

## Histological and immunohistochemical study of the possible protective effect of folic acid on the methotrexate-induced cardiac muscle toxicity in male albino rats

Doha S. Mohamed and Eman K. Nor-Eldin

Department of Histology, Faculty of Medicine, Sohag University, Sohag, Egypt

### ABSTRACT

**Introduction:** Methotrexate is widely used as a chemotherapeutic agent in cancers, ectopic pregnancies and rheumatic arthritis. Folic acid is found in dark green leafy vegetables. Methotrexate is folic acid antagonist that prevents its conversion to tetrahydro folic acid.

**Aim:** This research aimed to study the protective effect of folic acid on the histological changes produced by methotrexate in cardiac muscles.

**Materials and Methods:** Thirty adult male albino rats were divided into three equal groups. Group I (control group) 10 rats injected intraperitoneally with saline. Group II rats were intraperitoneally injected with methotrexate at a dose of 5mg/kg/day for one month. Group III: were intraperitoneally injected with methotrexate at a dose of (5mg/kg/day) with concomitant oral folic acid at a dose of (0.1mg/kg/day) for one month. After 24 hrs of the last dose, the animals were dissected. Hearts were processed for haematoxyline and eosine stain, immunohistochemical stains and electron microscopic examination.

**Results:** Both degenerative and apoptotic changes were observed in the cardiac muscles in the methotrexate-treated group, these changes were attenuated by administration of folic acid.

**Conclusion:** Folic acid is beneficial to the cardiac muscles during methotrexate treatment and should be used concomitant with methotrexate treatment.

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**Key Words:** Cardiac muscles, folic acid, methotrexate.

**Corresponding Author:** Doha Mohamed Saber, M.D., Department of Histology, Faculty of Medicine, Sohag University, Sohag, Egypt, Tel.: 01007843762, E-mail: dohasaber@yahoo.com

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### INTRODUCTION

Methotrexate is widely used as a chemotherapeutic agent for leukemia and other malignancies<sup>[1]</sup>. It is currently the most common anti-rheumatic drug prescribed for the treatment of rheumatoid arthritis and other rheumatoid disorders<sup>[2]</sup>. In addition, methotrexate is used as an alternative to surgical management of ectopic pregnancy especially in selected patients with small, unruptured tubal pregnancies<sup>[3]</sup>. It affects the normal tissues that have a high rate of proliferation, including the hematopoietic cells of the bone marrow and the actively dividing cells of the gut mucosa<sup>[4]</sup>.

Folic acid is present in dark green leafy vegetables such as kale, spinach and dried peas. It is also present in liver, kidney and brewer's yeast. It is eventually converted to its active biological form tetra hydro folic acid by a cellular enzyme called dihydrofolate reductase, tetrahydrofolic acid is required for manufacturing the building blocks used

in the synthesis of DNA and RNA (5).

Methotrexate is a folic antagonist, binds to dihydrofolate reductase and prevents the conversion of folate to tetrahydrofolic acid. This competitive inhibition of dihydrofolate reductase reduces the amount of active folate in cells and interferes with the production of DNA and RNA so folic acid supplement is used to counteract methotrexate effects on healthy tissues<sup>[6]</sup>. Some cardiac dysfunctions could be detected by previous studies after using methotrexate.

Since it is essential drug for treatment of many diseases, we could not dispense methotrexate and so trials were done to attenuate its side effects especially on the vital organs as the heart. Physiological research aimed at studying the benefits of folic acid. Administration of folic acid could reverse cardiac dysfunction induced by a variety of cardio toxins<sup>[7]</sup>. Folic acid could prevent the cardiomegaly, increased catalase activity and decreased lipid-peroxidation induced by some toxic agents<sup>[8]</sup>.

Our objective was to explain the histopathological basis of methotrexate-induced cardiac dysfunctions and the possible benefit of using concomitant folic acid.

## MATERIALS AND METHODS

### *Animals used*

Thirty adult male albino rats (200-250 gm) obtained from the Animal House of Assiut Faculty of Medicine and reared under the standard conditions of feeding, light-dark ratio and temperature. The animals were divided into three groups each of them consists of 10 rats. Group I (control group), 10 rats were injected intraperitoneally with saline. Group II, rats were intraperitoneally injected with methotrexate (Haupt Pharma GmbH) in the form of vials 50mg/2ml under trade name of Methotrexate Mylan at the dose 5mg/kg/day for one month [8]. Group III injected intraperitoneally with methotrexate at the aforementioned dose (5mg/kg/day) together with concomitant folic acid orally (Mepa-co Medifood) in the form of tablets 500 mcg under trade name of Folic acid which dissolved in distilled water) at the dose of 0.1mg/kg /day using nasogastric tube. Administration of folic acid started 24hrs after methotrexate injection for one month [8].

### *Methods*

After 24 hours from the last dose, rats were anesthetized using ether inhalation, sacrificed, carefully dissected and specimens from the left ventricle were processed for Haematoxyline and eosin, P.A.S , immunohistochemical stains and electron microscopic examination .

### *Preparation of the specimens for light microscopic examination*

Perfusion fixation was used and the specimens were fixed in 10% neutral buffered formalin and processed for light microscopic study. Paraffin sections of 6 $\mu$ m thickness were obtained for haematoxyline and eosin, PAS reaction and immunohistochemical stains [9].

### *Immunohistochemical methods*

Immunohistochemical staining was carried by avidin biotin peroxidase complex method. The specimens from cardiac muscles were processed. Caspase-3, (mouse monoclonal antibody and anticleaved caspase-3 rabbit polyclonal antibody (Neo Markers Fermont, CA 94539, USA) were used for detection of apoptosis . The reaction appeared brownish either cytoplasmic or nuclear.

Sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted. Tonsils were used as the positive-control tissues. Negative control were performed after omitting the primary antibody [10].

### *Electron microscopic technique*

At the end of the experiment, rats were anesthetized using ether inhalation. Perfusion fixation was used. The rats

were sacrificed, carefully dissected to take cardiac muscles from left ventricle. They were fixed in 2.5% glutaraldehyde at 4°C, washed in three to four changes of cacodylate buffer (pH 7.2) for 20 min at every change, and post-fixed in 1% osmium tetroxide for 2 hours, thereafter, dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. These samples were kept in an incubator at 35°C for 1 day, then at 45°C for another day, and finally at 60°C for 3 days. Semithin sections (1- $\mu$ m-thick) were prepared using an LKB (Bromma, Sweden) ultramicrotome, stained with 1% toluidine blue, and examined by means of a light microscope. Ultrathin sections (500–800 Å) were stained with uranyl acetate and lead citrate to examine using an electron microscope (Jeol JEM 1010, Tokyo, Japan) at 80 kV at an electron microscopic unit, at the Faculty of Medicine, Sohag University [11].

### *Morphometric studies*

The numbers of caspase 3 positive stained cells as well as the PAS density in cardiac muscles were detected in ten high power fields in each case in all groups. The light microscope Leica ICC50 Wetzlar (Germany) The digital photos were captured in the Microscopic Photography Unit (Histology Department, Faculty of Medicine, Sohag University) using fifty different relevant fields from each animal group that were selected randomly. The measurements were performed using Digimizer PC image analysis and imagej software (Leica Q 500 MC program, Wetzlar, Germany).

### *Statistical analysis*

Analysis of Variance (ANOVA) with a statistical significance of  $P < 0.05$ . Computations were performed with STATA version 9.2 software. All the analyses were performed in a blinded fashion.

## RESULTS

### *Control group (Group I)*

Light microscope showed cardiac myocytes in longitudinal section with prominent striations. No striations in the perinuclear region were seen. They displayed a single centrally located nucleus. The connective tissue stroma invested the myocytes and the capillary endothelial cells. Each muscle fiber was surrounded by an endomysium of delicate connective tissue with a rich capillary network. Fibroblast nuclei tend to be more flattened and darker staining than those of cardiac muscle cells and were peripherally located (Fig.1). Cross sections of cardiac muscle appeared irregular. The nuclei of cardiac fibers were found near the middle. Sometimes paler perinuclear regions could be noted. Connective tissue was seen in between bundles of the muscle cells. Fibroblast nuclei were found within the connective tissue or at the periphery of a muscle fiber. Many capillaries were seen among the cardiac muscle fibers (Fig. 2). Glycogen granules could be

detected by PAS stain in the perinuclear region. Intercalated discs had strong positive reaction (Fig. 3).

Ultrastructurally, the ordinary organization of sarcomere appeared between two z lines with dark and light bands with central M line and euchromatic nucleus with regular outlines. There were numerous mitochondria inside the cardiomycocyte (Fig. 4 and 5).

#### **Methotrexate-treated group (Group II)**

Animals appeared malaised from the first day following injection with diminished activity. The muscle fibers showed interstitial oedema and degeneration in some areas with less obvious striation. Some fibers had disarrangement manner with karyorrhexis of their nuclei. Myocytes were vacuolated (Figs. 6 and 7). Endomysium is thickened and has cellular infiltration with widening of the spaces in between the muscle fibers.

There were hemosiderin-laden-macrophages (brown pigmentation has star like appearance) (Fig. 7). Some cardiac muscle fibers had brown atrophy in the form of brown pigmentation inside the muscle fibers with extensive degenerative changes especially around the nuclei (Fig. 7). PAS reaction showed increase in glycogen granules in the perinuclear region. Cardiomyocytes had indistinct intercalated discs (Fig. 8)

Ultrastructurally; there were degeneration of mitochondria with loss of the ordinary organization of sarcomere and loss of banding pattern. Increased glycogen granules, and vacuolation of the cytoplasm were observed. Lipofuscin pigments were observed in the cytoplasm. The nuclei had irregular outline with chromatinolysis (Fig. 9 and 10).

In Group III; methotrexate-treated group combined with folic acid, most of cardiac muscle fibers attained their ordinary appearance and arrangement as control heart. Myocytes are cylindrical, branched and striated with central vesicular nucleus. Small areas still have degenerative changes and some cellular infiltrate. Endomysium appeared more or less as the control group (Fig. 11). No fibrosis, macrophage infiltration or brown atrophy was observed. PAS stain revealed mild reaction in the perinuclear region. Striation and intercalated discs were preserved. (Fig. 12). Ultrastructurally, the sarcomere appeared with its ordinary organization. Some mitochondria are intact others appeared destructed (Fig. 13).

#### **Immuno histochemical reaction for caspase 3**

In the control group, few cells had minimal reaction (Fig. 14). In methotrexate-treated group; there was increased number of immune positive cells compared to the control group (Fig. 15). Folic acid-treated group combined with methotrexate showed increased number of immune positive cells compared to the control group but still less

than the previous group (Fig.16).

#### **Morphometric results**

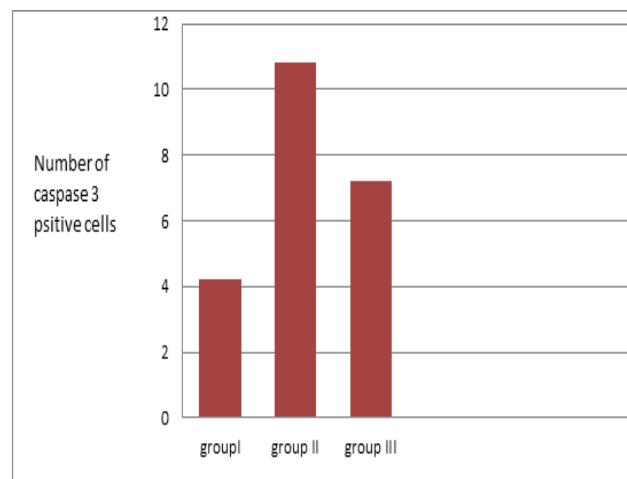
The morphometric studies and statistical analysis of the mean density of PAS reaction in cardiac muscles revealed a very highly significant difference (\*\*\*\*) between group II versus the control group as regard to the mean PAS reaction (*p value* <0.0001).

There were significant (S) difference between group III and the control group (*p value* <0.05). The summary of the means values of these results are shown in table 1 and histogram 1.

The morphometric studies and statistical analysis of the mean of positive cells for caspase-3 stain were performed on the cardiac muscle sections. The study revealed very highly significant difference (\*\*\*\*) between group II versus the control group as regard to the mean of positive cells for caspase 3 (*p-value* <0.0001). Significant (S) difference was obtained between group III and the control group (*p-value* <0.05). The summary of the means values of these results are shown in table 1 histogram 2.

**Table1:** Mean ± SE number of caspase-3 positive cells and PAS density of cardiac muscles in the study groups

Groups	Mean number of caspase-3 positive cells	Mean PAS density of cardiac muscles	P value
Group I	4.2±0.21	9.628±0.54	
Group II	10.8±0.32	22.0503±0.43	,<0.05 (****)
Group III	7.2±27	16.019± 0.56	<0.0001(****)



**Histogram 1:** Number of positive cells for caspase-3 antibody

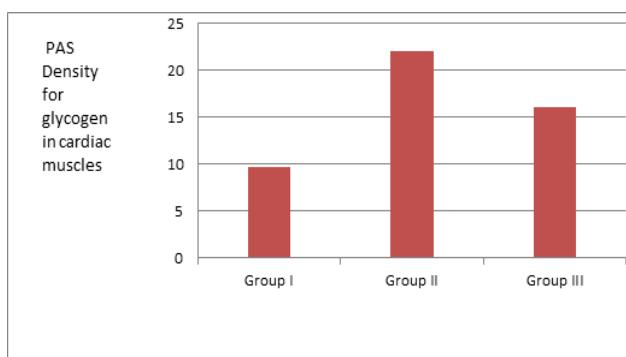


Fig. 2: PAS density of glycogen in cardiac muscles.

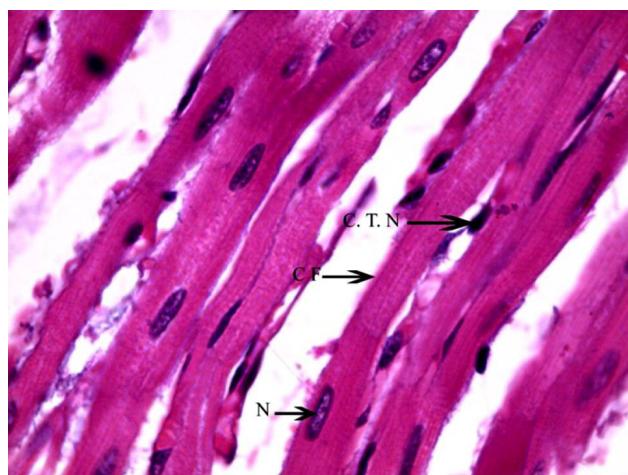


Fig. 1: A photomicrograph of L.S cardiac muscles of an adult control rat showing; longitudinally arranged cardiac muscle fibers which have acidophilic cytoplasm and central, vesicular and oval nuclei (N). They have obvious longitudinal and transverse striation cardiac fiber (C.F). The fibers are branching and anastomose with each other. There are connective tissue cells with their dense flattened nuclei (C.T.N).

Group I H&E X1000

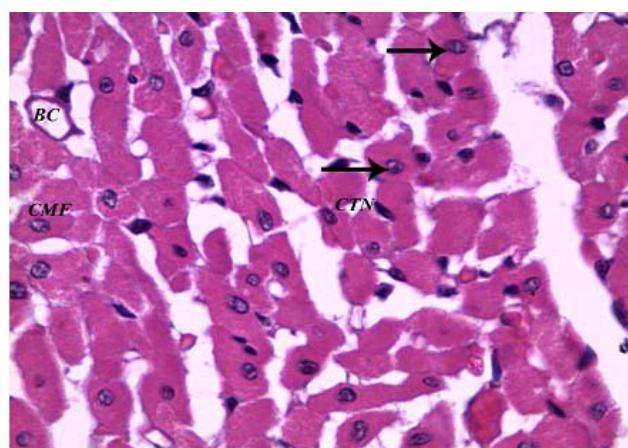


Fig. 2: A photomicrograph of cardiac muscles of adult control rat showing; cross section of myocytes (CMF) have central vesicular nuclei (arrows). Connective tissue fibers and nuclei are observed in between muscle fibers (CTN).

Note: Blood capillaries (BC) are observed.

Group I H&E X1000

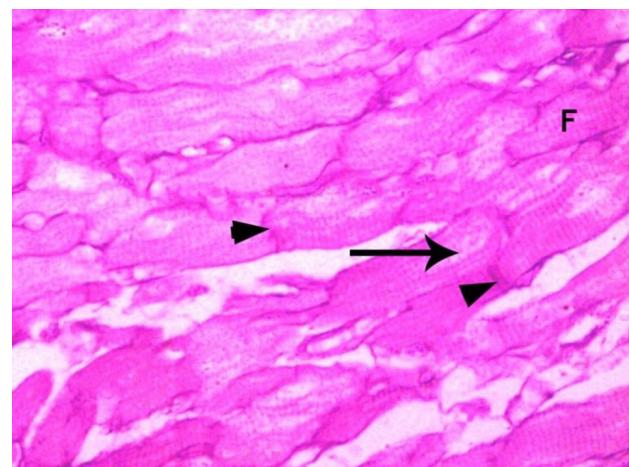


Fig. 3: A photomicrograph of cardiac muscles of adult control rat showing; positive PAS for glycogen granules in perinuclear region (Arrow) well defined intercalated disc (arrow heads) Note, well defined transverse striation (F)

(Group IPAS reaction X1000)

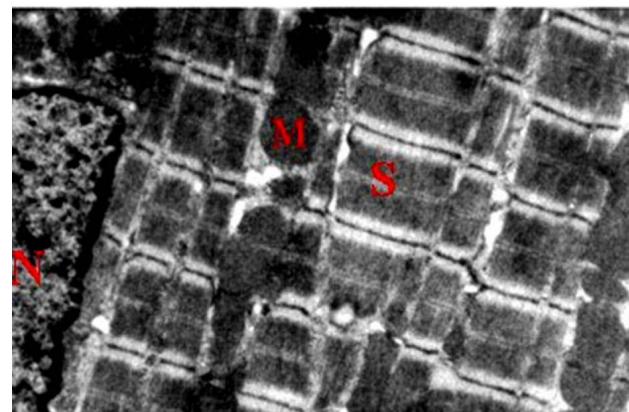


Fig. 4: An electromicrograph of cardiac muscle fibers of an adult-control rat showing; the ordinary organization of sarcomeres with dark and light bands (F) euchromatic nucleus with regular outlines (N). Note , numerous mitochondria (M).

Group I TEM X6000

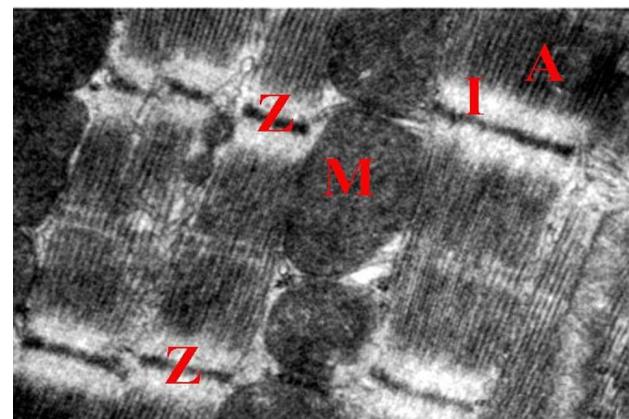
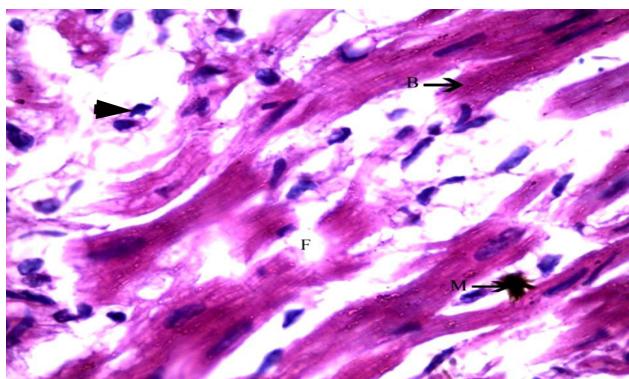
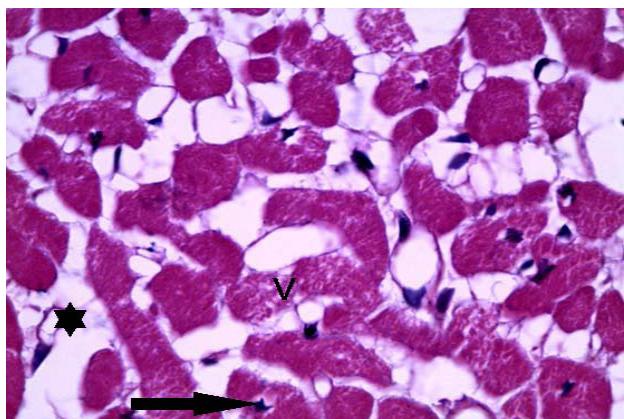


Fig. 5: An electromicrograph of cardiac muscle fibers of an adult control rat showing; the ordinary organization of sarcomere between z lines(Z) with dark (A) and light bands(I) note, the numerous mitochondria( M) note, M line (arrow head). Note, euchromatic nucleus with regular outlines.



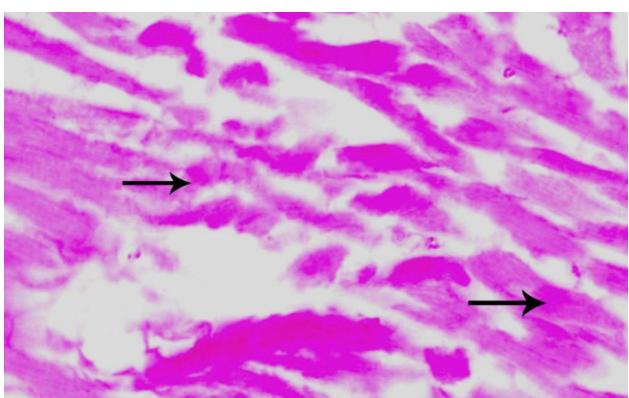
**Fig. 6:** A photomicrograph of cardiac muscles of rats treated with methotrexate showing; extensive necrosis and interstitial oedema are seen in between the fragmented cardiac muscle fibers. (F). Brown pigmentation (B) of cardiac cells is seen. There is hemosiderin-laden-macrophage (M) in between the fibrous tissue. Note, cellular infiltrations (arrowhead).

Group II H&E X1000



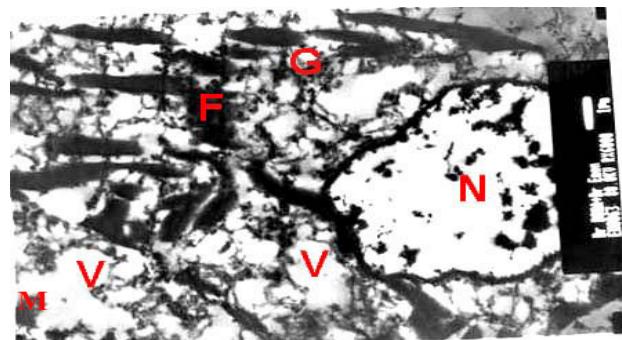
**Fig. 7:** A photomicrograph of heart of a methotrexate-treated rat showing; Cross section of cardiac muscle fibers with some vacuolations especially around the nuclei(V). The nuclei are condensed and have irregular fragmented nuclei (arrow). There is increase in interstitial connective tissue (Star).

Group II H&E X1000



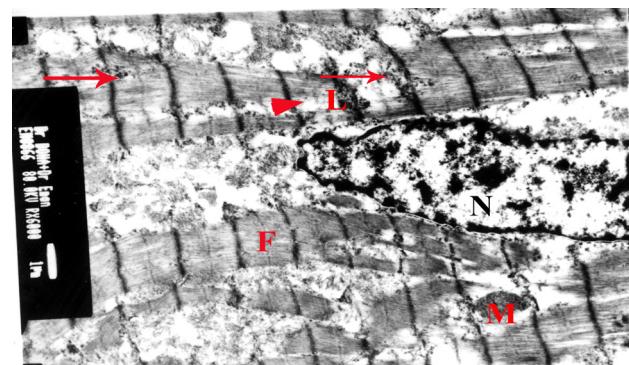
**Fig. 8:** A photomicrograph of heart of methotrexate-treated rat showing; increase in intensity of PAS reaction in the perinuclear region(arrow) ill-defined intercalated discs and myofibrils.

Group II PAS reaction X1000



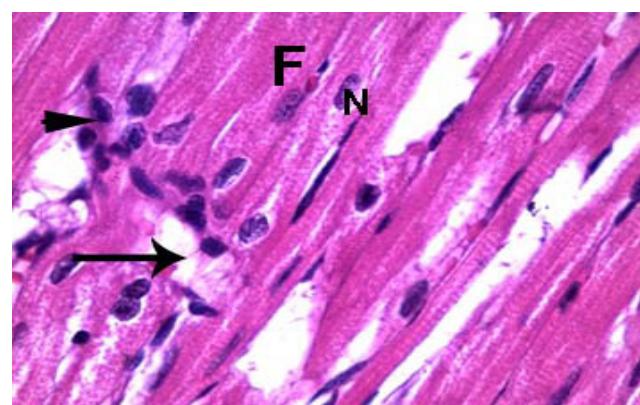
**Fig. 9:** An electromicrograph of cardiomyocyte of a methotrexate-treated rat showing; degeneration of mitochondria with loss of the ordinary organization of sarcomere and loss of banding pattern (F), increased glycogen granules (G), vacuolation of the cytoplasm (V). Irregular outline of the nucleus (N),

Group II TEM X6000



**Fig. 10:** An electromicrograph of cardiomyocyte of a methotrexate-treated rat showing; degeneration of mitochondria (M). Unclear banding pattern of sarcomeres (F) with areas of degeneration(arrowhead) increased glycogen granules (arrows) and lipofuscin pigments (L), Irregular outline of the nucleus (N),

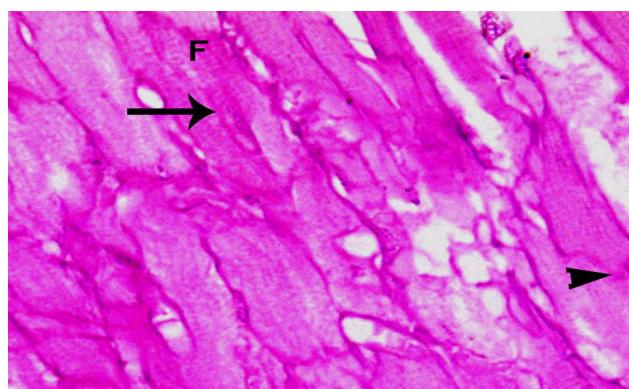
Group II TEM X6000



**Fig. 11 :** A photomicrograph of cardiac muscles of animals-treated with methotrexate concomitant with folic acid showing; most of myocytes are more or less as the control group (F). The nuclei (N)are more or less as the control with fragmentations in some areas are seen (arrow). Some cellular infiltrate are seen (arrow head).

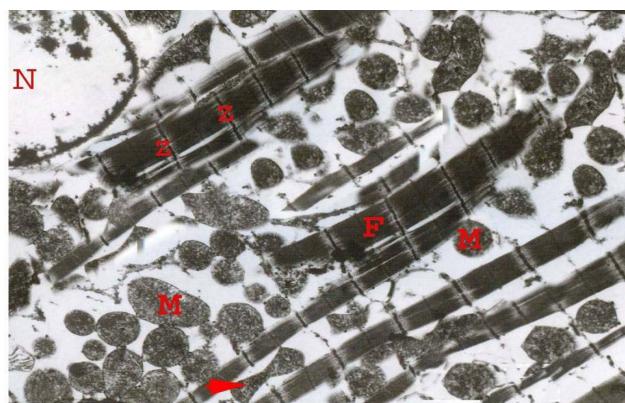
Note: transverse striations are seen.

Group III H&E x1000



**Fig. 12:** A photomicrograph of cardiac muscles of an animal treated with methotrexate concomitant with folic acid showing, moderate reaction for glycogen in the perinuclear region (Arrow ), well defined intercalated disc (arrow head). Striation is preserved in the myocytes (F).

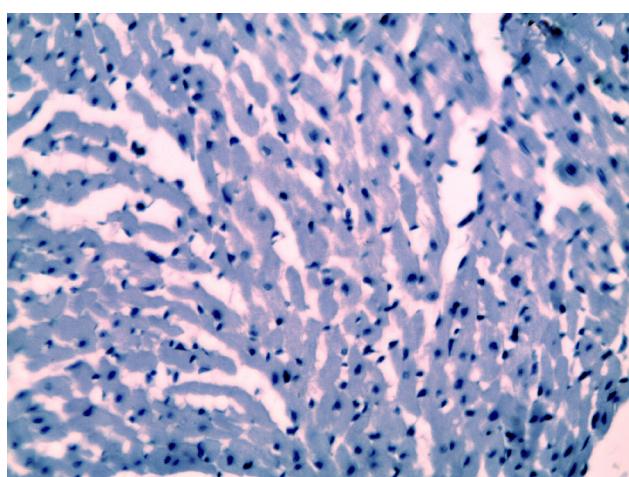
Group III X1000 PAS reaction



**Fig. 13:** An electronmicrograph of cardiac muscles of rats treated with folic acid together with methotrexate showing, some myofibrils have ordinary structure of sarcomere with well defined two z lines(Z) dark (A) and light bands(I). Some of mitochondria has destructed cristae (M). Preserved sarcoplasmic reticulum (arrow) .

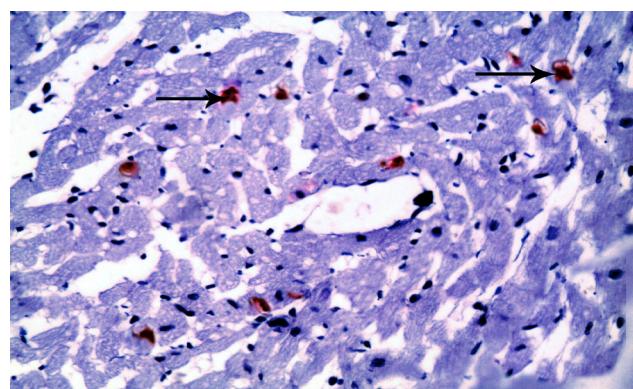
Note: the nucleus of fibroblast (N)

Group III TEM x 5000



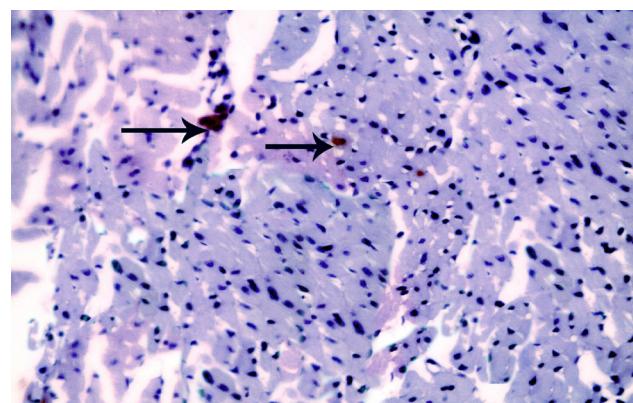
**Fig. 14:** A photomicrograph of cardiac muscles of group I showing no expression of caspase-3.

Group I Caspase-3 X400



**Fig. 15:** A photomicrograph of immunohistochemical staining of cardiac muscles of group II showing increased expression of caspase-3 positive cells compared to group I.

Group II Caspase-3 X400



**Fig. 16 :** A photomicrograph of immunohistochemical staining of cardiac muscles of group III showing moderate increase of expression of positive cells compared to group I. Group III caspase-3 X400

## DISCUSSION

In this work, animals received methotrexate appeared malaise from the first day following injection with diminished activity. These results are in agreement with previous studies whereas rats injected with methotrexate showed obvious malaise from the day following injection, anorexia, weight loss, and diminished activity<sup>[12, 13]</sup>. Many changes were noted both by light and electron microscopes. In the methotrexate-group, both necrosis and apoptosis were observed together with interstitial oedema in between the fragmented cardiac muscle fibers. Besides, brown pigmentation of cardiac cells was observed. Moreover, there was macrophage containing hemosiderin in between muscle fibers in addition to increase in interstitial connective tissue. Walter JB and Israel M. (1996) attributed the methotrexate-induced degenerative changes to increased levels of prostaglandin synthesis, which resulted in smooth muscle relaxation with subsequent vasodilatation<sup>[14]</sup>. Also inflammatory cellular infiltration is a reaction of methotrexate on microcirculation characterized by movement of fluids and leukocytes from the blood into the extravascular tissue<sup>[15]</sup>. It was

proved that methotrexate was a trigger for macrophage activation<sup>[16]</sup>. Despite the immunosuppressive effect of high dose of methotrexate, it does not suppress the cytokine release from peritoneal macrophages. Brown atrophy of the heart is due to accumulation of lipofuscin pigments which may be secondary to excessive destruction of mitochondria<sup>[17]</sup>. Previous studies proved that excessive accumulation of lipofuscin pigments has been shown to arise from peroxidative destruction of polyunsaturated lipid membranes<sup>[18]</sup>. Methotrexate has anti-proliferative actions. This action works by inhibition of enzyme dihydrofolate reductase, this enzyme is necessary to make some of the building blocks needed for DNA production. Upon dihydrofolate reductase inhibition, methotrexate interferes with a cell ability to repair and replicate itself<sup>[5]</sup>. This also means that methotrexate has a nonspecific mechanism of action, which explains the numerous side effects of methotrexate<sup>[19]</sup>. Methotrexate induced alterations of cell metabolism as it acts by inhibiting the enzyme dihydrofolate reductase and this in turn reduces the cellular supply of pyrimidine deoxythymidine triphosphate<sup>[20]</sup>. This impairs DNA and RNA synthesis which leads to decreased protein content in the cells as a result reduced cells activities<sup>[21]</sup>. By PAS we noticed increased in the reaction in methotrexate treated group especially around the nucleus which may explained the perinuclear vacuolation. A previous study proved that mitochondrial disorders of cardiac muscles is a leading factor for accumulation of glycogen in perinuclear region and associated with hypertrophic cardiomyopathies due accumulation of free radicals<sup>[22]</sup>.

Research on the effects of methotrexate on liver cells proved that methotrexate caused observed vacuolation of the cytoplasm explained by accumulation of toxic metabolites caused damage of the cell membranes with subsequent hydropic degeneration and vacuolation of the hepatocytes<sup>[23]</sup>. Electron microscopic findings support the light microscopic studies and our previous explanations whereas we observed destruction of mitochondria with loss of the ordinary organization of sarcomere, loss of banding pattern, increased glycogen level, vacuolation of the cytoplasm and irregular outline of the nuclei. Methotrexate therapy proven to induce mitochondrial dysfunction and decrease in activities of the mitochondrial electron chain complex which lead to reduced adenosine triphosphate synthesis<sup>[24]</sup>. By immunohistochemistry, we observed that methotrexate led to mild increase of apoptosis as detected by caspase 3 antibody as a marker for apoptosis. Previous researches considered apoptosis as one of the possible mechanisms of action of methotrexate. In this study we tried to study the protective effect of folic acid against the cardiovascular toxicity of methotrexate. Folic acid, a member of the B-vitamin family, is essential for amino acid metabolism. Adequate intake of folic acid is vital for

metabolism, cellular homeostasis, and DNA synthesis<sup>[25]</sup>. In our study, we observed that many cardiotoxic effect induced by methotrexate could partially reversed by the use of folic acid both at light and electron microscope level also both necrosis and apoptosis were less compared to the group treated with methotrexate.

Alike, Soliman (2009) reported that administration of folic acid with methotrexate revealed marked protection of the liver cells from the degenerative changes caused by methotrexate treated animals<sup>[26]</sup>. Folic acid supplement is used to counteract methotrexate's effects on healthy tissues as it is important for synthesize DNA and RNA, aiding rapid cell division and growth. Besides, folic acid supplementation is essential for development of the heart and its deficiency led to ventricular septal defects<sup>[27]</sup>. Many physiological studies were done to study the benefits of folic acid and reported that folic acid supplementation could reverse endothelial dysfunction in patients with cardiovascular disease<sup>[25]</sup>. The mechanism of folic acid in protection of endothelial cells suggest that folic acid and its active metabolite 5-methyl tetrahydrofolate improve nitric oxide bioavailability by increasing endothelial nitric oxide synthase coupling and nitric oxide production as well by scavenging superoxide radicals and so improve endothelial function, thereby preventing or reversing the progression of cardiovascular diseases<sup>[25]</sup>. In previous studies, folic acid had proven its important role in ischemia reperfusion. Folic acid can also prevent myocardial dysfunction preserve high energy phosphate and reduce reactive oxygen production<sup>[28]</sup>. Administration of folic acid could reverse cardiac dysfunction induced by insulin resistance in the form of decrease in cell length, peak shortening, and velocities of shortening and re-lengthening<sup>[7]</sup>. Folic acid could prevent the ethanol-induced-cardiomegaly, increased catalase-activity and decreased lipid peroxidation<sup>[29]</sup>.

Dietary folic acid supplementation diminishes the cardiomyocyte apoptosis in streptozotocin-induced diabetes<sup>[30]</sup>. Folic acid increased coronary flow, increased nitrite outflow, decreased superoxide anion production, and increased index of lipid peroxidation<sup>[31]</sup>.

From all these previous studies we could predict that as folic acid of benefit in preventing many cardiac dysfunctions whereas the histopathological side effects induced by methotrexate were ameliorated.

## CONCLUSION

We concluded that folic acid might attenuate the histological changes that were induced by methotrexate and it is beneficial to the cardiac muscles during methotrexate treatment and it should be used preliminary to methotrexate treatment .

**CONFLICT OF INTEREST**

There are no conflicts of interest.

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## الملخص العربي

### دراسة التغيرات النسيجية والمناعة الهستوكميائية للحماية المحتملة لحمض الفوليك على التأثير السمي للميزوتريكسين على عضلة القلب في ذكور الجرذان البيضاء

ضحي صابر محمد ، إيمان خليفة أحمد

قسم الهستولوجي، كلية الطب، جامعة سوهاج ، سوهاج ، مصر

المقدمة: يستخدم الميزوتريكسين على نطاق واسع كعقار كيماوي معالج. إلا أنه "الميزوتريكسين" مضاد لحمض الفوليك فيمنع تحوله الفوليك الثلاثي لحمض هيدروفوليك ومن المعروف أن حمض الفوليك يوجد بتركيز كبير في النباتات الورقية شديدة الخضار.

الهدف من البحث: دراسة الحماية المحتملة لحمض الفوليك على التغيرات الهستولوجية الناتجة عن استخدام الميزوتريكسين.  
مواد وطرق البحث: تم تقسيم ثلاثة من ذكور الفئران البيضاء البالغة لثلاث مجموعات متساوية. المجموعة الأولى استخدمت مجموعة ضابطة . المجموعة الثانية تم حقنها بالميزوتريكسين في الغشاء البريتوني بجرعة 5 مجم/كجم/اليوم لمدة شهر. المجموعة الثالثة: تم حقنها بكل من الميزوتريكسين بجرعة 5 مجم/كجم/اليوم متزامنا مع حمض الفوليك بجرعة 0.1 مجم/كجم/اليوم مع الميزوتريكسين لمدة شهر. و تم تشریح الفئران بعد أربع وعشرين ساعة من آخر جرعة وتجهيز عينات من القلب ليتم صباغتها بالصبغات العامة واختبارات المناعة الهستوكميائية وكذلك فحصها بالميكروسkop بالإلكتروني.

النتائج : ظهرت كل من مظاهر التحلل والضمور وكذلك الموت المبرمج في المجموعة التي تلقت الميزوتريكسين منفردا. في حين أظهرت عينات المجموعة التي عولجت بحمض تحسنا ملحوظا.

الاستنتاج : يجب استخدام حمض الفوليك كعلاج أولى مع الميزوتريكسين نظرا لأهميته لعضلة القلب.