The Hepatotoxic Effect Induced by Methotrexate Therapy and Protective Role of Bone Marrow-Derived Mesenchymal Stem Cells in Adult Male Albino Rats. Histological and Ultrastructural Study

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Abstract: Background: One of the significant side effects of methotrexate (MTX) was hepatotoxicity, for this reason the clinical use of this drug was restricted. The present study was intended to research the hepatoprotective impacts of bone marrow-derived mesenchymal stem cells against methotrexate, based on histological, immunohistochemical and ultrastructural parameters. **Material & methods:** Twenty four adult male albino rats were randomly divided into four experimental groups. Group I represented as control. Male adult albino rat received intramuscular (IM) injection of MTX (10 mg/ kg B wt.) once weekly for 6 weeks (group II) and others held for another 6 weeks without therapy then were sacrificed (group III). The rats received single dose of BM-MSC sintraperitoneal injection after induction of hepatic damage with MTX served as group IV. Light and electron microscopy were done to evaluate the histopathological changes. **Results:** Suppressive effect of BM-MSCs on activated hepatic stellate cells (HSC) was evaluated using immunohistochemical staining. Several histopathological changes were observed in liver cells of MTX-treated animals; when compared with hepatocytes of control rats, had depletion of glycogen, distortion of hepatocyte and infiltration with some inflammatory cells. **Conclusion:** Our finding proved that of BM-MSCs injection after induction of hepatic damage with MTX induced therapeutic effects. Moreover, it reduced the hepatic lesions and significantly minimized the MTX-induced histological alterations and nearly restored the normal architecture of liver.

[Eman E. Elwakeel and Amira Z. Mohamed. **The Hepatotoxic Effect Induced by Methotrexate Therapy and Protective Role of Bone Marrow-Derived Mesenchymal Stem Cells in Adult Male Albino Rats. Histological and Ultrastructural Study.** *J Am Sci* 2018;14(5):91-98]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 14. doi:10.7537/marsjas140518.14.

Keywords: Methotrexate, Bone marrow, Hepatic toxicity, Stem cells

1. Introduction

Liver is the main vital organ in the body as well the drug-induced hepatic injury can be described as a main issue that oppose the track of the drug treating and shorting its salutary role as aniticancer drug (1). Liver injuries caused by drugs include various pathways, comprise immune response and coordinate poisonous effect with the formation of dynamic metabolites. Several disorders, including autoimmune diseases, malignant tumors, and inflammatory disorders has been treated by using methotrexate (MTX) (2,3).

Dihydrofolate reductase has been prevented via MTX (4). A broad range of clinical applications could be deal with MXT like "psoriasis, psoriatic arthritis, and acute lymphocytic leukemia, ectopic and ulcerative colitis (5). MTX therapy has a side effects and the critical one is liver toxicity frommild hepatitisto acute liver failure (6). Mechanisms implicit MTX which result to hepatotoxicity didn't fully comprehend. But, some of researches gave a certainstatement that MTX conducts excess generation of reactive oxygen species with the synthesis of lipoperoxidation and nitric oxide (NO) in the liver (7). As well, study for finding medications to be utilized as reciprocal treatment beside the MTX therapy was beneficial to minimize the hepatotoxicity (6).

Mesenchymal stem cells were discussed initially by Friedenstein et al. (1970) (8), the isolation for those adult stromal cells from several sources considering bone marrow (BM), umbilical cord blood, adipose tissue, and other tissues (9, 10). The population of cells were described by Friedenstein et al. (1970), whom depict multipotential stromal precursors, which present spindle-shaped morphology. Bone marrow–derived MSCs, were categorized as hematopoietic stem cells and stem cells of non-hematopoietic tissue, variously known as mesenchymal stem cells. Multipotential stem cells are qualified to distinguish into both mesenchymal and nonmesenchymal cell lineages. Whether BM-MSCs participate to repair the tissue by differentiation into tissue-specific cell types (11, 12) or by producing trophic factors at the place of injury (13) to catalyze repairing tissue and to minimize tissue damage mediated by the immune system remains unknown (14). Their ability for tissue repair and immune modulation make them promising candidates for use in regenerative medicine and solid organ transplantation.

Based on the previous background, the present work was designed to declare the histological, immunohistochemical and ultrastructural changes of adult albino rat's liver after injection with therapeutic methotrexate doses and the possible protective function of bone marrow-derived mesenchymal stem cells on such liver toxicity.

2. Materials and methods

2. 1. Animals

Twenty four healthy male albino rats (weighing 200 ± 10 g) were obtained from the laboratories of ministry of agriculture. Rats were divided into four groups and placed in separate cages at a regulated environment (12-h dark/light cycle, 25 ± 2 °C temperature and 50 ± 5% humidity). Rats were fed with standard diet (El-Nasr Company, Abou Zaabal, Cairo, Egypt) with grant reach to water and libitum. Experimental ethics and procedures in accordance with the international ethical guidelines for animal carewere followed and approved by the ethical committee, Faculty of medicine, Benha University, Egypt.

2.2. Drugs, chemicals and reagent kits

2.2. 1. Methotrexate

MTX was presented as a solution ready for injection. 10 mg of methotrexate was present in each 1ml of the solution (The medication was prepared by dissolving MTX in isotonic saline. It was purchased from Algomhoria Company. It was given to rats with a dose of 10 mg/ kg Body weight once weekly for 6 weeks by IM injection (15).

2.2. 2. Bone marrow-derived mesenchymal stem cells

Rat bone marrow-derived MSCs were purchased in the form of vials from the Biochemistry Department, Kasr Al-Ainy Medical School. Each vial contained 3×10^6 BM-MSCs suspended in 0.5 ml PBS. The vials were maintained in an ice box until injection within 5 h (16).

2.3. Experimental design

After the acclimatization period, twenty four male albino rats were divided into four groups as follows:

Group I (Control group): This group included 6 rats and represented the experimental control group and did not receive additional treatment other than the standard diet.

The rats in this group were then sacrificed after 6 weeks.

Group II (MTX treated group): This group included 6 rats that were IM injected with MTX (10 mg/ kg B wt.) once weekly for 6 weeks (15).

Group III (Withdrawal group): This group included 6 rats that received MTX for 6 weeks and were held for another 6 weeks without therapy then they were sacrificed.

Group IV (MTX and BM-MSCs treated group): This group included 6 rats that were IM injected with MTX once weekly for 6 weeks and then were injected with a single intraperitoneal injection of 3×10^6 BM-MSCs suspended in 0.5 ml PBS. The rats were sacrificed 4 weeks following stem cell therapy (the total time of sacrifice was after 10 weeks).

2.4. Histological and immunohistochemical study

At the end of the experiment, the experimental animals were anesthetized by using chloroform inhalation and dissected. The technique was performed by collecting fresh specimens of liver and immediately immersion in 10% buffered formalin solution in normal saline for 24 h, then washing by distilled water, after that dehydrated with serial dilutions of alcohol and finally dehydrated using xylene and embedded in paraffin for 24 h in a hot air oven at 56 °C respectively. Tissue blocks were made using paraffin beeswax by sledge microtome, followed by deparaffination. slides were stained by hematoxylin and eosin , by modified Mallory's trichrome and modified avidin–biotin immunoperoxidase for α smooth muscle actin (α-SMA). Positive immunoreactivity for α-SMA appeared as brown cytoplasmic staining of varying degrees. The cancelation of step which involved the primary antibody led to acquiring the negative control (17) for histological examination. Microscopic examination of the stained sections was done using an Olympus BX60 light microscope (18).

2.5. Ultrastructure examinations

For Transmission Electron Microscope (TEM) examination, small liver specimens (1 mm^3) were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 h, postfixed in 1% osmium tetroxide in a 0.1 M phosphate buffer (4 °C, 1.5 h), then the sample was immersed in serial dilution of ethanol (50, 70, 90, 95 and four times 100%, each for 15 min) for dehydration and dehydrated by acetone for 30 minutes. Finally, the fixed specimens were embedded in epoxy resin (Epoxy Embedding Medium Kit; Sigma). Semi- and ultra-thinsections were cut on LEICA Ultra microtome. Semi-thin sections (0.8 m thick) were stained with 1% toluidine blue in 0.5% borax and observed using an Olympus BX60 light microscope. Ultra-thin sections were cut at 70 nm and stained with uranyl acetate as a principal stain and lead citrate as a counter stain and finally the sections were examined using JEM 100SX (JEOL, Japan) transmission electron microscope at the electron microscopic unit, operate at 80 kV, Faculty of medicine, Tanta University, Egypt (19).

3. Results

Pathological finding

1. Hematoxylin and Eosin stain:

The liver of control rats showed a normal histological appearance of hepatocyte and sinusoidal architectures (Figure 1). However, in methotrexate-treated rats showed distortion of hepatocytes architecture. Also cytoplasmic vacuolation and congested blood sinusoids could be observed (Figure 2). In withdrawal group, the normal radial arrangements of hepatocytes from central vein were severely disrupted with severe cholestasis. Congestion of some blood capillaries and hemorrhage of others could be seen throughout the hepatic parenchyma (Figure 3). While in rats treated with MTX and BM-MSCs showed significant reduction in the histopathological changes that induced by MTX alone or individually absent and restored to the normal appearance (Figure 4).

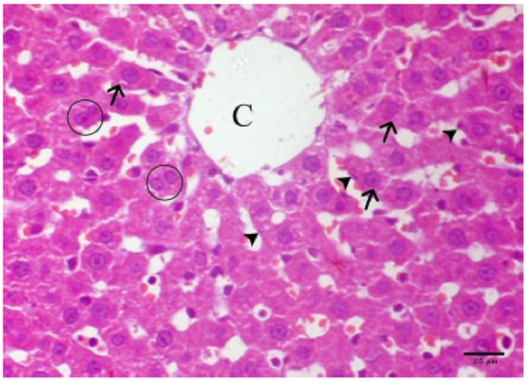

Figure (1): A photomicrograph of a section in the liver from control group showing radiating cords of hepatocytes from the central vein (C). The hepatocytes have central, rounded, vesicular nuclei and acidophilic cytoplasm (arrow). Some of the cells appear bi-nucleated (circle). Notice the lining cells (arrow head) of the blood sinusoids. (H & E x400).

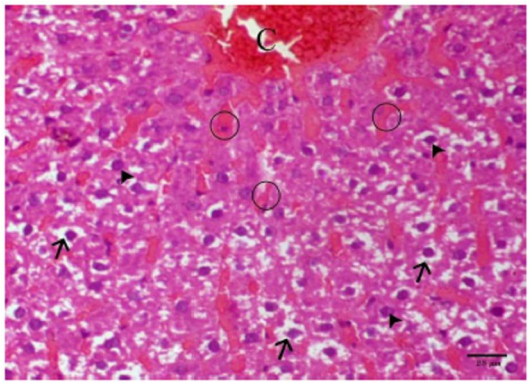

Figure (2): A photomicrograph of a section in the liver from group II showing loss of architecture of radiating cords of hepatocytes from the central vein (C). The hepatocytes are vacuolated and lacking acidophilic cytoplasm (arrow) with deeply stained nuclei (arrow head). Notice the congestion (circle) of the blood sinusoids. (H & E x400).

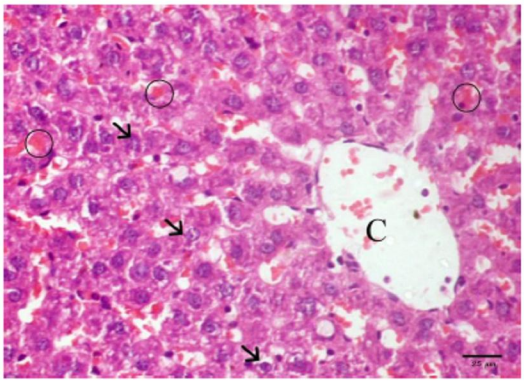

Figure (3): A photomicrograph of a section in the liver from group III showing disruption of architecture of radiating cords of hepatocytes from the central vein (C). Some hepatocytes are vacuolated and lacking acidophilic cytoplasm (arrow). Notice the congestion (circle) of the blood sinusoids. (H & E x400).

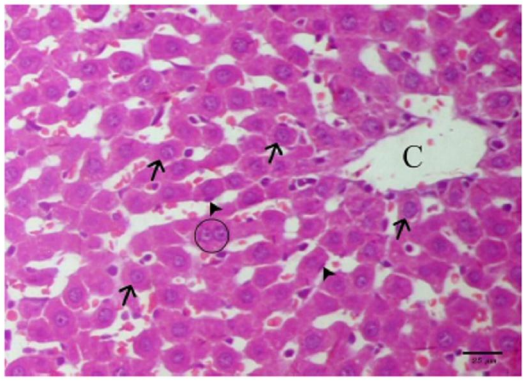

Figure (4): A photomicrograph of a section in the liver from group IVshowing most probably normal structure of liver, hepatocytes from the central vein (C), acidophilic cytoplasm of more or less normal hepatocytes (arrow). Some of the cells appear bi-nucleated (circle). Notice the lining cells (arrow head) of the blood sinusoids. (H & E x400).

2. Mallory's trichrome stain

Mallory's trichrome stain wasone of the most recognized known special stains performed to liver sections. About this stain, collagen appears in blue color against a red background of hepatocytes and other structures. A normal liver (group I) showed normal distribution of collagen fibers around the portal tracts and vessel walls (Figure 5). While the liver treated with MTX and the liver of withdrawal group showed increased collagen fibers surrounding the central veins and in portal area (Figure 6 and 7). In contrast, the treated-group with MTX and BM-MSCsshowed few collagen fibers surrounding the central vein and in portal area similar to control group (Figure 8).

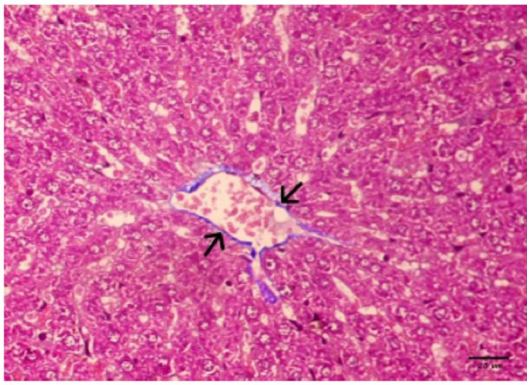

Figure (5): A photomicrograph of a section in the liver from control group showing minimal collagen fibers surrounding the central vein and in portal area (arrow). (Mallory's trichrome x 400).

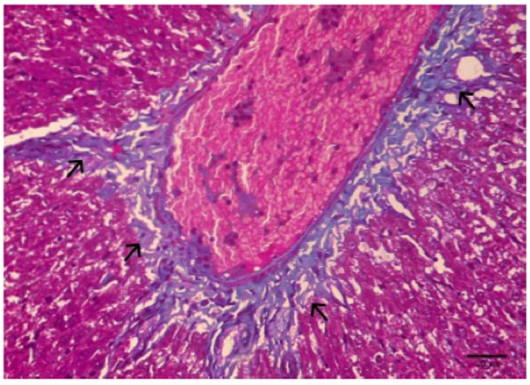

Figure (6): A photomicrograph of a section in the liver from group II showing numerous collagen fibers surrounding the central veins and in portal area (arrow). (Mallory's trichrome x 400).

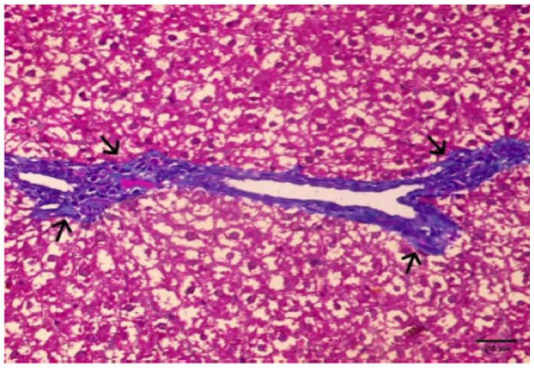

Figure (7): A photomicrograph of a section in the liver from group III showing increased collagen fibers surrounding the central veins and in portal area (arrow). (Mallory's trichrome x 400)

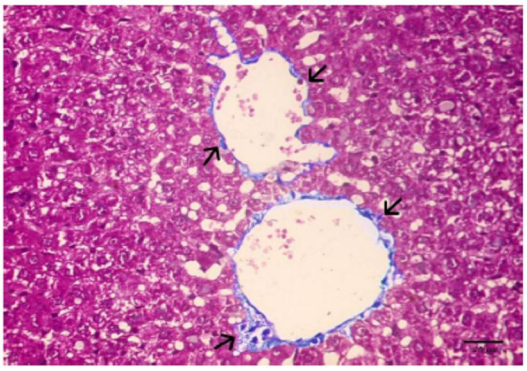

Figure (8): A photomicrograph of a section in the liver from group IVshowing few collagen fibers surrounding the central vein and in portal area (arrow). (Mallory's trichrome x 400).

3. Immunohistochemicalα-SMA stain:

The data of immunohistochemical stained sections of livers were given in Figure (9-12). A normal liver (group I) showed normal expression of α-SMA positively stained brown hepatic stellate cells (Figure 9). The immunohistochemical stained sections of liver treated with MTX and the withdrawal group showed strong immunoreactive expression of α- SMA represented by brown color of α- SMA positively among hepatic stellate cells mainly around center veins and forming intra-acinar thick bands (Figure 10 and 11). In contrast, the group IV showed deceased the number of positive stained cells comparable with the MTX (Figure 12).

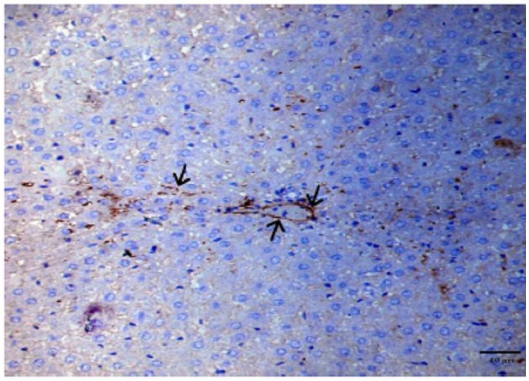

Figure (9): A photomicrograph of a section in the liver from control group showing minimal α-SMA positive cells (arrow) around the central vein and in-between the hepatocytes. (α-SMA x 250)

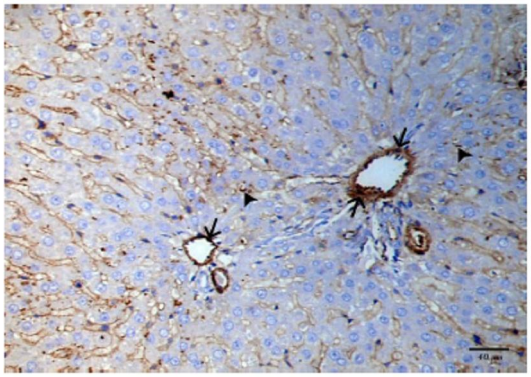

Figure (10): A photomicrograph of a section in the liver from group II showing an apparent increase in α-SMA positive cells (arrow) around central vein and in-between the hepatocytes (arrow head). (α-SMA x 250).

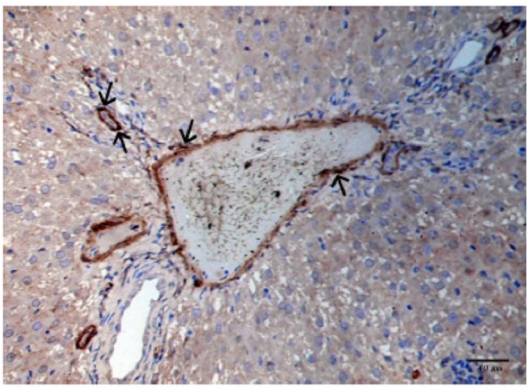

Figure (11): A photomicrograph of a section in the liver from group IIIshowing an apparent increase in α-SMA positive cells (arrow) around central vein. (α-SMA x 250)

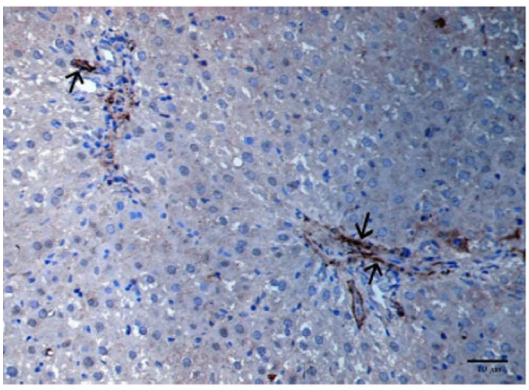

Figure (12): A photomicrograph of a section in the liver from group IV showing few α-SMA positive cells (arrow) around the central vein and in-between the hepatocytes.
(α-SMA x 250)

4. Electron microscopic examination of liver

Examination of control sample of liver (group I) revealed that each hepatocyte had double membrane which formed the envelope of hepatocyte interrupted by nuclear pores surrounded large central nucleus; two types of chromatin were possessed in the nucleus (light euchromatin and dark heterochromatin). Large number of mitochondria, rough endoplasmic reticulum, lysosomes and glycogen granules were present in the cytoplasm of the hepatocytes (Figure 13). Examination of the hepatocytes of rats of group II showed ultrastructural alterations such as disorganization in the hepatic system in general. The nucleus of some hepatocytes showed indentation. Moreover, cytoplasm of hepatocytes was rarefied and vacuolated (V). Also the glycogen was depleted. Inaddition to presence of infiltration with inflammatory cell in wide intracellular space between hepatocytes (Figure 14). While the ultrastructural changes of rats of group III showed pyknosis in the nucleus of some hepatocytes and slightly peripheral condensation of heterochromatin (Figure 15). Moreover, transmission electron microscopic examination of liver of group IV revealed partial improvements. The liver restoredits normal structure; the nuclei appeared oval with normal chromatin distribution. The cytoplasm of hepatocytes showed variable shapes and sizes of mitochondria with electron-dense matrix. Also the cytoplasm of hepatocyte was slightly rarified as well as disappearance of the fat droplets and finally, the rough endoplasmic reticulum and glycogen granules retained their normal appearance (Figure 16).

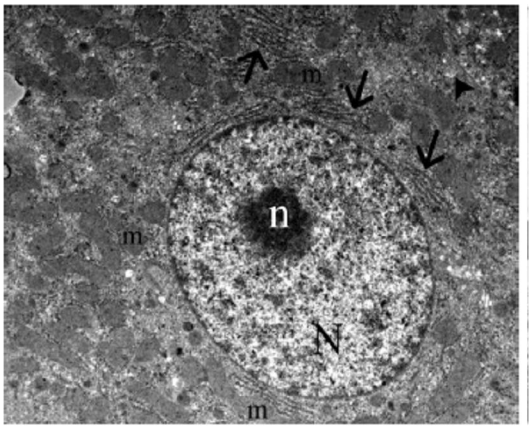

Figure (13): Transmission electron micrograph of liver from control group showing a hepatocyte with euchromatic nucleus (N) and prominent nucleolus (n). The cytoplasm contains many mitochondria (m), rough endoplasmic reticulum (arrow) and lysosomes (arrow head). (x 8000)

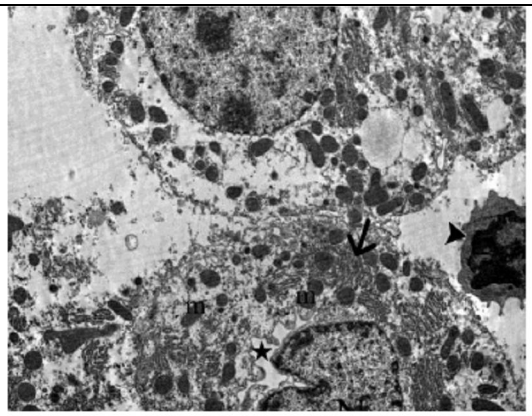

Figure (14): Transmission electron micrograph of liver from group II showing indentation in the nucleus (N) of hepatocyte. The cytoplasm is rarefied (astric) and contains many mitochondria (m), rough endoplasmicreticulum (arrow) and large vacuoles (V). Glycogenisdepleted. The intracellular space is widened with inflammatory cells (arrow head). (x 8000)

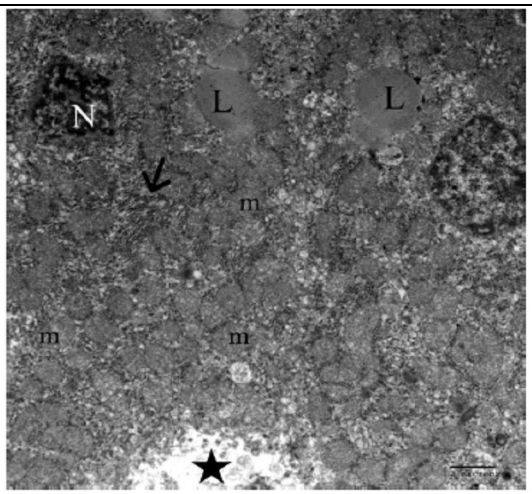

Figure (15): Transmission electron micrograph of liver from group III showings hrunken nucleus with slightly peripheral condensation of heterochromatin (N). The cytoplasm contains mitochondria (m), RER (arrow), small lytic areas (astric) and some lipid droplets (L). (x 8000)

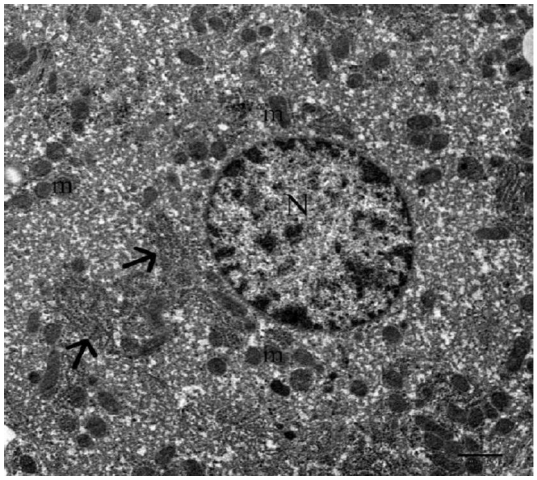

Figure (16): Transmission electron micrograph of liver from group IV showing more or less normal hepatocyte with euchromatic nucleus (N). The cytoplasm contains many mitochondria (m), rough endoplasmic reticulum (arrow). (x 8000)

5. Discussion

Understanding the pathogenesis of MTX-induced hepatotoxicity encourages the development of variable therapeutic interventions. The current study was designed to study the hepatoprotective effects of bone marrow-derived mesenchymal stem cells against MTX.

Methotrexate has been successfully used against various forms of tumorigenesis, inflammatory conditions and autoimmune diseases. However, the most common side effects associated with administration of MTX were immune suppression and hepatotoxicity [20, 21]. The hepatotoxicity of liver has been demonstrated in the current study via inspection process with considering methotrexate injection before inspection by light and electron microscopic. Those outcomes clarify the correlation between continuity of administration of cytotoxic drugs and ultrastructure alteration of liver. These results go in hand with Ali et al. (2014) (22) and Tousson et al. (2014) (23) who evaluated hepatocellular necrosis, fatty degeneration and inflammatory infiltrations which induced with methotrexate.

In the present study, animals injected with MTX showed distortion of hepatocyte, hepatic fibrosis, infiltration of hepatocyte with inflammatory cells, vacuolation and rarification of the cytoplasm and depletion of glycogen. These results agreed with Asmaa and Yasser (2018) (24).

Immunohistochemical examination in the present study showed that MTX hepatotoxicity hasjoined by α- SMA positive reaction among hepatic stellate cells mainly around center veins and forming intra-acinar thick bands. Those results referred to a likely participation via those mediators in MTX-induced hepatic injury. Overexpression of the pro-inflammatory mediators and other immunohistochemical reaction in MTX-induced tissue damage has been prior reported in various models (25).

MSC therapy has gained considerable interest as a promising approach for regenerative medicine in liver disease and considered a safe and potentially relevant therapeutic strategy for patients with chronic liver disease (26). Indeed and at first, mesenchymal stem cells (MSCs) has been created from bone marrow (BM), so there is no wonder that bone marrow originated mesenchymal stem cells (BM-MSCs) was the golden standard in MSCs experiments.

The mesenchymal stromal cells (MSCs) had a therapeutic potential effect in the liver fibrosis according to their immunosuppressive properties. In spite of the therapeutic mechanisms of MSC transplantation is not totally descried, all the data about this transplantation showed that several trophic factors excreted by MSCs perform the main therapeutic roles in renovation by alleviating inflammation, apoptosis, and fibrosis as long as stimulating angiogenesis and tissue renewal in damaged liver (27).

In this study, animals injected with MTX and then treated with BM-MSCs showed improvement in the architecture of liver and retained to most probably normal structure. These results go in line with Haldar et al. (2016) (28), Kuo et al. (2008) (29), Wu and Tao, (2012) (30) who studied the protective effects of MSCs and focused on its capacity for modulation of several components of the immune system. Further those immunomodulatory effects, MSCswere presented to reduce liver injury by ameliorating

oxidative stress throughfreeing of antioxidants and through anti-fibrotic effects. Further, MSCs were reported to be capable to distinguish into hepatocyte-like cells, which commitment for augmenting liver regeneration.

It could be concluded that bone marrow-derived mesenchymal stem cells represented a successful treatment against hepatotoxicity resulting by methotrexatetherapy (anticancer drugs) which has a deleterious effect on structure of liver.

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5/25/2018