PCR thermal cycler (Veriti Thermal Cycler, Thermo Fisher Scientific, Waltham, USA): 1 cycle at 94℃ for 3 min, 35 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec; and 1 cycle at 72℃ for 10 min. Finally, electrophoresis exhibited the bands utilizing DNA-safe stain in 1.5% agarose gel medium and gel documentation system (UVtec, Cambridge, UK).

Table 1: The sequences of mesenchymal and hematopoietic markers.

Gene	Primer sequence	Size (base pair)
CD73	Forward:5'-TGCATCGATATGGCCAGTCC-3'	208
	Reverse:5'-AATCCATCCCCACC GTTGAC-3'	
CD90	Forward:5'-GACCCAGGACGGAGCTATTG-3'	177
	Reverse:5'-TCATGCTGGATGGGCAAGTT-3'	
CD34	Forward:5'-GCCATGTGCTCACACATCA-3'	257
	Reverse:5'-CAAACACTCGGGCCTAACCT-3'	
CD45	Forward:5'-CCAAGAGTGGCTCAGAAGGG-3'	450
	Reverse:5'-CTGGGCTCATGGGACCATTT-3'	

To characterize by adipogenic induction, ADSCs were put in 6-well plates of complete culture medium. When reaching 80% confluence, media change happened containing adipogenic medium of a complete culture media mixed with 15% FBS, 100 nM dexamethasone, 200 µM indomethacin, and 100 µM ascorbic acid (Sigma Aldrich, USA) until 3 weeks. The final cells were fixed in 10% formalin for 20 min and washed 3 times with deionized water. After that, the cells were stained for 2 hours with 0.5% Oil Red-O (Sigma-Aldrich, USA) dissolved in 2-propanol solution (Merck, Germany), which should cause differentiated cells to show up in red due to the presence of oil droplets in the cells.

In osteogenic induction of cell characterization, ADSCs were placed in 6-well plates till reaching an 80% confluence. Change of culture medium to osteogenic medium containing complete culture medium in addition to 15% FBS, 50 µM ascorbic acid (Merck, Germany), 100 nM dexamethasone (Sigma Aldrich, USA), and 10 mM glycerol 3-phosphate (Merck, Germany) was undertaken for 21 days while changing media occurred every 3 days. After 3 weeks, 10% formalin was added to the cells for 20 min, washed three times with deionized water, and stained with 1.4% Alizarin Red solution (solved in deionized water at pH of 4.1, Sigma-Aldrich, USA), while the differentiated cells should appear in red color denoting to the presence of calcium deposit in the cells and a further calcification in the differentiation process.

Animal Grouping

ADSCs (2×10^6 cells/1 mL in phosphate buffered saline: PBS) were preconditioned for 24 hrs in a normal culture medium and then at the beginning of week 8th were injected in the tail vein under anesthesia and sterile condition. Four equal groups made up of all 32 rats were randomly selected. The control group 1 received just 1 mL/kg of distilled water (IP), twice a week for 8 weeks. The control group 2 was injected with 1 mL/kg of olive oil (IP), twice a week for 8 weeks. The sham group (group 3) was administered with 1 mL/kg of CCl4 dissolved in 1 mL of olive oil (IP)

twice a week for 8 weeks to induce hepatic injury. A blood sample was prepared at the beginning of the 8th week. The experimental group was treated with 1 mL/kg of CCl_4 dissolved in 1 mL of olive oil (IP) twice a week for 8 weeks to induce acute hepatic injury. Then, 2×10^6 ADSCs were injected into the tail vein. A blood sample was collected for liver function test and histological assessment 4 weeks after hepatic injury induction.

Liver Function Test

After anesthetizing the rats using a mixture of 2% xylazine (Alfasam, Netherlands) and 10% ketamine (Alfasam, Netherlands), blood samples were provided for liver function tests, 4 and 8 weeks after hepatic injury induction (Experimental 1 and 2 groups, respectively) and put into a chelate tube. The whole blood was kept at room temperature for 2 hrs and centrifuged at 4000 rpm for 10 min to get serum based on the manufacturer's instructions. To assay the function of the liver, serum activities of Aspartate Aminotransferase (AST) or Serum Glutamic Oxaloacetic Transaminase (SGOT), alanine transaminase (ALT), or Serum Glutamic Pyruvic Transaminase (SGPT) using Randox (UK) kits and Alkaline Phosphatase (ALP) using Parsazma (Iran) kits were evaluated.

Histological Assessment

Liver tissue processing and section preparation were conducted 4 and 8 weeks after hepatic injury induction by fixing in 10% formalin and embedding in paraffin. Blocks were cut at 5 µm thickness and stained with hematoxylin-eosin (H and E) and Masson's trichrome and observed for histological changes utilizing an optical microscope (Leica DMRBE).

Statistical Analysis

SPSS statistical package for Windows (version 11.5, Chicago, IL, USA) was employed for statistical analysis. The Kolmogorov-Smirnov test was used to assess the normality of the distribution of the serum data. The Tukey test was performed to compare the variations in the group mean values. The differences in the mean values of the groups were compared using a one-way analysis of variances. P values lower than 0.05 were regarded as significant.

RESULTS

Cell Characterization

ADSCs were negative for hematopoietic markers of CD34 and CD45 and positive for MSC markers of CD73 and CD90 (Figure 1A and 1B). They had a fibroblast-like spindle-shaped morphology and were adhered to culture flasks (Figure 1C). For adipogenic induction, ADSCs stained with Oil Red-O revealed intracellular lipid droplets in red color (Figure 1D). ADSCs stained with Alizarin Red revealed calcium deposits with a red color during osteogenic induction (Figure 1E). These findings confirm the mesenchymal properties of isolated ADSCs.

Liver Function Test

Regarding SGOT, a significant increase was observed in the average concentration of the enzyme in CCl_4 (1 mL/kg) group compared to other groups ($p \leq 0.0001$). There was a significant decrease in experimental groups 1 and 2 ($p=0.001$ and $p \leq 0.001$ after 4 and 8 weeks, respectively) compared to the CCl_4 (1 mL/kg) group. Regarding SGPT, a significant increase in the enzyme was noticed in CCl_4 (1 mL/kg) group in comparison to the control and sham groups

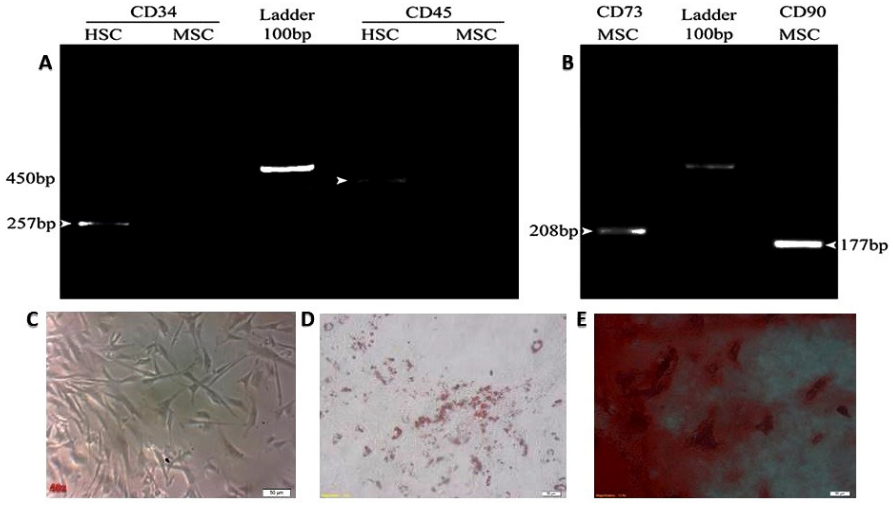

Figure 1: *Characterization of rat adipose tissue stem cells. RT-PCR with absence of hematopoietic markers of CD34 and CD45 and the presence of mesenchymal markers of CD73 and CD90 (A and B). Spindle shape cell morphology at 3rd passage (C, 40x), Adipogenic induction in red color (D, 20x), Osteogenic induction in red color (E, 20x).*

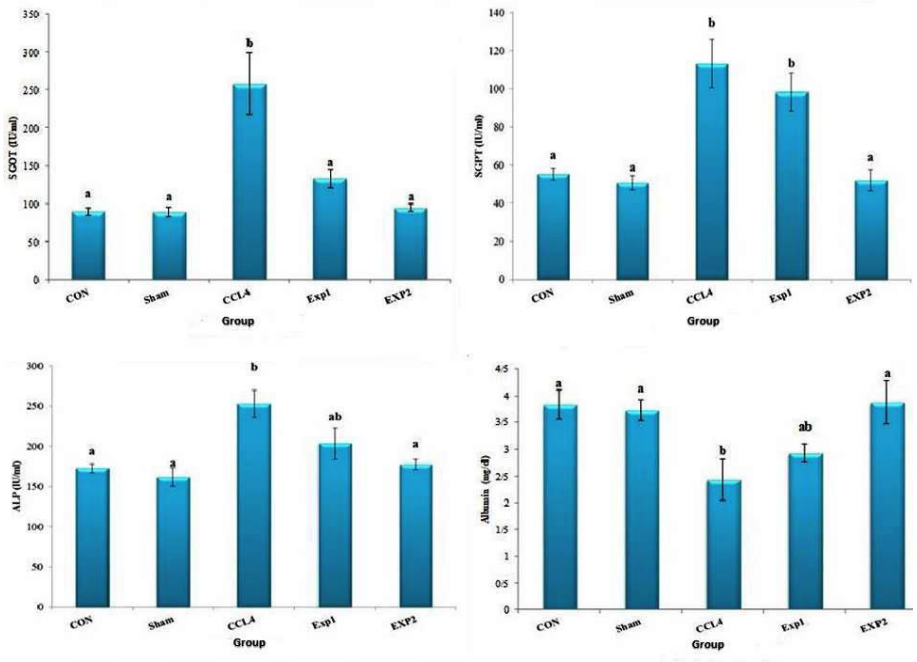

Figure 2: *Comparison of liver function enzymes between groups. Aspartate aminotransferase (AST) or serum glutamic oxaloacetic transaminase (SGOT), alanine transaminase (ALT) or serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP). Regarding SGOT, a significant increase was observed. Letter b denotes to significance of p ≤ 0.0001. For SGPT, a significant increase in the enzyme was noticed. Letter b denotes to significance of p ≤ 0.0001. Regarding ALP, a significant increase was visible. Letter b denotes to significance of p<0.01 and Letter ab denotes to significance of p<0.0001. For albumin, a significant decrease was exhibited. Letter b denotes to significance of p<0.01 and Letter ab denotes to significance of p<0.05. Liver function tests were conducted 4 and 8 weeks after hepatic injury induction presented as experimental 1 and 2 groups, respectively. Con: Control, Exp 1: Experimental 1, Exp2: Experimental 2.*

(p<0.0001). In experimental group 2, a significant decrease in the enzyme was shown (p<0.0001, after 8 weeks) when compared to CCl_4 (1 mL/kg) group. Regarding ALP, a significant increase was visible in the enzyme in the CCl_4 (1 mL/kg) group when compared to the control and sham groups (p<0.01 and p<0.0001, respectively). In experimental group 2, a significant decrease in the enzyme was demonstrated (p<0.01, after 8 weeks) compared to CCl_4 (1 mL/kg) group. Regarding albumin, a significant decrease was exhibited for the albumin in the CCl_4 group in comparison to the control and sham groups (p<0.01). Experimental group 2 illustrated a significant increase in the enzyme (p<0.05, after 8 weeks) compared to the CCl_4 group.

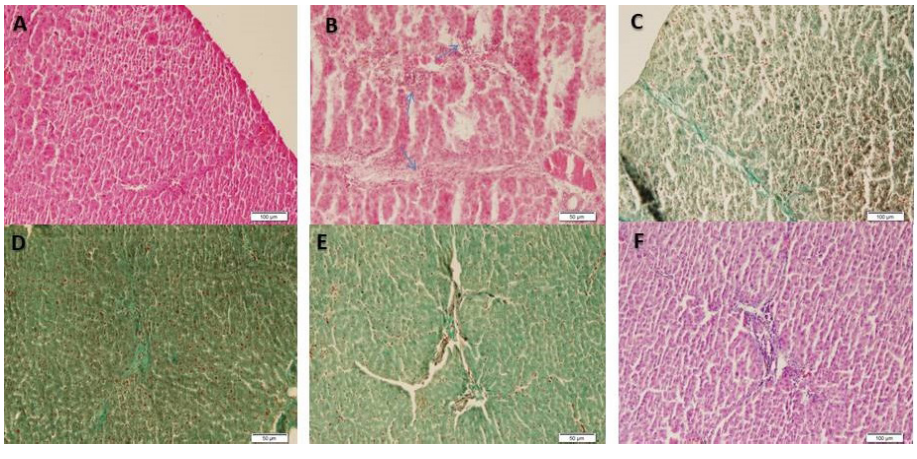

Figure 3: *Histological changes after administration of CCL_4 (1 mL/kg) and ADSCs in the liver: Normal liver (A: H&E, 40x), Interstitial hepatitis and hepatic fibrosis 4 and 8 weeks after cell transplantation (B: H&E, 40x; C: Masson's trichrome, 40x, respectively), Hepatic repair 4 weeks after cell transplantation (D: Masson's trichrome 8 weeks of after cell transplantation, 40x), Hepatic repair 8 weeks after treatment with ADSCs (E: Masson's trichrome, 40x), Hepatic repair 8 weeks after treatment with ADSCs (F: H&E, 40x).*

Histological Assessment

In the control group, normal tubular and vascular structure with hepatocyte and sinusoidal bands was visible (Figure 3A). In the sham group, nodule formations surrounded, and migration of inflammatory cells was noticed revealing interstitial hepatitis and hepatic fibrosis (Figure 3B and 3C). It was shown that hepatic injuries ameliorated 4 and 8 weeks after transplantation of ADSCs when compared to the sham group (Figure 3D-3F).

DISCUSSION

Liver diseases impose a huge burden worldwide. Among hepatic injuries, liver fibrosis is a chronic liver disease manifested by the accumulation of Extracellular Matrix (ECM) protein that can result into cirrhosis or hepatocellular carcinoma. Liver fibrosis is caused due to several reasons such as liver cell damage, inflammatory responses, and apoptosis. Antiviral drugs and immunosuppressive therapies were mentioned as available therapies for liver fibrosis, but with limited efficacy [19,20]. Although the liver can regenerate, but when liver fibrosis happens, tissue recovery is not possible, and liver transplantation is necessary. Based on reduced organ availability, a search for alternative solutions to treat liver injuries is essential. Regenerative medicine by employing transplantation of stem cells has opened a new window and as a therapeutic alternative strategy for the treatment of hepatic injuries [3].

MSCs are considered a promising therapeutic selection in liver fibrosis, as they have immunomodulating properties, promote liver regeneration, and inhibit the activation of Hepatic Stellate Cells (HSC) that participate in disease development. Several studies have illustrated that MSCs can inhibit liver fibrosis in preclinical animal models, revealing their potential application in remedy of hepatic injuries including Bone Marrow Derived Stem Cells (BMSCs) [17-21], Umbilical Cord Stem Cells (UCSCs) [22], and ADSCs [18]. ADSCs have a proven liver differentiation potential both *in vitro* and *in vivo* to acquire hepatocyte-like cell morphology and hepatocyte-specific markers [23].

Transplantation of UCSCs by inhibiting activated Kupffer cells and Hepatic Stellate Cells (HSCs) was demonstrated to significantly improve liver fibrosis. These cells by ameliorating liver microcirculation could decrease portal pressure too. Downregulation of TGF-β/SMAD signaling thus inhibits the fibrotic process and potentially opposes liver fibrosis [24]. It was found that incubation of MSCs with some specific growth factors such as the Hepatocyte Growth Factor (HGF) and basic Fibroblast Growth Factor (bFGF) can lead to hepatocyte differentiation [25]. Therefore, MSCs with the ability to differentiate into hepatocyte-like cells can be considered as a promising source of liver regeneration. In this relation, ADSCs were also shown to have high expression of anti-fibrotic factors such as IGF-1, IL-10, and HGF to protect hepatic injury and apoptosis and improve hepatic function [7]. Gene modification of MSCs by introducing various genes and microRNAs into MSCs via viral or non-viral vectors was illustrated to improve their differentiation, immune regulation, homing ability, and other repair-related capabilities [26].

Numerous studies have demonstrated that the transplantation of BMSCs has the potential to dramatically improve liver fibrosis recovery and to regenerate hepatic damage [17,21]. Transplantation of BMSCs could significantly improve liver synthesis (prothrombin time, albumin, and ascites) and secretion functions (total bilirubin) by down-regulating the lnc-BIHAA1/rno-miR-667-5p signaling pathway [27]. By using UCSCs in liver cirrhosis, it was found that inflammation and fibrosis scores were lower after cell transplantation together with a largely improved liver cirrhosis degree and lower Child-Pugh scores [28]. Gharbia et al. found that ADSCs could attenuate inflammation and microvesicular steatosis, diminish collagen deposits, and ameliorate liver fibrosis [29]. The impact of MSCs in the treatment of experimental CCL_4-induced liver fibrosis in rats denoted to anti-inflammatory, anti-fibrotic, and pro-angiogenic properties of MSCs to promote the resolution of liver fibrosis [30].

Khosrojerd et al. showed that MSCs and hepatocyte-derived exosomes along with imipenem in mice model reduced inflammation in the liver and increased the TCD8+ and Treg populations and ultimately increasing the survival rate [31]. Zhang et al. reported the key mechanism of ADSCs-derived exosomes from anti-hepatic fibrosis to be the inhibition of PI3K/AKT/mTOR signaling pathway impacting the changes of metabolites in lipid metabolism, and mainly regulation of choline metabolism. ADSCs were shown to attenuate hepatic stellate cell activation and suppress the progression of liver fibrosis, which holds the significant potential of stem cells in the treatment of chronic liver diseases [32]. Yan et al. found that the transplantation of ADSCs into the acute liver injury model had a certain therapeutic role in the recovery of the liver in canines [33]. The findings of these studies are in agreement with our results revealing that ADSCs are immune-compatible and demonstrate hepatocyte differentiation properties in the absence of any post-transplantation rejection. They can be considered as a novel approach for hepatic regeneration and hepatocyte differentiation and support hepatic function

in hepatic injuries. The limitation of the study was that the underlying working mechanisms involved in the regeneration process were not assessed as the aim of the present study was to evaluate the therapeutic effect of ADSCs on the reversal of hepatic injury; so further studies should target elaborating the relationship between the mechanisms involved for regeneration process of ADSCs and treatment of liver injuries in greater details.

CONCLUSION

Our study revealed that ADSCs could promote liver function and regeneration that can be beneficial in ameliorating and repairing hepatic injuries. These data can be added to the literature to open a new era for researchers to facilitate the treatment of hepatic injuries when ADSCs are targeted. So it may have the potential to relieve the symptoms of patients with liver disease and improve their quality of life and prognosis.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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