

Ultrastructural Perturbations in Hepatic Cells of Rats Induced by Methotrexate and the Prophylactic Role of Folic Acid Administration

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ABSTRACT

Injection of rats with a low dose (10 mg/kg b.w.) and a high dose (25 mg/kg b.w.) of methotrexate for 2, 4 and 6 weeks induced fine structural alterations in hepatic cells of rats. These were represented by disintegration in the cisternae of the RER and primary enlargement of mitochondria followed by pleomorphism appearing by increasing the dose or the time of methotrexate treatment. The cytoplasmic architecture was abnormal and sometimes acquired a dusty or spongy-like appearance reflecting coagulation. Also, degenerative or vacuolated cytoplasmic regions were demonstrated after methotrexate injection. The nuclear euchromatin of hepatic cells showed a deteriorated organization especially at the high dose for long periods of treatment (6 weeks). However, the present finding indicated a protective role played by low dose (2.5 mg/kg b. w.) or high dose (5 mg/kg b. w.) of folic acid when administered 24 hours after low and high dose of methotrexate injection, respectively. Marked reduction in the cellular hepatic lesions at the ultrastructural level was recorded. The ultrastructural picture of liver cells was to a large extent similar to control condition. Results suggest the necessity of concomitant folic acid administration during prescribed courses of methotrexate injection. Following such recommendation is important to overcome the dangerous effects of methotrexate in liver.

Key words: Methotrexate; liver; folic acid, ultrastructure

Introduction

Methotrexate (MTX) is a methyl analog of folic acid, which is widely used in the treatment of various neoplastic and nonneoplastic diseases such as leukemia; lymphoma; osteosarcoma; carcinomas of the head and neck, breast, and lung; choriocarcinoma; hydatidiform mole; rheumatoid arthritis; and psoriasis (Thomson, 2003).

Earlier, Hopwood and Nyfors (1976) reported bile ducts damage in high dose methotrexate-treated rats. Numerous endothelial cells with spindle shaped nuclei lining blood sinusoid were seen. Inflammatory infiltrates of neutrophils and monocytes were also detected together with many enlarged lysosomes and microvesicular bodies. In MTX- treated psoriatics, Horvath *et al.* (1978) reported that detachment of desmosomes was a conspicuous finding. They also demonstrated the on-specific mitochondrial alterations, such as pleomorphism, gigantism, and paracrystalline inclusions. Fragmentation and partial degranulation of the granular endoplasmic reticulum were revealed in the study of Burk *et al.* (1995) in the low dose methotrexate treated rats. Prodromos *et al.* (2004) demonstrated pathologic changes resulted from treatment of patients with high dose of MTX. These changes included widespread damage to mitochondria, hypertrophy and dilatation of the Golgi apparatus, dilatation of the lateral intercellular spaces with presence of debris and zones of duplication of basement membranes. The effects of a high dose of methotrexate on the ultrastructure and metabolic activity of isolated rat livers were examined by Al-Ali *et al.* (2006). The cytoplasm of such cells was packed with altered mitochondria exhibiting fragmentation, peripheral displacement or partial lysis of mitochondrial cristae, pleomorphism, proliferation of mitochondria, swelling, and increase or decrease in the density of matrices. The RER cisternae suffered from partial degranulation, whereas there was an increase in number of profiles of the smooth endoplasmic reticulum (SER) and transformation into either tubular or vesicular cisternae. Soliman (2009) found that after treatment with methotrexate hepatocytes rats revealed lysis of the cytoplasm especially in the perinuclear area where most of the cell organelles were absent. In addition, some mitochondria appeared with broken cristae while others were densely stained and clumped together. Dilated or broken RER could be also seen. Bokhari *et al.* (2011) reported that scanning and transmission electron micrographs demonstrated concentration dependent changes in liver cells structure subsequent to treatment with MTX.

The liver is the main organ of folate storage and metabolism. Folic acid is involved in the transformation of certain amino acids as well as in the synthesis of nucleic acid (DNA) required by rapidly growing cells. Tetrahydrofolate is the biologically active form of folic acid, which is produced by the enzyme dihydrofolate

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reductase (Lan *et al.*, 2007). Folic acid (Folate, B9) is a vitamin of special importance in normal cellular functions (Czeczot, 2008). Soliman (2009) reported that the concomitant administration of folic acid with MTX was accompanied by marked protection of the liver from the degenerative changes.

The liver is the principle organ responsible for MTX metabolism and, given the diversity and extent of today's use of MTX therapy. Some investigators pointed out that changes in the liver following MTX administration are minimal, inconsistent and clinically insignificant (Kremer *et al.*, 1995; Bessler *et al.*, 1996 and Ros *et al.*, 2002). Others have implicated MTX in hepatotoxicity, including structural changes, in both human and experimental animals (Hendel *et al.*, 1985).

Based on these considerations, the present work was designed to declare the Ultrastructural changes in hepatocytes of rat's liver following injection with therapeutic methotrexate doses for variable durations in addition to evaluate the possible protective role of folic acid on such liver toxicity.

Materials and Methods

Experimental animals:

A total of 130 adult male albino rats, ageing approximately 8 weeks and weighing 165-185 grams were used. After acclimatization for 2 weeks, rats were housed in the animal house of Faculty of Science, Zagazig University in glass cages (50 x 40 x 30 cm). Each cage contained 10 animals in an air-conditioned room at temperature of 24±2°C and a relative humidity of 55±5% with approximately alternate 12h periods of light and dark during the whole period of experimentation. Rats were allowed free access to food on an ordinary rodent diet (food rodent pellets) and they were provided tap water *ad-libitum*.

Treatment:

The experimental animals were divided randomly into 5 groups. The control group consisted of 10 rats and 30 rats for each treated group. Each group was treated as follow:

Contol group (Group 1):

Rats (n=10) of the control group received an equivalent amount of saline I.M. once weekly for two ,four and six weeks.

Low-dose methotrexate group (Group 2):

Comprised rats (n=30) which received the low dose of the drug (10 mg/kg b.w) I.M once weekly at a dose of 10 mg/kg b.w for two, four and six weeks. It was obtained from Ebewe Pharma. MTX is available in the form of solution ready for injection, each 1ml of it contains 10 mg methotrexate (The drug was prepared by dissolving MTX in isotonic saline.

Low-dose methotrexate followed by low-dose folic acid group (Group 3):

Animals (n=30) were concomitantly treated with low dose of MTX (10 mg/kg b.w.) and folic acid (2.5 mg/kg b.w.) 24 hours after methotrexate injection once weekly for two, four and six weeks.

High-dose methotrexate group (Group 4):

Rats (n=30) of this group were given I.M high dose methotrexate (25 mg/kg body weight) once weekly for two, four and six weeks.

High-dose methotrexate followed by high-dose folic acid group (Group 5):

The animals (n=30) received I.M high dose of methotrexate (25 mg/kg body weight) followed by folic acid at a dose of 5 mg/kg 24 hours after methotrexate injection once weekly for two, four and six weeks.

Ultrastructural study:

For electron microscopic examination, specimens were dropped into the preliminary fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at (pH 7.2 - 7.4) for 24 h, at 4°C. Specimens were then post-fixed with osmium tetroxide. The specimens were washed with buffer, dehydrated in ethanol and passed through propylene oxide and embedded in an Epon-812 resin at 60°C. Semi-thin sections (0.5-1 µ) were cut from each block and stained with 0.1 toluidine blue and examined by light microscopy. Ultrathin sections (80-100 nm) were cut with a diamond knife on an ultramicrotome (KLP Ultratome) and stained with saturated uranyl acetate followed by lead citrate (Reynolds, 1963). The sections mounted on copper grids were examined at 80 KV with JEOL 1200 EX II transmission electron microscope (Histology Department, Faculty of Medicine, Zagazig University and in National Cancer Institute (N.C.I), Cairo University.

Results

Control Hepatic Cells:

Hepatic cells are polyhedral with well defined boundaries. Each cell is characterized by the presence of a central spherical nucleus with a distinct nuclear membrane possessing many nuclear pores. Each nucleus has one or more prominent nucleoli (Figs. 1, 2 & 3).

The cytoplasm of hepatic cells harbors abundant cisternae of rough endoplasmic reticulum. This reticulum forms aggregates dispersed in the whole cytoplasm (Figs. 1-3). Cavernous membranes of smooth endoplasmic reticulum were distributed diffusely throughout the cytoplasm. Elements of Golgi apparatus could be frequently seen (Fig. 1).

Abundant spherical, oval or rod – shaped mitochondria were widely distributed throughout the cytoplasm. These mitochondria were characterized by moderate number of transverse cristae. Lysosomes and varied quantities of lipid droplets were also seen in the cytoplasm of hepatic cells (Figs. 1-3).



Fig. 1: Electron micrograph showing control hepatic cell characterized with a spherical nucleus (N) harboring an obvious nucleolus (nu). The cytoplasm of hepatic cells harbors abundant cisternae of rough endoplasmic reticulum (RER), numerous mitochondria (M) with transverse cristae and lysosomes (Ly). Golgi apparatus (G) cisternae could be frequently seen. Bar= 10 microns.

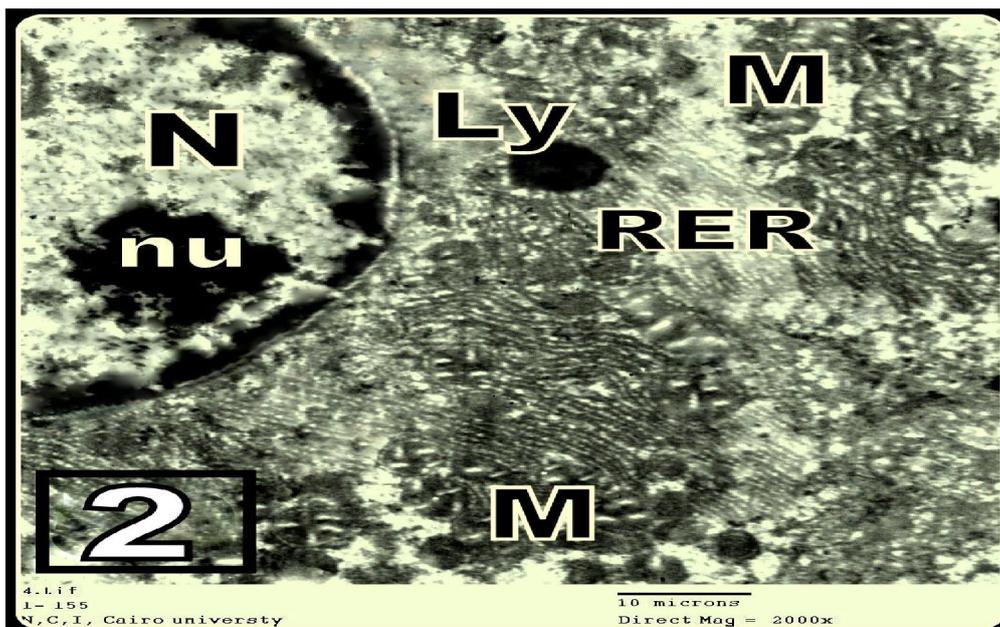


Fig. 2: Electron micrograph showing control hepatic cell with its prominent nucleus (N) possessing an obvious nucleolus (nu). The cytoplasm is occupied with abundant cisternae of the rough endoplasmic reticulum (RER), abundant mitochondria (M) and some lysosomes (Ly). Bar= 10 microns.

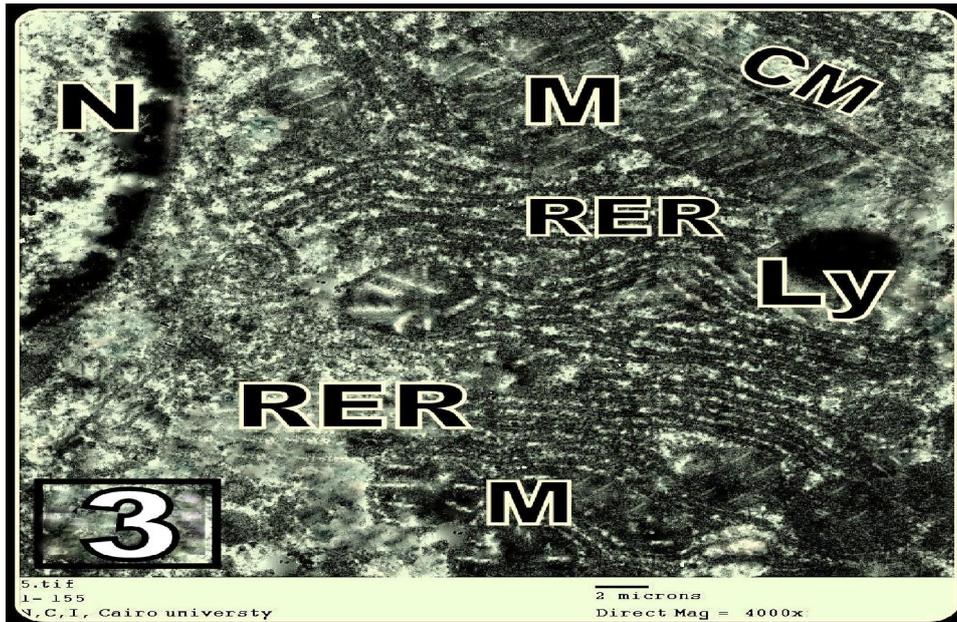


Fig. 3: Higher magnification of a control hepatic cell showing nucleus (N) and clumps of heterochromatin resting on the inner surface of the nuclear membrane. The cytoplasm of the hepatic cell is loaded with abundant cisternae of rough endoplasmic reticulum (RER), mitochondria (M) and lysosomes (Ly). The cell membrane (C.M) is clearly detected. Bar= 2 microns.

Treated Groups:

First: Low Dose:

Low dose of methotrexate (10 mg/kg bw) for 2 weeks:

Electron micrographs obtained from the liver of rats injected with low dose of methotrexate (10 mg/kg bw) for 2 weeks showed nuclei with a moderate osmiophilic nucleoplasm, with moderate-sized clumps of heterochromatin scattered on a moderate osmiophilic nucleoplasm. The cytoplasm was vacuolated and perforated by small or large spherical degenerations. Some mitochondria appeared pleomorphic and were characterized by increased density of matrices without discrimination of their cristae. Some giant mitochondria were seen and others had degenerated cristae. The mitochondria were surrounded with fragmented cisternae of the rough endoplasmic reticulum. Fragmentation and partial degranulation of some of the granular endoplasmic reticulum were observed. The nuclear membrane was thinned out and appeared undistinguished into inner and outer leaflets and the heterochromatin resting on the inner surface of the inner nuclear envelope appeared slightly reduced (Fig. 4).



Fig. 4: An electron micrograph of liver section of a rat treated with low dose of methotrexate for 2 weeks showing nucleus (N) with a moderate osmiophilic nucleoplasm. There is a destruction in the rough-endoplasmic reticulum (RER) and presence of a little number of pleomorphic mitochondria (M). Patches of cytoplasmic disintegration (Disin) and degeneration (DG) are randomly detected. The cell membrane is thinned (C.M). Bar= 2 microns.

Low dose of methotrexate (10.0 mg/kg bw) followed by low dose of folic acid (2.5 mg/kg bw) for 2 weeks:

Electron micrographs from liver sections of rats injected with the low therapeutic dose of methotrexate (10.0 mg/kg bw) followed by low dose of folic acid (2.5 mg/kg bw) for 2 weeks showed to a large extent a control-like fine structure. The Mitochondrial appearance had a nearly normal shape as there was no pleomorphism or gigantism with clear discrimination of their cristae and most of them were normal in size. The cytoplasm appeared normal except small degenerations. The nucleus appeared with homogenated nucleoplasm and obvious heterochromatin layer lining its inner membrane. No abnormality was detected in the nuclear membrane that appeared distinguished into inner and outer membranes. The endoplasmic reticulum was mildly dilated and disintegrated but the Golgi apparatus was unremarkable. Still small spherical cytoplasmic vacuolizations were prominent associated with partial fragmentations and partial disintegrations in the cisternae of rough endoplasmic reticulum (Fig. 5).

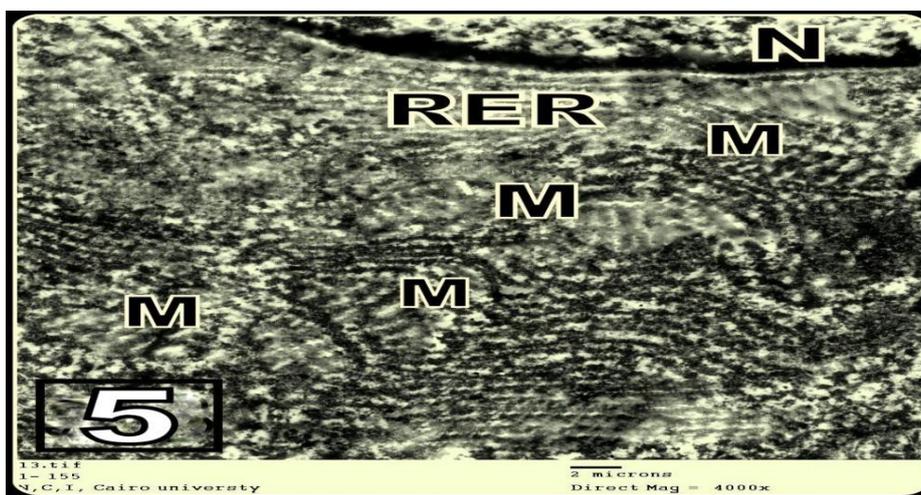


Fig. 5: An electron micrograph of liver section of a rat treated with a low therapeutic dose of methotrexate (10.0 mg/kg bw) followed by low dose of folic acid (2.5 mg / kg bw) for 2 weeks. Mitochondria (M) are nearly with normal shape. The nucleus (N) has homogeneous nucleoplasm but the rough-endoplasmic reticulum (RER) showig partial fragmentations. Bar= 2 microns.

Low therapeutic dose of methotrexate (10 mg/ kg bw) for 4 weeks:

Injection of rats with the low dose of methotrexate (10 mg/ kg bw) for 4 weeks resulted in ultrastructural perturbations (Fig. 6). Cytoplasmic vacuolizations were prominent and there was just residual patches of cytoplasm acquiring a dusty granular architecture. Focal areas of degenerative regions were observed throughout the cytoplasm. Nuclei of such cells showed homogenated euchromatin without large clumps of heterochromatin distributed in the nucleoplasm but moderate to small-sized clumps were demonstrated. The nuclear membrane is thinned out and the heterochromatin resting on the inner surface of the inner nuclear envelope is slightly reduced. Mitochondria noted in variable sizes from small, moderate and elongated and were mostly pleomorphic without clear discrimination of their cristae. Clumped pleomorphic mitochondria were demonstrated throughout the sever vacuolated cytoplasm. Mitochondria were surrounded by the disintegrated rough endoplasmic reticulum and compressed with each other toward the nucleus leaving large patches of cytoplasmic vacuolizations perforated with small round degenerated areas and devoid of glycogen granules. Remnants of RER cisternae were seen surrounding the scattered mitochondria (Fig. 6).

Low dose of methotrexate (10.0 mg kg /bw) followed by low dose of folic acid (2.5 mg/kg /bw) for 4 weeks:

Ultrastructural examination of liver cells from treated rats with a low therapeutic dose of methotrexate (10.0 mg/kg bw) followed by low dose of folic acid (5.0 mg kg /bw) after 24 hour for 4 weeks showed that the cytoplasm of hepatocytes appeared more or less normal. No cytoplasmic vacuoles appeared and some hepatocytes showed healthy moderate electron dense mitochondria with transverse cristae but others appeared elongated or degenerated and faintly osmophilic. Rough and smooth endoplasmic reticulum appeared normal in regions and degenerated in others. The nuclei appeared with obvious nucleoli and containing well distributed euchromatin with marked heterochromatin attached to the inner nuclear envelope (Fig. 7).

Low therapeutic dose of methotrexate (10 mg/kg bw) for 6 weeks:

Hepatocytes inspected from ultrathin sections obtained from rats treated with a low therapeutic dose of methotrexate (10 mg/kg bw) for 6 weeks suffered many pathological alterations. Massive cytoplasmic vacuolization, degeneration and focal areas of necrosis were detected and the residual patches of cytoplasm acquired a dusty granular architecture. The nucleus appeared with distinguished nuclear membrane and homogenated nuclear sap and the heterochromatin was resting on the inner surface of the inner nuclear envelope

(Fig. 8). Mitochondria appeared pleomorphic characterized by increased density of matrices and without discrimination of their cristae.

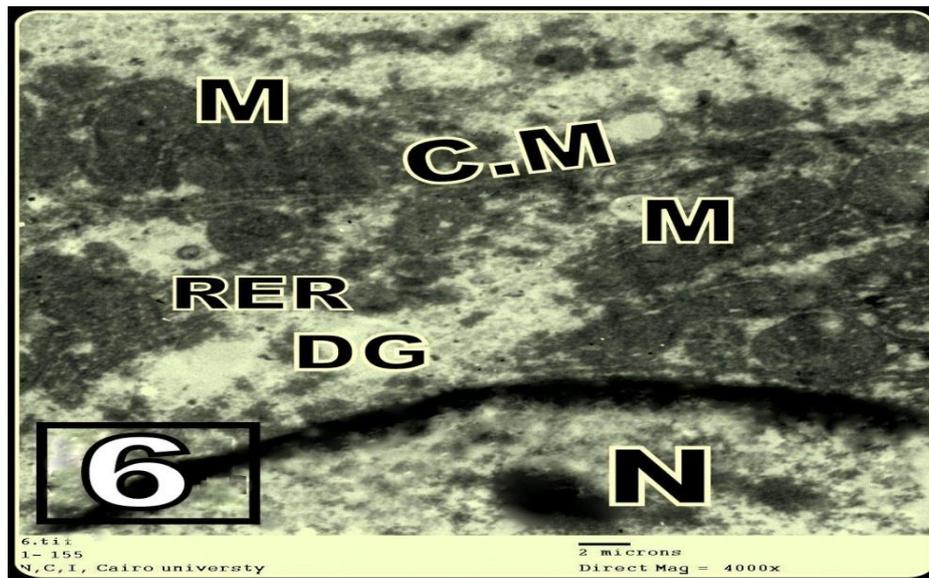


Fig. 6: An electron micrograph of liver section of a rat treated with a low therapeutic dose of methotrexate (10 mg/kg bw) for 4 weeks showing thinned out nuclear membrane. There is a destruction in the rough endoplasmic reticulum (RER) and mitochondria (M) are noted in variable sizes and are mostly pleomorphic. The nuclear membrane is contiguous to a focal area of degenerative region (DG). The cell membrane (CM) between the two cells is hardly detected. The cytoplasm of the above cell is highly perforated. Bar= 2 microns.



Fig. 7: An electron micrograph of liver section of a rat treated with a low therapeutic dose of methotrexate (10.0 mg/kg bw) followed by low dose of folic acid (2.5 mg/kg bw) for 4 weeks. The nucleus (N) is normal with an obvious nucleolus. Rough endoplasmic reticulum (RER) is seen healthy in regions and degenerated in others. Moderate electron dense mitochondria (M) are noted in variable sizes and some of them are pleomorphic. The cell membrane (C.M) is detected at the lower left corner of the micrograph. Bar= 2 microns.

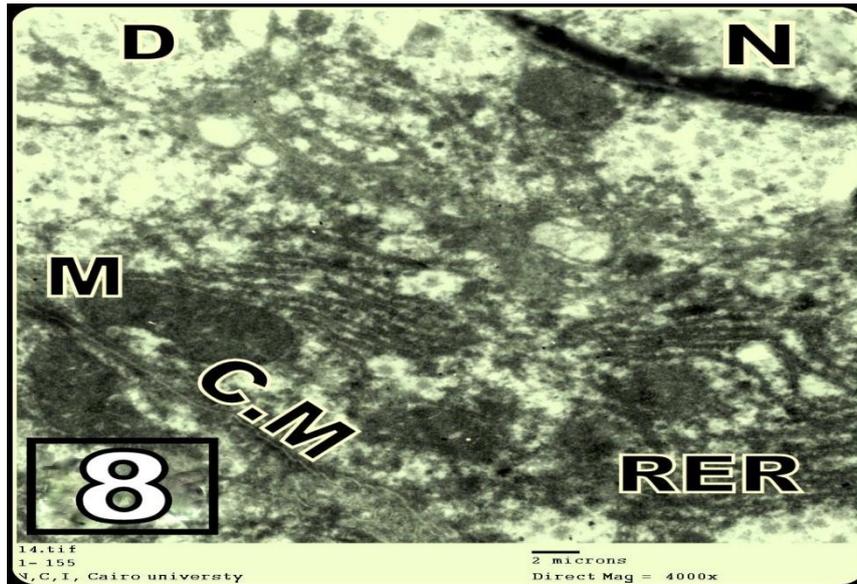


Fig. 8: An electron micrograph of liver section of a rat treated with a low therapeutic dose of methotrexate (10 mg/kg bw) for 6 weeks. The nucleus (N) appeared with little heterochromatin resting on the inner surface. Focal area of cytoplasmic degenerations (DG) is prominent. Severe rough endoplasmic reticulum (RER) cisternae are disintegrated (RER). Mitochondria (M) are pleomorphic while the cell membrane (C.M) is visible with a desmosome. Bar= 2 microns.

Some of pleomorphic mitochondria were giant (Fig. 9). The granular endoplasmic cisternae were disintegrated, fragmented and coagulated with each other while others were aggregated leaving a spongy degenerated gap around the nucleus (Fig. 8).

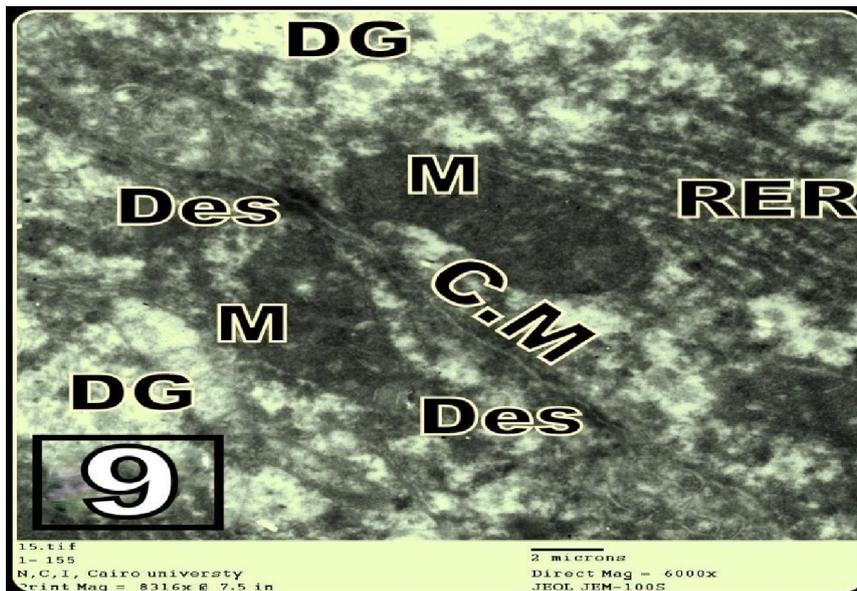


Fig. 9: An enlarged part of the previous electron micrograph showing cytoplasmic degeneration (DG) and severe rough endoplasmic reticulum (RER) disintegration. Mitochondria (M) revealed gigantism and have pleomorphic texture. The cell membrane (C.M) between the two hepatocytes is remarkable and each one share by half a desmosome (Des). Bar= 2 microns.

Low dose of methotrexate followed by low dose of folic acid (2.5 mg/kg bw) for 6 weeks:

Rats treated with a low therapeutic dose of methotrexate followed by low dose of folic acid (5.0 mg/kg bw) for 6 week showed good protection as nearly no pathological changes were remarkable in the liver. The hepatocytes appeared with well defined cytoplasm containing mitochondria with different shapes and sizes as well as obvious cristae and the rough endoplasmic reticulum with partially attached ribosomes. The nucleus appeared rounded with distinct well defined inner and outer membranes and prominent nucleolus. The heterochromatin lying on the inner surface of the inner nuclear membrane appeared increased and slight amount distributed through the homogenated nucleoplasm. Golgi apparatus was flourish and well developed (Fig. 10).



Fig. 10: An electron micrograph of liver section of a rat treated with a low therapeutic dose of methotrexate followed by low dose of folic acid (2.5 mg/kg bw) for 6 weeks. Mitochondria (M) have different shapes and sizes and with intact obvious cristae. The rough endoplasmic reticulum (RER) are seen with partially attached ribosomes. Golgi (G) apparatus is flurished and exemplified by saccules and vacuoles. The nucleus (N) has abundant heterochromatin clumped at the inner nuclear membrane and the nucleolus (nu) is prominent. Bar= 500 microns.

Second: High Dose:

High dose of methotrexate (25 mg/kg bw) for 2 weeks:

Following injection of rats with a high dose of methotrexate (25 mg/kg b.w) for two weeks hepatic cells revealed drastic pathological alterations. Cytoplasmic vacuolization was seen in a sever manner and the cytoplasmic patches acquired a dusty granular architecture. Mitochondria were highly atrophied and aggregated in randomly distributed clumps circumscribing with the remnants of fragmented rough endoplasmic reticulum. Endoplasmic reticulum cisternae were aggregated in a network shape surrounding the scattered groups of crowded small mitochondria. These were pleomorphic and characterized with a dense matrix whereas mitochondria tended to be dense oval with significantly reduced mean diameter. The heterochromatin exhibited condensation, margination and resting on the inner surface of the inner nuclear envelope and was greatly reduced and of high osmiophilia. No large clumps of heterochromatin were seen distributed in the nucleoplasm but a dusty like appearance was demonstrated. A marked amount of vacuolation occurred, possibly reflecting presence cellular of degeneration. Some degenerative cytoplasmic regions of vesicular shape were seen contiguous to the nuclear membrane. The nuclear membrane was thinned out in a part and distinguished in another part but the cell membrane was still healthy (Fig.11).

High dose of methotrexate followed by high dose of folic acid (5.0 mg/kg bw) for 2 weeks:

Ultrastructural examination of hepatic cells from rats treated with high dose of MTX followed by high dose of folic acid for 2 weeks showed good amelioration where hepatocytes seemed to be rather normal. The cytoplasm appeared homogenous with small vacuolations. Mitochondria appeared normal in number and shape with normal cristea, but others were elongated and mostly surrounded with the RER. Normal patches of rough endoplasmic reticulum were extended throughout the cytoplasm but with partially degranulated cisternae. Lysosomes appeared in a normal shape. The nucleus looked to be healthy with distiguished nuclear membrane and with normal distribution of euchromatin and heterochromatin on a homogenous nuclear sap (Figs. 12).

High therapeutic dose of methotrexate (25 mg/ kg bw) for 4 weeks:

In liver sections investigated by the electron microscope from rats injected with the high dose of methotrexate for 4 weeks hepatic cells revealed drastic pathological alterations. The hepatic cells suffered large vacuolations and were perforated with small degenerations and obvious focal areas of cytoplasmic degenerations. Dense osmiophilic granules or inclusions of various sizes and densities were scattered on the degraded cytoplasm (Fig. 13). Cells in the affected zone showed nearly complete degeneration of the granular endoplasmic reticulum and few RER still appeared with degranulation. Megamitochondria were detected and others were pleomorphic and coagulated with the remnants of the disintegrated rough endoplasmic reticulum (Fig. 13). Nuclei of such cells

showed moderate amount of heterochromatin resting on the inner leaflet of the nuclear envelope. The nucleoplasm suffered slight degenerations (Fig. 13).

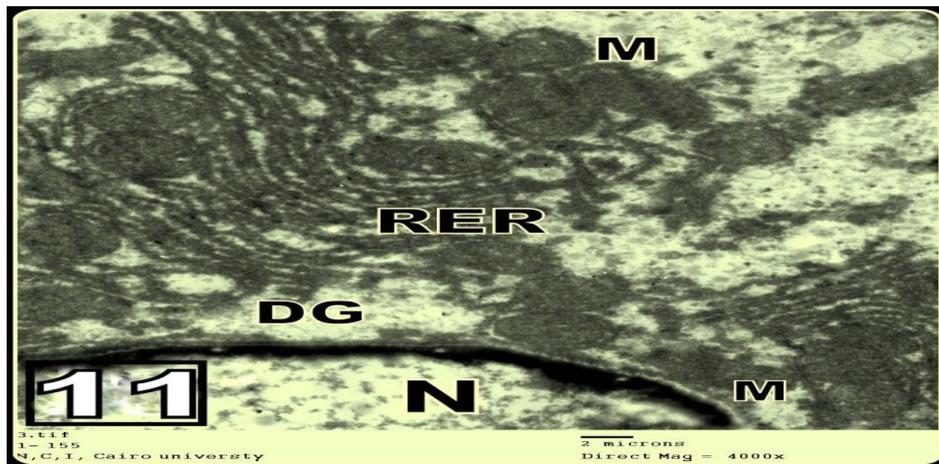


Fig. 11: An electron micrograph of liver section of a rat treated with the high dose of methotrexate (25 mg/kg bw) for 2 weeks showing atrophied pleomorphic mitochondria (M) aggregated in clumps. The rough endoplasmic reticulum (RER) is fragmented. The nucleus (N) harboring thin layer of heterochromatin on the inner surface leaving the rest of nucleoplasm translucent. Cytoplasmic degeneration (DG) is observed near the nucleus. Bar= 2 microns.

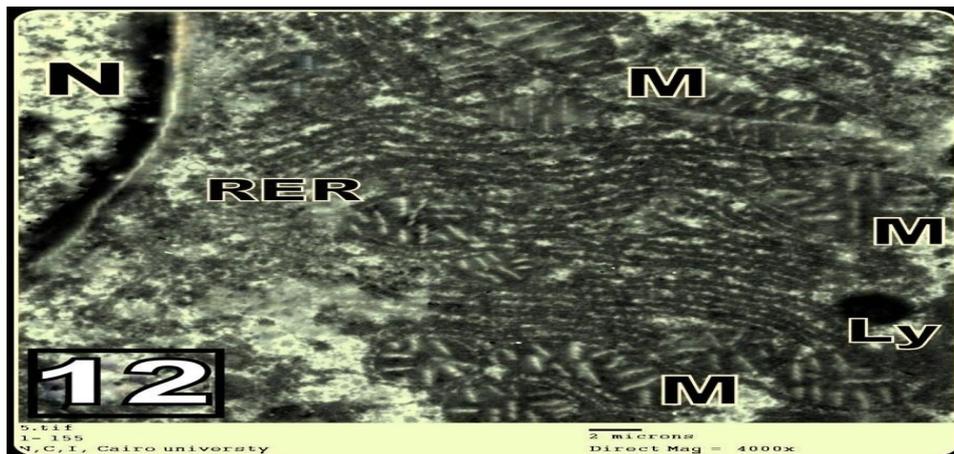


Fig. 12: An electron micrograph of liver section of a rat treated with a high dose of methotrexate followed by high dose of folic acid (5.0 mg/kg bw) for 2 weeks. The mitochondria (M) have normal shape and with normal cristae. Normal distribution of cisternae of the rough endoplasmic reticulum (RER) and lysosomes (Ly) are demonstrated. The nucleus (N) looked to be healthy with homogenous nuclear sap. Bar= 2 microns.

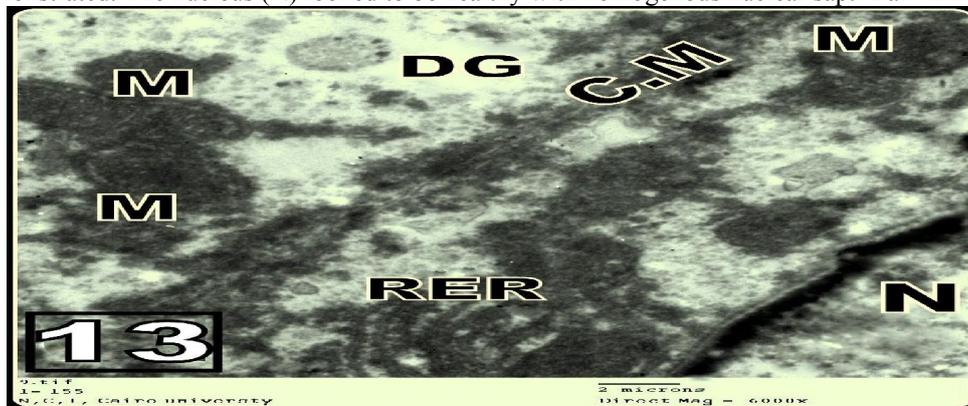


Fig. 13: An electron micrograph of liver section of a rat injected with the high therapeutic dose of methotrexate (25 mg/kg bw) for 4 weeks. Mitochondria (M) are of normal size but pleomorphic. The rough endoplasmic reticulum (RER) is clearly disintegrated. The nucleus (N) has a moderate amount of heterochromatin. The cell membrane (C.M) is abnormal and regions of degenerations (DG) are seen. Bar= 2 microns.

These nuclei exhibited clumping and margination of the nuclear chromatin together with hypertrophy of nucleolar elements. Electron – lucent euchromatin regions were demonstrated in the nucleoplasm which possessed moderate to small-sized heterochromatin clumps. Glycogen granules were crowded and distributed throughout the vacuolated cytoplasm (Fig. 14).



Fig. 14: Another electron micrograph of liver section of a rat treated with a high dose of methotrexate (25 mg/ kg bw) for 4 weeks. Mitochondria (M) are of reduced size and pleomorphism. Rough endoplasmic reticulum (RER) suffered massive fragmentation and degeneration. The nucleus (N) harbors a nucleolus (nu) and translucent nucleoplasm. Bar= 2 microns.

High dose of methotrexate (25 mg/kg bw) followed by high dose of folic acid (5.0 mg/kg bw) for 4 weeks:

Hepatocytes investigated from rats administered a high dose of methotrexate (25 mg/kg bw) followed by high dose of folic acid (5.0 mg/kg bw) for 4 weeks showed remarkable protection. Most endoplasmic reticulum cisternae appeared normal and parallel to each other but fragmented parts appeared ensheathed some of mitochondria. Nucleus appeared rounded with large nucleolus and a moderate amount of chromatin appeared throughout the nucleoplasm. Peripheral heterochromatin was clearly attached to the inner nuclear envelope but appeared reduced. The nucleoplasm showed slightly translucent background giving the nuclear sap a dusty – like appearance. Glycogen granules were scattered throughout the cytoplasm. Mitochondria appeared with dense matrices and obvious cristae. The cytoplasm appeared homogeneous but small regions were disintegrated and other parts were vacuolated (Fig. 15).

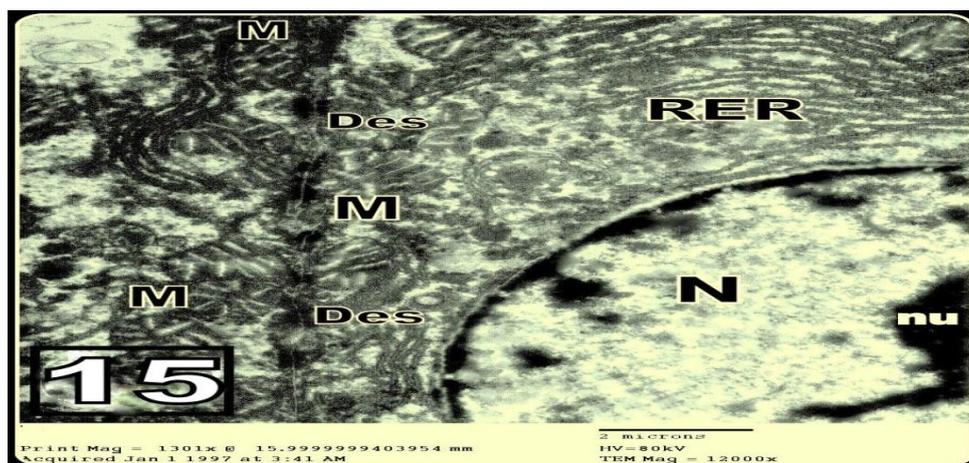


Fig. 15: An electron micrograph of liver section from a rat treated with a high dose of methotrexate (25 mg/ kg bw) followed by high dose of folic acid (5.0 mg/kg bw) for 4 weeks. Mitochondria (M) are of normal shape and with dense matrices and obvious cristae. Most rough endoplasmic reticulum (RER) cisternae appeared normal and parallel to each other except few disintegration. The nucleus (N) has a large nucleolus (nu) and a moderate amount of chromatin. Bar= 2 microns.

High dose of methotrexate (25 mg/ kg bw) for 6 weeks:

Upon treatment with this regimen the hepatic cells appeared severely vacuolated and granulated. All organells appeared coagulated with each other throughout the cytoplasm (Fig. 16).

Features such as cytoplasmic alterations were prominent and were associated with complete disintegrations in the cisternae of the rough endoplasmic reticulum. Condensation of the nuclear chromatin was apparent leaving the nucleoplasm trans-lucent and vacuolated. Filamentous nucleoli were demonstrated through the necrotic nucleoplasm that exhibited electron – lucent euchromatin. The heterochromatin layer appeared margined on the inner surface of the nuclear membrane together with condensations of nuclear chromatin giving moderate-sized clumps of chromatic material appeared through the nucleoplasm. In some hepatic cells, small rounded regions of the cytoplasm were degenerated and revealed cytoplasm perforation. In such cells only remnants of cytoplasm were demonstrated forming just a rim surrounding the pleomorphic atrophied mitochondria. Most cytoplasmic patches acquired a dusty granular architectur. Mitochondria appeared with oval shape and fragmented cristae as seemed to be filled with degenerated inclusions and with small spherical vacuolated parts (Fig. 17).

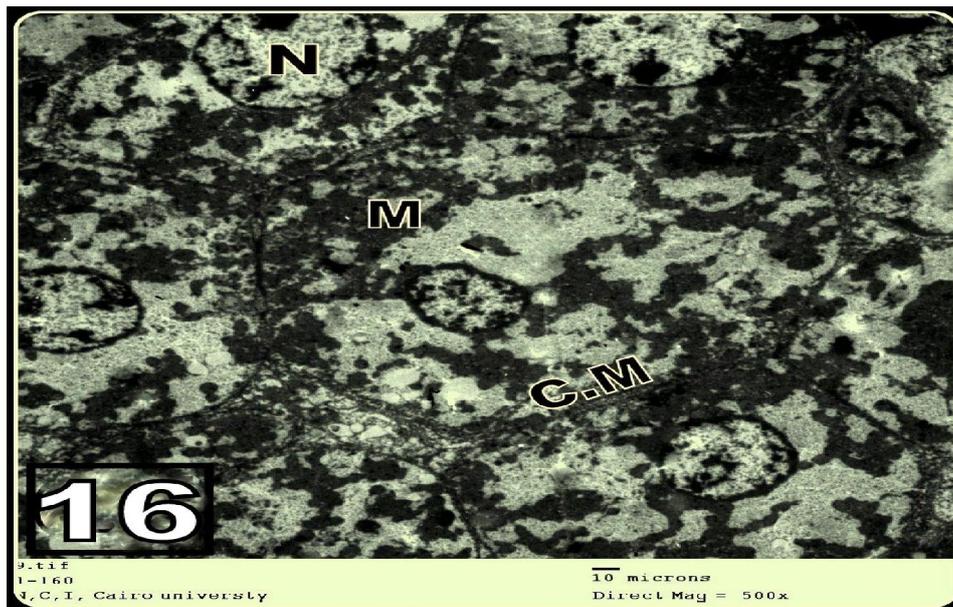


Fig. 16: An electron micrograph of liver section of a rat treated with a high dose of methotrexate (25 mg/ kg bw) for 6 weeks. A group of hepatocytes are observed each one surrounded by a cell membrane and has a nucleus (N). All organells including mitochondria (M) appeared coagulated with each other through the dusty-like cytoplasm. Bar= 10 microns.



Fig. 17: An electron micrograph of liver section of a rat treated with a high dose of methotrexate (25 mg/ kg bw) for 6 weeks showing complete disintegration of the cisternae of the rough endoplasmic reticulum. Mitochondria (M) have an oval pleomorphic shape with fragmented cristae and seem to be filled with degenerated inclusions. The nucleus (N) with abnormal margined heterochromatin and eccentric nucleolus (nu). Cytoplasm is obviously degenerated (DG) and the cell membranes (C.M) are deteriorated. Bar= 10 microns.

High dose of methotrexate (25 mg/kg bw) followed by a high dose of folic acid (5.0 mg/kg bw) for 6 weeks:

Ultrastructural examination of liver cells from rats treated with high dose MTX followed by high dose folic acid for 6 weeks revealed obvious protection for the hepatocytes. Cytoplasm of these cells appeared granulated with nearly normal mitochondria with clear discrimination of their cristae. Cisternae of the rough endoplasmic reticulum were aggregated in bundles near the nucleus in a normal shape except minute disintegrations or degradations that were observed surrounded the nuclei with normal appearance and distribution of glycogen (Fig. 18). In some hepatic regions binucleate hepatocyte were detected. Prominent nucleoli appeared in hepatic cells and clumps of peripheral heterochromatin were clearly attached to the inner nuclear envelope and moderate clumps existed through the nucleoplasm. The nuclear membranes of appeared normal with distinguished inner and outer membranes.

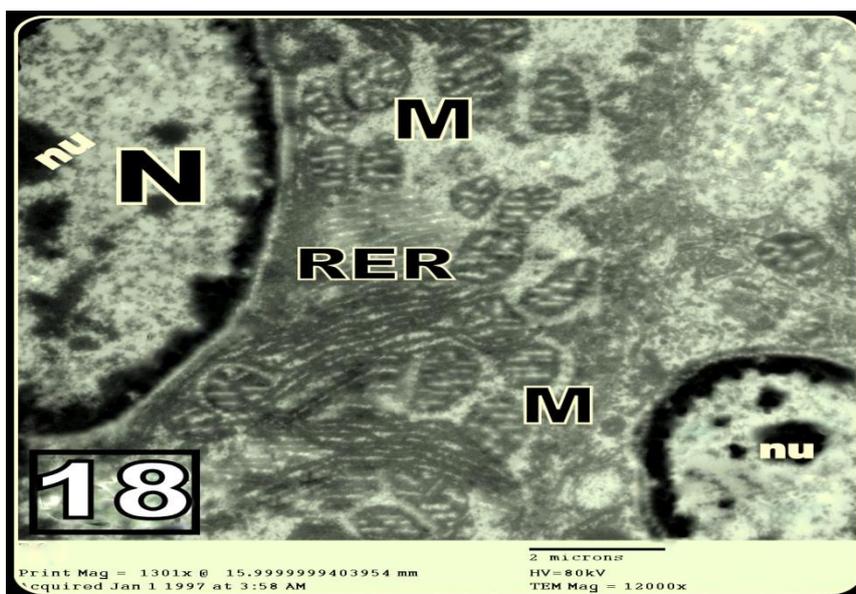


Fig. 18: An electron micrograph of liver section of a rat injected with the high therapeutic dose of methotrexate (25 mg/kg bw) followed by a high dose of folic acid (5.0 mg/kg bw) for 6 weeks. The two Nuclei (N) are to large extent healthy with prominent nucleoli (nu). Mitochondria (M) are almost normal in shape and size with clear discrimination of their cristae. Rough endoplasmic reticulum (RER) is observed in aggregated bundles. Bar= 2 microns.

Discussion

Electron micrographs of liver section of rats injected with low dose of methotrexate (10 mg/kg bw) for 2 weeks showed nuclei with a moderate osmiophilic nucleoplasm and moderate-sized clumps of heterochromatin. Cytoplasm showed large degenerations. Mitochondria appeared pleomorphic. Giant mitochondria were also detected. Also, there were fragmentation and partial degranulation of some of the granular endoplasmic reticulum.

In the present ultrastructural study, a variety of mitochondrial abnormalities was present. Rats treated with a low therapeutic dose of methotrexate (10 mg/kg bw) for 2, 4 and 6 weeks showed pleomorphic mitochondria characterized by increased density of matrices and without discrimination of their cristae. Giant mitochondria (gigantism) appeared in an elongated shaped. Other mitochondria suffered degenerated matrices cristae and lysis of cristae similar to paraerystalline inclusions bodies. Deteriorated mitochondria were surrounded with fragmented cisternae of the rough endoplasmic reticulum. Rubin (2001) suggested that such findings could be attributed to the oxygen metabolites with deficiency of the protective enzymes from the liver following drug treatments.

A similar view was previously announced by Leedle and Aust (1990) who demonstrated also the relationship between peroxidation of membrane phospholipids and reduced glutathione (GSH) concentration. Glutathione depletion in the liver may be a causative factor for liver necrosis (Burk *et al.*, 1995). Moreover, the role of GSH in the mitochondria is believed to be critical in the maintenance of vital mitochondria and cellular functions through the metabolism of reactive oxygen derivatives generated within the electron transport chain and through the regulation of mitochondrial inner membrane permeability by maintaining calcium (Ca²⁺) homeostasis (Martensson and Meister, 1989).

In rats treated with a high dose of methotrexate (25 mg/kg b.w.) for 2 and 4 weeks mitochondria were highly atrophied and aggregated in randomly distributed clumps, ensheathed with the remnant fragmented rough endoplasmic reticulum. Endoplasmic reticulum cisternae aggregated in a net shape surrounding the scattered groups of small mitochondria which were crowded, pleomorphic and characterized with a dense matrix.

Megamitochondria were also detected. These findings considerably support those presented by Prodromos *et al.* (2004) who demonstrated pathologic changes resulted with high dose of MTX. The authors recorded widespread damage to mitochondria. Non-specific mitochondrial alterations, such as pleomorphism, gigantism, and paracrystalline inclusions have been reported in MTX-treated psoriatics by various researchers (Hopwood and Nyfors, 1977; Horvath *et al.*, 1978; Oogarah *et al.*, 1995).

In the present study, rats treated with a high dose of methotrexate (25 mg/ kg /bw) for 6 weeks the nucleoplasm suffered slight degenerations. Nucleus of such deteriorated cell were conspicuously had segmented small marginated nucleoli or the nuclei may exhibit clumping and margination of the nuclear chromatin, and hypertrophy of nucleolar elements. Prodromos *et al.* (2004) recorded anisonucleosis and multinucleation of hepatocytes in patients who had received low dose methotrexate over 15 or more years.

In rats treated with the high dose of MTX for 6 weeks mitochondria have fragmented cristae as seemed to be filled with degenerated inclusions and with small spherical vacuolated parts. According to Bessler *et al.* (1996), all these mitochondrial changes, including the presence of flocculent wooly focal inclusions, were strongly suggestive of toxic injury resulting probably from a biochemically defective mitochondrial membrane. The accumulation of many altered mitochondria and the increased oxygen consumption following HD-MTX infusion may be either a compensatory reaction occurring at the organelle level or the result of the lesion's high demands upon the cell's energy resources (Myagkaya *et al.*, 1985).

Following injection of rats with low and high doses of methotrexate (10 and 25 mg/kg b.w) hepatic cells revealed drastic pathological alterations such as cytoplasmic vacuolization. Cytoplasmic vacuolations and perforation with small degenerations were observed and the highly vacuolated cytoplasm revealed obvious focal areas of cytoplasmic degeneration. Large patches of cytoplasmic degeneration were randomly detected and large cytoplasmic regions were translucent or acquired a dusty – like appearance and most cells became necrotic in low dose methotrexate treated rats for 4 and 6 weeks. These findings could receive support from Soliman (2009) who demonstrated the ultrastructural alterations in liver specimens following high dose methotrexate treatment. The marked cytotoxicity was displayed by severe degeneration of cell organelles, the majority of hepatocytes revealed lysis of the cytoplasm especially in the perinuclear area where most of the cell organelles were absent. Ultrastructural study carried out by Dixon *et al.*, 1994 confirmed that cytoplasmic vacuolation is known to develop when cellular semipermeability is increased but not totally destroyed. In the present study, the cytoplasm of some hepatic cells showed large degenerations. Focal areas of necrosis became more prominent showing residual patches of cytoplasm acquiring a dusty granular architecture in rats treated with methotrexate especially for 6 weeks. Cytoplasmic vacuolation and cell swelling occur in certain hepatopathies in humans and experimental animals and degenerations may lead to cellular perforations (Crawford, 2005; Kumar *et al.*, 2005).

Fragmentation and partial degranulation of the granular endoplasmic reticulum are obvious events observed in the present ultrastructural study in the low dose methotrexate treated rats. Fragmented and dilated cisternae were seen in some cells and remnants of the disintegrated rough endoplasmic reticulum were appeared in both LD-MTX and HD-MTX treated rats for 2,4 and 6 weeks. In this respect, Slater (1984) reported that the rough endoplasmic reticulum is particularly liable to the free radical attack, not only because it is considered as a site of radical production but also due to the enrichment of its membrane with polyunsaturated fatty acids.

Ultrastructural alterations were noticed in the study of Al-Ali *et al.* (2005) after application of a high dose of methotrexate to examine its effects on the ultrastructure and metabolic activity of isolated rat livers. The authors recorded an increase in number of profiles of the smooth endoplasmic reticulum (SER) and transformation into either tubular or vesicular cisternae. Fragmentation and partial degranulation of the granular endoplasmic reticulum were also shown in the study of Burk *et al.* (1995) in the low dose methotrexate treated rats.

The ultrastructural observations in the present liver specimens after two, four and six weeks using low dose and high dose MTX followed by folic acid showed a control like fine structure. This concomitant administration of folic acid with MTX was accompanied by marked protection of the liver from the previous degenerative changes. Electron microscopic examination revealed reduction in the structural impairment as the majority of the cells showed a relatively normal ultrastructure. Hepatocytes cytoplasm appeared homogenous with more or less hypertrophied mitochondria, ruptured endoplasmic reticulum still evident. Nucleus appeared rounded with distinct outer and inner membranes whereas the peripheral heterochromatin was clearly attached to the inner nuclear envelope. The cytoplasm appeared granulated, mitochondria appeared almost normal in shape and size, well organized rough and smooth endoplasmic reticulum were observed with normal appearance of glycogen. In this concern, Soliman (2009) pointed out that folic acid supplementation should therefore be routinely prescribed to every patient taking MTX as a sole treatment agent to protect the liver against adverse effects of the used drug. Controlled studies in adult patients with rheumatoid arthritis carried out by Shiroky *et al.* (1993) and Morgan *et al.* (1994) and indicated that supplementation with either folic or folinic acid may reduce the frequency and severity of side effects. These findings correlate with the reports of Lan *et al.* (2007) who pointed out that folate deficiency showed increased oxidative stress of the liver and alters hepatic methionine metabolism which is associated with increased hepatocellular apoptosis. Several studies carried out by Duhra (1993) and Griffith *et al.* (2000) have

shown that supplementing methotrexate therapy with either folic acid or folinic acid (leucovorin a synthetic form of reduced folate) can reduce methotrexate toxicity (specifically, hepatotoxicity).

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