Original Article

A Comparative Histological Study On The Therapeutic Effect of Mesenchymal Stem Cells Versus Exosomes on CCL4 Induced Liver Injury in Adult Male Albino Rats

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ABSTRACT

Introduction: Liver diseases are major global health burden which lead to many complications if left untreated.

Aim of Work: Evaluating and comparing the therapeutic effect of bone marrow mesenchymal stem cells (BMMSCs) versus Exosomes in CCL4 induced liver injury rat model.

Materials and Methods: Thirty-two adult male albino rats were divided into:Group I (Control),Group II (CCL4), Group III (Recovery),Group IV (MSCs),Group V(Exosomes). 0.1ml/kg of CCL4 in 0.1 ml olive oil was administered intraperitoneally (IP) twice weekly for six weeks for all experimental groups. MSCs were injected in the tail vein of group IV at a dose1×106 after last CCL4 injection. Exosomes were injected in the tail vein of group V at a dose 250 µg after last CCL4 injection. Animals of group II were sacrificed 6 weeks from the start of experiment. while groups(I&III&IV&V) were sacrificed 10 weeks after. ALT&AST were measured at the end of 6thand 10th week. Hematoxylin and eosin, Masson's trichrome stain,α-SMA and Caspase 3 immunostaining were performed. Mean values of AST&ALT,mean area % of collagen, mean number of α-SMA&caspase3 immunopositive cells &mean optical density of caspase 3 immunoreactivity were measured.

Results: In groups II& III, hepatocytes showed cytoplasmic vacuolations with dark pyknotic nuclei.A significant increase in mean values of liver enzymes,mean area % of collagen, mean number of α-SMA & caspase 3 immunopositive cells and mean optical density of caspase 3 in group II&III compared to control group and other groups. In group IV&V hepatocytes were apparently normal, few cells showed dark pyknotic nuclei. A significant decrease in mean values of liver enzymes, mean area % of collagen, mean number ofα-SMA &caspase 3 +ve cells and mean optical density of caspase 3 in group IV&V compared to groups II&III.

Conclusion: MSCs was effective as Exosomes with significant reduction of fibrosis in liver fibrosis rat model.

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Key Words: CCL4, exosomes, fibrosis, liver, MSCs.

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INTRODUCTION

Liver diseases are major health burden, causing numerous complications and contributing significantly to high morbidity and mortality rates. Many reasons can induce liver damage, including excessive lipid deposition, toxicant and medication toxicity, viral infections, biliary tract diseases, autoimmune disorders, and congenital or hereditary abnormalities[1,2].

Hepatocyte injury leads to the fibrogenic and inflammatory condition known as liver fibrosis, which is characterised by distortion of liver architecture. Excessive matrix tissue may accumulate as a result of chronic injury, and the functioning of the liver may be compromised. Immune infiltrations and angiogenesis occur in conjunction with fibrosis. Collectively, these activities deteriorate the structure of the liver and decrease its elasticity[3,4].

Transplantation of MSCs is a recent therapy in liver disorders because they are able to regenerate damaged tissue, inhibit the fibrosis and immune regulate the immunity. They can degrade ECM by producing matrix metalloproteinases that can degrade type I and III collagen, which is the key collagen in scars of liver cirrhosis. They can also increase the phagocytosis of debris[5].

Furthermore, exosomes may offer a novel therapeutic approach because of their benefits over MSCs[6]. They are one of three categories of extracellular vesicles secreted from many types of cell. They are spherical particles protected by a bilayer of phospholipids[7].

Exosomes have a critical role for a variety of pathological events as pulmonary, cardiac and neurological disorders[8]. They are beneficial in treatment of liver fibrosis by inhibition of macrophage activation and cytokine

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secretion, remodelling extra cellular matrix production, reducing fibrous scars and inactivating hepatic stellate cells (HSCs) which are major myofibroblast population[9].

Cell survival, regenerative capacity, immunological rejection, and tumour differentiation are problems for MSC-based therapies. By employing exosomes as a cell-free therapy, these issues can be avoided. Even after allogeneic injection, the likelihood of immunological rejection is extremely low due to the low amount of exosome membrane-bound proteins. Moreover, because exosomes do not replicate, tumour development is not a possibility[10].

Carbon tetrachloride (CCL4) is a hepatotoxin which cause liver damage because of the cytochrome P450 conversion to a very high reactive free radicals which cannot be eliminated quickly, leading to oxidative stress and cell death[11]. CCL4-induced hepatic injury or fibrosis mouse model is probably the best representative experimental model for elucidating the different roles of new treatments in response to liver injury[12].

AIM OF WORK

This study aimed at is evaluating and comparing therapeutic effect of MSCs versus Exosomes in induced liver injury caused by CCL4 in adult male albino rats, by the use of histological, serological, Immuno-histochemical and morphometric studies.

MATERIALS AND METHODS

Materials

Drugs

1- Carbon tetra chloride: It was purchased from Company Sigma Aldrich (St. Louis, USA) in a liquid state. 100 ml of olive oil added to100 mg of CCL4 to form the solution for injection. 0.5 mg/ Kg of CCL4 was injected[13].

2- Bone-marrow mesenchymal stem cells (BMMSCs): PKH26 labelled BMMSCs were purchased from Biochemistry Department, Cairo University. One million Paul Karl Horan 26 (PKH26) labelled BMMSCs were suspended in phosphate buffered saline (PBS) (0.5ml) and injected via tail vein.

3- Mesenchymal stem cells derived exosomes (BMMSCs-Exos): PKH26 labelled exosomes were purchased from the Biochemistry Department. PKH26 Labelled exosomes (250µg) were suspended in 0.5ml PBS, injected via tail vein.

Animals

Thirty two albino rats, adult and males of 180-220 g in weight & aged about 12-14 weeks were purchased and raised in Animal House (kasr El Ainy Hospital). The study was carried out in conformity with the Animal Ethics Committee's ethical guidelines and rules of Kasr Alainy, Cairo University with approval number (Cu III F920).

The rats were classified as follows

Group I (Control): 8 rats were subdivided to four subgroups:

- Subgroup IA (2 rats): Rats were used as a negative control group and sacrificed after 10 weeks from the start.
- Subgroup IB (2 rats): Each rat was IP injected by olive oil (0.1 ml) two times per week for six weeks. They were sacrificed after six weeks from start of experiment.
- Subgroup IC (2 rats): Each rat was IP injected by olive oil (0.1 ml) two times per week for six weeks. They were sacrificed after ten weeks from start of experiment.
- Subgroup ID (2 rats): Each rat was IP injected by olive oil (0.1 ml) two times per week for six weeks. They received a single IV injection of PBS (0.5 ml) via the tail vein. They were sacrificed ten weeks from start of experiment.

Group II (CCL4 treated group, 5 rats): Each rat was IP injected by CCL4 (0.1 ml) in 0.1 ml of olive oil (0.1 ml) two times per week for six weeks[13]. They were sacrificed after six weeks from start of experiment.

Group III (Recovery group, 5 rats): Each rat received0.1 ml of CCL4 dissolved in 0.1 ml olive oil by IP injections two times per week for six weeks. They were sacrificed after ten weeks from start of experiment.

Group IV (MSCs treated group, 7 rats): Each rat was IP injected by CCL4 (0.1 ml) in 0.1 ml of olive oil (0.1 ml) two times per week for six weeks. Then rats received a single IV injection of BM-MSCs ($1{\times}10^6$) suspended in PBS (0.5 ml) through tail vein[14]. One week later two of the rats were randomly selected and sacrificed to detect homing of MSCs and the rest were sacrificed ten weeks from start of experiment.

Group V (Exosomes treated group, 7 rats): Each rat was IP injected by CCL4 (0.1 ml) in 0.1 ml of olive oil (0.1 ml) two times per week for six weeks then received an IV injection of MSCs-Exos at dose 250 µg harvested from 1×10^6 MSCs in 0.5 ml (0.5 ml) via tail vein[15]. One week later two rats were randomly selected and sacrificed to detect homing of exosomes and the rest were sacrificed ten weeks from the start of experiment.

Methods

preparation of BMMSCs.

Tibia and femur of male Wistar 8-week-old rats used to get bone marrow. Bone marrow was flushed with glucose low Dulbecco's Modified Eagle's Medium (DMEM) [Gibco, Gainesville, MD, USA] and foetal bovine serum [FBS, GIBCO/BRL]. The cells were grown in 1% penicillin/streptomycin (GIBCO) containing medium. They kept at as primary culture for 2 weeks at 37°C.

The cells were PBS washed at 80% confluence. During five minutes at 37°C, they were trypsinized with 0.25% trypsin in ethylenedi-amine-tetra-acetic acid. Then they were centrifuged, immersed in serum-containing media (10% FBS) then cultured on Falcon flasks measuring 50 cm^2. To increase the cell population, the subculture passage was continued until the third passage[16]. BMMSCs were distinguished by their ability to adhere, fusiform shape, and surface marker identification by flow cytometry (CYTOMICS FC 500, Beckman Coulter, Champaign, IL, USA). They were positive for CD105 & CD90 and negative for CD34 & CD45)[17].

Labelling of BMMSCs

The MSCs were harvested from 3rd passage then labelled with PKH26, which is red fluorescent dye (lipophilic) (Cat. # MINI26, Sigma Aldrich, St. Louis, MO, USA). The BMMSCs were centrifuged and washed 2 times. Then, they were incubated for 1 h (5% CO2 at 37°C) in a solution containing of serum-free DMEM and PKH26 dye solution. Fluorescent microscope CKX53 (Olympus, Tokyo, Japan) was used to examine the labelled cells. Cells were injected IV into rats of groups IV &V tail veins[18].

Preparation of Exosomes from BMMSCs.

Exosomes were produced in FBS by ultracentrifugation overnight at 140,000 g. 15-cm plates containing MSCs had their cells grew to 70–80% confluence before MSCs were added. After that, the standard medium was taken out and an exosome-depleted medium (10% FBS) was introduced. After 2 days, MSCs medium was collected. For separation of exosomes from MSCs, differential centrifugation was used. The following were the initial centrifugations: 500 g for 10 mins, 2000 g (10 minutes), 12,000 g (30 minutes) at 4°C. The supernatant was centrifuged 70 mins (4°C) and the pellet was immersed in PBS , followed by ultracentrifugation at for 70min (4°C). Last pellet was re-immersed in 200-ml PBS[19].

Labelling of exosomes with PKH-26 fluorescent linker dye (Sigma, USA, Catalog Number MIN126)[20].

Biochemical study

Using capillary tubes, samples for blood were withdrawn from tail vein. ALT &AST levels in the serum were measured for all rats in all groups at the 6th and 10thweek to prove the liver injury and to detect effect of treatment. Measurements were done at Biochemistry Department.

Histological study

The rats were sacrificed at the end under anesthesia through IP phenobarbital injection (80mg\kg)[21]. Livers of each group were dissected from the animals. Samples from the right lobe of the liver were taken for all studied groups. They were kept in (10%) of formal saline for one day, dehydrated in ethanol ascending grades, cleared with xylol and immersed in paraffin. 5-7 micrometer thickness sections were cut serially then mounted in glass slides while those used for immunohistochemistry were mounted on charges slides. These sections were used in following studies:

A) Light Microscopic study

1. Hematoxylin & eosin (H&E) stain: for histological evaluation of liver[22].
2. Masson trichrome : collagen fibers detection[22].
3. Immunohistochemical study[22] by using:
    - Anti α-SMA to detect hepatic stellate cells.
    - Anti-caspase 3 antibody as a marker for apoptosis.

Sections used in immunohistochemical stains were boiled (10min) in citrate buffer(10mm) at pH 6 to retrieve the antigen. Followed by incubation of these sections with primary antibody for one hour. Anti α-SMA was the primary antibody. It is a mouse monoclonal antibody (Thermo Fischer, CA, USA, catalogue NO MS-113-R7) and Anti caspase 3 was the primary antibody. It is rabbit polyclonal antibody (Thermo Fisher Scientific, Fremont, USA, catalog number(94538).It was supplied as 7.0ml of ready to use antibody (pre-diluted in 0.05mol/L Tris-HCL,PH 7.6 containing stabilizing protein and 0.015mol/L sodium azide). To complete immunostaining, Ultra-vision detection system (TP-015-HD) was used and for counterstaining Mayer's Hematoxylin (TA-060-MH) was used. Citrate buffer, Ultravision detection system and Mayer's hematoxylin were purchased from Lab vision Thermo Scientific, Fremont, California, USA. Same steps were done for negative controls except for adding primary antibodies step was skipped. Brown deposits in cytoplasm of anti αSMA and anti-caspase 3 antibody positive cells appeared in positive reaction.

B) Fluorescent Microscopic Study for detection of PKH26 labelled BMMSCs and BMMSCs-Exos

Morphometric Study

Ten non-overlapping fields randomly chosen x 400 magnification; measuring frame: 116964.91µm2from sections of each rat from all groups were investigated for the measurement of:

- Mean area percent of collagen fibers in sections stained with Masson's Trichrome .
- Mean number of anti αSMA immunoreactivity.
- Mean number of anti-caspase 3 immunoreactivity.
- Mean optical density of anti caspase3 immunoreactivity.

"Leica Qwin 500C" image analyzer computer system Ltd (Cambridge, UK) was used. (Histology department, Cairo University).

Statistical study

ANOVA then post-hoc Tukey test was performed to compare the quantitative data which was summarised as, mean ± standard deviation (SD)[23]. Differences were considered to be statistically significant when probability value of P is < 0.05. SPSS version 16 was used to make the calculations.

RESULTS

Thirty two albino rats males were included in present study with no mortality observed throughout the whole experimental duration.

Biochemical Results:(Table 1, Histograms 1,2)

At end of 6th week, ALT&AST levels increased with high significance in all experimental groups in comparison to group I. While no significant difference was found between the other experimental group when compared with each others.

Meanwhile, At end of 10th week, ALT&AST levels increased with high significance in in group III while there was no significant difference was found in group IV and V when compared to control group.

Values of group III showed a highly significant increase in ALT&AST levels when compared to groups IV and V. While no significant difference was found between groups IV and V.

Histological Results

A- Light Microscopic Study

Hematoxylin and eosin results (Figures 1,2): Sections stained with H&E in group (I) exhibited normal parenchyma of liver and preserved liver architecture with indistinct hepatic lobules where hepatocyte cords radiating from central vein. Hepatocytes appeared polygonal in shape with eosinophilic cytoplasm & large, central, rounded pale nuclei with prominent nucleoli. Some binucleated cells were also seen. The sinusoids between plates of hepatocytes are lined with endothelial cells. A portal tract is noted (Figures 1a,b).

Group II showed vacuolations of hepatocytes and dilatation of central hepatic vein. In other fields hepatocytes had pyknotic nuclei. Mononuclear cellular infiltration in the portal area was observed. Dilation of blood sinusoids was also noted (Figures 1c,d).

Group III revealed hepatocytes with marked cytoplasmic vacuolations and some exhibited dark pyknotic nuclei. Dilated congested portal veins could be detected in portal areas. Dilation of blood sinusoids were also noted (Figures 2a,b).

Group IV showed apparently preserved hepatic architecture where the hepatocytes radiated from central veins. They were separated by blood sinusoids. Most of hepatocytes around central veins and portal areas were polygonal with acidophilic cytoplasm and pale nuclei. Some hepatocytes appeared binucleated while few hepatocytes showed dark pyknotic nuclei. Portal areas appeared normal with few cellular infiltration (Figures 2c,d).

Meanwhile, group V section revealed apparently normal architecture. Most of hepatocytes around central vein and portal area were apparently normal. Hepatocytes exhibited eosinphilic cytoplasm & large, central, vesicular, rounded nuclei. Some hepatocytes appeared binucleated while few hepatocytes had pyknotic dark nuclei. No cellular infiltration could be detected (Figures 2e,f).

Masson's trichrome stain results (Figures 3,4): Group I showed few collagen fibers around central veins & in portal areas. (Figures 3a,b).

Group II revealed multiple collagen fibers in C.T septa in between lobules. Other fields showed multiple collagen deposition in the portal areas (Figures 3c,d). Similarly the examination of sections from group III revealed multiple collagen fibers in septa outlining hepatic lobule and portal areas and also in between the hepatocytes (Figures 4a,b).

Meanwhile, group IV &group V revealed few collagen fibers around central veins (Figures 4c,e) and in the portal areas (Figures 4d,f).

Immunohistochemical Results

a- Anti α - SMA immuno-stained liver sections (Figure 5)

Liver sections group I showed positive cytoplasmic immune-reaction to anti α-SMA in few cells in wall of blood vessels in the portal areas (Figure 5a).

Group II showed positive cytoplasmic immunoreaction to anti α-SMA in central vein wall and in many branched cells around blood sinusoids (Figure 5b).

Group III showed postive cytoplasmic immunoreaction to anti α-SMA in wall of blood vessels of the portal tracts and in many cells around blood sinusoids (Figure 5c).

Groups IV &group V showed positive cytoplasmic immunoreaction to anti α-SMA in few cells in the wall of blood vessels of the portal tract and central vein. No positive reaction could be detected around the wall of blood sinusoids (Figures 5d,e).

b- Anti Caspase 3 immunostained liver sections (Figure 6)

Liver sections of group I, showed few hepatocytes with faint positive cytoplasmic anti caspase 3 reaction around central veins (Figure 6a). Groups II & III, showed many hepatocytes with strong positive cytoplasmic anti caspase 3 reaction. (Figures 6b,c). Liver sections of groups IV & V showed moderate positive cytoplasmic anti caspase 3 reaction in few hepatocytes. (Figures 6d,e).

B) Immunofluorescent Microscopic Study

PKH26 labelled liver sections of rats in groups IV &V exhibited multiple fluorescent labelled cells among the hepatocytes (Figure 7a,b).

Morphometric & statistical results (Table 2, Histograms 3-6)

As compared to control group, mean area % of collagen increased with high significance in groups II & III. While no significant difference was recorded in groups IV & V. A highly significant increase was recorded in groups II & III when compared to groups IV &V. However There was no significant difference between group II and group III. In

addition there was no significant difference between group IV and group V.

Anti α SMA positive cells mean number increased with high significance in group II & III when compared to group I & treated groups (group IV & V). No significant difference was found in group IV &V in comparison with control group. No significant difference was found between group II & group III, also between group IV & V.

The mean number and mean optical density of caspase +ve cells increased with high significance in groups II & III when compared to group I. Also there was a significant increase in group IV& V. Values in group II & III increased with high significance in comparison with groups IV & V. While, no significant difference was recorded between groups II & III. No significant difference was recorded between groups IV &V.

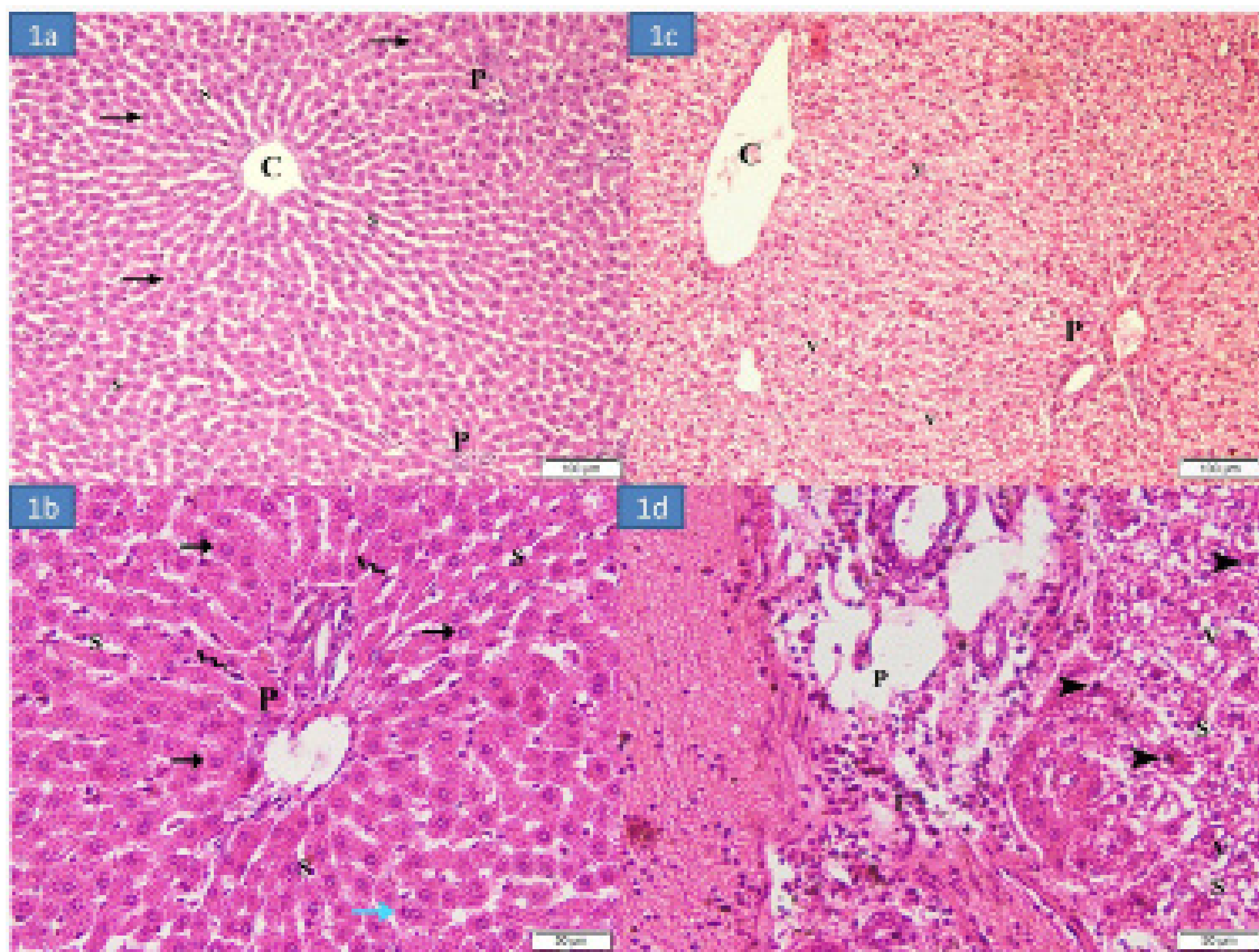


Fig. 1: Photomicrographs of sections in the liver of rats stained with H&E, (1a): group I showing hepatocytes (black arrows) arranged in cords radiating from the central vein (C) and separated by blood sinusoids (S). Portal areas (P) are also noted (x100).(1b):group I showing hepatocytes (black arrows) that are polygonal with eosinophilic vacuolated cytoplasm and large, central, rounded vesicular nuclei. Some hepatocytes appear binucleated (blue arrows). The sinusoids (S) appeared between plates of hepatocytes and lined by endothelial cells (zigzag line). A portal tract is noted (P)(x200). (1c):group II showing dilated central hepatic vein (C). Hepatocytes show cytoplasmic vacuolations (V). A portal tract (P) is also noted (x100). (1d):group II showing hepatocytes with dark nuclei (arrow heads) and marked cytoplasmic vacuolations (V). Notice the mononuclear cellular infiltrations (I) in the portal area (P),sinusoids (S) are apparently dilated (x200).

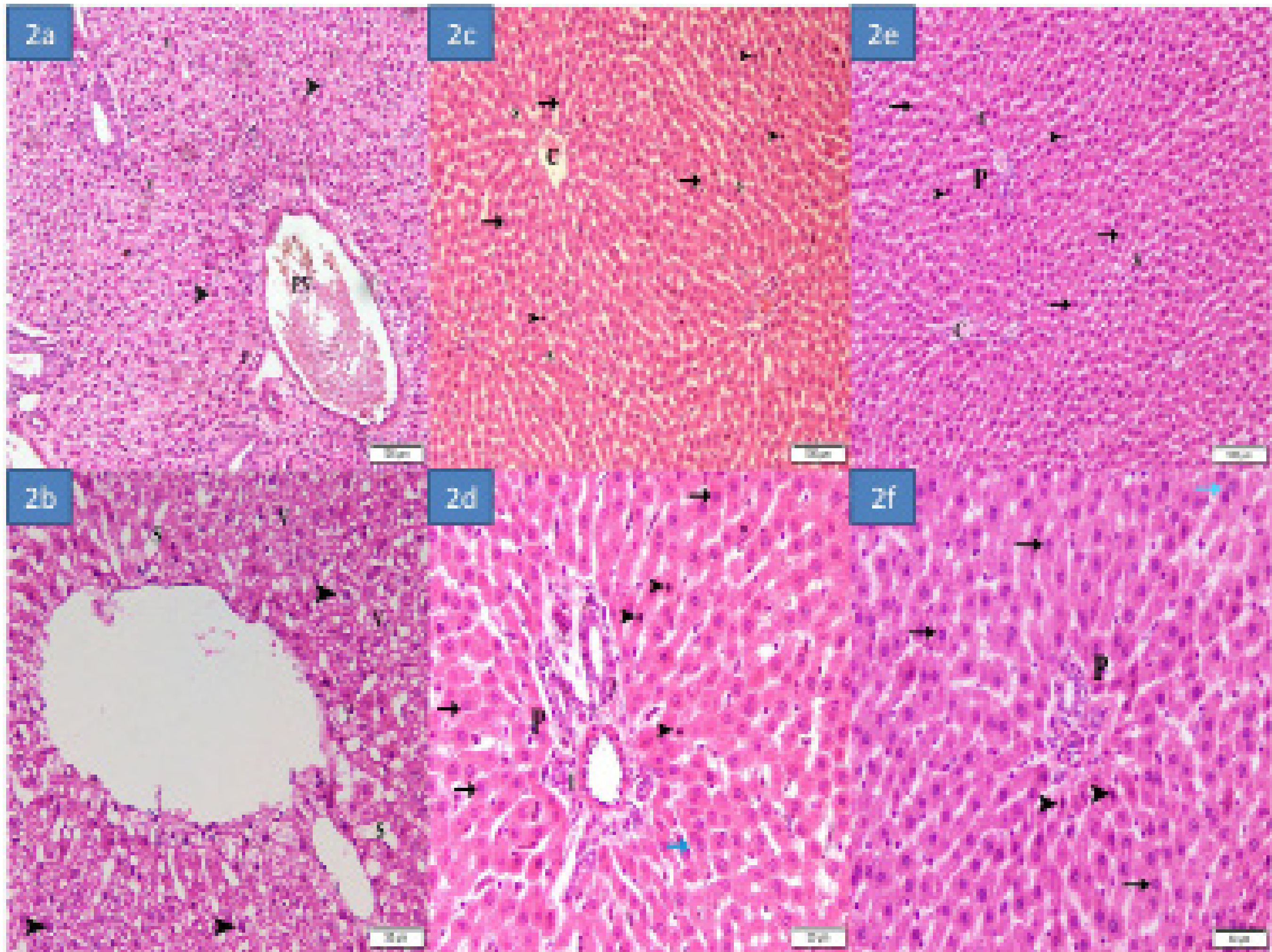


Fig. 2: Photomicrographs of sections in the liver of rats stained with H &E, (2a): group III showing dilated and congested portal vein (PV) in portal tract (P). Hepatocytes exhibit cytoplasmic vacuolations (V) with dark pyknotic nuclei (arrow heads) (x100). (2b): group III showing hepatocytes with marked cytoplasmic vacuolations (V) and dark pyknotic nuclei (arrow heads). Note: sinusoids (S) are apparently dilated(x200).(2c): group IV showing indistinct classic hepatic lobules. Hepatocytes (arrows) radiate from central vein (C) and are separated by blood sinusoids (S). Some dark nuclei (arrow heads) are noticed.(2d): group IV showing that most of hepatocytes are polygonal with vesicular nuclei and acidophilic cytoplasm (black arrows), some hepatocytes are binucleated (blue arrow). Few hepatocytes have dark nuclei (arrow heads). Few cellular infiltration (I) aroundportal area (P) is also noted(x200). (2e): group V showing indistinct classic hepatic lobules with portal area (P). Hepatocytes (arrows) appear radiating from central vein (C) and are separated by blood sinusoids (S). Some hepatocytes have dark nuclei (arrow heads) (x100). (2f):group V showing hepatocytes (black arrows) around portal area (P). Hepatocytes are polygonal with eosinophilic cytoplasm and large, central, rounded vesicular nuclei. Some hepatocytes appeared binucleated (blue arrow) while few hepatocytes show dark nuclei (arrow heads) (x200).

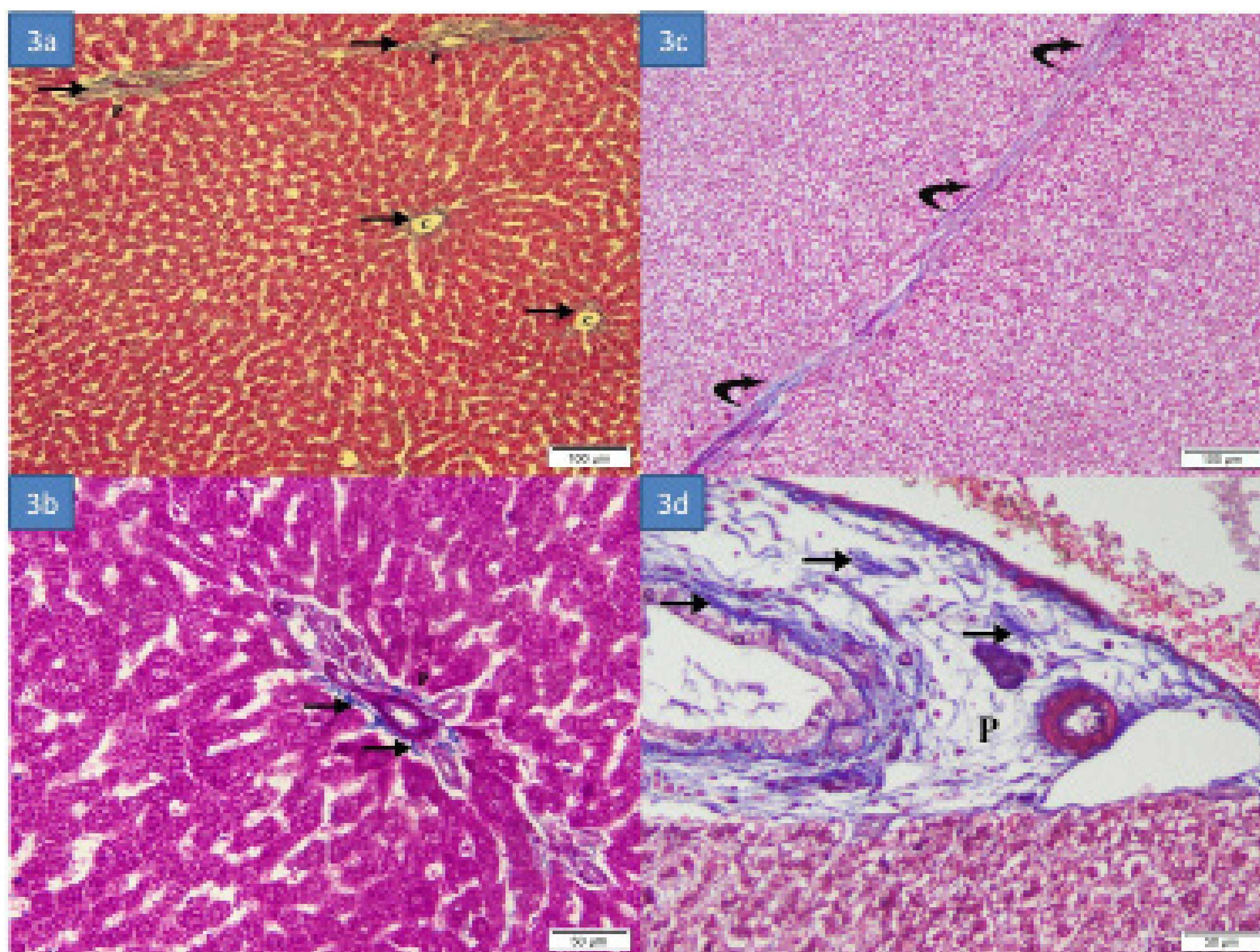


Fig. 3: Photomicrographs of sections in the liver of rats stained with Masson's Trichrome. (3a): group I showing few collagen fibers (arrows) in portal areas (P) and around central veins (C)(x100).(3b): group I showing few collagen (arrows) at the portal area (P) (x200). (3c): group II showing multiple collagen fibers (curved arrows) in the C.T septa outlining the hepatic lobule (x100). (3d): group II showing multiple collagen fibers (arrows) in portal area (P) (x200).

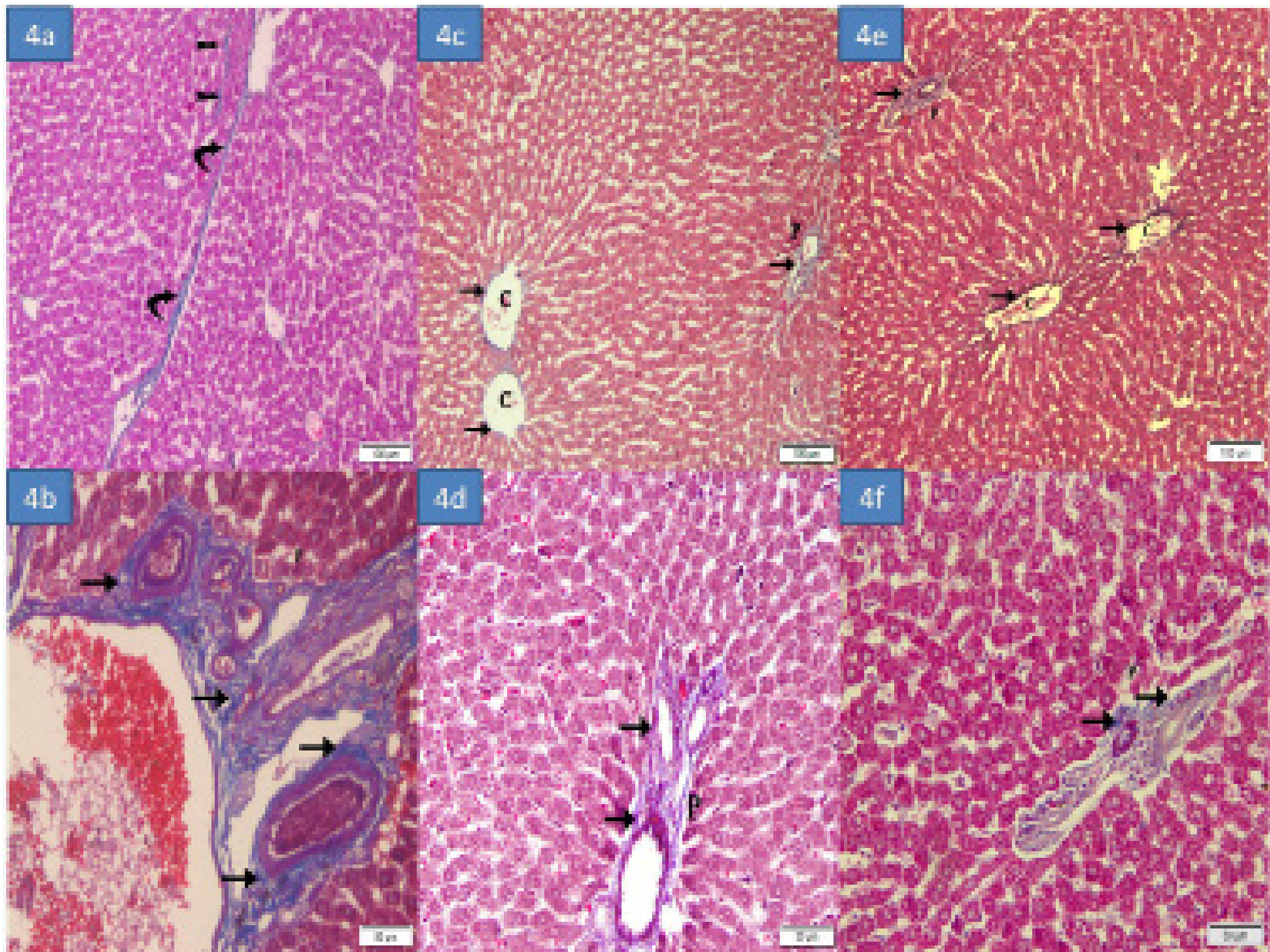


Fig. 4: Photomicrographs of sections in the liver of rats stained with Masson Trichrome. (4a): group III showing multiple collagen fibers (curved arrows) in C.T septa and (bifid arrows) in between the hepatocytes (x100). (4b): group III showing multiple collagen fibers(arrows) in the portal tract (P) (x200). (4c): group IV showing few collagen fibers (arrows) in portal tract (P) and around central veins (C) (x100). (4d):group IV showing few collagen fibers(arrows) in portal tract (P) (x200). (4e): group V showing few collagen fibers (arrows) around portal area (P) and central veins (C) (x100). (4f):group V showing few collagen fibers (arrows) in the portal area (P) (200).

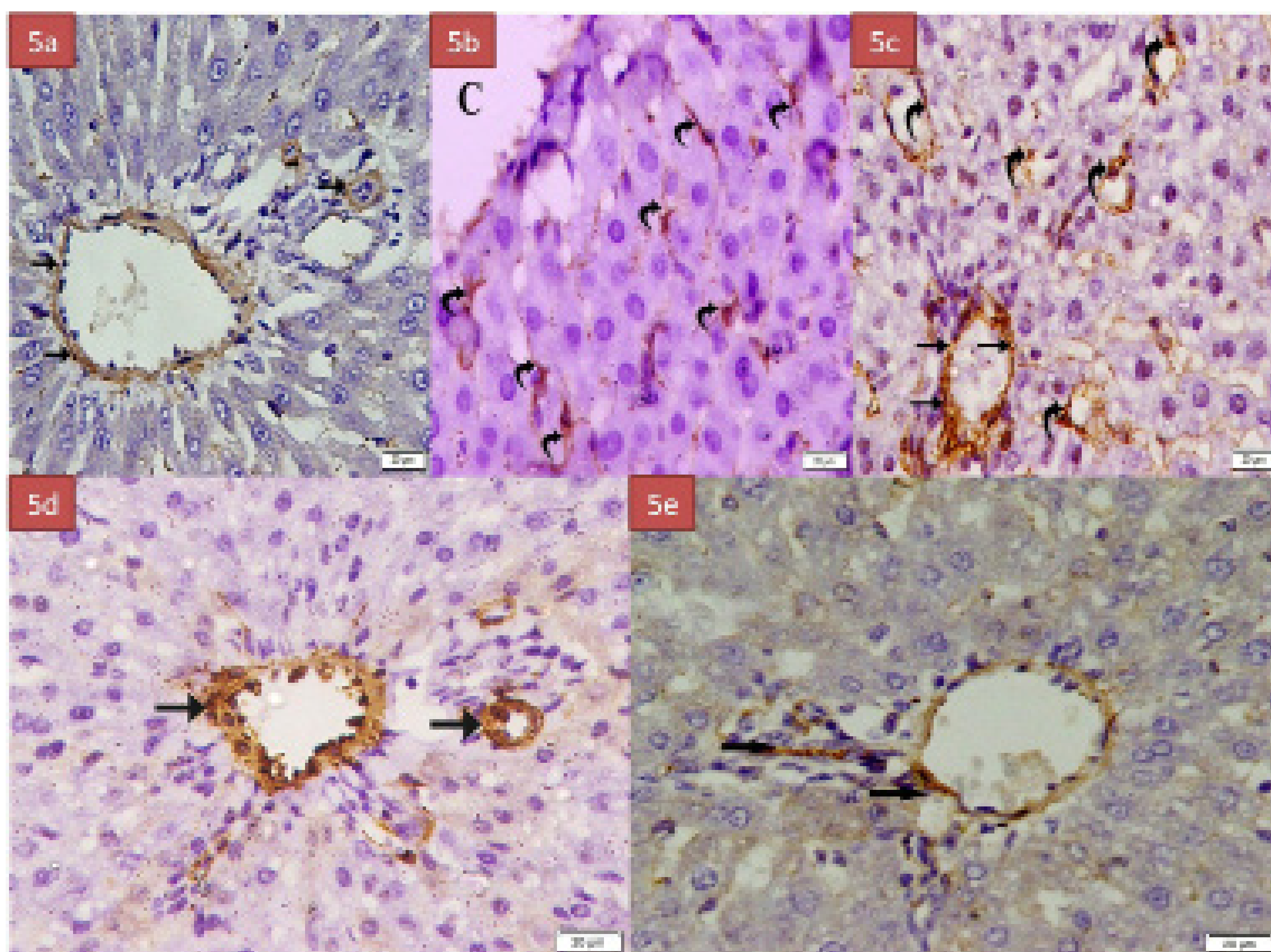


Fig. 5: Photomicrographs of sections in the liver of rats stained with anti α-SMA immunostaining (5a): group I showing positive cytoplasmic reaction for anti α-SMA in the wall of the blood vessels at the portal area (arrows) (x400).(5b): group II showing positive cytoplasmic reaction for anti α-SMA in many cells around the blood sinusoids (curved arrows)around central vein (C). Note branched appearance of the cells (x400). (5c): group III showing positive cytoplasmic reaction for anti α-SMA in many cells is in the wall central vein (arrows) and around the wall of sinusoids (curved arrows)(x400).(5d): group IV showing positive cytoplasmic reaction for anti α-SMA reaction in few cells in the wall of the vessels (arrows) at the portal area. No positive reaction could be seen around the wall of blood sinusoids (x400). (5e):group V showing positive cytoplasmic reaction for anti α-SMA in few cells in the wall of the vessels (arrows) at the portal area. No positive reaction could be seen around the wall of blood sinusoids (x400).

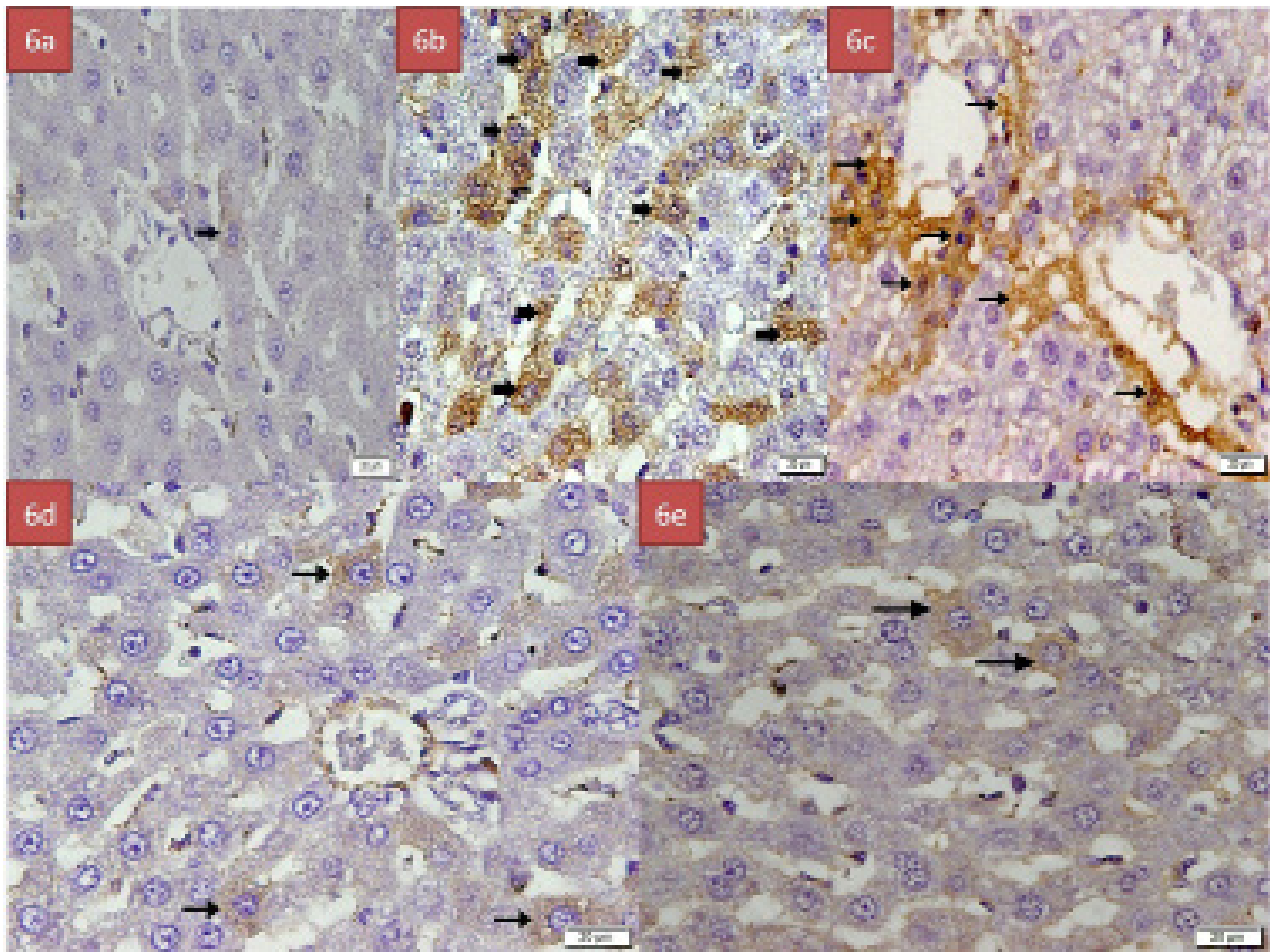


Fig. 6: Photomicrographs of sections in the liver of rats stained with anti caspase 3 immunostaining (6a):group I showing one hepatocyte with faint positive cytoplasmic anti caspase 3 reaction (arrow) around the portal area (P)(x400).(6b): group II showing many hepatocytes with strong positive cytoplasmic anti caspase 3 reaction (arrows)(x400).(6c):group III showing many hepatocytes with strong positive cytoplasmic anti caspase 3 reaction (arrows).(6d):group IV showing few hepatocytes with moderate positive cytoplasmic anti caspase 3 reaction (arrows) (400).(6e):group V showing few hepatocytes with moderate positive cytoplasmic anti caspase 3 reaction (arrows)(x400).

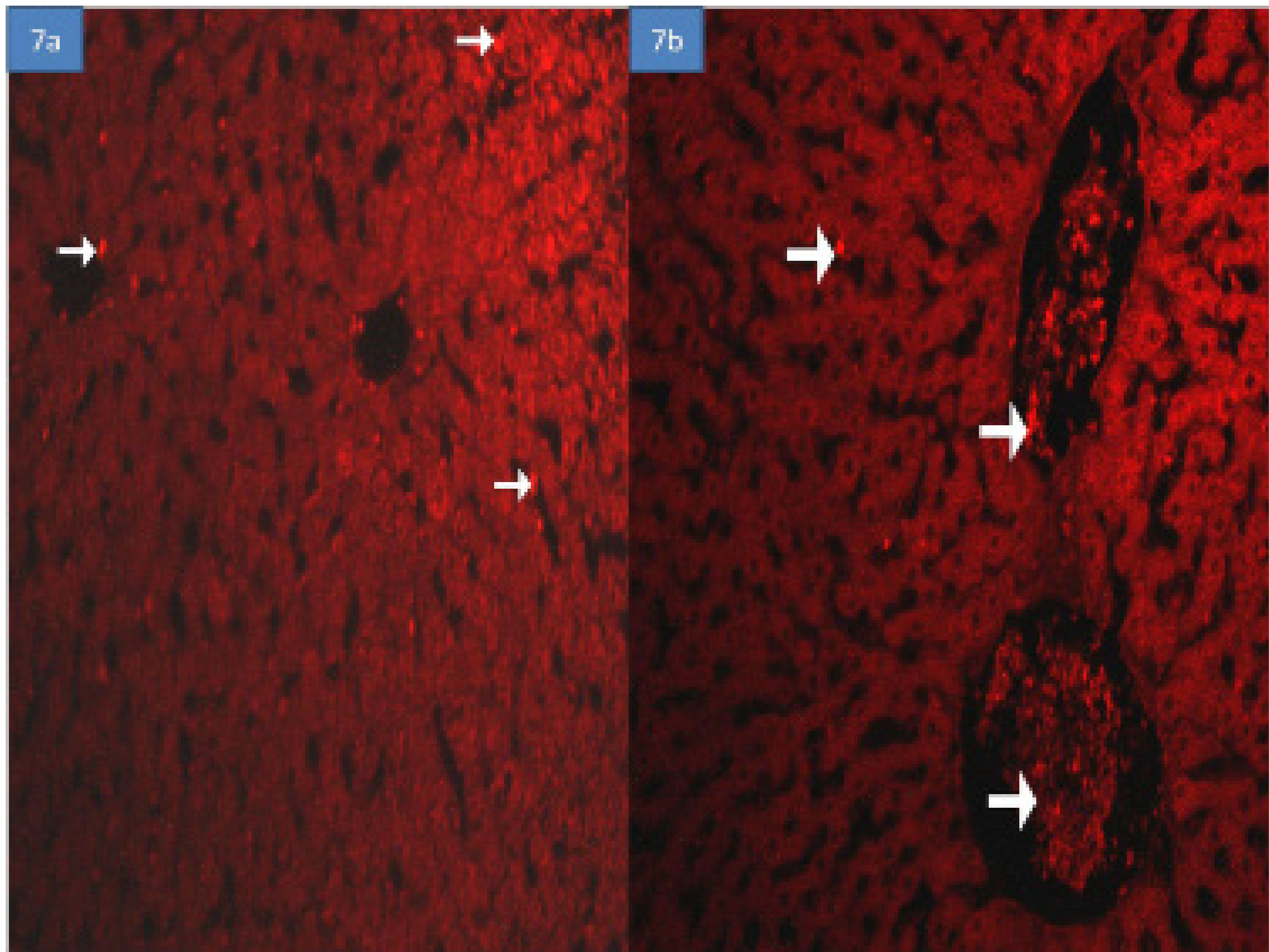


Fig. 7: Photomicrographs of sections in the liver of rats (immunofluorescent) (7a): group IV showing multiple fluorescent labelled cells (white arrows) (x100). (7b): group V showing multiple fluorescent labelled cells (white arrows) (x100).

Table 1: Mean values (IU/L) ± SD of ALT &AST in control &experimental groups at end of 6th week and 10th week

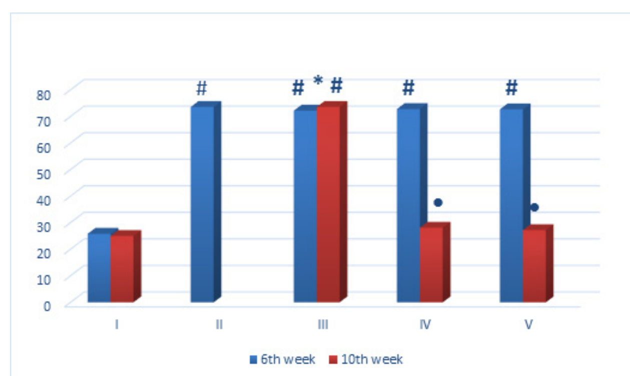
Parameters and group	Group I	Group II	Group III	Group IV	Group V
Mean value of ALT at end of 6th week	25.8±1.92	73.6±2.3#	72.2±2.48#	72.8±1.98#	72.7±2.58#
Mean value of ALT at end of 10th week	25±1.98		73.6±3.23*#	28.1±2.33•	27.2±1.81•
Mean value of AST at end of the 6th week	116.8 ±2.16	166.4 ±1.67#	166.2 ±2.16#	164.8 ±2.34#	165.42 ±3.36#
Mean value of AST at end of 10th week	118.3±3.36		163.8±3.61*#	120.8±3.99•	119±4.08•

significant *P value* in comparison to control group (*P*<0.05).
* significant *P value* in comparison groups IV &V (*P*<0.05).
• significant *P value* in comparison to group III (*P*<0.05).

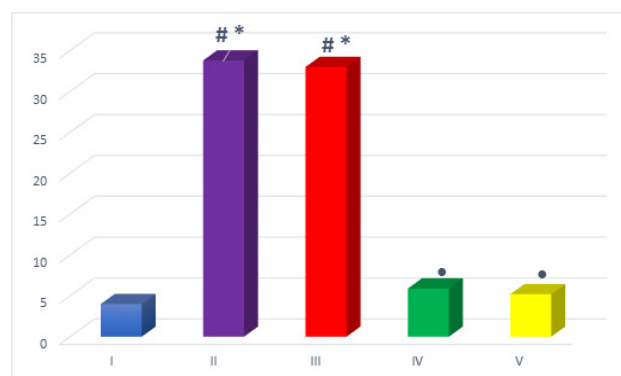
Table 2: The mean ± SD of area % of collagen fibers , mean number of anti α-SMA positive cells, mean number and mean optical denisty of anti caspase 3 +ve cells of control & experimental groups

Parameters and groups	Group I	Group II	Group III	Group IV	Group V
Area % of collagen fibers	9.11±0.73	62.93±1.5#*	62.07±1.72#	12.38±2.71•	11.81±1.81•
Mean no of anti α-SMA positive cells	4.00±1.15	33.8±2.34#*	33.0±1.82*#	5.8±0.99•	5.2±1.31•
Mean no of anti caspase 3 positive cells	3.0±0.81	22.4±1.83*#	22.2±1.58*#	5.3±1.15•#	5.0±0.81•#
Mean optical density of anti caspase 3 positive cells	0.40±0.02	0.87±0.06*#	0.85±0.05*#	0.48±0.04•#	0.47±0.05•#

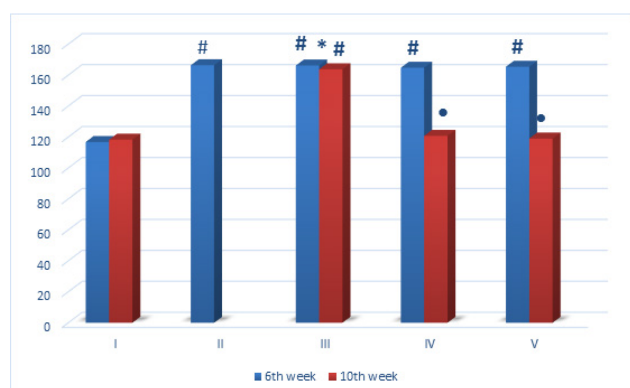
significant *P value* in comparison to control group (*P*<0.05).
* significant *P value* in comparison groups IV &V (*P*<0.05).
• significant *P value* in comparison to group III (*P*<0.05).



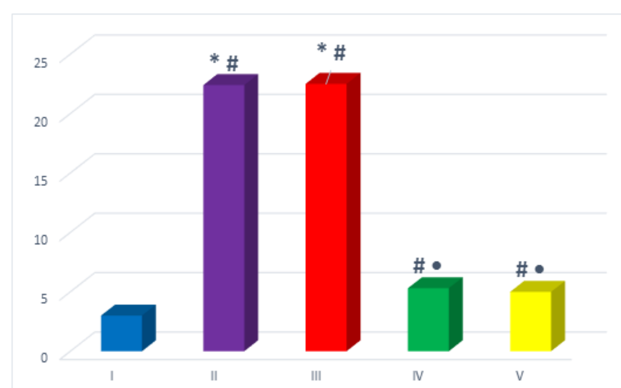
Histogram 1: The mean values± SD of ALT in control and experimental groups at end of 6th week and 10th week
significant in comparison to control group (P<0.05).
* significant in comparison groups IV & V (P<0.05).
• significant in comparison to group III (P<0.05).



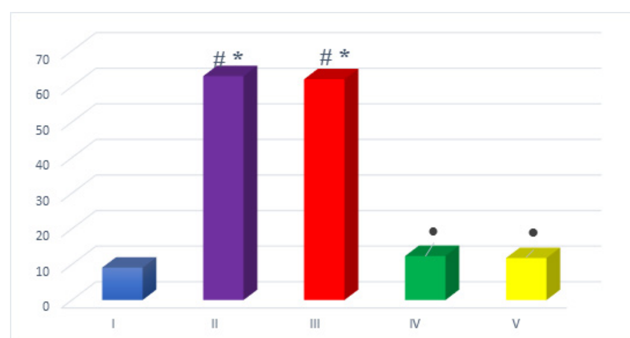
Histogram 4: Mean number± SD of anti α-SMA positive cells in the control & experimental groups
significant *P value* in comparison to control group (P<0.05).
* significant *P value* in comparison groups IV & V (P<0.05).
• significant *P value* comparison to group III (P<0.05).



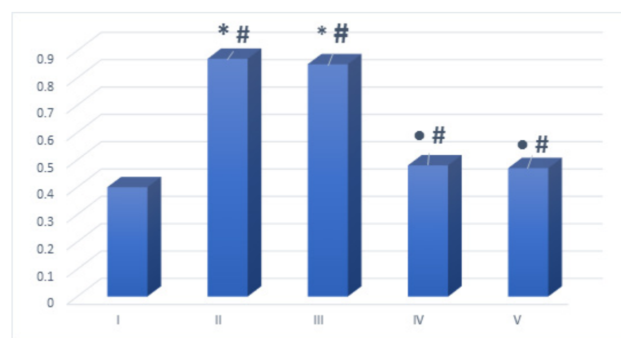
Histogram 2: The mean values ± SD of AST level in control & experimental groups at end of 6th week and 10th week
significant *P value* in comparison to control group (P<0.05).
* significant *P value* in comparison groups IV & V (P<0.05).
• significant *P value* comparison to group III (P<0.05).



Histogram 5: Mean number ± SD of anti caspase 3 positive cells in the control & experimental groups
significant *P value* in comparison to control group (P<0.05).
* significant *P value* in comparison groups IV & V (P<0.05).
• significant *P value* comparison to group III (P<0.05).



Histogram 3: The mean ± SD area % of collagen fibers in control & experimental groups
significant *P value* in comparison to control group (P<0.05).
* significant *P value* in comparison groups IV & V (P<0.05).
• significant *P value* comparison to group III (P<0.05).



Histogram 6: Mean optical denisty ± SD of anti-caspase 3 positive cells in control & experimental groups
significant *P value* in comparison to control group (P<0.05).
* significant *P value* in comparison groups IV & V (P<0.05).
• significant *P value* comparison to group III (P<0.05).

DISCUSSION

Liver fibrosis progresses eventually to liver cirrhosis, one of the most common death causes worldwide. It complicates to serious conditions, as portal hypertension, liver cell failure and liver cancer resulting in destruction of liver structure and ultimately death[24].

Mesenchymal stem cells administration could decrease liver fibrosis and improve functions of the liver primarily through differentiation to hepatocytes, regulation of immune system, production of cytokines, hepatocyte apoptosis reduction and hepatocyte regeneration promotion(5). Exosomes have important roles in acute or chronic liver diseases. Furthermore, many studies showed that exosomes could protect the liver, improve antioxidation pathways and enhance drug sensitivity in liver diseases[25].

This current study evaluated and compared stem cells versus exosomes therapeutic effect in liver injury caused by CCL4 in rats. It was reported that CCL4 liver fibrosis model was appropriate for simulating human liver diseases It can cause hepatocyte inflammation and damage through generation of free radical species leading to liver fibrosis[26].

In the present study 0.1 ml of CCL4 was administered IP twice/ week for 6 weeks to rats to evoke liver injury. In accordance Wu *et al*., (2020)[27] demonstrated that intoxication with CCL4 inflicted liver fibrosis in a dose & time-dependent manner. Bao *et al*, (2021)[28] stated that four-weeks repeated CCL4injections could cause early stages of hepatic fibrosis while the injections for 6-8 weeks might lead to progression in the pathological changes. The disease progression over 12 weeks resulted in cirrhosis of mice with high mortality.

The mean values of serum ALT and AST measured at end of 6th week increased significantly in all the groups in comparison to group I. The previously mentioned changes indicated liver injury and damage which affected the liver function. Going with, Wang *et al*., (2020)[29] commented that there was an increase in ALT & AST levels, after CCL4 IP injection twice per week in a rat model for 6 weeks as a liver injury result.

Han *et al*, (2019)[30] explained that following CCL4 liver injury hepatocytes were destructed, permeability of cell envelope increased and large amounts of transaminases, as ALT & AST which are main indicators for early liver injury would be secreted into the blood.

In group III, the mean values of serum ALT and AST at the 10th week indicated a significant increase in comparison to group I & treated groups. Such finding suggested that liver function would not improve if left without treatment. Similarly, it was demonstrated that blood serum ALT, AST levels of CCL4-treated group with liver fibrosis were raised compared to group I in a rat model[31].

In groups IV& V, the mean values of ALT and AST levels at end of experiment decreased significantly when compared to groups II&III. Going with previous studies which stated that stem cells & exosomes could significantly enhance hepatic function and liver enzyme levels by improving fibrosis caused by CCL4 injection in liver of rats[32,33].

Rong *et al*., (2021) stated that the serum levels of ALT and AST were significantly suppressed in the MSCs and MSCs-Ex-treated groups in CCl4 induced liver fibrosis rat model. They explained that the MSCs and MSCs derived exosomes are believed to alleviate pathological changes through the transfer of their cargo to injured cells. They could inhibit inflammation and contribute to hepatocyte regeneration by decreasing inflammatory cytokines, which are one of the main factors inducing liver fibrosis and promoting liver tissue repair by reducing the inflammatory response, enhancing the recovery of liver functionality[14].

In group II, H&E sections showed vacuolations in hepatocytes and dilatation of central hepatic vein. Other fields showed hepatocytes with pyknotic nuclei. Mononuclear cellular infiltration in a portal vein was also observed. The previously mentioned changes indicated inflammatory degenerative process developed in the liver. Similar changes were illustrated by Shi *et al*., (2020)[34] as they found severe pathological changes including vacuolation, inflammation and degeneration in liver tissue after CCL4.

It was reported that there was inflammatory cell infiltration after CCL4 administration in the liver of a mouse model[27]. Also it was revealed that CCL4 resulted in destruction of the tissue & infiltration of inflammatory cells within lobules and portal tracts of the liver[35].

Congestion and Dilatation in blood vessels & portal vein was noted in CCL4induced liver injury and it was caused by portal hypertension and direct effect of CCL4 on blood vessels which lead to secretion of nitric oxide relaxation factor from the endothelial cells[36]. It may be also explained as a part of the inflammatory process[37].

CCL4 was broken down by cytochrome P-450 in liver which lead to production of free radicals. This caused changes in the endoplasmic reticulum membrane and other cellular membranes leading to increase the calcium ion permeability of membrane which affect the homeostasis of calcium. This in turn, activated proteases of the cells, degraded protein& phospholipids and led to inflammation and cytotoxicity[37].

Liver sections of group III showed no improvement where hepatocytes exhibited marked vacuolations in the cytoplasm with dark pyknotic nuclei. Congested dilated portal veins could be detected in portal areas. Dilation of blood sinusoids was also noted. Similarly a previous study reported that following the injection of CCL4 twice weekly for 8 weeks, the injected group showed fibrosis with degeneration of hepatocytes and many vacuoles in the liver tissue. Liver injury was indicated by the observation of cell death around central vein and the disorganisation of blood sinusoids[38].

Abdel Aal *et al*., (2019)[39] reported that the dilatation of sinusoids might be due to the peri-sinusoidal cells activation which had contractile properties in drug induced liver injury.

Group IV and group V exhibited multiple fluorescent PKH26 labelled cells which confirmed the homing of MSCs and exosomes. In agreement, Lee *et al*., (2021)[40] reported that induction and migration of cells to damaged area was the primary feature for effective stem cell therapy. The ability of MSCs to migrate to the liver following administration was critical to their regenerative potential in liver disease. Stress signals that were generated by tissue damage attract stem cells to the area of injury. MSC surface-expressed molecules made it easier for the cells to adhere, activate, and migrate into injured tissue.

Liver sections of group IV showed regression of degenerative changes. most of hepatocytes appeared normal while few hepatocytes showed dark pyknotic nuclei. These findings were concomitant with Abdel Aal *et al*., (2019)[39] who reported marked improvement in histology of the liver of CCL4 rat model following MSCs treatment.

Also it was reported that, MSC therapy showed decline in apoptosis of the hepatocytes, veins congestion and collagen deposition. Histopathological examination showed that activity of the liver increased following treatment with MSCs by recovering liver fibrosis, fat alterations and inflammatory changes[41].

Khalil *et al*., (2020)[42] explained that the MSCs secreted cytokines and growth factors to encourage regeneration, prevent inflammation, and slow down the production of ECM and the breakdown of the intrahepatic excess in ECM. They also added that BMMSCs enhanced antioxidant defenses, inhibited oxidation factors and able to differentiate into hepatocytes.

Liver sections of group V showed apparently preserved hepatic architecture. In accordance Jiang *et al*., (2018)[43] stated that the treatment with exosomes showed preserved liver architecture with no fibrosis or cellular infiltration. They explained that they possessed anti-fibrotic, anti-inflammatory and proangiogenic features promoted it to reverse fibrosis in livers of the rats treated by CCL4.

It was reported that treatment with exosomes improved hepatocyte viability, reduced the production of pro-fibrotic molecules, as collagen, α-SMA and increased collagenases, as MMP-9. It also inhibited infiltration of inflammatory cells, hepatocyte apoptosis in liver fibrosis[44].

liver sections stained with Masson's trichrome showed that collagen mean area percent of group II& III significantly increased in comparison to group I. In agreement, Mahmoud *et al*., (2019) & Shi *et al*., (2020) [45,34] revealed expansion in fibrous tissue in portal and central vein areas on group treated CCL4. They also stated that there was a significant increase in collagen in comparison to control.

It was explained that prolonged release of TGFβ (one of the strongest profibrosis factors), associated with CCL4induced hepatocyte destruction activated quiescent HSCs to convert to the star-shaped stellate cells or to myofibroblast-like cells, which in turn released growth factors and inflammatory signal factors in the liver microenvironment, synthesized large amounts of ECM including collagen. They could also lead to an imbalance of MMPs and tissue inhibitor of metalloproteinase ending in liver fibrosis[46].

Group IV showed regression of the CCL4 induced fibrotic changes, which was confirmed statistically as mean area perecnt of collagen decreased signifiacntly in comparison to groups II & III. Hermansyah *et al*., (2021) [47] found similar results and indicated that in the liver fibrosis animal model, MSCs reduced significant collagen fibers by causing active HSCs apoptosis, as well as by inhibiting profibrotic genes and elevating antifibrotic hepatic factors like MMP-9.

The ability of MSCs to modulate immune responses may also have contributed to the remission of fibrosis. By regulating the polarisation of inflammatory cells and cytokines secretion, MSCs were able to control the pro-fibrotic environment[48].

Group V revealed that collagen mean area percent decreased significantly in comparison with groups II &III. In agreement, Rong *et al*., (2019)[14] demonstrated that the liver area which positively stained for collagen showed a significant reduction in rats treated with BMMSCs-Exos when compared to the CCL4 group. They explained that the BMMSCs-Exos treatment downregulated the expression of several proteins, which inhibited HSCs and myofibroblastic activation and led to liver fibrosis reduction.

Groups II & III showed that the mean number of αSMA immunoreactivity at portal area & around blood sinusoids increased significantly in comparsion to control. Similarly, Wang *et al*., (2019)[49] reported that there was a significant over-expression of α-SMA in CCL4 injected rats. They also stated that α-SMA is the marker of HSCs and the levels of its expression in liver tissues could be used to detect the liver damage extent.

CCL4 induced liver injury resulted in HSC activation, propagation and progression into a myofibroblast-like phenotype which could release many ECM components as collagen and express α-SMA ending in fibrosis and increased collagen deposition[50].

Liver sections in groups IV&V revealed that mean number of α-SMA positive cells decreased significantly in comparison with groups II &III. No significant difference was detected as between group IV & V. Similarly, Mazhari *et al*., (2020)[51] stated that treatment with stem cells led to in the reduction of α-SMA expression in comparison to CCL4 treated group.

Furthermore, the direct & indirect effects of MSCs in inhibition of HSCs activation and growth which might inhibit collagen production. The direct interaction between MSCs and HSCs aided in the inhibition of HSC proliferation by stimulating arrest of G0/G1 cell-cycle. MSCs, also contain high levels of milk fat globule-EGF factor 8, which reduces TGF-1 receptor expression on HSCs, so thus prevents HSCs activation[52].

Rong *et al*., (2019)[14] documented a lower α-SMA immunoexpression by exosomes treatment in CCL4 induced liver toxicity in a rat model. Similarly, it was reported that the treatment with exosomes decreased αSMA expression significantly through inhibition of proliferation of HSCs &activation and prevention of the fibrotic myofibroblast phenotype which resulted in degradation of collagen and reduction of fibrosis of the liver[53].

Caspase 3 immunostained sections in group II& III showed many hepatocytes with strong positive caspase cytoplasmic reaction. Statistically by a the mean number & mean optical density of caspase 3 immunoreactivity increased significantly in groups II &III when compared to group I and the treated groups.

Chang *et al*., (2021)[54] stated that the caspase 3 expression was very high in CCL4 groups. Han *et al*., (2019) &Lim *et al*., (2021)[30,55] explained that the oxidative stress was thought to be important pathology of liver damage caused by CCL4. So when the body stimulated by harmful factors, it would react by producing a lot of highly-active substances, as reactive oxygen and nitrogen species, leading to an imbalance between the oxidation and antioxidantion defence mechanisms. They added that infiltration with neutrophils and the large number of oxidative intermediates led to tissue damage and apoptosis. When DNA damage occurred, the tumor suppressor p53 promoted cell death by increasing pro-apoptotic genes as caspase 3 & caspase 9 and decreasing antiapoptotic genes a Bcl-xL& Bcl-2.

Group IV showed that the mean number & mean optical density of anti-caspase 3 immunoreactivity decreased significantly in group IV when compared to groups II & III. These findings were concomitant with previous study which reported that the caspase 3 staining expression was decreased after MSC treatment as it has the potential to reverse oxidative stress-induced apoptosis in liver damage caused by CCL4[43].

Lee *et al*., (2021)[40] explained that Superoxide dismutase, a key antioxidant defence that shields bodily tissues from oxidative stress, was generated by MSCs. MSCs increased antioxidant and cytoprotective activities to lower hepatocyte apoptosis and decreased ROS in damaged liver cells. They were able to release many growth factors, as hepatic and nerve growth factor, which inhibited apoptosis in hepatocytes and were crucial for liver regeneration.

Group V showed that the mean number and mean optical density of anti-caspase 3immunoreactivity decreased significantly in comparison to groups II &III. It might be explained that the antiapoptotic effects of exosomes on hepatocytes. it prevented death of hepatocytes , increased the liver regeneration & improved survival rate in rats after severe hepatic failure caused by drugs. It can also secrete growth factors and anti-inflammatory cytokines[56].

Bruno*et al*., (2020)[57] demonstrated increase in Bcl-xL expression with decrease in caspase 3/7 expression in the drug induced liver-injury group received exosomes. This suggested that exosomes can alleviate mitochondrial dysfunction as part mechanisms in the anti-apoptosis pathway. They claimed that exosomes could potentially activate proliferative, regenerative and antiapoptotic hepatic responses after liver injury.

Finally, in the light of the present results, it was found that treatment with MSCs and exosomes showed nearly the same serological & histological improvement which was confirmed statistically.

CONCLUSION

Liver injury caused by CCL4 , in form of inflammatory and degenerative changes which progressed to fibrosis. MSCs and exosomes therapy proved definite therapeutic effect by regression of degenerative and fibrotic changes, effect was nearly the same.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية مقارنة علي التأثير العلاجي للخلايا الجذعية الوسيطة مقابل الاكسوزومات علي إصابة الكبد المستحدثة برابع كلورايد الكربون في ذكور الجرذان البيضاء البالغة

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الخلفية: تعد امراض الكبد عبئ صحي عالمي كبير وتؤدي الي الكثير من المضاعفات اذا تركت بدون علاج.
الهدف: تقييم و مقارنة التأثير العلاجي للخلايا الجذعية المستخلصة من نخاع العظم مقابل الاكسوزمات علي اصابة الكبد المستحدثة برابع كلورايد الكربون في الجرذان
الطرق: تم استخدام اثنين وثلاثون من ذكور الجرذان البيضاء البالغة و قد قسمتالي خمس مجموعات: المجموعة الاولي)المجموعة الضابطة(, المجموعة الثانية (مجموعة عقار رابع كلوريد الكربون),المجموعة الثالثة (مجموعة الشفاء التلقائي), المجموعة الرابعة (المجموعة المعالجة بالخلايا الجذعية الوسطية),المجموعة الخامسة (المجموعة المعالجة بالاكسوزومات).تلقي كل جرذان المجموعات التجريبية ٠,١مل من عقاررابع كلوريد الكربون (CCL٤) مذاب في زيت الزيتون مرتين اسبوعيا بالحقن البريتوني لمدة ستة اسابيع. ثم تم حقن (١×١٠٦) من الخلايا الجذعية الوسطية عبر الوريد الذيلي لجرذان المجموعة الرابعة بعد اخر جرعة من CCL٤، تم حقن الاكسوزوماتعبر الوريد الذيلي لجرذان المجموعة الخامسة بعد اخر جرعة من CCL٤.تم التضحية بحيوانات المجموعات الثانية في نهاية الاسبوع السادس بينما حيوانات المجموعة الاولي والثالثة والرابعة والخامسة في نهاية الاسبوع العاشر . تم تعيين مستوي انزيمات الكبد ناقلة امين الاسبارتات وناقلة امين الالانين في الدم نهايةالاسبوع السادس و الاسبوع العاشر.
تمت صباغة القطاعات بالهيماتوكسيلين والايوسين, ماسون ثلاثي الالوان, الصبغ النسيجي الكيميائي المناعي: الفا اكتين العضلات الملساء ,كاسبس ٣. تم قياس مستوي ناقلة امين الاسبارتات وناقلة امين الالانين متوسط المساحة المئوية لألياف الكولاجين و متوسط عدد الخلايا المتفاعلة إيجابيا مع الأجسام المضادة لاكتين العضلات الملساء الفا و كاسبس ٣ ومتوسط الكثافة الضوئية للخلايا الموجبة لكاسبس
النتائج: في المجموعة الثانية كان هناك فجوات في سيتوبلازم خلايا الكبد و ظهرت انوية داكنة منكمشة في خلايا الكبد.كان هناك ارتفاعا ذا دلالة احصائية في متوسط قيمة انزيمات الكبد و متوسط المساحة المئوية لألياف الكولاجين و متوسط عدد الخلايا المتفاعلة إيجابيا مع الأجسام المضادة لاكتين العضلات الملساء الفا و كاسبس ٣ ومتوسط الكثافة الضوئية الخلايا الموجبة لكاسبس في المجموعة الثانية والثالثة مقارنة بالمجموعة الضابطة والمجموعات الاخري.
في المجموعة الرابعة و الخامسة ظهرت خلايا الكبد طبيعية و القليل من الخلايا كان لديها انوية داكنة منكمشة . كان هناك انخفاض ذا دلالة احصائية في متوسط قيمة انزيمات الكبد و متوسط المساحة المئوية لألياف الكولاجين و متوسط عدد الخلايا المتفاعلة إيجابيا مع الأجسام المضادة لاكتين العضلات الملساء الفا و كاسبس ٣ ومتوسط الكثافة الضوئية للخلايا الموجبة لكاسبس في المجموعة الرابعة والخامسة مقارنة بالمجموعة الضابطة الثانية والثالثة.
الاستنتاج:أحدث العلاج بالخلايا الجذعية الوسيطة والاكسوزومات تأثيرا ملحوظا في تراجع التليف في نموذج تليف الكبد في الجرذ.