Original Article

Ultrastructural Alterations of Liver Tissue Cells in Methotrexate-Treated Balb/c Mice

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Abstract

Aim: The current study investigated the efficacy of methotrexate (MTX) on liver tissue cells of Balb/c mice at the ultrastructural level using transmission electron microscopy. **Background:** This agent is well known and used as a chemotherapeutic agent for a long time and not selective for cancer cells so, healthy cells beside cancer cells are also affected by MTX. **Materials and Methods:** Experimental animals were divided into two groups; the first group was kept without treatment and served as the control, the second group was treated with 115 mg/kg MTX i.p. once weekly for 4 weeks and sacrificed under anesthesia after the 4th week. The liver tissues were osmium fixed and embedded in araldite, sectioned and observed under transmission electron microscope. **Results:** Normal cell ultrastructure was determined in the control group whereas the liver cells of the MTX-treated group revealed ultrastructural alterations, such as the increase in lipid droplets, discontinuity of rough endoplasmic reticulum cisternae and vacuole formation. In addition, the loss of cytoplasmic material in hepatocytes was also evident. Condensation of nuclear chromatin and fusion of nucleic membranes were observed in the liver cells of the treated group. **Conclusions:** Results of the study indicated that MTX, used for different types of medical treatment, disturbed liver cell ultrastructure.

Keywords: Hepatocyte, liver, methotrexate, transmission electron microscope, ultrastructure

Introduction

Methotrexate (MTX) is a folic acid antagonist[1] and plays a cytotoxic role in the cell cycle, inhibits DNA synthesis, stops cell proliferation, and leads cell to apoptotic death. It is well known and used as a chemotherapeutic agent for a long time and not selective for cancer cells. Healthy cells and cancer cells are affected by methotrexate (MTX).[2] MTX is also used in the treatment of psoriatic arthritis,[3] rheumatoid arthritis,[4] Crohn's disease, and colitis.[5] MTX causes several deficits in different organs and systems.[6,7] Pinkerton *et al*. reported that MTX showed jejunal crypt abnormalities in children with acute lymphoblastic leukemia.[8] A study in the small intestine of rats revealed proliferation, differentiation, and cell death in goblet and paneth cells.[9] Yuncu and Kanter determined that MTX induced mucosal damage in the small intestine of mice.[10] Another study in MTX-treated intestines by Elbakary *et al*. detected ultrastructural changes in the form of dilation of rough endoplasmic reticulum (rER) cisternae, vacuolation of the cytoplasm, focal accumulation of lipid droplets, swollen mitochondria with destroyed cristae, short sparse microvilli with focal loss of microvillus border, widening of the intercellular space, indentation of nuclei, and variable degrees of corrugation in nuclear membrane.[11] Al-Ali *et al*. determined ultrastructural changes in rat livers perfused *in vitro* and *in vivo* with high doses of MTX, such as disorganized endoplasmic reticulum, dispersion of polyribosomes, a variety of mitochondrial changes, and glycogen redistribution.[12] Soliman also reported tissue damage in thin sections of MTX-treated liver such as severe degeneration of cell organelles, especially in mitochondria and rER.[13] Hepatotoxicity and hematologic toxicities were seen in patients using MTX.[14] It was mentioned that these effects resulted from binding to the enzyme dihydrofolic reductase, preventing the conversion of folic acid to its active form, folinic acid. Thus, nucleic acid and amino acid synthesis are blocked, and protein production is affected. Electron microscopic

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observations of Choi *et al*. detected vesiculation and dilation of cisternae in the endoplasmic reticulum and Golgi complex, appearance of autophagic vacuoles, and, hyperplasia in pancreatic acinar cells.[15] As a result, organelles and plasma membranes of hepatic cells are damaged and hepatic function is corrupted.[16] Sargin *et al*. also stated that MTX impaired mouse kidney cell structure.[6]

As shown, a number of studies have collected evidence for MTX-mediated tissue damage. Therefore, the present study was planned to reflect the changes in liver cells exposed to high dose of MTX in Balb/c mice.

Materials and Methods

Experimental animals

This study was performed with the permission of ethical commission of Ahi Evran University, Kırsehir, Turkey. As Balb/c mice are widely used in oncological research, they ($n = 10$) were preferred in the study. Mice were maintained under controlled conditions of light (12:12 h; light: dark), temperature (22°C ± 3°C), and humidity (65%). Before assays, animals were quarantined for 10 days and then divided into two groups: control group ($n = 5$) and MTX-treated group ($n = 5$).

Methotrexate administration

A dosage of (115 mg/kg) physiological saline solution was administered to the control group by intraperitoneal injection. Methotrexate Ebewe (MTX) (Sandoz Drug Company, Istanbul, Turkey) was injected in the same dosage of 115 mg/kg i.p. once weekly for 4 weeks to the treated group.[17] After 4 weeks, mice were sacrificed under pentobarbital anesthesia (i.p.) overdose (>120 mg/kg).[18]

Transmission electron microscope preparation

Liver tissues were removed and immersed in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium phosphate and held for 2 h for the first fixation. After rinsing with sodium phosphate buffer, tissues were postfixed in 1% osmium tetroxide solution for 2 h. Tissues were dehydrated in a series of graded ethanols, placed into propylene oxide, and embedded in araldite.[19] Tissues were cut with an ultramicrotome to ultrathin sections of 60–80 nm and stained with uranyl acetate and lead citrate and then examined under transmission electron microscope (TEM, JEOL 100 CXII, JEOL Company, USA) at 80–100 kV in Electron Microscope Laboratories of Ankara University, Department of Biology, Faculty of Science.

Results

Ultrastructural investigations of the control group liver revealed a normal histological appearance. Polygonal-shaped hepatocytes with centrally localized nuclei, sinusoids, and bile canaliculus were observed in liver tissue sections [Figure 1].

MTX-treated livers removed at the end of 4 weeks displayed structural deformations in hepatocytes and irregular-shaped erythrocytes in the sinusoid. Hepatocytes contained lipid droplets, loss of cytoplasmic material, and pyknotic nuclei [Figure 2].

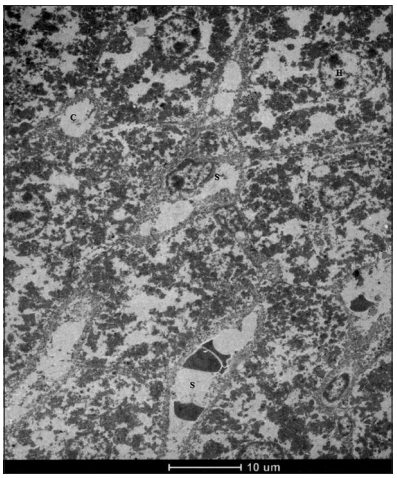

Figure 1: Liver tissue of control group mice. Hepatocyte (H), sinusoids (S), bile canaliculus (C). Bar: 10 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03980

Due to effects of MTX, lipid droplets were seen to be numerous in hepatocytes. In addition, neutrophils were present in the hepatic sinusoid [Figure 3].

Lipid droplets and hyalinization of cytoplasmic material in cell cytoplasm were distinctive features in the MTX-treated group [Figure 4].

Most hepatocytes had a centrally placed single nucleus, but a binucleated hepatocyte was seen in this micrograph. One of the structural alterations of MTX was the fusion of nuclear membranes in the hepatocyte. In addition to this cellular disorder, cytoplasmic material loss and melting of chromatin material were seen. Note the irregular-shaped mitochondria in hepatocytes [Figure 5].

Another conspicuous finding in this group was the difference in the appearance of the hepatocyte nucleus such as chromatin clumping. Cytoplasmic material melting around the nuclear envelope was also observed [Figure 6].

Furthermore, discontinuous rER cisternae were seen among the mitochondria, which are different in size due to the effect of MTX. Abundant hepatic sinusoids were determined in treated liver [Figure 7].

Discussion

MTX is a therapeutic agent widely used to treat chronic inflammatory and neoplastic diseases.[20] In the present study, ultrastructural pathological lesions, which altered the cell structure, were determined in the liver tissues of MTX-treated mice. In his study, Al-Motabagani reported that there were focal areas of liver cell necrosis with abnormal hepatic structures in MTX-treated adult male albino rats. Glycogen content was seen to decrease.[16] Similar data were observed in the current study. Yuncu and Kanter mentioned that MTX

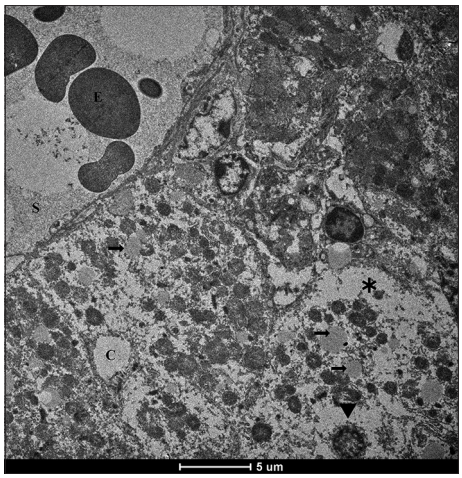

Figure 2: Liver section of methotrexate-treated mice. Loss of cytoplasmic material (asterisk), pyknotic nucleus (arrow head), bile canaliculus (C), lipid droplets (arrows), sinusoid (S), erythrocytes (E). Bar: 5 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03981

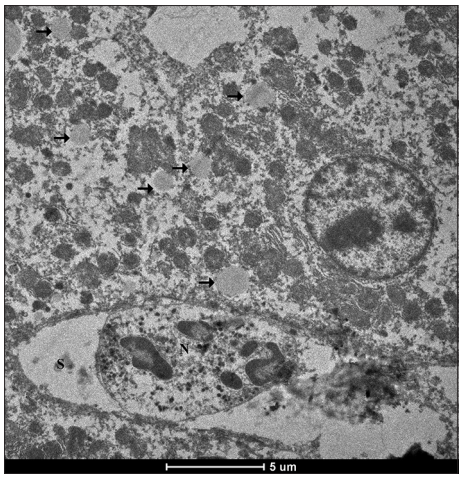

Figure 3: Neutrophil (N) in hepatic sinusoid (S) in methotrexate-treated mouse liver. Accumulation of lipid droplets (arrows). Bar: 5 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03982

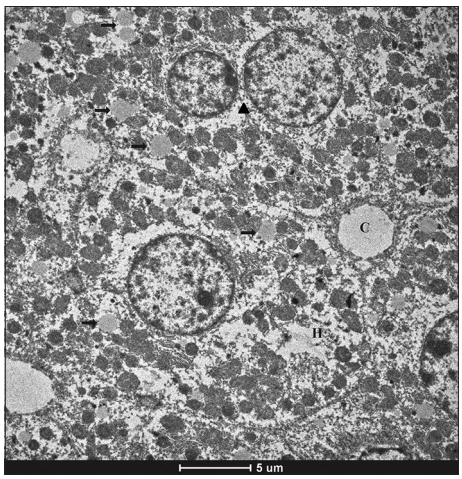

Figure 4: Another section of methotrexate group liver tissue with binucleated hepatocyte (arrow head), accumulation of lipid droplets (arrows), hyalinization (H) of cytoplasmic material. Bile canaliculus (C). Bar: 5 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03983

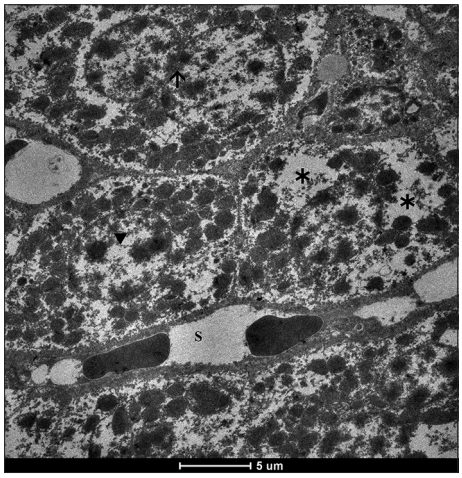

Figure 5: Fusion of nuclear membranes (arrow) in binucleated cell and cytoplasmic material loss (asterisk) in hepatocyte methotrexate group liver were seen in the micrograph. Sinusoid (S) and melting of chromatin material (arrow head). Bar: 5 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03984

caused swelling in mitochondria and dilation of endoplasmic reticulum in mouse small intestine mucosa.[10] Contrary to these data, we observed shrinkage of mitochondria and no dilatation but discontinuity in the endoplasmic reticulum. Choi *et al*. determined dilatation in endoplasmic reticulum of rat pancreatic acinar cells;[15] however, we did not observe dilation in the endoplasmic reticulum of hepatocytes. They also reported that there were no alterations in the nuclei of pancreatic acinar cells of MTX-treated rats. In contrast to this study, our data showed alterations in nuclei of MTX-treated mice, such as both melting and clumping of chromatin material and fusion of the nuclear membrane. In another study, numerous vacuoles were present in MTX-treated mouse kidneys.[6] It was shown that paneth cells showed striking structural alterations with vacuolar dilatation of the cytoplasm.[8] As can be seen from literature, MTX causes different changes in different tissues in terms of cell ultrastructure. If we look again at liver tissue, Al-Ali *et al*. introduced MTX at a dose of 0.5 mg/kg to rat livers perfused *in vitro* and *in vivo* and examined under electron microscope.[12] TEM studies revealed disorganized endoplasmic reticulum, dispersion of the polyribosomes, a variety of mitochondrial changes, and glycogen redistribution. In addition to these findings, Soliman concluded that MTX treatment gives rise to the degeneration of liver cell organelles, especially in mitochondria and rER.[13] We described similar observations in the present study such as the disruption of rER and change in the distribution of nuclear chromatin. Al-Motabagani introduced MTX to adult male rats and examined by light microscopy.[16] According to

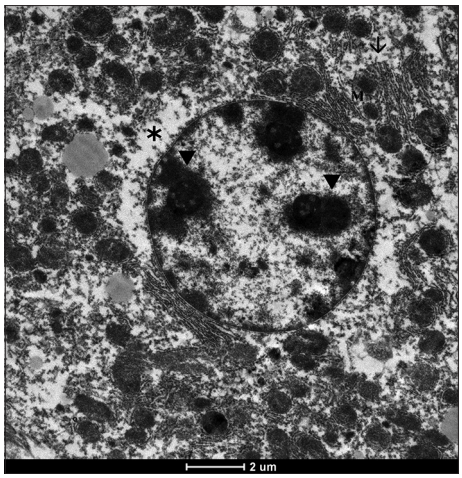

Figure 6: Ultrastructural alteration of treated liver cells. Chromatin clumping (arrow heads), melting cytoplasmic material (asterisk), discontinuous rough endoplasmic reticulum cisternae (arrow) around shrinked mitochondria (M). Bar: 2 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03985

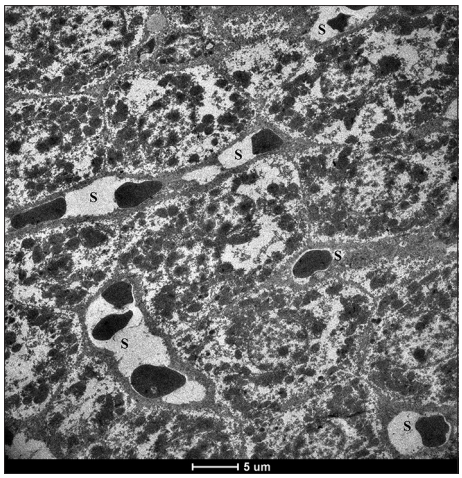

Figure 7: Abundant hepatic sinusoids (S) in methotrexate-treated liver. Note the rarefied and vacuolated cytoplasm of hepatocytes. Bar: 5 μm. A high-resolution version of this slide for use with the virtual microscope is available as eSlide: VM03986

their observations, mononuclear cell infiltration was present in MTX-treated livers. Even though their observations were at the light microscopy level, their findings shed light on ours as we noticed neutrophil cells in the hepatic sinusoids as well. The presence of this type of cells suggests pinocytotic activity in order to destroy the effects of MTX. Hall *et al*. and Patel *et al*. indicated that high doses of MTX therapy (200/150/100 μg/kg and 0.250 mg/kg, respectively) can cause hepatotoxicity.[21,22] We used 115 mg/kg of MTX in our study and determined that MTX has toxicological effects on hepatocytes of Balb/c mice. High doses of MTX treatment seem to cause hepatotoxicity, and a consequence of changes in the histology of the mice liver is consistent with previous studies.[21,22] These findings may be a sign of the metabolizing process of degenerative effects of MTX on Balb/c mouse hepatocytes precisely because Balb/c mice are known as sensitive to carcinogens and play an important role in oncological researches. Another study presented vacuolation and degeneration of hepatocytes in MTX-administered rats.[23] A study of Asci *et al*. found out that MTX has a direct effect on the liver structure alterations and causes cytotoxicity of hepatocytes with vacuolation of hepatic cytoplasm.[24] Similarly, in different tissue, Elbakary *et al*. described rarefied and vacuolated cytoplasm, focal accumulation of lipid droplets, and swollen mitochondria in their assay on MTX-mediated tissue damage of intestine.[11] Similar to the findings above, we also determined changes in the structure of mouse liver cells treated with MTX, such as vacuolation and degeneration in the meaning of cytoplasmic material loss. It was mentioned that MTX treatment leads to nuclear variability in liver cells[25] and also indented the nucleus in enterocytes.[11] Consequently, we made the same observations such as nuclear alterations in cells of the liver, such as melting and clumping of chromatin material. As MTX reduces cell proliferation, we suggest that these alterations in liver cell nuclei may be explained by the antimitotic effect of MTX. However, Quintin *et al*.[26] revealed that the direct role of long-term low-dose MTX in the genesis of a MTX-specific liver change with dystrophic nuclei in hepatocytes was very rarely observed. On the other hand, Kremer *et al*.[27] revealed that, even at low doses, MTX is associated with hepatic disorders like elevated liver enzymes. It should not be an unpredictable state that we expect cellular changes related with the rise of enzymes.

We hope that our findings shed light on understanding the mechanism of this multipurpose drug.

Conclusion

The present study examined the ultrastructural changes of liver cells in high-dose MTX-treated Balb/c mice. When all observations were evaluated, we can suggest that, despite the chemotherapeutic properties of MTX, it seems to alter liver tissue cells destructively. We think that this finding generates a biological significance as well.

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Conflicts of interest

There are no conflicts of interest.

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