

Biochemical and Molecular Studies on Bone Marrow Derived Stromal Stem Cells on Liver Injuries in Rats

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Abstract

Liver is a remarkable vital organ, it is responsible for metabolizing the different food elements, filtering and detoxifying poisons in the blood to remove numerous toxic compounds. Liver affections are essentially caused by harmful chemicals (certain anti-infection, paracetamol, carbon tetrachloride, aflatoxins, per oxidized oil and so on.), overabundance utilization of liquor, immune system issue and disease. Up till now, orthotropic liver transplantation is the most powerful treatment of liver infections. Because of the deficiency of donors, high costs, the number of patients who can profit by this methodology is extremely constrained. Recently, cell based treatments have been explored as other option to entire liver transplantation. Thirty rats were separated into three equivalent groups (n=10, each). Group 1: control; Group 2: was infused peritoneally with acetaminophen (300 mg/kg); Group 3: was injected intra peritoneal with acetaminophen (300 mg/kg), then injected intra-venous with mesenchymal stromal stem cells (1 million BM-MSCs/kg). Blood samples for estimation of liver functions, and liver samples for detection of homing labeled MSCs with red fluorescent cell linker dye (PKH26), gene expression of matrix metalloproteinase (MMP2), tissue inhibitor metalloproteinase (TIMP) and histopathological examination were collected. The infusion of BM-MSCs enhanced liver capacities test, MMP2 and TIMP. Histopathological examination of liver tissue demonstrated critical antifibrotic impact, improvement in the hepatic structure as compared to the second group. It could be concluded that BM-MSC is effective in treatment of liver damage by upgrading hepatocyte recovery through enhancing the liver stress and inflammatory signaling by improving MMP2 and TIMP, besides it has a significant antifibrotic effect. The MSCs stem cell is an attractive cell source for regenerative medicine.

Keywords: Acetaminophen, Bone Marrow, Liver Injury, Mesenchymal Stromal Stem Cell.

Introduction

Stem cells are single cell population with the ability of self-renewal and differentiation into dissimilar cell lines. They were documented as possible tool for advanced therapeutic plans development. Stem cell therapy is a part of a new medical branch called "Regenerative medicine" [1]. There are two types of stem cells according to the origin, the adult stem cell (ASC) and the embryonic stem cell (ESC). The ASC are used in a number of fields with extreme success, especially in cases of hematological malignancies. *In vitro* and *in vivo* studies revealed that ASC from one tissue or organ can differentiate into the cells of other organs [2]. Bone marrow-derived stem cells (hematopoietic or mesenchymal) are types of ASC which have important implications with no need for ethical concern inherent to fetal

embryonic tissue [3]. The adherence ability of bone marrow stem cells to a plastic support, resulted in an expanded use and culture of these cells. They differentiate into multiple mesenchymal cell types, as well as cartilage, bone, adipose, fibrous tissues and myelosupportive stroma under appropriate experimental conditions [4]. This has led many investigations to explore the potential of their therapeutic applications [5]. Many scientists believe that ESC might be a future solution to most of the diseases. However, it is under research, because of the ethical barriers related to its usage [6].

It is expected that stem cells will be the basis for what is known as cell based therapy for treating various diseases. Cells-based therapy relies on replacing diseased or dysfunctional cells with healthy functioning

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ones [7]. Scientists primarily studied ESC or ASC of animals and humans.

Embryonic stem cells can be isolated from the embryo at a gestation age of 3-5 days (blastocysts). Their use in research and therapy is limited due to ethical considerations. Adult stem cells can be isolated from various tissues such as muscles, liver, gastrointestinal tract, adipose tissue and bone marrow [8].

The ASC has two types of stem cells; the hematopoietic stem cells (HSCs) and the mesenchymal (non-hematopoietic) stem cells (MSCs). Bone marrow mesenchymal stem cells are also referred to as marrow stromal cells [9], colony creating unit fibroblast and marrow stromal stem cells [10], and as multipotential mesenchymal progenitor cells (MPCs) [11].

The HSCs give rise to all circulating blood cells, while MSCs can be induced to differentiate into different mesenchymal lineages such as osteoblastic, chondroblastic and adipocytic lineages, when cultured into appropriate environments, *in vitro* [9]. The bone marrow represents the first source of MSCs, because they can be easily obtained after aspiration from the sternum or the iliac crest of the human volunteer donors after informed consent [12].

Applications of stem cells include many fields of medicine such as cardiology, neurology, hematology, hepatology, immunological diseases, and diabetes mellitus and as a cell model for drug trials [13]. MSCs distinguish into osteoblasts, adipocytes, chondrocytes [14], monocytes [15], astrocytes, oligodendrocytes, neurons [16] and hepatocytes [17]. Consequently, the cells are tested for their use in cell and gene therapy for a number of diseases [18]. The ability of MSCs to distinguish into hepatocytes *in vitro* [17] also *in vivo* has been recently reported [19]. Moreover, injuries, lung, liver or heart can be repaired by MSCs administration [19].

In the field of hepatology, stem cells differentiated into hepatocytes introduce new functioning liver cells into diseased organs, supporting intrinsic liver regeneration. New clinical studies also showed that hepatocyte transplantation may be effective in treating children with liver-based metabolic diseases

[20]. The purpose of the current study was to evaluate the protective and therapeutic effects of MSCs in injured- liver rats induced by acetaminophen.

Materials and Methods

Isolation of MSCs from femur and tibiae of rats and cultivation of MSCs in Dulbecco modified Eagle medium supplemented with 10% fetal bovine medium was performed. MSCs in culture were characterized by their adhesiveness and fusiform spindle shape [21], and then they were identified by flow cytometer. Briefly, MSCs were washed and suspended in phosphate-buffer saline, CD45 and CD90 monoclonal antibodies (vendor) were added to cells and kept for 1 h at 4°C, incubated with anti-mouse immunoglobulin G for (vendor) 45 min on ice, then analyzed on FACS caliber flow cytometer. After that, we labeled the isolated MSCs with PKH26 red fluorescent dye (Sigma Aldrich) and injected them in rat tail vein for indicating homing of MSCs in rat liver. Thirty rats of 130-170 gm body weight were divided into three equal groups. Group 1: control; Group 2: were injected I/P with acetaminophen (300 mg/kg BW); Group 3: were injected I/P with acetaminophen (300 mg/kg BW), then injected with mesenchymal stromal stem cells I/V [22]. After 2 weeks from injection of stem cells, the animals were fasted overnight. Blood samples were collected from the retro-orbital venous plexus for assessment of liver functions (ALT, AST, ALP, total protein, albumin, direct bilirubin and indirect bilirubin) and liver samples were also collected for the detection of MSCs homing after its labeling with red fluorescent cell linker dye (PKH26) dye by inverted microscope, for histopathological examination, and for gene expression of matrix metalloproteinase (MMP2), tissue inhibitor metalloproteinase (TIMP) [23].

Results and Discussion

Liver is a remarkable vital organ in both form and function. It is responsible for performing more functions than any other organ in the body. Liver illnesses are mainly initiated by toxic chemicals (certain antibiotics such as amoxicillin in a large dose, aflatoxin, carbon tetrachloride, paracetamol in a large dose, chlorinated hydrochloride, etc.), excessive alcohol, infections and autoimmune

disorders [24]. Some chemicals or viral infections often lead to liver fibrosis, which subsequently causes an impairment of liver function that required medical intervention [25]. Acetaminophen was originally used as an analgesic in therapeutic dose, however, at higher doses; it causes fatal hepatic necrosis [26, 27]. Injury to the liver is not due to the drug itself, but due to a toxic metabolite (N-

acetyl-p-benzoquinone imine (NAPQI) created by cytochrome P-450 enzymes in the liver [28]. Normally, detoxification of this metabolite occurs by conjugation with glutathione. In overdose, a large amount of NAPQI is generated impairing the process of detoxification and then results in liver cell injury. In addition, Nitric oxide has a role in inducing toxicity [29].

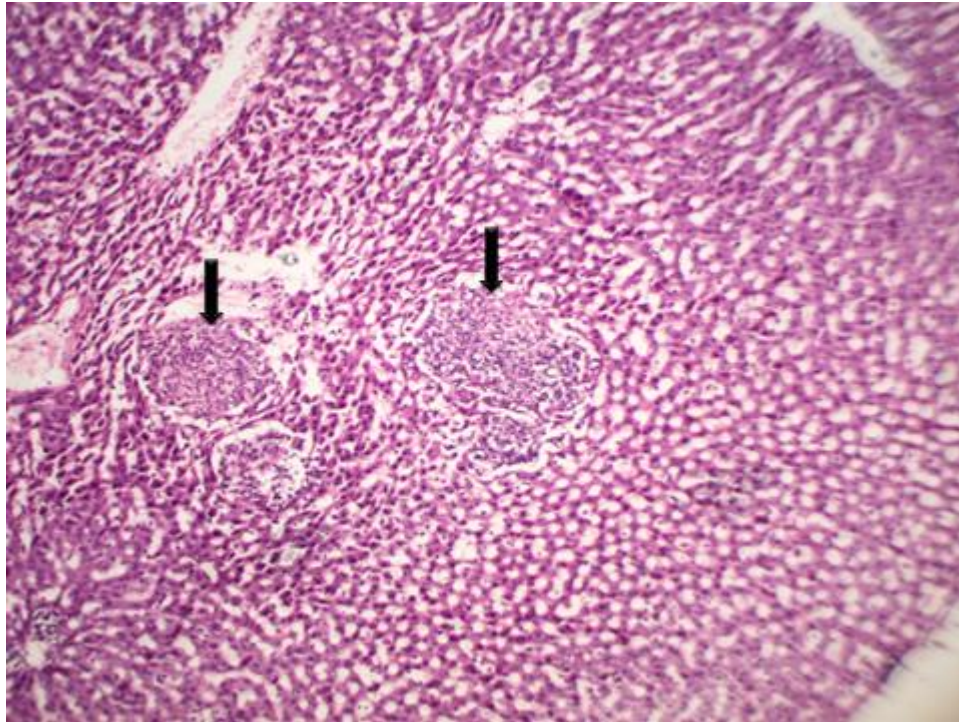


Figure 1: Liver of rat (acetaminophen) showing multiple focal areas of coagulative necrosis (arrows) H&E (X100).

In this study, when rats were injected with acetaminophen (300 mg/kg BW) intra peritoneal [30], the liver showed multiple focal areas of coagulative necrosis of variable sizes infiltrated by mononuclear cells; some portal areas contain numerous bile ductules and fibrous tissue infiltrated with few lymphocytes and hyalinization in portal vein (Figure 1). This result is in agreement with Davidson and Eastham [31]. Hepatotoxicity of acetaminophen was reported in rodents treated with overdose in different studies [32,33]. Acute liver failure (ALF) causes both high morbidity and mortality rates (> 80%). Liver transplantation is the only viable approach to the treatment of ALF. This has significantly improved the survival in patients [34]. However, the major limitations of liver

transplantation are non-availability of the donor liver, major surgical approach, high cost and long-term immunosuppression [35]. Cell therapy by transplantation of hepatocytes or bone marrow cells is also attractive approach by stimulation of endogenous or exogenous regeneration by stem cells [36]. In the present study, BM-derived MSCs were isolated from adult rats and they were characterized in culture by their adhesiveness and fusiform shape (Figure 2). This was consistent with another study [36]. The possible therapeutic advantage of MSCs can only be understood through their homing proficiency to the obligatory site. The homing of MSCs in this study was noticed by PKH26 dye which was used to label MSCs [37,38].

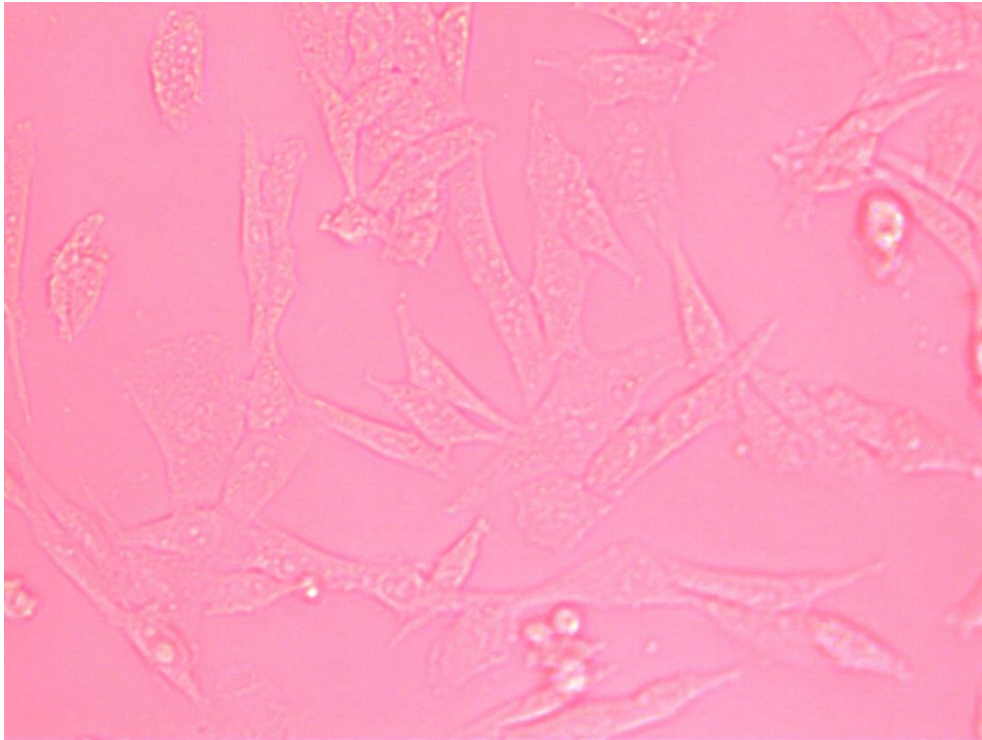


Figure 2: Dulbecco's modified Eagle's (DMEM) culture media change of 10 days age showing bone-marrow mesenchymal stem cells (BM-MSCs) of rat appeared as spindle shape cells using inverted microscope.

Munoz *et al.* [39] labeled bone marrow MSCs with PKH26 dye and engaged it as red fluorescence of MSCs. Identification of MSCs of antibody markers using molecular detection of the expression of the specific markers CD45 (leukocyte marker) and CD90 (mesenchymal marker) by RT-PCR was carried out (Figure 3). The results showed that MSCs were positive for CD90, and negative for CD45; these results indicated that relatively purified bone marrow- derived MSCs were isolated. This was in agreement with many investigators who isolated MSCs from bone marrow and

characterized it by different combination of CD markers. Wu *et al.* [40] found that BM-MSCs were typical of spindle and fibrocystic-like adherent monolayers with high CD90, CD29 positive rate and very low CD45 expression.

This study assessed the protective effects of infusion BM-derived MSCs to rats on the advancement of liver injuries using the experimental model of acetaminophen induced liver injuries in rats.

Table 1: The activities of some liver enzymes and MMP2, TIMP mRNA expression after two weeks of MSCs administration (mean values \pm SE)

Enzyme	Group 1 (Control)	Group 2 (acetaminophen)	Group 3 (Acetaminophen+ MSCs)
ALT	18.36 \pm 1.952 ^b	62.00 \pm 8.515 ^a	32.00 \pm 3.010 ^b
AST	14.80 \pm 1.908 ^c	71.80 \pm 5.928 ^a	38.75 \pm 3.250 ^b
ALP	124.7 \pm 4.024 ^b	372.9 \pm 18.559 ^a	187.8 \pm 9.966 ^b
Total proteins	8.120 \pm 0.317 ^a	4.682 \pm 0.401 ^c	7.266 \pm 0.333 ^b
Albumin	4.968 \pm 0.284 ^a	2.542 \pm 0.222 ^c	3.920 \pm 0.225 ^b
Direct bilirubin	0.280 \pm 0.025 ^b	0.556 \pm 0.0635 ^a	0.310 \pm 0.015 ^b
Indirect bilirubin	0.722 \pm 0.054 ^b	1.362 \pm 0.1684 ^a	0.862 \pm 0.054 ^b
MMP 2	1.006 \pm 0.014 ^c	11.88 \pm 0.800 ^a	3.805 \pm 0.946 ^b
TIMP	1.050 \pm 0.072 ^a	0.276 \pm 0.048 ^c	0.788 \pm 0.107 ^b

Mean carrying different super scripts within the same raw are significantly different at $P < 0.05$, based on Duncan's multiple comparisons and the highest value is letter (a). MMP2: matrix metalloproteinase, TIMP: tissue inhibitor metalloproteinase.

In this work, liver functions (serum ALT, AST, ALP, total proteins, albumin, total bilirubin, direct bilirubin and indirect bilirubin) were investigated in all groups (Table 1). Serum protein and albumin were decreased, while ALT, AST, ALP, bilirubin levels were increased in the injured groups. These results were in agreement with Roberts *et al.* [41] and Hu and Colletti [42] who reported significant increases in serum AST, ALT in mice after administration of acetaminophen. Prescott and Critchley [43] summarized the biochemical changes after an acetaminophen overdose to be a dramatic rise in serum ALT and AST activities, mild hyperbilirubinemia and raised prothrombin time [44].

In the present study, when rats were injected by MSCs next to injection of acetaminophen, the liver functions were enhanced. The total serum proteins and albumin levels were increased in the group of rats which received MSCs, while ALT, AST, ALP, direct and indirect bilirubin were decreased (Table 1). These findings were consistent with the results reported by El-Khayat *et al.* [45] who showed that MSCs management have significant enhancement in liver functions of the treated group compared to the fiber-optic group. Jang *et al.* [46] found that liver function of mice received the

mesenchymal stromal stem cells were recovered as early as two days after transplantation of MSCs.

Transplantation of bone marrow stromal stem cells enhances the ability of hepatocytes *in vivo* and *in vitro* as they enhance the viability and phenotypes (maintenance of hepatocytes) where the BMSc act as feeders for the liver cells [47]. Also, transplantation of bone marrow mesenchymal stromal stem cells can distinguish into hepatocytes, reestablish the plasma albumin level, and overwhelm transaminase activity and liver fibrosis in trial model of liver injury [38,48]. The improvements of liver function are explained by the fact that the MSCs lower the fibrosis and allow the repopulation of the liver with normal hepatocytes [49].

This study showed that transplantation of mesenchymal stromal stem cells decreased AST, ALT and prothrombin time to level observed in control rats (Table 1). This was comparable with other studies [30, 49-50].

The degree of fibrosis was monitored in the liver tissue by detection of MMP2 and TIMP (fibrous gene expression). MSCs have important antifibrotic influence as indicated by the decrease in MMP2 and increases TIMP (Table 1).

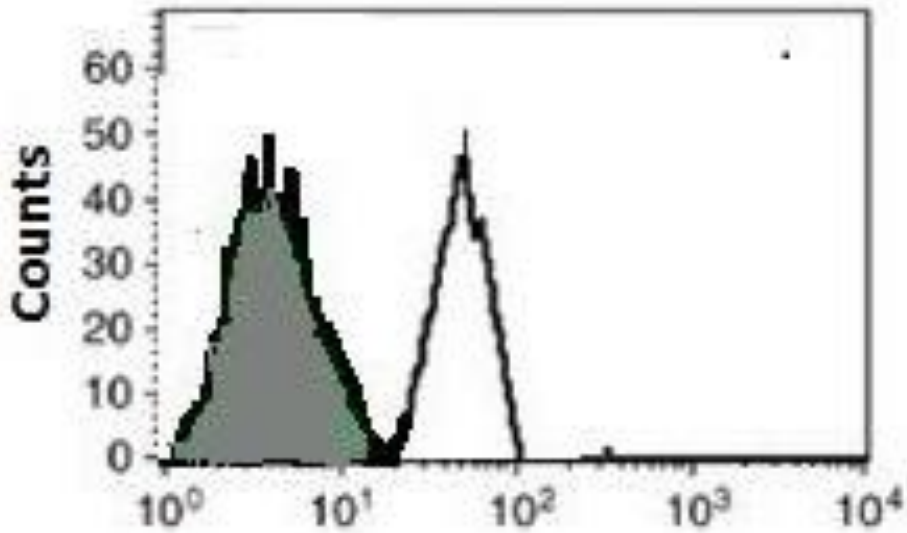


Figure 3: Flow Cytometer analysis of BM-MSCs surface markers Cells were positive to CD90.

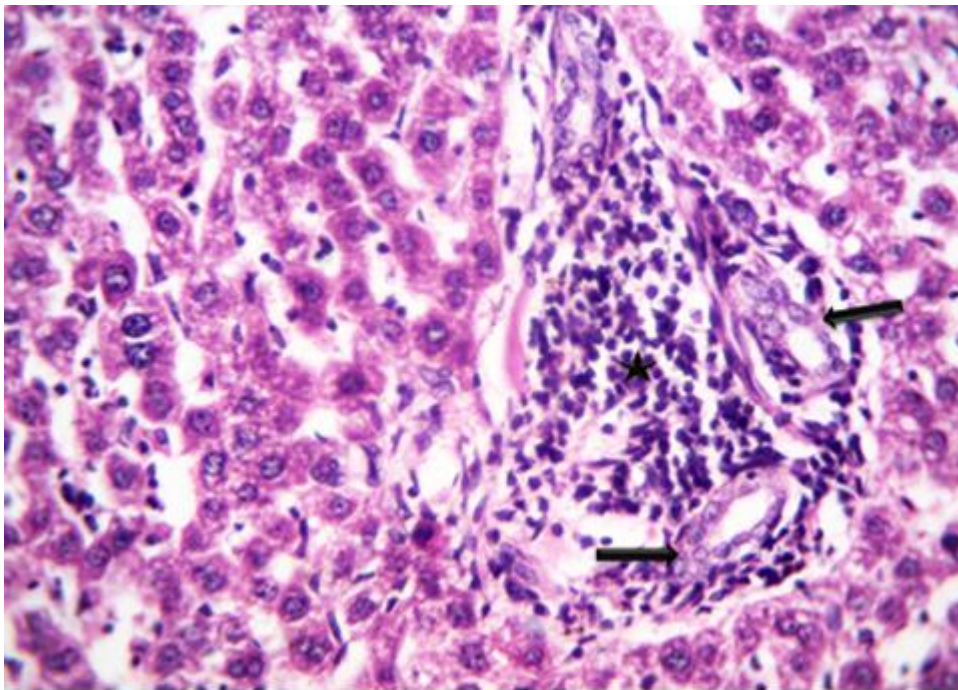


Figure 4: Liver of treated rat (Acetaminophen+MSCs) showing portal area infiltrated by lymphocytes (star) and numerous bile ductules (arrows) beside normal hepatic cords. H&E (X400).

Matrix-degrading metalloproteinase enzyme and specific matrix degrading metalloproteinase inhibition [51,52] may reveal that the distillation of BM derived MSCs may have a protective influence in contradiction of liver fibrosis. This is in agreement with the result reported by Salomon *et al.* [30] who showed that mesenchymal stromal stem cell transplantation is effective in treating acetaminophen liver injury by enhancing hepatocyte regeneration and

inhibiting liver stress and inflammatory signaling. The accumulation of ECM proteins interferes with the hepatic architecture by creating fibrous scar. Accretion of ECM is originated from both increased synthesis and decreased degradation [53]. The reduction in ECM activity removing factors, e.g. Matrix Metalloproteinase (MMP) are mainly due to over expression of their specific inhibitor such as tissue inhibitor of metalloproteinase (TIMP).

Liver pathology serves as an important tool for identifying and characterizing liver injury. The main pattern of liver injury through hepatotoxicity may contain zonal necrosis, hepatitis, cholestasis, granuloma, steatosis, vascular lesion [54]. In this study, samples were taken from the liver of rats of different groups. Histological examination of liver tissue of the control group indicated normal hepatic parenchyma, while in the acetaminophen group, liver showed multiple focal area of coagulated necrosis infiltrated by mononuclear cells scattered in the hepatic parenchyma, as reported by Chandrasekaran *et al.* [55]. Other hepatic cells suffered from different types of cell injuries or accumulation mainly microsteatosis with dilated hepatic sinusoids and pressure atrophy of cords compared with the control group. Some portal areas showed fibrous tissue infiltrated by lymphocytes and hyalinization of the portal vein. While, liver investigation of the treated group with BM mesenchymal stromal stem cells showed improvement in the hepatic structure compared with the control group which had normal hepatic cord and few portal areas of fibrous tissue infiltrated with lymphocytes, as shown in Figure (4). These results were comparable with Ries *et al.* [56] who found that BM MSCs has a significant antifibrotic effect. Also, liver section of intoxicated rat with acetaminophen showed lobular necrosis and diffuse vascular degeneration, whereas, in rat transplanted with mesenchymal stromal stem cells, liver injury was almost absent. Stock *et al.* [57] showed that MSCs reduced the inflammatory response and support liver regeneration following acute liver injury induced by acetaminophen. Therefore, MSCs may serve as a novel source of the hepatocytes and are suitable for the cell therapy of acute liver diseases.

Conclusion

From the current study, it could be concluded that transplantation of BM-MSCs is effective in treating acetaminophen liver injury by enhancing hepatocyte regeneration, inhibiting liver stress and inflammatory signaling by improving matrix metalloproteinase (MMP2) and tissue inhibitor metalloproteinase (TIMP). The MSCs stem cells is an attractive cell source for regenerative

medicine. Further studies are needed to apply the effect of stem cells in liver disease.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

دراسات كيميائية حيوية جزيئية على الخلايا الجذعية للنخاع العظمي لمعالجة اضرار الكبد بالفئران

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يعتبر الكبد من الأعضاء الحيوية في الجسم ذلك لما يؤديه من عمليات حيوية عديدة لها دور هام في عملية التمثيل الغذائي وكذلك التخلص من السموم والأدوية بالدم لذلك يعتبر الكبد مصفى للدم ومنظم لمستويات بلازما الدم ، الجلوكوز ، الأمونيا ، الكلوستيرول وله كذلك وظائف أخرى عديدة. تعتبر أمراض الكبد واحدة من أخطر المشاكل الصحية التي تواجه الانسان. وتعتبر الكيماويات السامة سبب من الأسباب الرئيسية لأمراض الكبد ومن أمثلتها (المضادات الحيوية ، الباراسيتامول ، رابع كلوريد الكربون) وفيروس سى والاسراف في تناول الكحوليات أيضا من الأسباب الرئيسية لتليف الكبد وقصوره في أداء وظائفه وتتنوع الاصابة حسب درجة التعرض لهذه السموم. حالياً تعتبر عملية زراعة الكبد الحل الأمثل لعلاج أمراض الكبد ، لكن تواجهها بعض الصعوبات منها قلة عدد المتبرعين ، التكلفة العالية ، وعدم القبول في بعض الأحيان من المنقول إليهم الكبد. لذا وجب البحث عن حل آخر لعلاج أمراض الكبد ، ففي السنوات الأخيرة أصبح العلاج باستخدام الخلايا الجذعية هو البديل لزراعة الكبد. لذا صممت هذه التجربة لمحاولة فصل الخلايا الجذعية من النخاع العظمي وحقتها في الفئران لمعرفة تأثيرها العلاجي ضد السمية الكبدية المحدثة بالاسيتومينوفين. تم تخصيص عدد ٣٠ من الجرزان البيضاء تتراوح أوزانها ١٣٠-١٧٠ جرام. تم تقسيمهم عشوائيا الى ثلاث مجموعات متساوية على النحو التالي: المجموعة الأولى: مكونة من ١٠ جرزان استخدمت كمجموعة ضابطة. المجموعة الثانية: مكونة من ١٠ جرزان تم حقنهم في الغشاء البريتوني بالاسيتومينوفين بجرعة ٣٠٠مجم/كجم. المجموعة الثالثة: مكونة من ١٠ جرزان تم حقنهم في الغشاء البريتوني بالاسيتومينوفين بجرعة ٣٠٠مجم/كجم ثم تم حقنهم بالوريد الذيلي بالخلايا الجذعية بجرعة واحدة (١ مليون خلية/كجم). بعد حقن الخلايا الجذعية بأسبوعين تم تجميع عينات الدم من الثلاث مجموعات وفصلها لإجراء التحاليل المختلفة لها. كذلك تم أخذ نسيج الكبد وقطعه الى ثلاثة اجزاء . جزء للتأكد من وصول الخلايا الجذعية المصبوغة الى الكبد، جزء تم اجراء بعض التحاليل الجينية له وقياس MMP2,TIMP والجزء الاخر للفحص الهيستوباثولوجي . وقد أظهرت النتائج التالي: ١- حدوث تلف في نسيج الكبد للمجموعة الثانية والتي حقنت بالاسيتومينوفين وخلل في نتائج التحاليل المختلفة لهذه المجموعة مقارنة بالمجموعة الضابطة. ٢- تحسن كبير وملحوظ في نسيج الكبد في المجموعة الثالثة التي تم معالجتها بالخلايا الجذعية ، كذلك تحسن في نتائج التحاليل المختلفة لهذه المجموعة الى ما يقارب المجموعة الضابطة.