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The regenerative effect of stem cells on acetaminophen-induced hepatotoxicity in male albino rats

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Abstract

Background: Acetaminophen overdose is the leading cause of acute liver injury (ALI) and acute liver failure (ALF) in the developed world. We aimed at studying the therapeutic potential of bone marrow mesenchymal stem cells (MSCs) in accelerating healing of acetaminophen-induced hepatotoxicity.

Results: This prospective study included 50 male albino rats divided into 2 groups: hepatotoxic group and non-hepatotoxic group. Hepatotoxicity was induced in experimental rats by acetaminophen and then stem cells were transplanted into the rats and their effects on the liver cells were assessed. After injection of BM MSCs, the cells reached the targeted tissues. They were established in the central veins and blood sinusoids in the liver tissue. The hepatotoxic liver showed degeneration and loss of normal hepatic architecture as well as necrotic areas and congestion mainly in the portal tract vessels, dilation of blood sinusoids, and infiltration by inflammatory cells around the central veins. In addition, there were abnormal nuclei either irregular in shape or showing loss of open face compared to the normal control group. The liver tissue in BM MSC-treated group showed restoration of normal architecture of the liver tissue.

Conclusion: Administration of MSCs has hepato-therapeutic effect on acetaminophen-induced hepatotoxicity in rats. The mechanism of this hepatoprotective effect may be through anti-inflammatory, anti-apoptotic, and immunomodulatory actions of MSCs.

Keywords: Acute liver failure, Stem cells, Acetaminophen-induced hepatotoxicity

Background

Acute liver failure (ALF) is a complex and often catastrophic illness that usually occurs due to rapid decline of liver functions in a patient without preexisting liver disease [1]. Arterial hypotension, coagulopathy, renal failure, sepsis, acute lung injury, encephalopathy, brain edema, hemorrhagic tendency, and end-organ dysfunction are all frequent complications of ALF [2].

Hepatic injury and the resulted hepatic failure due to acetaminophen overdose has affected patients for decades. The pathophysiology, course of the disease, and management of acute liver failure due to acetaminophen toxicity remain to be precisely elucidated, and adverse outcomes with increased morbidity and mortality continue to occur [3].

N-acetylcysteine (NAC) therapy is considered as the mainstay therapy, but liver transplantation (LT) might represent a life-saving procedure for selected patients. However, serious problems are associated with LTx: lack of donors, surgical complications, rejection, and high cost. Regenerative therapies have the potential to

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provide minimally invasive procedures with few complications [4].

Stem cells (SCs) field of research, named "regenerative medicine," has emerged rapidly with huge interest among scientists and clinicians [5]. Stem cell therapy can be mediated by a direct contribution to the functional hepatocyte population with embryonic, induced pluripotent, or adult stem cells (ASCs) or by promotion of endogenous regenerative processes through the bone marrow (BM)-derived SCs [6]. SCs are unspecialized cells with an extraordinary ability to self-renew, capable of differentiating into one or more specialized cell types playing a crucial role in homeostasis and tissue repair. Based on the SC origin, SCs are categorized as embryonic stem cells or as postnatal SCs/ASCs/somatic SCs [7].

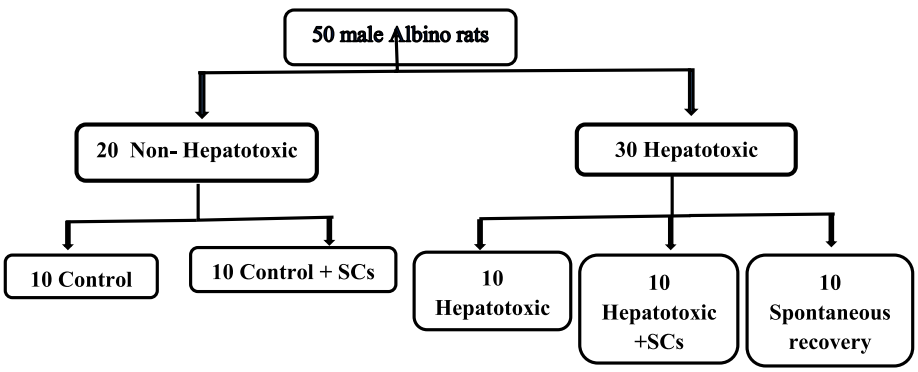
Mesenchymal SCs (MSCs) have been isolated from many adult and fetal tissues, including BM, adipose tissue, amniotic fluid, placenta, umbilical cord blood, and breast milk. Particularly, BM-derived stromal cells (BMSCs) which have superior multipotency [8]. After transplantation of SC, it will home in the liver and various organ tissues after approximately 16 days, and regeneration effect takes place within days [9].

The aim of the current work was to study the therapeutic potential of BM MSCs in accelerating healing of acetaminophen-induced hepatotoxicity as well as trying to elucidate the possible underlying mechanisms leading to this, monitored by histological and immunohistochemical methods in adult male albino rats.

Methods

This prospective study included 50 male albino rats of matched weight (150–200 g). Experiments on animals were performed in the Animal House of Faculty of Medicine, Al-Azhar University. A written informed consent was obtained to use the animals in our study from the legal autorities in the faculty. Animal procedures were carried out in accordance to the National Institute of Health guidence for care and use of the laboratory animals (NIH Publication No. 85-23, revised 1996). Rats were housed in cages maintained in a 12-h light/dark cycle at 22 ± 2 °C. The rats had received tap water and a standard rat chow. An adaptation period of 1 week was allowed. Experiments on animals were performed at the Animal House of Faculty of Medicine, Al-Azhar University, according to the standard guidelines for care of laboratory animals. The idea of this research was based on inducing hepatotoxicity in experimental rats and then transplanting stem cells into the rats to see their effect on the liver damage.

The animals were randomly selected and divided into the following groups: *Non hepatotoxic* (*n*:20): normal control (*n*:10) and SC control (*n*:10). (1) Normal control—ten rats: each received 5ml of distilled water by an oral gastric tube and were sacrificed at the same time as the corresponding pathological control group after 12–24 h according to Shahid and Subhan, 2014 [10]. (2) SC control—another ten rats were injected intravenously with stem cell culture media and PKH26-labeled MSCs, and sacrificed after 15 days as SCs reach the organs after transplantation around this time [11]. *Hepatotoxic* (*n*:30): pathological control (*n*:10), spontaneous recovery (SR) (*n*:10), and SC-treated group (*n*:10). (1) Pathological control—ten rats in which hepatotoxicity was induced by acetaminophen (Paracetamol drops and acetaminophen powder from Arab Drug Comp) (800mg/kg body weight, intra-peritoneal (I.P) according to many protocols [10], and were sacrificed at the same time as the control subgroup (12–14 h). (2) Spontaneous recovery—ten rats in which hepatotoxicity was induced by acetaminophen and left for SR, these rats were sacrificed at the same time as stem cell and hepatotoxic subgroup. (3) SC-treated group—ten rats were given SCs labeled by PKH26 dye once I.V. in the caudal vein within 24 h of induction of hepatotoxicity. This group was sacrificed within 15 days after investigating liver function test by sampling from rat tail vein.



ALT, AST, and INR were done for all rats before induction of hepatotoxicity and before sacrification.

Isolation, propagation, identification, and labeling of BM-derived MSCs

(A) Isolation and propagation of BM-derived MSCs; BM was obtained from the long bones of 6-week-old male white albino rat (150–200 g) after sacrification by cervical dislocation, under sterile conditions. The femurs and tibiae of rats were excised, and all connective tissues attached to the bones were removed gently. BM was harvested by flushing the tibiae and femurs with DMEM, centrifuged 1000 rounds per minute (RPM) for 15 min, and washed by PBS three times, and nucleated cells were collected with a density gradient cell separation technique using Ficoll/Paque, then suspended in complete medium composed of DMEM supplemented with 10% fetal bovine serum and 1% Penicillin G/streptomycin.

Cells were plated in T75 cell culture flasks at a seeding density of 7.5×10^6 viable cells/cm^2 and incubated at 37°C in 5% humidified CO_2 for 12–14 days as a primary culture or upon large colony formation. When large colonies developed (80–90% confluence), cultures were washed twice with PBS and cells were trypsinized with 0.25%trypsin in 1ml ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C. After centrifugation for 20 min at 2400 RPM, cells were resuspended in serum-supplemented medium and incubated in culture flasks. The resulting cultures were named as first-passage cultures. On day 14, adherent colonies of cells were trypsinized, isolated, and observed by phase contrast microscope to be transformed to flowcytometry step [12].

Morphological identification of BM-derived MSCs

The morphology of the cultures was studied by optic and phase contrast (inverted) microscopy. MSCs in culture were characterized by their fusiform shape and adhesiveness.

Twenty-four hours after the primary culture (passage 0 = P0) of bone marrow-derived mesenchymal stem cells, the cultured cells appeared crowded and suspended. They were variable in size and shape. Most of the cells appeared rounded. After 3 days of the primary culture, the native MSCs became attached to the culture flasks sparsely and sporadically. Some MSCs appeared as spindle-shaped and some began to form processes.

The hematopoietic stem cells were not attached to the culture flasks, remained suspended, and appeared rounded and refractive. In contrast, MSCs were arranged in the form of small colonies. They were of variable shapes (spindle, star, and triangular) with many processes, granular cytoplasms, and rounded vesicular nuclei.

Flowcytometry identification of cells

This step was done at the Flowcytometry Unit, Department of Clinical Pathology, Faculty of Medicine (Kasr al-Aini, Medical School), Cairo University. The MSCs were washed and re-suspended in phosphate-buffered saline. All monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added directly to the cells and were kept for 1 h in 4°C. The cells were then incubated with anti-mouse immunoglobulin G fluorescein-conjugated secondary antibody (Millipore Corp, Temecula, CA) for 45 min in ice. Cell suspensions were washed twice and analyzed on a fluorescence-activated cell sorting (FACS) caliber flow cytometer. Cluster of differentiation (CD) 44, CD90, and CD105 (+ve markers for MSCs) in addition to CD 34 and CD45 (−ve markers for MSCs) were analyzed.

Labeling of SCs with PKH26 dye

MSCs were harvested during the 4th passage and were labeled with PKH26 fluorescent linker dye (final concentrations of 2×10^6 M PKH26 dye and 1×10^7 cells/ml in a 2-ml total volume was stained according to Sigma protocol steps). PKH26 is a red fluorochrome, with 567 nm emission and 551 nm excitation. The linkers are stable physiologically and show no to little toxic side-effects to the cell. Labeled cells preserve both biological and proliferating activity, and are suitable for in vitro proliferation studies, and in vivo cell tracking. When the cells divide, the dye is stable and will divide equally. After staining with PKH dye, one can observe as many as 8 divisions depending on how brightly the cells were stained initially and the amount of the dye on the surface area of the cells. Most commonly, 4–6 divisions can be visualized.

Three millions of undifferentiated MSCs were injected intravenously into rat tail vein

Detection of homing of injected cells in rat liver tissue After 15 days, liver tissues were examined with a fluorescent microscope to detect the cells stained with PKH26 dye to ensure homing and to trace the injected cells in the liver tissues.

Liver tissues were harvested for histopathological study The following investigations were performed: histopathological assessment of rats' liver with the following techniques: The animals were killed by cervical dislocation, and the liver from each animal was removed and fixed immediately in 10% neutral-buffered formalin.

The tissues were dehydrated in graded ethanol solutions (50, 70, 80, 90, two changes each of 100 %), cleared in 100% xylene, and infiltrated and embedded in paraffin wax. Tissue blocks were sectioned at 4 μm on a rotary microtome and were stained with Harris hematoxylin and eosin (H & E) for microscopic observation. Assessment of the fibrosis stage was done according to Ishak fibrosis staging scale [13].

Immunohistochemical study of cyclooxygenase (COX)-2 in rats' liver COX-2 was detected in paraffin-embedded liver sections using specific antibodies and an avidin–biotin complex immunoperoxidase method (Santa-Cruz Biotechnology). Endogenous peroxidase activities were blocked through treating the sections with 3% hydrogen peroxide. After blocking with 10% nonimmunized goat serum, the primary goat antiserum was applied and incubated for 2 h at room temperature for COX-2 detection (1:150 dilution). Primary antibodies were omitted, and nonimmunized goat serum was used for negative controls. Avidin–biotin complex and horseradish peroxidase (Dako A/S, Glostrup, Denmark) were applied after rinsing the biotinylated secondary antibodies. Peroxidase activities were visualized thrpogh applying diaminobenzidine to the sections, which then were counterstained by hematoxylin.

Statistical analysis

Data were entered, coded, and analyzed using SPSS, version 18.0 (SPSS Inc., Chicago, Illinois, USA). Data were entered as numerical or categorical, as appropriate. Descriptive results were expressed as mean ± standard deviation (mean ± SD) or number and percentage. For quantitative data, significance between 2 groups was tested either by independent sample t-test or Mann–Whitney *U* test according to the nature of the data. ANOVA test and Kruskal-Walis test were used for comparing more than 2 sets of parametric and non-parametric quantitative data, respectively. The significance between qualitative and categorical data was assessed by the χ^2 test or Fisher's exact test. Results were considered significant when P value < 0.05.

Results

Seven days from the primary culture, the MSCs of cultured flasks proliferated, exhibited different shapes with well-developed cytoplasmic processes, granular cytoplasms, and vesicular nuclei. Twelve days from the primary culture, the adherent cells reached 70–90% confluency and appeared triangular, star-shaped, and spindle-shaped. Before injection of BM MSCs, they were labeled with PKH26 dye (Fig. 1).

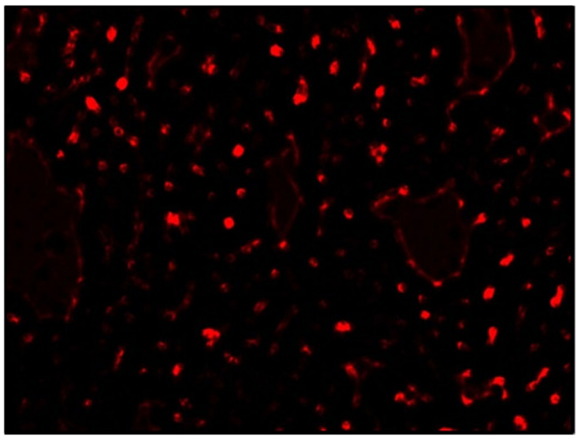


Fig. 1 Florescent micrograph of labeling stem cells. The stem cells appeared shiny and red under florescent microscope after labeling with PKH26 dye referred to by yellow arrow (×100)

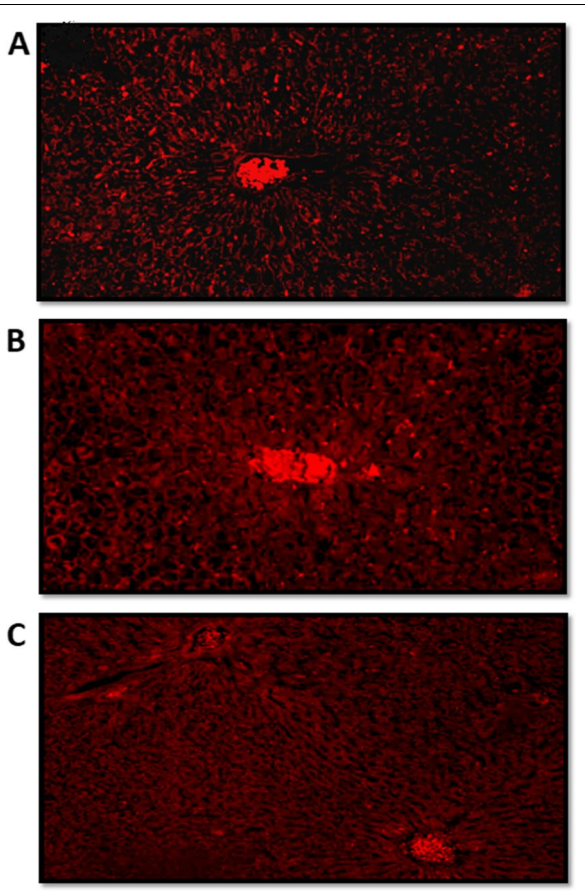


Fig. 2 Fluorescence microscopic study of the liver showing MSCs labeled with PKH26 fluorescent dye in and around central vein in **A** and **B** and central vein adjacent portal tract **C**; confirming that cells can homing into the liver tissue (×100)

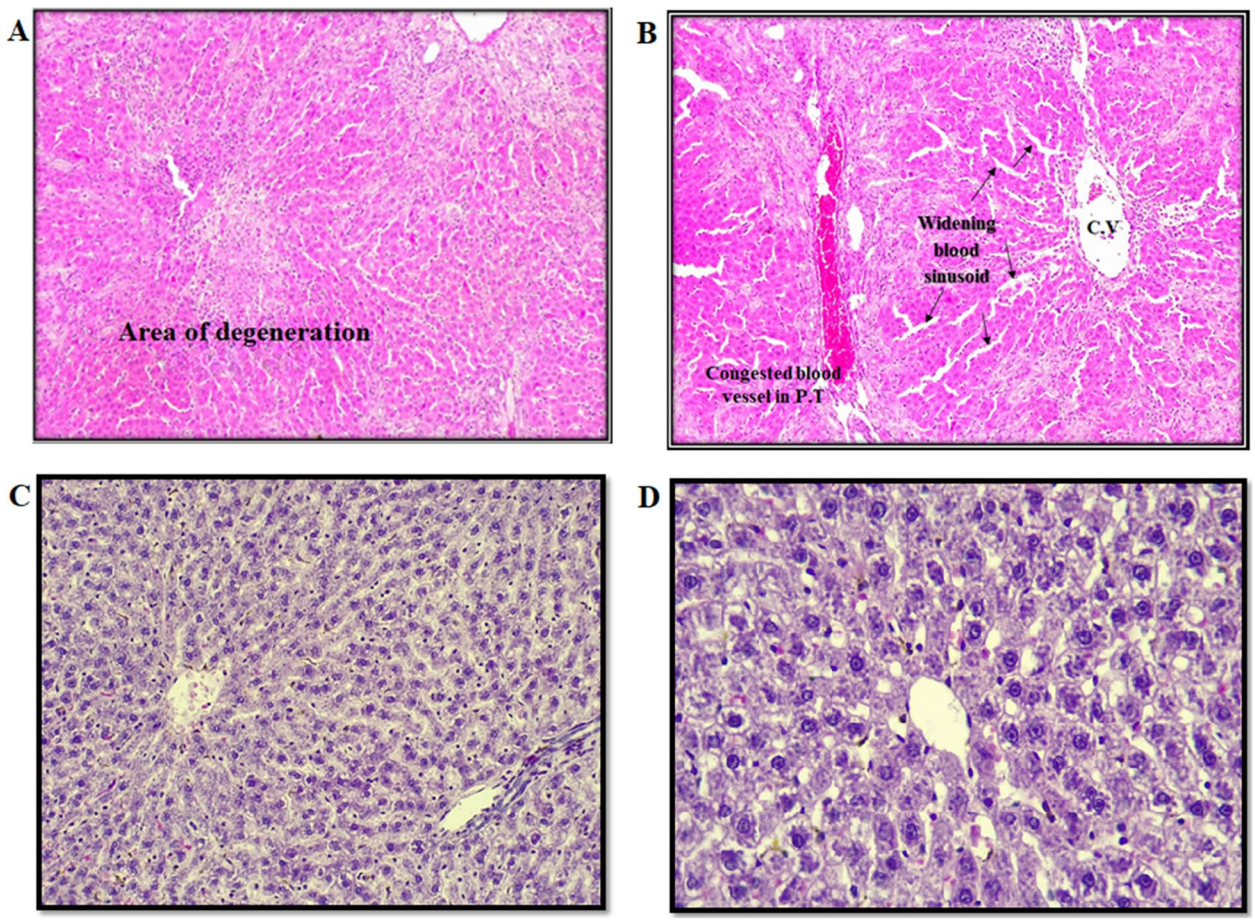


Fig. 3 **A**, **B** The liver tissue of the pathological control group showed area of degeneration and loss of hepatic architecture with irregular shaped nuclei. Widening of central vein and blood sinusoid with marked congestion of the blood vessel in portal area. **C, D** The liver tissue in (SC-treated group) showed more or less normal central vein, restoration of normal hepatic architecture with radiating regenerated cords of hepatocytes, and reappearence of open face nucleus and normal blood sinusoids but still there is presence of infiltration by inflammatory cells

MSCs homing in the liver tissue

After injection of BM MSCs, the cells reached the targeted tissues. They settled in the central vein and blood sinusoids in the liver tissue (Fig. 2).

On routine hematoxylin & eosin (H & E) staining technique, the hepatotoxic liver tissue showed degeneration and loss of normal hepatic architecture in some areas as well as necrotic areas and congestion mainly in the portal tract vessels, dilation of blood sinusoids, and infiltration by inflammatory cells. In addition, there were abnormal nuclei either irregular in shape or losing the open face, Fig. 3A, B compared to the normal control group. The liver tissue in BM MSC-treated group showed restoration of normal architecture of the liver tissue, Figs. 2D and 3C.

The percentage of fibrosis was higher in the pathological group than the normal control, MSC-treated group, and SR group. In addition, the fibrosis percentage was lower in the MSC-treated group than the SR group (Fig. 4).

In the normal control group, COX-2 immunohistochemical study showed normal distribution in the liver tissue. In the acetaminophen-induced hepatotoxicity group, the reaction and distribution of COX-2 were increased. In the hepatotoxic MSC-treated group, COX-2 reaction and level appeared near normal. The SR group showed slight decrease immunostaining reaction of COX-2 (Fig. 5).

On comparing the LFTs between groups, ALT, AST, and INR were significantly lower in the SC-treated group than the SR and pathological control groups (P value <0.001), although it was still significantly higher in the SC-treated group than the normal control and SC control groups (P value <0.001). In addition, ALT, AST, and INR of the pathological control group were significantly higher than the other groups (P value <0.001) (Table 1).

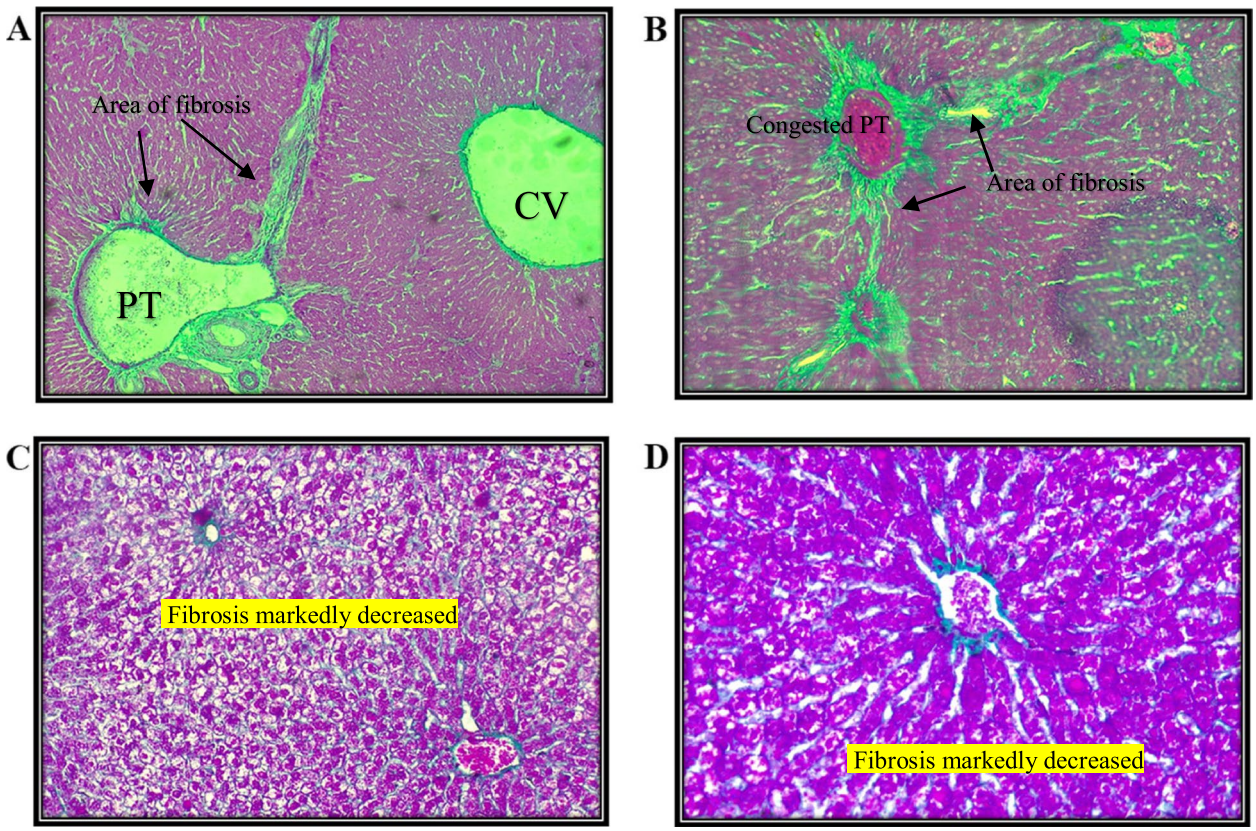


Fig. 4 The liver tissue of pathological control (Masson trichrome stain) showed **A**, **B** deposition of collagen fibers around portal area (PT) area and around central vein (cv) with extension to PT. **C, D** A photomicrograph of a section in BM-MSC-treated rat liver showing marked decrease in deposition of collagen fibers

On analyzing the results of liver function tests, pre- and post-treatment in the SC-treated group and in the SR group pre- and post-recovery, the rate of improvement was significantly higher in the SC-treated group compared to those who were left to recover spontaneously (P value < 0.05).

On assessing the histopathological findings in the studied groups, there was a statistically significant difference between the studied groups regarding the centrilobular fibrosis, portal fibrosis, hemorrhage, and areas of necrosis (P value < 0.05) (Table 2). Centrilobular fibrosis and hemorrage were present in 100% of the pathological control group. The portal fibrosis was more prevelant in the spontenous recovery group (80%). The areas of necrosis was higher in the pathological control group (80%).

Discussion

Acute liver failure is a rare but life-threatening critical illness that presents a unique challenge in clinical management. Acute hepatotoxicity has many etiologies, one of them is induction by drugs as acetaminophen, particularly in children, and it is considered the most common drug causing ALF [3]. Acetaminophen is metabolized in hepatocytes by cytochrome P450 to the reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI). After therapeutic dose, the glutathione (GSH) detoxifies NAPQI efficiently; however, in overdosage, the total hepatic GSH will be depleted. Under this condition, NAPQI reacts with available sulfhydryl group on proteins to form 3 cysteinyl acetaminophen—acetaminophen APAP-Cys protein which correlates with hepatic toxicity [14].

Liver transplantation is considered the main effective management in severe liver injuries. However, because of the hazards, difficulties, and complications, alternative strategies are urgently needed. Human hepatocytes are commercially available; however, maintaining them in vitro culture is very difficult [15].

In the previous years, an extrahepatic cell populations which potentially impact the liver diseases were discovered. The potential candidate SCs for therapy of an injured liver are MSCs, based on the idea of scientific research saying that SCs can differentiate into liver SCs

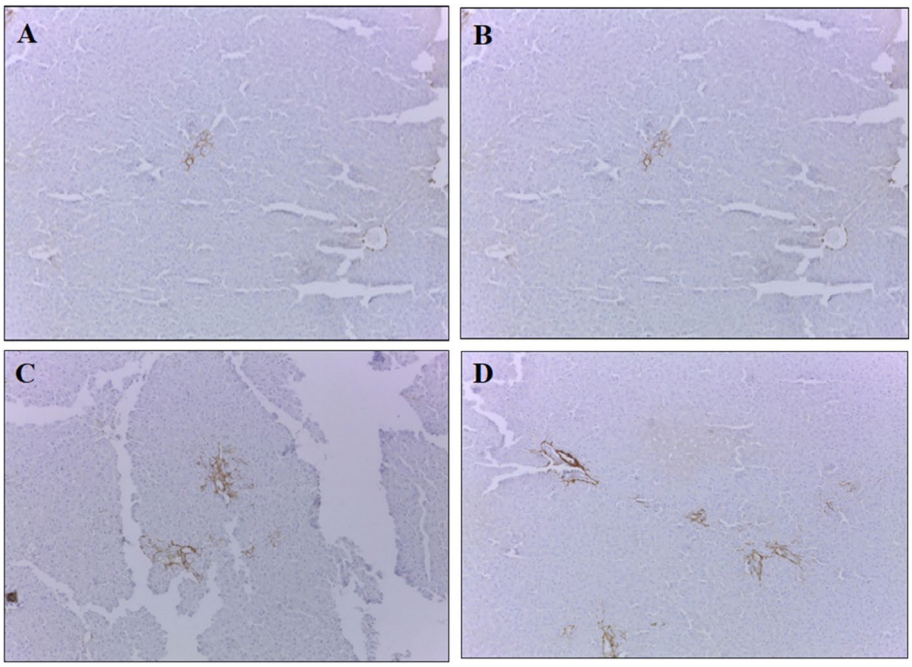


Fig. 5 Immunohistochemical study of COX-2 in the liver tissue of the control group (**A**), hepatotoxic group (**B**), BM-MSC-treated group (**C**), and spontaneous recovery group (**D**) (magnification ×40)

Table 1 Estimation of serum ALT, AST, and INR levels among studied groups

	Normal control *n*=10	Pathological control *n*=10	SC control *n*=10	SC-treated group *n*=10	Spontaneous recovery group *n*=10	*P* value
ALT (U/L)	10.18±2.4	253.2±47.6	18.7±7.6	64.4±25.2	166.4±29.5	<0.001
AST (U/L)	11.38±3.1	344.8±61.3	16.87±7.2	78.5±26.9	244.5±33.4	<0.001
INR	0.62±0.09	2.89±0.6	0.655±0.11	1.8±0.45	2.38±0.36	<0.001

Table 2 Comparison between the studied groups regarding their histopathology

Histopathology	Normal control *n*=10	Pathological control *n*=10	SC control *n*=10	SC-treated group *n*=10	Spontaneous recovery group *n*=10	Fisher's exact test	*P* value
Centrilobular fibrosis:							
Yes	0 (0%)	10 (100%)	0 (0%)	2 (20%)	6 (60%)	32.15	<0.001
No	10 (100%)	0 (0%)	10 (100%)	8 (80%)	4 (40%)		
Portal fibrosis							
Yes	0 (0%)	6 (60%)	0 (0%)	5 (50%)	8 (80%)	22.01	<0.001
No	10 (100%)	4 (40%)	10 (100%)	5 (50%)	2 (20%)		
Hemorrhage							
Yes	0 (0%)	10 (100%)	0 (0%)	1 (10%)	8 (80%)	39.21	0.001
No	10 (100%)	0 (0%)	10 (100%)	9 (90%)	2 (20%)		
Areas of necrosis							
Yes	0 (0%)	8 (80%)	0 (0%)	0 (0%)	6 (60%)	30.153	<0.001
No	10 (100%)	2 (20%)	10 (100%)	10 (100%)	4 (40%)		

and liver cells under certain circumstances resulting in repair of liver injury and liver reconstruction [16].

This research adopted the idea of the ability of transplanted SCs to home at the site of the injury to begin their action as soon as they arrive. This was confirmed by Yagi et al. who found in their research that increased inflammatory chemokine concentration at the site of inflammation is a major factor causing MSCs to preferentially migrate to these sites [17].

In this work, we used MSCs due to their multipotent character, being easily isolated from different tissues and easily expand in vitro, as well as their wide range of therapeutic potential in clinical trials. The transplanted MSCs were isolated from completely different rats than those used in the experimental groups; however, no immune reaction or sings of rejection were noticed. This may be attributed to the inability of SCs as precursor cells to give an antigenic immune reaction [17].

In our study, the results elucidated the regenerative effect of MSCs on the livers of (SC treated group) when it was compared to (SR group) not only at the level of histopathological examination where the shape and arrangement of cells were improved as well as the congestion of the blood vessels were reduced, but also, at the level of biochemical investigations (ALT, AST, INR) in which liver functions regained nearly their normal values.

In addition, the protective effect of MSCs appeared with the decreased reaction and level of COX-2 in the treated animals which appeared near normal, while in acetaminophen-induced hepatotoxicity group, the reaction and distribution of COX-2 were increased, and in the spontaneous recovery group, there was slight decrease in immunostaining reaction of COX-2.

Cyclooxygenase-1 and -2 catalyze the first step in the biosynthesis of prostanoids. COX-1 is expressed in many tissues. It seems to be involved in prostanoids' housekeeping function. The inducible isoform of COX-2 increases prostaglandin levels in response to different inflammatory stimuli, growth factors, and hormones. In addition, COX-2 expression was associated with regulation of cell growth, tissue remodeling, and carcinogenesis. The expression of COX-2 under response to pro-inflammatory challenges is restricted to non-hepatocyte cell population. However, under chronic pro-inflammatory condition, hepatocytes express this isoenzyme and the contribution of the increased synthesis of prostanoids to liver pathology is a current subject of research [18].

Animals deficient of COX-2 exhibited full recovery of the liver function and mass after partial hepatectomy but with delayed proliferation. However, the simultaneous absence of COX-2 and other genes relevant for liver regeneration, such as nitric oxide synthase-2 resulted in an impaired liver mass recovery after partial hepatectomy leading to animal death [18].

Meanwhile, SCs do no harming effect if they are injected in a normal body, this was illustrated when we injected MSCs into the stem cell—control group; the values of liver function tests (ALT, AST, INR) did not change in this group, and the *P* values were insignificant between the normal control group and this group—these results in turn are confirming that any changes in the lab investigations and histopathological changes after stem cell transplantation were due to the therapeutic effects of SCs.

Additional confirmatory study was done, when we compared the results of liver function tests pre- and post-treatment in the SC-treated group and in the SR group pre- and post-recovery, where the results revealed high significance of the *P* values in the SC-treated group compared to those who were left to recover spontaneously.

The mechanism of therapeutic potential of MSCs is intricated and not completely understood. It is believed that the unique ability to differentiate is not the only characteristic feature that makes these cells attractive for therapeutic purposes, but also the secretion of a broad range of bioactive molecules, such as growth factors, cytokines, and chemokines, that have biologically significant role under injury conditions; this makes the properties of MSCs in vivo an issue of therapeutic concern [19].

The concept of MSCs discussed above has been broadened to include the secretion of biologically active molecules that exert beneficial effects on other cells. This shifts the paradigm centered on the differentiation of MSCs to a view in which MSCs can exert a therapeutic effect even if they do not engraft or differentiate into tissue-specific cells. MSC paracrine effects can be divided into trophic "nurturing," immunomodulatory, anti-scarring, and chemoattractant, this significantly increases the range of MSC therapeutic applications.

Conclusion

Administration of MSCs has hepato-therapeutic effect on acetaminophen-induced hepatotoxicity in rats. The mechanism of this hepatoprotective effect may be through anti-inflammatory, anti-apoptotic, and immunomodulatory actions of MSCs.

Abbreviations

ALF: Acute liver failure; ALI: Acute liver injury; ASCs: Adult stem cells; BM: Bone marrow; BMSCs: Bone marrow-derived stromal cells; MSCs: Bone marrow mesenchymal stem cells; COX: Cyclooxygenase; EDTA: Ethylenediaminetetraacetic acid; FACS: Fluorescence-activated cell sorting; GSH: Glutathione;

I.P: Intra peritoneal; LT: Liver transplantation; MSCs: Mesenchymal SCs; NAC: *N*-acetylcysteine; NAPQI: *N*-acetyl-p-benzoquinone imine; RPM: Rounds per minute; SR: Spontaneous recovery; SCs: Stem cells.

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Authors' contributions
SA and AF performed the experiments on animals and the histological examination. HA, MA, SA, AF, HM, and GA collected, analyzed, and interpreted the data and wrote the manuscript. The authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee of the Al-Azhar University. The committee's reference number is not available.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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