The possible protective effect of folic acid against methotrexate induced ovarian damage in female albino rats. « Light and electron microscopic study»

Abdel Aziz Abd Allah Shohda, Ahmed Kamal El-banna and Fayez Mohammed Abdel Fattah

Anatomy and Embryology Department, Faculty of Medicine, Al-Azhar University

ABSTRACT

Methotrexate (MTX) is a chemotherapeutic drug that is widely used in the treatment of malignant tumors and rheumatic disorders. This work aims to study the protective effect of folic acid on the ovary affected by methotrexate. ***Materials and Methods:*** *Sixty healthy, adult female albino rats were classified into six groups (10 animals each).* ***Group I:*** *Served as control group.* ***Group II:*** *The rats received folic acid in a daily oral dose of (250ug/kg b.w) for four weeks.* ***Group III:*** *Included rats which received an I.P injection of MTX (1mg/kg b.w) once weekly for four weeks.* ***Group IV:*** *The animals were concomitantly treated with MTX and FA as the same previous doses, period and routes of administration.* ***Group V:*** *The animals were concomitantly treated with MTX and FA as the same previous doses, routes of administration for eight weeks.* ***Group VI:*** *Included rats which received an I.P injection of MTX (1mg/kg b.w) once weekly for four weeks then left without treatment for another successive four weeks.* ***Results:*** *The following results from our study were decreased number of ovarian follicles, multiple degenerated and atretic follicles with vacuolation of the oocyte cytoplasm, disturbed oocyte in some follicles with marked apoptotic bodies inside the oocyte and granulosa cells. On the other hand, rats received folic acid (FA) following treatment with MTX revealed more or less apparent normal architecture.* ***Conclusion:*** *FA proved to have remarkable protective effect against toxicity of MTX by minimizing the previous degenerative changes.*

Key Words: *ovaries, methotrexate, folic acid, rats.*

Introduction

Methotrexate (MTX), a folic acid antagonist is widely used as a cytotoxic chemotherapeutic agent for malignancies as well as in the- treatment of various inflammatory diseases **(Cetinkaya et al., 2006).** It is now prescribed worldwide to at least 500,000 patients with rheumatoid arthritis **(Swierkot and Szechinski, 2006).** It has proven efficacy as a sole agent in the treatment of such inflammatory disorder**3** despite the availability of new treatments such as biological agents **(Ranganathan et al., 2003)**. MTX is generally well tolerated although there are a number of potentially serious adverse effects of these hematopoietic suppression, hepatotoxicity and pulmonary toxicity are the more severe **(Swierkot and Szechinski, 2006).** It is not known whether the effects of MTX are due to immunosuppressive and/or anti-inflammatory actions **(Hornung et al., 2000).**

Folic acid is a vitamin of special importance in normal cellular functions **(Czeczot, 2008).** The liver is the main organ of folate storage and metabolism **(Lan et al., 2007).** 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 reductase **(Branda et al., 1998).** Folate deficiency results in neural tube defects, megaloblastic anemia and acceleration of arteriosclerotic process **(Czeczot, 2008).**

The aim of the present study is to verify the effective role of folic acid on ovarian toxicity induced

experimentally in rats with MTX treatment as the therapy with methotrexate is a dynamic process, therefore maintaining a delicate balance between benefits and risks is urgently needed.

Materials and Methods:

The experiment was conducted on sixty adult female albino rats .the weight of the rats ranging from 200-250 gms. The animals were classified into six groups (ten animals each) as follows:

Group I: Served as control group. The animals received an equivalent amount of saline intraperitoneal (I.P) once weekly for four weeks.

Group II: Rats were given folic acid which was provided by The Nile Company of Egypt. It was available in the form of tablets. The content of the tablets were dissolved in distilled water and given by gavage in a daily oral dose of 250 ug/kg body weight for four weeks **(Ardeshir and Mohsen, 2003).**

Group III: Comprised rats which received MTX- Mylan manufactured by Haupt pharma, Germay and imported by Ramco. The drug was supplied as vials each were 2ml and contained 50 mg MTX. Each vial was diluted in 48 ml of isotonic saline, so each 1 ml contained 1 mg of methotrexate. Methotrexate was injected I.P once weekly at a dose of 1mg/kg b.w1 for four weeks **(Cetin et al., 2008).**

Group IV: Animals were concomitantly treated with MTX and FA as the same previous doses, period and route of administration.

Group V: The animals were concomitantly treated with MTX and FA as the same previous doses, routes of administration for eight weeks.

Group VI: Included rats which received an I.P injection of MTX (1mg/kg

b.w) once weekly for four weeks then left without treatment for another successive four weeks. At the end of the experiment rats of each group were anesthetized then sacrificed and the ovaries were dissected for histological and ultra-structure studies.

Results

1-Group I: (Control group)
Light microscopic finding:

Histological examination of the ovaries of the control group revealed that, the ovary of the adult rat appeared containing ovarian follicles at different stages of maturation including primary, growing and Graafian follicles. The corpora lutea were also noticed in the ovarian stroma. The medulla consists of a richly vascularized loose connective tissue containing blood vessels (Fig. 1).

B- Electron microscopic Finding:

Electron microscopic study of the ovary of the adult rat of the control group showed that, the Graafian follicle was formed of several layers of granulosa cells surrounding oocyte and separated from it by zona pellucida. The granulosa cells have round or oblong shaped nuclei, prominent nucleolus, well-formed nuclear membrane, granular cytoplasm contains normal regular mitochondria in different shapes and rough endoplasmic reticulum (Fig. 2).

2-Group II: (Folic acid only)
A- Light microscopic finding:

Histological examination of the ovaries of adult albino rats of the Folic acid only group revealed that, the ovary was formed of an outer cortex and inner medulla. The cortex contained numerous follicles at different stages of development, primary, secondary and Graafian follicles. The medulla

contained blood vessels. (Fig.3).

B- Electron microscopic Finding:

Electron microscopic study of the ovary of the adult rat of the folic acid group revealed that, the Graafian follicle was formed of several layers of regular granulosa cells surrounding oocyte and separated from it by zona pellucida. The granulosa cells have round shaped nuclei, prominent nucleolus well distinct nuclear membrane and granular cytoplasm contains normal regular mitochondria in different shapes, ribosomes and rough endoplasmic reticulum (Fig.4)**.**

3-Group III: (Methotrexate for four weeks)

A- Light microscopic finding:

The ovaries of the methotrexate treated group were apparently reduced in size if compared with those of the control rats. Histological examination of the ovaries of adult albino rat of methotrexate treated group showed that, the cortex, contained multiple degenerated and atretic follicles with degenerated thin lining and no oocyte**.** Some of the degenerated follicles appeared with necrotic desquamated lining, corpora lutea with central cystification while the medulla contained multiple dilated blood vessels and cavities (Fig.5).

B- Electron microscopic Finding:

Electron microscopic study of the ovary of adult rat of methotrexate treated group showed the granulosa cells of the Graafian follicle of this group appeared apoptotic and distorted in shape with irregular shaped nucleus, peripheral segregation of chromatin content into component of variable densities and pale cytoplasm containing ill-defined organelles and

multiple vacuoles, with marked apoptotic bodies inside granolosa cells. Some of the granulosa cells also appeared as dark shrunken cells with irregular shaped pyknotic nucleus; their cytoplasm appeared with swollen, vacuolated mitochondria with loss of its cristae and marked distribution of vacuoles (Fig.6).

4-Group IV: (Methotrexate and folic acid for four weeks

A- Light microscopic finding:

Histological examination of the ovaries of adult albino rats of this group revealed that, the ovary of the adult rat appeared as a small bean shaped body composed of a surface epithelium covering fibrous stroma. The ovarian follicles at different stages of maturation were observed within the fibrous stroma of the ovary, including primary, growing and Graafian follicles with average lining and containing oocyte, and multiple corpora lutea in less congested and moderately thickened stroma (Fig. 7).

B- Electron microscopic Finding:

Electron microscopic study of the ovaries of adult albino rats of this group revealed that, the Graafian follicle was formed of layers of regular granulosa cells surrounding oocyte and separated from it by zona pellucida. Some of The granulosa cells had a round or oblong shaped nuclei with prominent nucleolus, granular cytoplasm which contain normal regular mitochondria, rough endoplasmic reticulum and well developed basement membrane. On the other hand some of the granulosa cells appeared with irregular and indentation of their nuclei, little vacuolation of their cytoplasm showed vacuolated mitochondria with loss of their cristae and

the others showed shrinkage and or absent nuclei (Fig. 8).

5-Group V: (Methotrexate and folic acid for eight weeks

A- Light microscopic finding:

Histological examination of the ovaries of adult albino rats of this group revealed that, the ovary of the adult rat appeared with different types of follicles including secondary, Graafian follicle and multiple corpora lutea in mildly congested and moderately thickened stroma(Fig9).

B- Electron microscopic Finding:

Electron microscopic study of the ovaries of adult albino rats of this group revealed that, the Graafian follicle which was formed of several layers of regular granulosa cells surrounding oocyte and separated from it by zona pellucida. The granulosa cells had a round or oblong shaped nuclei with prominent nucleolus, granular cytoplasm contained normal regular mitochondria in different shapes, ribosomes and rough endoplasmic reticulum. Some of the granulosa cells appeared with irregular and indentation of nuclei and little vacuolation of their cytoplasm including vacuolated mitochondria with loss of their cristae and the others show absent nuclei (Fig.10).

Group VI: (Methotrexate for four weeks then left without treatment for another four weeks)

A- Light microscopic finding:

Histological examination of the ovaries of adult albino rats of this group revealed that, the ovarian follicles at different stages of development including, primary, secondary, and Graafian follicles, surrounded by

mildly fibrotic stroma (Fig. 11).

B- Electron microscopic Finding:

Electron microscopic study of the ovaries of adult albino rats of this group revealed that, the Graafian follicle which was formed of layers of regular granulosa cells surrounding oocyte and separated from it by zona pellucida. The granulosa cells had a round or oblong shaped nuclei, prominent nucleolus, granular cytoplasm contained normal regular mitochondria, ribosome and rough endoplasmic reticulum (Fig.12).

Discussion

Methotrexate is widely used drug in treatment of many diseases such as rheumatoid arthritis, psoriasis and ectopic pregnancy. It is also used in the treatment of advanced stage non Hodgkin lymphomas and acute lymphoblastic leukemia. In our study we emphasize on the effect of methotrexate and folic acid on ultra-structures of the ovary of adult albino rats in order to put the bases for the best way of use of this drugs in treatment in multiple disease to avoid many hazards that may develop due to uncontrolled use of these drugs.

In the present study, the ovaries of the methotrexate treated group showed decrease in all categories of follicles with relative increase in the number of atretic follicles. Similar results were obtained by **Karri and vanithakumari (2011)**. These findings were confirmed by **McLaren et al., (2009)** who mentioned there was a time limited decrease oocyte yield during ovarian stimulation in woman previously treated

with MTX for ectopic pregnancy.

Also **Stern et al., (2006)** reported that MTX caused damage to the ovarian follicles and that MTX as an antimetabolite affected growing and Graafian follicles. This was reported by **Lower et al., (1999)** who notice the occurrence of amenorrhea in about 31% of patients during treatment of early stage breast cancer patient treated by methotrexate, but this percentage was higher after finishing treatment. On the contrary **Orvieto et al., (2007)** showed that MTX treatment in ectopic pregnancy did not influence the subsequent ovarian response to invitro fertilization (IVF) treatment.

Another study to **Oriol et al., (2008)** and **Benian et al., (2013)** demonstrated that MTX treatment to ectopic pregnancy had no effect on serum anti mullerian hormone concentrations which is produced by granulosa cells of small antral follicles of the adult ovary. In the present study there was degeneration of follicles and cortical necrosis in the ovary of methotrexate treated animals. These findings were in agreement with **Suzuki et al., (1998)** who explained degeneration of follicles and cortical necrosis associated with MTX treated animals is that the drug first causes damage to follicles. Due to localized disappearance of follicles, blood vessels are less attracted to that zone and the result is focal degeneration and necrosis.

Also these findings were in agreement with **Cetin et al., (2008)** who stated that MTX blocked the mitotic activity in epithelial cells. The temporary blockage of the regeneration of epithelial cells caused more intense degenerative changes in female genital system especially the tubal

and ovarian epithelium. The loss of follicles in ovary leads to a relative decrease in their steroid hormones secretion.

In the current work there were medullary congestion and dilation of blood vessels in methotrexate treated animals leading to focal cortical necrosis with loss of some follicles. These findings were in agreement with **(Tilly, 2004)** who explain vascular complications associated with MTX were due to drug induced endovascular damage. This results in shutdown of blood supply to certain areas of cortex, thus resulting in focal necrosis and neovascularization. It is possible that injury and obstruction of blood vessels cause local ischemia and destroy segmental regions of normal ovarian cortex with loss of follicles.

In the current work there were marked necrosis and apoptotic bodies in the granulosa cells of ovarian follicles. These findings were in agreement with **Ramadan et al., (2008).**

In the current study different types of changes in the ultra-structural morphology of cell organelles could occur following administration of methotrexate. These changes were in the form of ill-define cell organelles, vacuolated mitochondria with loss of its cristae, degranulation of the granular endoplasmic reticulum. These findings were in agreements with **Bayram et al., (2005, 2006)** who revealed that, cytoplasmic vacuoles, dilatation of rough endoplasmic reticulum (rER), nuclear irregularity with irregularity of basal membrane and loss of intercellular junctions after administration of methotrexate.

Also these changes were similar to those described by **Gol et al., (2009)** and **Karri et al., (2010)** who revealed progressive damage of mitochondria and irregularity of the nucleus of granulosa cells of the ovarian follicles in methotrexate treated animals.

In the current work, the apoptotic granulosa cells showed nuclear changes including shrinkage, irregular or elongated nuclei, chromatin migration and segregation of nucleolus. These findings were in agreement with **Vardi et al., (2010)**

In the present study the ovaries of the animals treated with methotrexate and folic acid showed reduction in the structural impairment of the cells organelles as mitochondria and rough endoplasmic reticulum have normal like appearance. The nuclei of cells containing well distributed euchromatin with marked heterochromatin attached to the inner nuclear envelope. These findings were in agreement with findings of **Soliman, (2009)** who detected a reduction in the structural impairment of hepatocytes from the livers of the rats received folic acid with methotrexate as the majority of the cells showed a relatively normal ultrastructure.

In conclusion, although patients with cancer or RA may drive much benefit from chemotherapeutic treatment, they are at risk for developing-life threatening complications. The present study confirms the earlier reports that (MTX) associated with ovarian damage, so folic acid supplementation might help patient taking MTX as a sole treatment agent to protect the ovary against adverse effects of the used drug.

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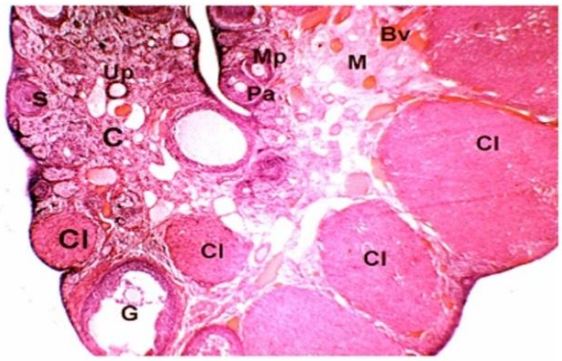

Fig.1: A photomicrograph in the ovary of adult albino rat, of the control group showing: the cortex(C) and medulla (M) of the ovary. The cortex harbors numerous follicles at different stages of development, unilaminar primary (Up), multilaminar primary (Mp), secondary (S), pre-antral (Pa), antral (Graafian), (G), and multiple corpora lutea (Cl) and the medulla containing blood vessels (Bv). **(H&E X 150).**

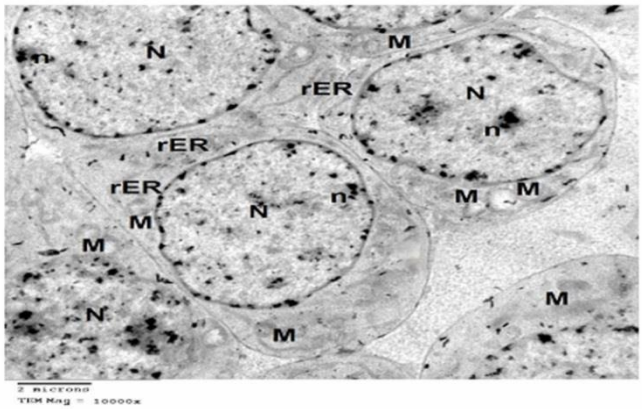

Fig. 2: Electron micrograph of the ovary of adult rat of folic acid treated group showing: A higher magnification of the granulosa cells, with oval shaped nuclei (N), finely dispersed chromatin and peripheral nucleoli (n). The cytoplasm contains normal regular mitochondria (M) in different shapes and rough endoplasmic reticulum (rER). **(TEM X10000)**

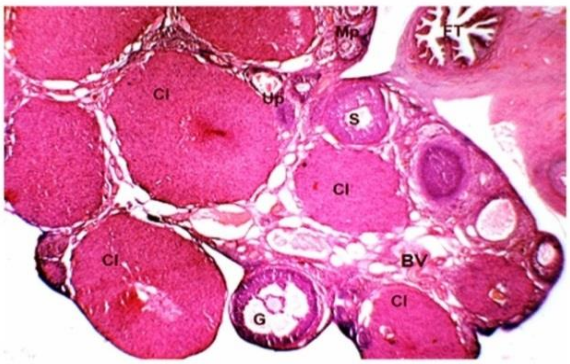

Fig. 3: A photomicrograph of the ovary of adult albino rat of folic acid group showing: Different types of follicles, unilaminar primary (Up), multilaminar primary (Mp), secondary (S) and Graafian follicle (G), and multiple corpora lutea (cl) in different stages and fallopian tube (FT). **(H&E X 150).**

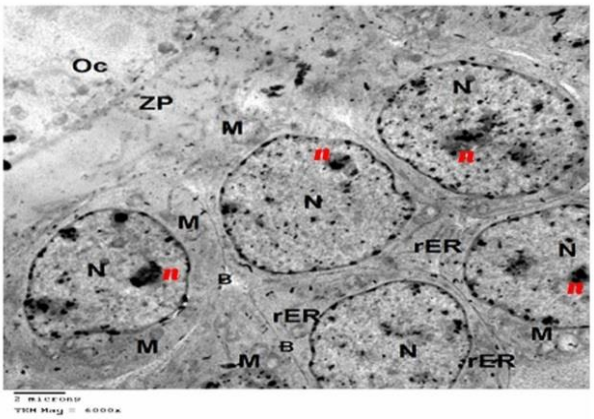

Fig. 4: Electron micrograph of the ovary of an adult rat of folic acid group showing: Graafian follicle which is formed of layers of regular granulosa cells surrounding oocyte (oc) and separated from it by zona pellucida(ZP). The granulosa cells have a round or oblong shaped nuclei (N), prominent nucleolus (n), granular cytoplasm contains normal regular mitochondria (M) in different shapes, ribosomes (r) and rough endoplasmic reticulum (rER), and well developed basement membrane. ***(TEM* X6000)**

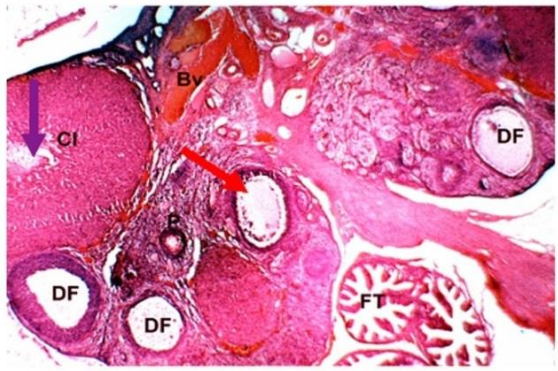

Fig. 5: A photomicrograph of the ovary of adult albino rat of methotrexate treated group showing: Few primary (P), Graafian follicles with degenerated thin lining and no oocytes (DF), one follicle showing necrotic desquamated lining (red arrow), corpora lutea (CL) with central cystification (violet arrow), in markedly congested stroma containing congested blood vessels (Bv) and fallopian tube with thin lining (FT). **(H&E X 150).**

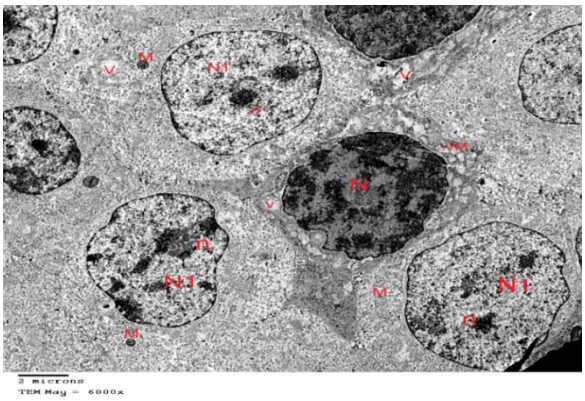

Fig. 6: Electron micrograph of the ovary of an adult rat of methotrexate treated group showing: the granulosa cells of the Graafian follicle of this group appeared dark, apoptotic and distorted in shape with irregular and indented shaped nucleus (N), peripheral segregation of chromatin content into component of variable densities and pale cytoplasm containing ill-defined organelles and multiple vacuoles, with marked apoptotic bodies inside granulosa cells. Some of the granulosa cells appeared regular with round or oblong shaped nuclei (N1), prominent nucleolus (n), and granular cytoplasm contains normal regular mitochondria (M) in different shapes. ***(TEM*** **X6000)**

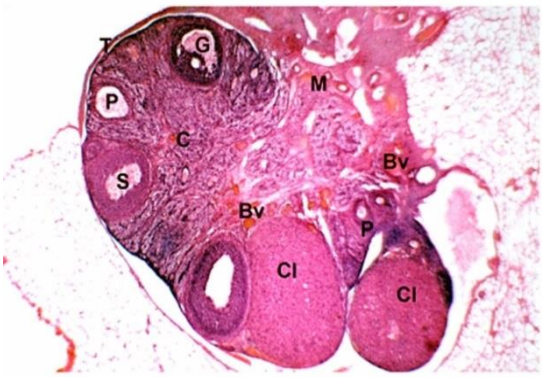

Fig. 7: A photomicrograph of the ovary of adult albino rat of methotrexate and folic acid treated group for four weeks showing: Cortex(C) containing Primary (P), secondary (S) , Graafian follicles showing average lining and containing oocyte (G), and multiple corpora lutea (Cl) in less congested stroma and medulla containing less congested blood vessels (Bv). **(H&E X 150).**

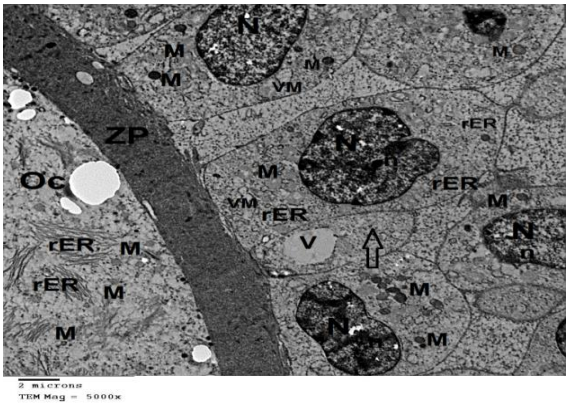

Fig. 8: Electron micrograph of the ovary of adult rat of methotrexate and folic acid treated group for four weeks showing: Higher magnification of Graafian follicle which is formed of multiple layers of regular granulosa cells surrounding oocyte (oc) and separated from it by zona pellucida (ZP).the cytoplasm of the oocyte show normal regular mitochondria (M) and rough endoplasmic reticulum (rER). Some of The granulosa cells have a round or oblong shaped nuclei (N) with segregation of its chromatin content, granular cytoplasm which contain normal regular mitochondria (M) and rough endoplasmic reticulum (rER), others appear with irregular and indentation of nuclei and little vacuolation of their cytoplasm showing vacuolated mitochondria (vm) with loss of their cristae and the others show absent nuclei (arrow). **(TEM X5000)**

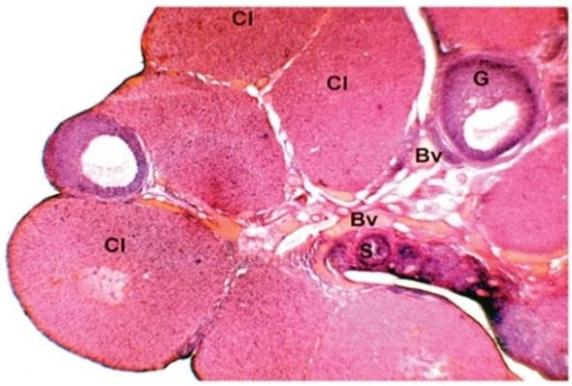

Fig. 9: A photomicrograph of a section in the ovary of adult albino rat of this group showing: Secondary (S), Graafian follicle showing average lining (G), and multiple corpora lutea (CL) in mildly congested stroma containing mildly congested blood vessels (Bv). **(H&E X 150).**

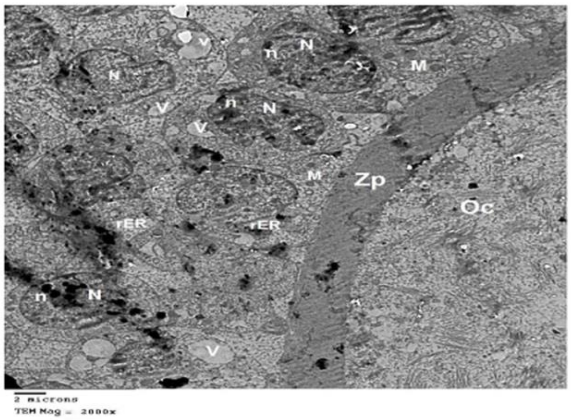

Fig. 10: Electron micrograph of the ovary of adult rat of methotrexate and folic acid treated group for eight weeks showing: Graafian follicle which is formed of layers of regular granulosa cells surrounding oocyte (Oc) and separated from it by zona pellucida (ZP). Some of The granulosa cells have a round or oblong shaped nuclei (N), prominent nucleolus (n), granular cytoplasm contains normal regular mitochondria (M) and rough endoplasmic reticulum (rER) ,some appears with irregular and indentation of nuclei and little vacuolation of their cytoplasm. **(TEM X2000).**

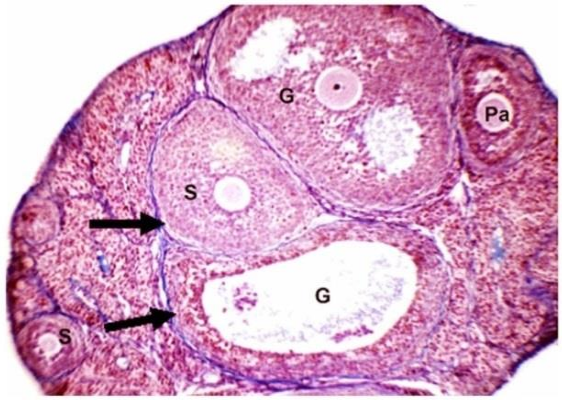

Fig. 11: A photomicrograph of the ovary of adult albino rat of this group showing: Primary (P), secondary (S), pre antral (Pa) and Antral (Graafian) follicles (G) surrounded by mildly fibrotic stroma (black arrows). **(Mallory stain X 360).**

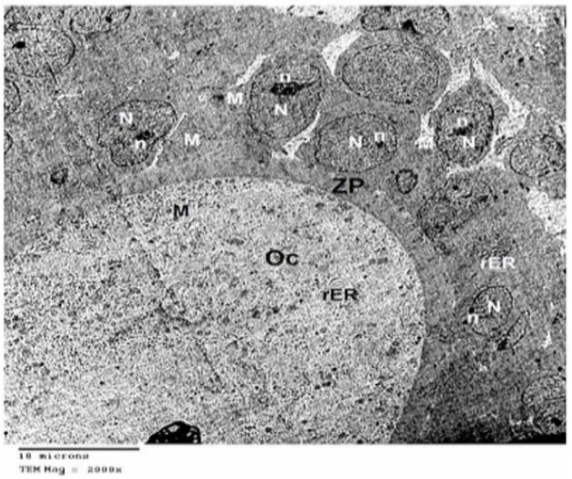

Fig. 12: Electron micrograph of the ovary of adult rat of (MTX) treated group for four weeks then left without treatment for another four weeks showing: Graafian follicle which was formed of layers of regular granulosa cells surrounding oocyte (oc) and separated from it by zona pellucida(ZP). The granulosa cells had a round or oblong shaped nuclei (N), prominent nucleolus (n), granular cytoplasm contains normal regular mitochondria (M) in different shapes, and rough endoplasmic reticulum (rER). **(TEM X2000)**

التـأثير الوقائـي المحتمل لحمض الفوليك ضد التلف الناجم عن عقارالميثوتركسيت في المبيض عند إناث الفئران البيضاء.« دراسة بالميكروسكوب الضوئي والإلكتروني»

عبد العزيز عبد الله شهدة و أحمد كمال البنا و فايز محمد عبد الفتاح
قسم التشريح وعلم الأجنة - كلية الطب بنين القاهرة – جامعة الأزهر

الخلاصة

*يعد عقار الميثوتريكسيت من أوائل الأدوية المستخدمة على نطاق واسع في علاج الأورام الخبيثة والاضطرابات الروماتيزمية. أجريت هذه الدراسة لإلقاء الضوء على التغيرات الهستولوجية والدقيقة التي تطرأ على التركيب الدقيق للمبيض بعد فترات زمنية مختلفة من حقن عقار الميثوتريكسيت وبيان الدور الذي يقوم به حمض الفوليك للحماية من هذا التأثير الضار. استخدم في هذا البحث ستون فأرا أبيض بالغ ، متوسط أوزانهم ٢٠٠ - ٢٥٠ جرام من الاناث وقد تم تصنيفهم إلى ست مجموعات كالآتي: **المجموعة الأولى: (الضابطة)**:كانت جميع الفئران في هذه المجموعة في صحة جيدة من حيث شهيتها للطعام وأظهرت الدراسة أن المبيض يحتوى على أنواع مختلفة من الحويصلات الطبيعية.**المجموعة الثانية: (المجموعة المعالجة بحمض الفوليك)**:أظهرت الدراسة لهذه المجموعة نتائج شبيهة للمجموعة الضابطة.**المجموعة الثالثة: (المجموعة المعالجة بالميثوتريكسيت)**:فئران هذه المجموعة التي تم حقنها بعقار الميثوتريكسيت بدا عليها الخمول والإعياء و فقدان الشهية. كما بدت المبايض المستخرجة أصغر حجما حيث ان الخلايا المحبية ظهرت أقل فى العدد وذات نواة صغيرة داكنة ليس فيها مظاهر النشاط ..ولقد لوحظ أيضا تغيرات فى أنوية الخلايا المحبية مثل تمزق بعض أنويتها وتغلظ فى بعضها.**المجموعة الرابعة: (مجموعة الميثوتريكسيت وحمض الفوليك لمدة اربعة اسابيع)** :فئران هذه المجموعة التي تم إعطاؤها حمض الفوليك بالإضافة إلى عقار الميثوتريكسيت كانت في حالة جيدة باستثناء بعض الخمول و الإعياء وقد اختزل حمض الفوليك التأثير الضارللميثوتريكسيت من الناحية الهستولوجية والدقيقة. **المجموعة الخامسة: (مجموعة الميثوتريكسيت وحمض الفوليك لمدة ثمانية اسابيع):**أظهرت الدراسة لهذه المجموعة نتائج شبيهة للمجموعة الرابعة.**المجموعة السادسة: (مجموعة الميثوتريكسيت لمدة اربعه اسابيع وايقافه لمدة اربعه اسابيع أخري):** فئران هذه المجموعة التي تم اعطاؤها عقار الميثوتريكسيت لمدة اربعه اسابيع وايقافه لمدة اربعه اسابيع اخري كانت في حالة جيدة بعد ايقاف الميثوتريكسيت .**الاستنتاج :** أظهرت الملاحظات من هذا البحث وجود علاقة سببية بين تعاطى الميثوتريكسيت وتأثر المبيض سلباً ويمكن تفادى تلك التأثيرات التي أحدثها عقار الميثوتريكسيت علي المبيض عن طريق اعطاء حمض الفوليك معه .*

الكلمات المفتاحية : *المبايض، الميثوتريكسيت، حمض الفوليك، الفئران .*