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Bone marrow derived mesenchymal stem cells suppress diethylnitrosamine induced liver cirrhosis in rats

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ABSTRACT

Aim: The present study was designed to explore the ability of bone marrow derived mesenchymal stem cells (BM-MSCs) to repair diethylnitrosamine (DEN)-induced liver cirrhosis (DILC) in rats. **Materials and methods**: Sixty male rats were divided into four groups (15 rats per group), the rats of the first group received saline, and the rats of the other three groups received DEN in drinking water. After thirty days, the rats of the third and fourth groups received $1.5x10^6$ and $2x10^6$ MSC infusions ,respectively. Liver function tests were estimated in serum of rats in all groups. The expression of albumin (Alb) and cytokeratin-18 (CK-18) genes were detected by reverse transcription-polymerase chain reaction (RT-PCR). Histopathological examination of liver tissue was performed. **Results:** Administration of BM-MSC of both doses ($1.5x10^6$ and $2x10^6$ cells) into rats with DILC resulted in ameliorating liver functions and histopathological features compared to that received DEN only. RT-PCR analysis revealed that the expression of albumin gene increased, while CK-18 decreased in liver tissue of rats with DILC which received $2x10^6$ BM-MSCs compared to DEN control group. **Conclusion:** BM-MSCs suppressed DEN-induced liver cirrhosis in rats as indicated by up regulation of albumin and CK-18 genes, as well as improvement of liver functions and architecture.

INTRODUCTION

Liver cirrhosis is a condition where scar tissue replaces the healthy tissue of the liver and regenerative nodules with surrounding fibrous bands develop as a result of the injury [1]. Cirrhosis is the common end of progressive liver disease of various causes, resulting in chronic liver failure entailing complications such as hepatic encephalopathy, spontaneous bacterial peritonitis, ascites, and esophageal varices [2]. Unfortunately, the majority of cases are usually in an irreversible state when diagnosed. Despite current advancements in its management [3,4], cirrhosis was the 14th leading cause of death worldwide in 2012 [5]. DEN is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models [6,7]. Exposure to DEN has also been associated with hepatocellular accumulation of reactive oxygen species (ROS), which may result in oxidative

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damage to DNA and other nucleophiles, a mechanism that may further enhance DEN-induced hepatocarcinogenesis [8]Some reports proved that there were similarities between DEN-induced liver cirrhosis and human cirrhosis[9,10]Currently, the most effective therapy for acute liver failure and advanced cirrhosis is liver transplantation, but its use is limited. Due to the presence of many obstacles during liver transplantation such as the difficulties in the finding of a suitable donor, as well as the immunological challenges of the receiver, the discovery of an alternative approach became necessary. During the last years, stem cell transplantationhas been presented in many studies as a promising therapy for liver cirrhosis[11-14].MSC has a potential role in liver regeneration due to its capability of self-renewal and differentiating to daughter hepatocyte [15].In order to detect the differentiation, several markers can be estimated in liver tissue such as albumin, alpha fetoprotein, cytokeratin 18 and gamma glutamyl transferase[16,17]. The objective of the present investigationwas to explore the mechanism by which BM-MSC potentiate to regenerate DEN-induced cirrhotic liver in rats.

2. MATERIALS AND METJODS:

2.1.Chemicals and Supplies

Phosphate Buffer Saline (PBS), ficoll (Lymphocyte separation medium), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS),penicillin-streptomycin ,absolute alcohol ,heparin, trypsin and trypan blue were purchased from Sigma Life Science Company. All chemicals have high grade and convenient for cell culture protocol. Disposable syringe, petridishes, sterile pipettes (5 and 10 ml),falcon tubes (15 and 50 ml) , cell culture flasks (75 cm^2) were purchased from Greiner bio-one (Germany).

2.2.Isolation of mesenchymal stem cell (MSCs) from bone marrow

3-5 ml of BM was aspirated from the femur and tibias bone from Sprague-Dawley rat using needle. BM was transferred into sterile petridish containing 500ml heparin and 5ml PBS, then cell suspension was transferred to falcon tube (50 ml) containing ficoll (v/v 3:1),and then mononuclear cells (MNCs) were isolated by density-gradient centrifugation. Mononuclear cells were plated in a 75cm^2 flask (Falcon, Franklin Lakes, NJ, USA) with low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin, and cultured at 37 °C in a 5%CO_2 atmosphere. After 3 days, non-adherent cells were removed by replacing the medium. When the cultures approached 80% confluence, the cells were harvested by trypsinization and replated in 75cm^2 flasks containing fresh medium. MNCs were propagated by serial subculture up to the fifth passage.

2.3.Animal model of DEN-induced liver cirrhosis

All animal experiments were performed according to the guidelines on the use of laboratory animals approved by ethical committee at Zagazig university, Egypt. 60 Male Sprague-Dawley rats, (age, 3-5 weeks; weight, 180-220 g) were purchased from the scientific and medical research center at the faculty of medicine, Zagazig university(Egypt). Rats were housed in an air-conditioned room at 25˚C with specific pathogen-free conditions and were subjected to a 12 h light/dark cycle with access to chow and water ad libitum. Liver cirrhosis in rats was established by addition of 0.01% (v/v) diethylnitrosamine (DEN) to the drinking water for 30 days.

2.4.Experimental design

Rats were randomly divided into four groups (15 rats per each) including normal control group, DEN control group and two treatment groups, all groups except normal control were induced with DEN for 30 days. In the 31th day, two treatment groups injected with 1.5 x 10^6 and 2 x 10^6 BM-MSC infusion respectively. After 15

days from treatment, blood samples were collected from orbital venous plexus of rats under anesthesia using diethyl ether, then samples were left at 37 C° for 10 minutes, after that serum was separated by centrifuging at 4000 rpm for 15 minutes and stored at -20 C° till use. Rats were sacrificed and liver tissue samples were harvested and placed in PBS at -80 C°. In addition, small pieces of liver were fixed in 10% buffered formalin for histological study.

2.5.Determination of biochemical parameters

Serum total bilirubin (T BIL) , Total protein (TP) , albumin (ALB) ,globulin (GLB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alpha feto protein (AFP) were analyzed using a semi-automated analyzer (Robonik,india).

2.6.Real-time quantitative analyses for albumin and CK-18 genes expression

Total RNA was extracted from liver tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA). cDNA was generated from 5 µg of total RNA extracted with 1 µl (20 pmol) antisense primer and 0.8 µl superscript AMV reverse transcriptase for 60 min at 37°C. Quantitation of gene expression was conducted using universal probe library sets based real time PCR (Roche diagnostics). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control housekeeping gene. FastStart Universal Probe Master mix was used in LightCycler® 480 Instrument (Roche Applied Science, Indianapolis, USA). Briefly, in the LightCycler®480, a total reaction volume of 20 µl was prepared, of which 2 µl of starting RNA material was included for RT-PCR, a final concentration of 0.5 µM of each forward and reverse primer and 0.2 µM of the TaqMan probe was used. Cycling conditions involve reverse transcription at 50°C for 30 min; enzyme activation at 95°C for 15 min, followed by 50 cycles of 95°C for 15 sec and 52°C for 60 sec, finally 72°C for 60 sec. LightCycler® 480 RT-PCR data were analyzed using LightCycler1.2 version 3.5 software using the second derivative maximum method. Successfully amplified targets are expressed in Ct values, or the cycle at which the target amplicon is initially detected above background fluorescence levels as determined by the instrument software. Each sample RT-PCR was performed minimally in duplicate, and the mean Ct value with standard deviation reported and the fold change related to GAPDH was calculated.

Primer sequences:

1- albumin: (The length of PCR product was 135 bp) .

- sense : 5'-CTTGTTTTGCACAGCAGTCAG-3'
- antisense:5'-CAAAAACATGTGTTGCTGATGA-3'

2- CK-18 :(The length of PCR product was 126 bp).

- sense : 5'-GTCAATCTGCAGAACGATGC -3'
- antisense: 5'-GAGCACTTGGAGAAGAAGGG -3'

2.7.Histopathological study

Liver tissue specimens were fixed in 10% buffered formalin overnight. Paraffin blocks were prepared and 5µm thick sections were cut using microtome for staining with hematoxylin and eosin (H&E), then examined histopathologically using light microscope.

2.8.Statistical analysis

The numerical results were statistically analyzed using SPSS software version 16 and the data were expressed as mean ± standard error. Independent-sample t test was carried out to detect the significant differences between the experimental groups and the probability was considered less than 0.05. Each sample RT-PCR was performed twice and the mean Ct value with standard deviation were reported.

3. Results

3.1. The effect of bone marrow derived MSC on liver function tests

Serum levels of total protein (TP) and albumin (ALB) demonstrated a significant increase in rats with DEN-induced liver cirrhosis which received the two doses of BM-MSCs when compared to that received DEN only, while no significant difference was detected in rats groups received both doses of MSCs after administration of DEN compared to normal control group. Conversely, serum levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TB),Direct bilirubin (DB), indirect bilirubin (IDB),alkaline phosphatase (ALP),alpha feto protein (AFP) demonstrated a significant decrease in rats groups with DEN-induced liver cirrhosis which received both doses of MSCs compared to rats group received DEN only (table 1).

3.2. Results of RT-PCR analysis of gene expression

Rats group received DEN only showed a fold decrease in the expression of CK-18 which reached 0.2±0.1($P \leq 0.001$) when compared to normal control group, while rats with DEN-induced liver cirrhosis which received BM-MSC infusion showed a significant increase in CK-18 gene expression when compared to DEN control group in a dose dependent manner(Figure1),where groups received $1.5x10^6$and $2x10^6$MSC showed fold increase in the expression of CK-18 which reached 0.8±0.22 ($P \leq 0.01$) and 1.52±0.13($P \leq 0.001$) respectively, when compared to DEN control group.Furthermore, the administration of$1.5x10^6$ and $2x10^6$MSC into rats with DEN-induced liver cirrhosis led to a significant fold increasein the expression of albumin gene which reached 9.92±2.21 and 14.3± 1.12 ($P \leq 0.001$)compared to rats group received DEN only(Figure2).

3.3. Results of histopathology

Histopathology of liver tissues of the animals that received DEN only showed mild congestion of the portal blood vessels or telangiectasia and mild portal round cells aggregations together with biliary proliferation. apoptotic, degenerative and necrotic changes were also recorded as shown in (Figure4).Improvement of histopathological picture after the administration of MSCs of two doses into rats with DEN-induced liver cirrhosis is demonstratedcompared to normal group(Figure3,5&6).

Discussion

Cirrhosis is the most common liver disease, causing the death of thousands every year. This disease results from successive cellular, biochemical, and molecular events that lead to changes in the hepatic parenchyma and increase collagen deposition. Formation of nodules, anatomical changes, and death of hepatocytes also occur [18]. The experimental models of cirrhosis currently used are induced by chemical substances such as DEN [19]Our results revealed that the liver function tests of the animals which received DEN had significantly increased levels for ALT,AST, TB, DB,IDB,ALP and AFP, while significantly decreased levels for TP and ALB when compared to normal control. The histological examination showed changes in the liver architecture. The administration of BM-MSCs to rats with DILC in both of $1.5\times10^6$and 2×10^6infusions achieved an improvement of liver functions, as well as ameliorated the histological pictures of liver in a dose dependent manner compared to DEN-control group.

When hepatocytes are chronically exposed to oxidative stress and toxic substances, they become ballooned, accumulate fat, show a disruption in the keratin intermediate filament network, and form Mallory bodies [20]. A Mallory body is composed of abnormally phosphorylated and cross-linked keratins, such as cytokeratin 8 and 18 and stress-induced proteins [21].CK-18 is considered the major intermediate filament protein in the liver—resulting in apoptosis [22].The degradation of CK-18 bycaspase 3 is the initial step of apoptosis.**Mandelia and**

coworkers (2016)reported that Plasma cytokeratin-18 fragment level, a marker of hepatocyte apoptosis, is significantly higher in children with NAFLD and liver fibrosis compared to those without liver fibrosis[23]. According to our results, the transplantation of BM-MSC into rats with DILCsignificantly raised the expression of cytokeratin-18 compared to rat group which received DEN only.

Albumin represents the majority of plasma proteins. Albumin consists of 585 amino acids and molecular weight 66 kDa encoded by a gene on chromosome 4 and is exclusively synthesized by hepatocyte, which release it directly into the blood stream without storage. Liver cirrhosis is characterizedby hypoalbuminemia due to reduced synthesis of albumin by hepatocyte as well as retention of water and sodium which dilutes the content of albumin in the extracellular space [24].

In the present study, RT-PCR analysis revealed that the expression of albumin significantly increased in liver tissue of DEN-injured rats received BM-MSC compared to rats received DEN only, however the stronger expression was recorded at the higher dose of BM-MSC ($P \leq 0.01$). Our result agreed to **Li et al. (2010)**who showed that the expression of albumin and CK 18 genes significantly increased in CCl_4-injured rats which implanted with BM-MSC at week 4[25].

According to our results, AFP level significantly decreased in serum of DEN-injured rats which received BM-MSC compared to rats group received DEN only ($P \leq 0.001$). Previous investigation revealed that BM-MSC implantation in CCl_4-injured rats reduced AFP expression [25].

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Tables and Figures

Table1: The effect of bone marrow derived MSC on liver function tests.

Groups / Indicators	Group I Normal control M±SE	Group II (DEN) M±SE	Group III (DEN+1.5x10^{6}MSC) M±SE	Group V (DEN+2x10^{6}MSC) M±SE
TP (g/dl)	6.57±0.18	5.47±0.11	6.35***±0.13	6.56***±0.14
Alb (g/dl)	3.48±0.18	2.40±0.15	3.32**±0.17	3.28**±0.18
Globulin (g/dl)	3.18±0.05	3.07±0.08	3.27±0.12	3.28±0.18
A/G ratio	1.15±0.04	0.97±0.03	1.32**±0.07	1.27*±0.10
TB (mg/dl)	0.70±0.02	0.96±0.04	0.77*±0.06	0.74**±0.04
DB (mg/dl)	0.23±0.01	0.44±0.04	0.23**±0.01	0.29**±0.01
IDB (mg/dl)	0.55±0.02	0.69±0.01	0.51***±0.02	0.57***±0.02
(AST) (U/L)	81.33±3.13	150.00±8.27	120.17**±2.41	125.67*±2.26
(ALT) (U/L)	19.17±1.66	50.83±2.30	28.33***±1.47	32.83***±1.35
(ALP) (U/L)	251.67±15.87	625.83±5.56	385***±18.40	270.83***±25.89
(AFP) (mg/dl)	0.13±0.01	0.54±0.02	0.27***±0.02	0.22***±0.02

Values are represented as Mean±SE (n=7), Where * = Significant difference between test groups and group II (DEN),*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

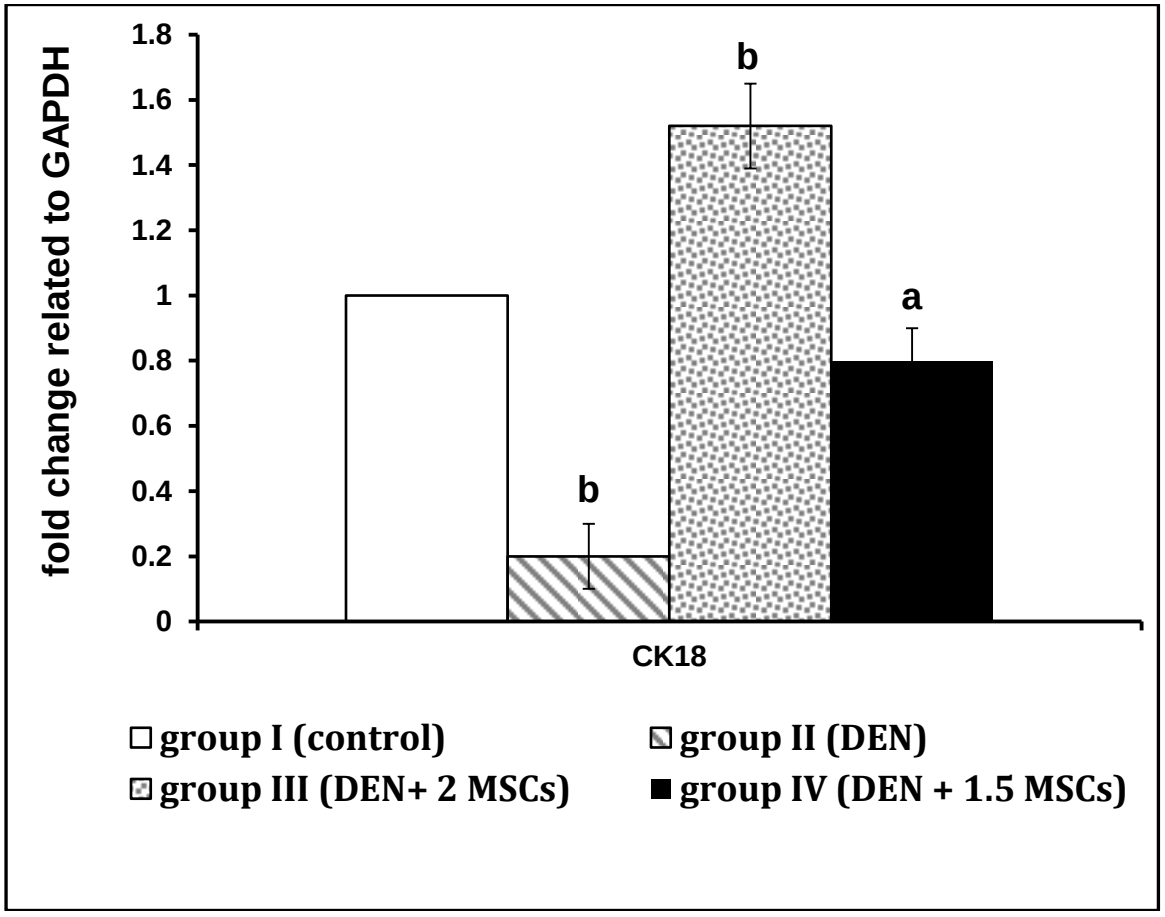

Figure 1: The effect of BM-MSC on the expression of CK-18 gene in liver tissue. The chart is reported as the means ± SD of three independent experiments using SPSS version 16 softwarewhile b and c represent *P*≤ 0.01 and 0.001 respectively.

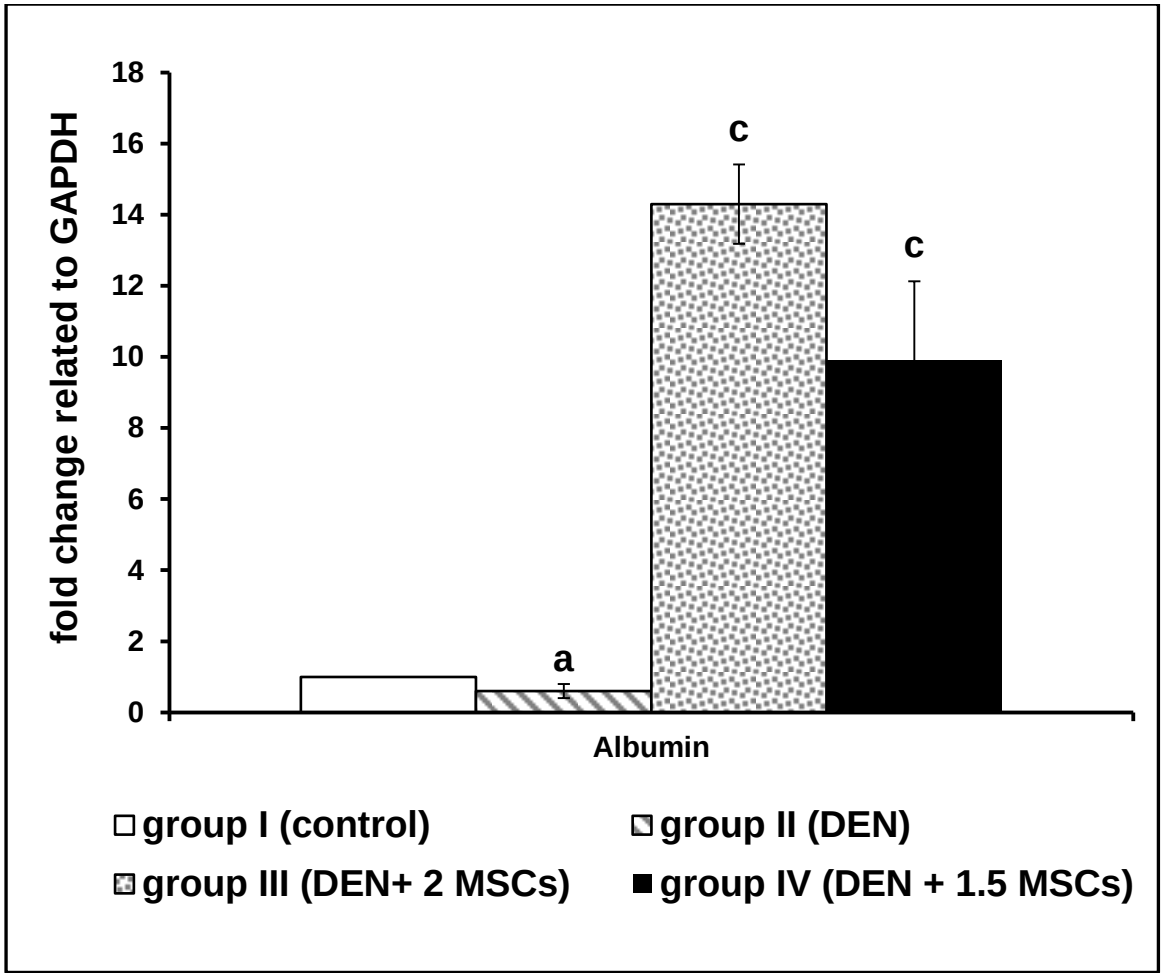

Figure 2: The effect of BM-MSC on the expression of albumin gene in liver tissue.The chart is reported as the means ± SD of three independent experiments using SPSS version 16 softwarewhile a and c represent $P \leq 0.05$ and 0.001 respectively.

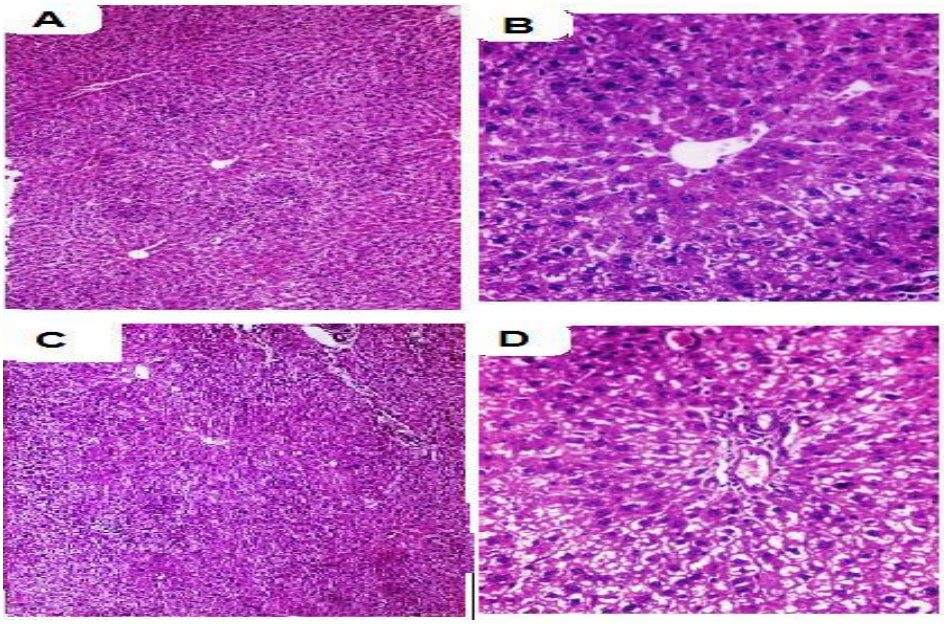

Figure 3: Histopathological picture of liver tissues in rats received saline showing normal hepatic parenchyma. H&E X 100 (A,C), 400(B,D).

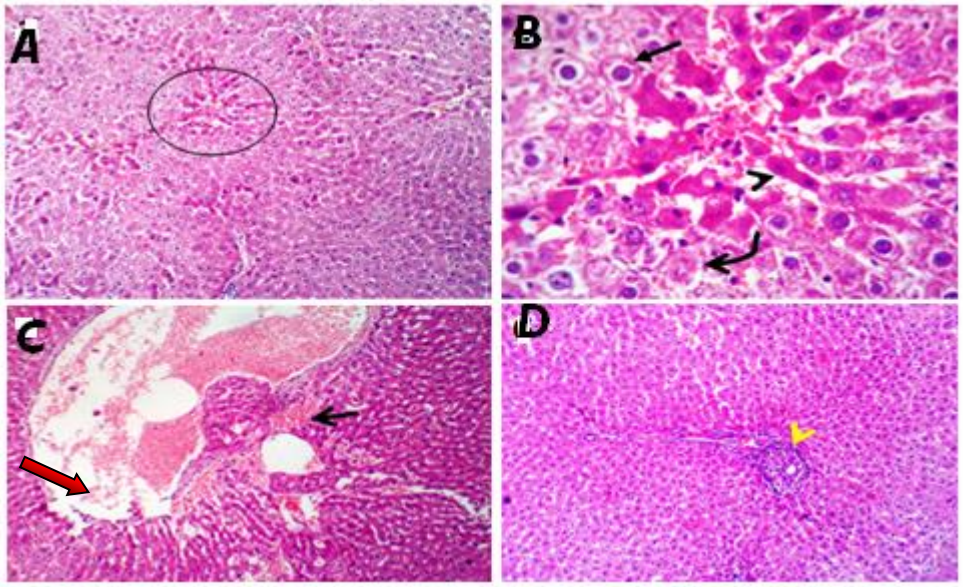

Figure 4: Histopathological picture of liver tissues in rats received DEN only showing telangictasis (C, red arrow) and mild portal round cells aggregations (D, yellow arrow head).Degenerative (B, closed arrow) and necrotic (A & B, circle, open and curved arrows) changes are seen . H&E X 100(A,D),200(C)400(B).

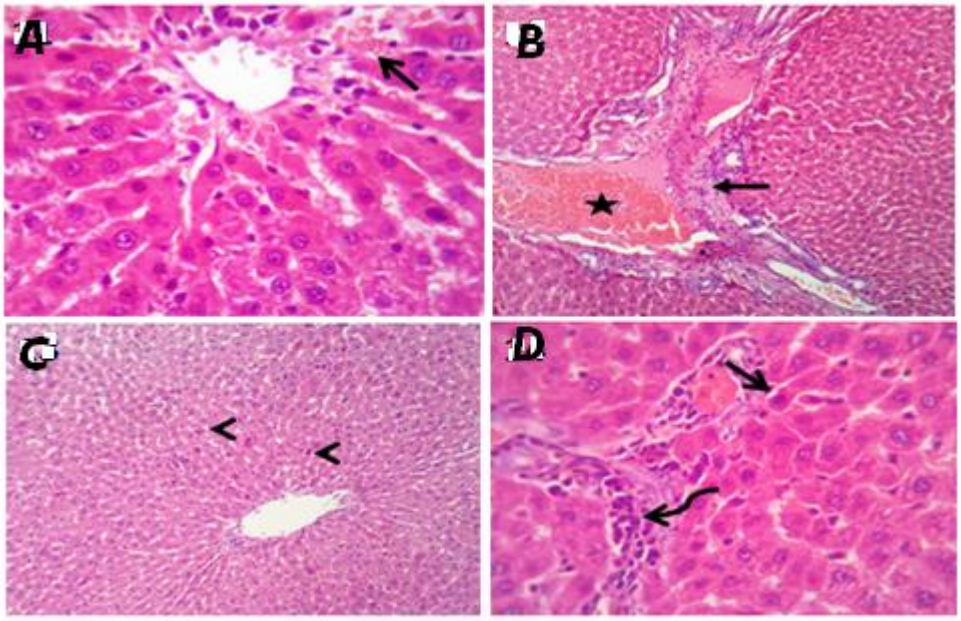

Figure 5: Histopathological picture of liver tissues in rats received $1.5x10^6$ MSC infusion after DEN administration, showing normal hepatic parenchyma with congestion of portal veins **(star),** dilatation of hepatic sinusoids **(open arrow).** Moderate congestion of the portal blood vessels and biliary proliferation beside fibroplasia **(closed arrow)** and round cells infiltration **(curved arrow**) In portal area. H&E X 100(B,C),400(A,D).

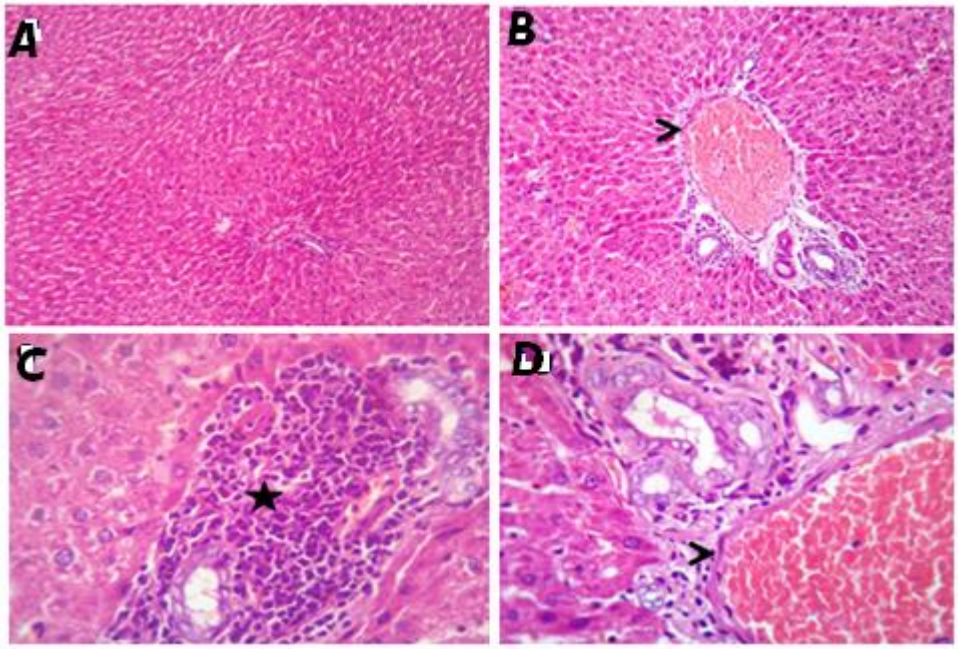

Figure 6: Histopathological picture of liver tissues in rats received $2x10^6$ MSC infusion after DEN administration showing normal hepatic parenchyma with congestion of portal veins (arrow heads) and round cells aggregation(star). H&E X 100(A,B), 400(C,D)**.**