Antioxidant Effects of Bone Marrow Mesenchymal Stem Cell against Carbon Tetrachloride-Induced Oxidative Damage in Rat Livers

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ABSTRACT

Background: Liver fibrosis results from excessive accumulation of extracellular matrix, which affects liver function over time and leads to its failure. In the past, liver transplant was thought to be the only treatment for end-stage liver disease, but due to the shortage of proper donors other medical treatments have been taken into consideration.

Objective: To evaluate the therapeutic effects of bone marrow derived mesenchymal stem cells (BM-MSC) in CCl4 damaged rats.

Methods: Liver damage in adult male Wistar rats was induced with carbon tetrachloride (CCl4). The rats were divided into normal control group, receiving CCl4, and those receiving CCl4 + marrow derived-MSC. Human BM-MSC was isolated, cultured, and characterized. The rats were injected with xenograft MSCs into the hepatic lobes of the liver. In the eighth week, blood samples were taken from all groups. Histological examination and biochemical analyses were used to compare the morphological and functional liver regeneration among different groups. Measurement of lipid peroxidation and glutathione transferase activity was also performed.

Results: Histopathology and biochemical analyses indicated that local injection of human BM-MSCs was effective in treating liver failure in the rat model. Furthermore, oxidative stress was attenuated by increased level of GSH content after MSC transplantation.

Conclusion: Evidence of this animal model approach showed that bone marrow-derived MSCs promote an antioxidant response and support the potential of using MSCs transplantation as an effective treatment modality for liver disease.

KEYWORDS: Liver failure; Mesenchymal stem cells; Transplantation; Carbon tetrachloride

INTRODUCTION

Liver transplantation is the most important treatment for patients suffering from liver failure, but this method has limitations due to the shortage of appropriate donors. Clinical and laboratory studies have considered alternative therapies for these patients. Recently, mesenchymal stem cell (MSC) has been investigated with the prospect of treatment of acute and chronic liver diseases. Some studies provide clinical and experimental evidence suggesting that MSC transplantation can restore the liver function in acute and chronic damages [1,2]. Other studies have concentrated on hematopoietic...
cells and stem cells derived from the bone marrow (BM-MSC), which have a great ability for division and implantation in the liver of transplant recipient [3].

In the bone marrow, there are main populations of stem cells including hematopoietic stem cells, MSCs and multipotent adult progenitor cells [4]. A number of studies have proven that under appropriate environmental conditions, cells derived from the bone marrow can differentiate into hepatocytes both in vivo [5,6] and in vitro [7]. We have previously found that BM-MSCs can participate in differentiation of hepatocytes. In vitro differentiated MSCs using IGF-I are able to display advanced liver metabolic functions supporting the possibility to use them as potential alternatives to primary hepatocytes [8].

Administration of MSC can decrease the injury in the liver, lungs and heart by reducing inflammation, collagen deposition and rearrangement [9]. Sakaida, et al [10], and Fang, et al [9], reported that bone marrow cells or MSCs can reduce hepatic fibrosis caused by carbon tetrachloride (CCl₄) in mice. However, the reversal of fibrosis by MSC is not completely known so far.

Stem cells have the ability not only to implant in the target tissues, but also to secrete many factors that could change or improve the function of the damaged tissue [11]. Given that after transplantation of the damaged tissues or organs, the stem cells are influenced by several factors such as inflammatory cytokines [12,13], and migration to the target organs after a successful transplantation; the cells can activate processes that lead to reconstruction of the damaged cells and tissues [14,15].

CCl₄ is a more efficient hepatotoxic substance, the toxicity of which is based on the change in its bio-structure into two free radicals and plays an important role in hepatotoxicity, tissue damage and cell death [16]. Oxidative stress contributes to the pathogenesis of various diseases. Tightly regulated defense systems such as glutathione production have evolved to combat these stresses.

There are many controversies on the main mechanisms through which cell-based therapy affects liver tissue repair. The possibility of using human MSCs to repair liver damage has not yet been evaluated. In this study, we investigated the therapeutic use of MSC transplantation on CCl₄-induced liver failure in rats.

**MATERIALS AND METHODS**

**Chemicals**

CCl₄; sodium dodecyl sulfate; ethylene diamine tetra-acetic acid (EDTA); 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB); tris, thiobarbituric acid (TBA); and trichloroacetic acid were purchased from Sigma Chemical Company, Germany. All other chemicals were of highest quality available in the market.

**Isolation of human MSCs (hMSCs)**

Human MSCs (hMSCs) were isolated using a method previously described [17]. Cells from human bone marrow (BM) were taken from several people from the posterior iliac crest bone by aspiration in Namazi Hospital. Then, they were mixed with complete medium (ratio of 1:1) and mononuclear cells were gently placed in a separate Falcon using density gradient method (Percoll, 1.073 g/mL). The mononuclear cells were then isolated from the BM by centrifuging at 2000 rpm for 20 min at 21 °C and were cultured in complete medium (Dulbecco’s modified Eagle’s medium [DMEM, Gibco/BRL]) with fetal bovine serum (10%). The cells were then incubated at 37 °C in a medium containing 5% humidity and CO₂ for 12–14 days; during this period, they began to form colonies. Once the colonies developed (filling 80%–90%), the media were washed twice in PBS and the cells were trypsinized with 25% trypsin in 1 mM EDTA (Gibco/BRL) for five minutes at 37 °C. After centrifugation, the cells were resuspended in supplemented medium with serum and incubated in a 25-cm² flask. Culture media were prepared for the first passage [18]. MSCs were identified in the culture medium by sticking and spindle deformation [19].

At each passage, the cells were counted and
analyzed for viability by trypan blue staining analysis. Flow cytometric analysis and functional ability of differentiation into osteocyte and adipocyte was achieved in response to specific culture conditions. Each experiment described here was replicated thrice.

Preparation of animals and experimental groups
Twenty-one adult male Wistar rats weighing 250–300 g were purchased from Razi Institute for Serum and Vaccine and categorized into three groups (7 rats in each group). Group 1 rats (negative control) did not receive any CCl\textsubscript{4}; they only received olive oil intraperitoneally twice per week for eight weeks. Group 2 rats (positive control) received CCl\textsubscript{4} diluted 1:1 in olive oil intraperitoneally (1 mL/kg) twice per week for eight weeks. Group 3 rats, in addition to receiving CCl\textsubscript{4} intraperitoneally (1 mL/kg), received the prepared cells (1\times10^6 cells in 1 mL PBS) by insulin syringe into several lobes of the liver in a completely sterile environment in the fourth week after CCl\textsubscript{4} injection. After the eighth week of CCl\textsubscript{4} injection, the rats were anesthetized using thiopental (50 mL/kg). Their blood was collected for providing serum and their liver was dried after being washed with normal saline. Segments of their dried liver were collected for assessment of the oxidative stress and the rest was fixed in formalin (10%). Histological sections (5 \mu m) of the liver-lobes were obtained, stained with hematoxylin, eosin and Masson’s trichrome stain, and transported to the lab.

Histopathological studies
A segment of the liver was excised from the animals and fixed in 10% formalin for at least 24 hours. Then, the paraffin sections were
prepared and cut into 5-µm sections by a rotary microtome. The sections were stained with hematoxylin-eosin and studied for histopathological changes, i.e., necrosis, fatty changes, ballooning degeneration, and inflammation. Histological damages were scored—0: “absent;” +: “mild;” ++: “moderate;” and +++: “severe.”

Measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin

Biocon standard kits and DAX-48 autoanalyzer were used to measure serum ALT, AST, and albumin, according to Wilkinson, et al., and Bessay, et al’s method [20,21].

Determination of lipid peroxidation

The extent of lipid peroxidation was assessed by measuring the amount of thiobarbituric acid-reactive substances (TBARs). In brief, 500 mg of the liver tissue was gently minced in 4.5 mL of 0.25 M sucrose. The minced tissue was gently homogenized and then centrifuged at 2000 rpm for 30 min. Afterwards, 0.1 mL of the supernatant was treated with a buffer containing 0.75 mL of thiobarbituric acid (0.8%, w/v), 0.75 mL of 20% acetic acid (pH 3.5) and 0.1 mL of sodium dodecylsulfate (8.1%, w/v). The solution was mixed up with 2 mL of distilled water and heated in a boiling water bath for 60 min. The absorbance was measured at 532 nm by a Beckman DU-7 spectrophotometer [22].

GSH determination

Glutathione reductase 5, 50-dithiobis-2-nitrobenzoic acid (DTNB) recycling procedure [23] was used to determine the reduced glutathione. In brief, 100 mg of the liver tissue was homogenized in a buffer containing EDTA (0.2 M) to obtain 4% (w/v) whole homogenate. Then, 1.5 mL of the suspension was taken and mixed with a buffer containing 2.5 mL distilled water and 0.5 mL of 50% TCA. The mixture was then centrifuged at 3000 rpm for 15 min, and 1 mL of the supernatant with 1 mL of tris buffer (0.4 M, pH 8.9) and 0.1 mL of DTNB (0.01 M). The absorbance was measured after 5 min at 412 nm using a Beckman DU-7 spectrophotometer [24].

Statistical analysis

The data were analyzed by Student’s t test and one-way ANOVA, followed by Graphpad Prism 5. A p value <0.05 was considered statistically significant.

RESULTS

Histological studies of the liver sections from control rats showed normal architecture, characterized by polyhedral shaped hepatocytes with small uniform nuclei. Hepatocytes were arranged in well-organized hepatic cords and separated by narrow blood sinusoids. Hepatic sections, followed by CCl₄ treatment, showed typical CCl₄-induced hepatic injury (Fig 1B). The sections revealed extremely vacuolated hepatocytes (apoptotic necrotic cells and a lot of foamy cells) adjacent to the central vein (arrows and arrowhead, Fig 1B), indicating chronic CCl₄-induced hepatotoxicity (with

| Table 1: Effect of the bone marrow-derived mesenchymal stem cell (BM-MSCs) on histopathological liver damages induced by CCl₄ in rats. |
|---|---|---|---|---|---|
| Groups | Ballooning Degeneration | Fatty change | Hepatocyte necrosis | Inflammation | Fibrosis |
| Control | 0 | 0 | 0 | 0 | — |
| CCl₄ | ++ | + | +++ | + | Many foamy macrophages and old necrosis |
| CCl₄ + MSC | 0 | 0 | 0 | 0 | — |

0: absent; +: mild; ++: moderate; +++: severe

Rats were injected with CCl₄ (1 mL/kg, ip 1:1 in olive oil) twice per week for 8 weeks. Four weeks after the first injection MSC was infused to their livers locally. The injection of CCl₄ was continued until the end of 8th week. Values are mean±SD of 6 rats per group.
+++ grade, Table 1), moderate inflammation (with + grade, Table 1), and fatty changes (with + grade, Table 1). Hepatocytes were significantly protected from $\text{CCl}_4$-induced toxicity after MSC infusion (Fig 1C), which demonstrated a normal appearance.

The section from liver in Figure 1 shows severe necrosis in the central vein of the rats treated with $\text{CCl}_4$ compared to the control group (with +++ grade, Table 1); there was no necrosis in the $\text{CCl}_4 + \text{MSC}$ treated group (with 0 grade, Table 1). Liver lobules in the $\text{CCl}_4 + \text{MSC}$ treated group had a relatively normal appearance, compared to the $\text{CCl}_4$ group with no fatty changes (with 0 grade, Table 1 and Fig 1C). The effects of MSCs on reducing necrosis (with + grade, Table 1) surrounding the central vein area (Fig 1C), and the absence of swollen hepatocytes were clearly shown in the H&E sections.

Histopathological examination of the liver revealed that MSC has anti-fibrosis effects by reducing the amount of collagen which is shown by Masson's trichrome stain compared to the group receiving $\text{CCl}_4$, which showed significant periportal fibrosis (Fig 2D).

The results showed slight increase in the serum albumin levels in the group receiving $\text{CCl}_4 + \text{MSC}$ compared to the group receiving $\text{CCl}_4$ alone (Fig 2A). Other liver enzymes also showed significant changes. For instance, AST and ALT in the group treated with $\text{CCl}_4 + \text{MSC}$ were significantly (p<0.01) lower compared to the group receiving $\text{CCl}_4$ alone (Figs 2B and 2C).

Lipid peroxidation in the liver showed a significant increase in the $\text{CCl}_4$ group compared to the control group (p<0.01); in the $\text{CCl}_4 + \text{MSC}$ treated group, a significant reduction was observed in lipid peroxidation levels (p<0.01) (Table 2).

Glutathione (GSH) was also assessed as an indicator of hepatic antioxidant enzymes. A significant...
significant increase was observed in GSH levels in CCl\textsubscript{4} + MSC treated group compared with the CCl\textsubscript{4} group (p<0.05). Moreover, a significant increase was observed in GSH levels in CCl\textsubscript{4} + MSC treated group compared with the control group (p<0.05) (Table 2).

**DISCUSSION**

Rats treated with CCl\textsubscript{4} are usually used as an in vivo model for studies on liver damage. CCl\textsubscript{4} usually produces free radicals that trigger a cascade of reactions leading to liver fibrosis. CCl\textsubscript{4} is converted to free radicals by cytochrome P450 which exerts its effects on the liver through lipid peroxidation \[25,26\].

Oxidative stress is a pathogenic mechanism in the initiation and progression of liver damage involved in many liver disorders. Cell damage occurs when reactive oxygen species concentration increases in the liver. The use of antioxidants reduces the amount of these free radicals \[27\].

Glutathione (l-γ-glutamyl-l-cysteinyl-glycine), which is present in all mammalian tissues, especially the liver, provides the reduction capacity for most reactions and plays a very important role in the detoxification of hydrogen peroxide, other peroxides and free radicals \[28\].

In the present study, the hepatic content of GSH was found to be decreased significantly in the CCl\textsubscript{4} intoxicated rats compared to the controls. Table 2 shows that MSC-based therapy significantly inhibited the CCl\textsubscript{4}-induced decrease of hepatic GSH content, and it was significantly increased in the MSC-treated group. The resulting hepatocellular toxicity has been demonstrated in numerous studies as reflected by increased liver enzymes.

Because the liver is considered the main organ for biological degradation and subsequently detoxification of harmful substances, it contains important enzymes for their biological actions. When CCl\textsubscript{4} is administered to rats, the activity of AST and ALT in plasma rises significantly with necrosis of and lipid accumulation in hepatocyte \[29\]. Both enzymes are indicators of liver injury. ALT is more sensitive to acute liver injury, whereas AST is more sensitive to chronic injury \[30\]. In this study, we investigated alteration of liver enzymes after transplantation of MSCs into the rat liver injured with CCl\textsubscript{4}. MSCs transplantation restored the increase of liver enzymes as well as the up regulation of albumin.

Recovery of liver function after MSCs transplantation was also examined by histological changes. MSCs reversed the hepatic necrosis, fatty changes, and inflammation. However, the mechanisms by which MSCs repair liver damage remain unclear. HGF is one of the factors produced by the MSC and its beneficial effects include mitogens, morphogens, and anti-tumor activities \[31\]. Specifically, it was determined that HGF exerts beneficial effects on liver damage. MSC transplantation regenerates the reduction in hepatic protective genes and shows its effects by reducing LP, as determined by the amount of GSH in the liver. It was also determined that in ischemic/perfusion damage to liver, MSC transplantation-
tion leads to suppression of oxidative stress and reduction in the amount of apoptosis in rats [39].

We have shown that MSC transplantation may protect liver injury by altering the oxidative effect of CCl₄ by increasing GSH content of the liver. In conclusion, we demonstrated the protective effect of MSCs, and suggest that bone marrow-derived MSCs could be a therapeutic approach for liver damage, particularly for those due to oxidative stress.

ACKNOWLEDGMENTS

This work has been supported financially by Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, under thesis proposal (No. 14:5). The authors would like to thank Mrs Zahra Kaviani, and Mrs Fatemeh Heydari for their kind assistant in conduction of this research. Also the authors would like to thank Dr. Vahedi, and Mr. Kohi Hossein-abadi at Animal Lab Center, Shiraz University of Medical Sciences, for their sincere assistance, and Dr. Nasrin Shokrpour from Center for Development of Clinical Research, Namazi Hospital for editorial assistance.

CONFLICTS OF INTEREST: None declared.

REFERENCES

1. Peng L, Xie Dy, Lin Bl, et al. Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: Short-term and long-term outcomes. Hepatology 2011;54:820-8.

2. Li L, Hu Z, Li W, et al. Establishment of a Standardized Liver Fibrosis Model with Different Pathological Stages in Rats. Gastroenterology res practice 2012;2012:560345.

3. Weil BR, Manukyan MC, Herrmann JL, et al. The immunomodulatory properties of mesenchymal stem cells: implications for surgical disease. J Surg Res 2011;167:78-86.

4. Muguruma Y, Reyes M, Nakamura Y, et al. In vivo and in vitro differentiation of myocytes from human bone marrow–derived multipotent progenitor cells. Exp hematol/ 2003;31:1323-30.

5. Okumoto K, Saito T, Hattori E, et al. Differentiation of bone marrow cells into cells that express liverspecific genes in vitro: implication of the Notch signals in differentiation. Biochem biophys res commun 2003;304:691-5.

6. Miyazaki M, Akiyama I, Sakaguchi M, et al. Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem biophys res commun 2002;298:24-30.

7. Petersen B, Bowen W, Patrene K, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168-70.

8. Maryam Ayatollahi, Masoud Soleimani, Seyed Ziaadin Tabei, Salmani MK. Hepatogenic differentiation of mesenchymal stem cells induced by insulin like growth factor- I. World J Stem Cells 2011 26:113-21.

9. Fang B, Shi M, Liao L, et al. Systemic infusion of FLK1+ mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 2004;78:83-8.

10. Terai S, Sakaida I, Yamamoto N, et al. An in vivo model for monitoring trans-differentiation of bone marrow cells into functional hepatocytes. J biochem 2003;134:551-8.

11. Khaldooyanidi S. Directing stem cell homing. Cell Stem Cell 2008;2:198-200.

12. Penn MS, Mangi AA. Genetic enhancement of stem cell engraftment, survival, and efficacy. Circ res 2008;102:1471-82.

13. Baer PC, Geiger H. Mesenchymal stem cell interactions with growth factors on kidney repair. Curr Opin Nephrol Hypertens 2010;19:1-6.

14. Bai X, Yan Y, Coleman M, et al. Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. Mol Imaging Biol 2011;13:633-45.

15. D’Agostino B, Sullo N, Siniscalco D, et al. Mesenchymal stem cell therapy for the treatment of chronic obstructive pulmonary disease. Expert Opin Biol Ther 2010;10:681-7.

16. Boll M, Weber LW, Becker E, Stampfl A. Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites. Z Naturforsch C 2001;56:649-59.

17. Ayatollahi M, Geramizadeh B, Zakerinia M, et al. Human Bone Marrow-derived Mesenchymal Stem Cell: A Source for Cell-Based Therapy. Int J Organ Transplantation 2011;3:32-9.

18. Alhadjlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. Stem cells dev 2004;13:436-48.

19. Rochefort GY, Vaudin P, Bonnet N, et al. Influence of hypoxia on the domiciliation of Mesenchymal Stem Cells after infusion into rats: possibilities of targeting pulmonary artery remodeling via cell therapies? Respir res 2005;6:125.

20. Wilkinson JH, Boutwell JH, Winsten S. Evalua-
tion of a new system for the kinetic measurement of serum alkaline phosphatase. Clin Chem 1969;15:487-95.

21. Bessay OA, Lowry OH, MJ. A method for rapid determination of alkaline phosphatase with five cubic milliliters of serum. J Biol Chem 1946;164:321-9.

22. Jamall IS, Smith JC. Effects of cadmium treatment on selenium-dependent and selenium independent glutathione peroxidase activities and lipid peroxidation in the kidney and liver of rats maintained on various levels of dietary selenium. Arch Toxicol 1985;58:102-5.

23. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502-22.

24. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent. Anal Biochem 1968;25:192-205.

25. Bruckner JV, Ramanathan R, Lee KM, Muralidhara S. Mechanisms of circadian rhythmicity of carbon tetrachloride hepatotoxicity. J Pharmacol Exp Ther 2002;300:273-81.

26. Janakat S, Al-Merie H. Optimization of the dose and route of injection, and characterisation of the time course of carbon tetrachloride-induced hepatotoxicity in the rat. J Pharmacol Toxicol Methods 2002;48:41-4.

27. Medina J, Moreno-Otero R. Pathophysiological basis for antioxidant therapy in chronic liver disease. Drugs 2005;65:2445-61.

28. Meister A, Larsson A. Glutathione synthetase deficiency and other disorders of the g-glutamyl cycle. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic bases of inherited disease, 6th ed. New York: McGraw-Hill, 1989:855-68.

29. Yachi R, Igarashi O, Kiyose C. Protective effects of vitamin E analogs against carbon tetrachloride-induced fatty liver in rats. J Clin Biochem Nutr 2010;47:148.

30. Wolf PL. Biochemical diagnosis of liver disease. Indian J Clin Biochem 1999;14:59-90.

31. Cho KA, Woo SY, Seoh JY, et al. Mesenchymal stem cells restore CCl4-induced liver injury by an antioxidative process. Cell Biol Int 2012;36:1267-74.

32. Jin G, Qiu G, Wu D, et al. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. Int J Molecul Med 2013;31:1395.

33. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22:696-9.