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A short-term comparison between the effect of two different doses of methotrexate on ovarian tissue and function in female albino rats

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Abstract

Background: Methotrexate is an antifolate that is widely used in cancers and inflammatory or auto-immune diseases, it is also known to be contraindicated in pregnancy, breast feeding and not recommended in patients planning to be parents since it has a harmful effect on fetus and gonads.
Ovarian function can be evaluated by certain parameters like the levels of female hormones or anti-Mullerian hormone which is considered as a good indicator for this purpose or histopathological examination of ovarian follicles especially the primordial follicles.

Objective: The aim of this study was to determine the effect of two different doses of methotrexate on ovarian tissue integrity and function in female albino rats.

Materials and methods: Adult female albino rats (n=30) were randomly divided in to 3 groups ; group A (n=10) in which animals were treated with intra-peritoneal injection of normal saline once weekly for four weeks, group B (n=10) in which animals were treated with *i. p.* injection of 1mg/ kg bw of methotrexate once weekly for four weeks, and lastly group C in which animals were treated with *i. p.* injection of 2.5 mg/ kg bw of methotrexate once weekly for four weeks. Then serum level of anti-Mullerian hormone were measured and histopathological examination of the ovaries were done to evaluate ovarian injury caused by methotrexate treatment.

Results: Current study demonstrated that methotrexate in the both 1mg and 2.5 mg / kg of BW doses did not significantly reduce the levels of anti- Mullerian hormone and the numbers of primordial and atretic follicles were also not significantly affected by the treatment of methotrexate in both doses. however histopathological examination revealed mild ovarian damage in case of treatment with 1mg/ kg bw of methotrexate and moderate damage in case of treatment with 2.5 mg/ kg bw of methotrexate.

Conclusions: There is no important difference between the effects of 1 and 2.5 mg/ kg of BW of methotrexate on ovarian function but there is a slight difference in the effects of the two different doses on the integrity of ovarian tissue.

Keywords: anti- Mullerian hormone, atretic follicles, methotrexate, ovarian reserve, ovarian tissue, primordial follicles.

Introduction:

Methotrexate can be considered as one of the most frequently prescribed drugs in chemotherapy and autoimmune conditions (3) and remained for a long time as the cornerstone treatment, alone or in combination with other drugs in the treatment of many different diseases whether they were malignant or non-malignant (4).Ovarian damage usually coincides with chemotherapy or radiotherapy and with different suggested mechanisms (like oxidative damage induction or direct DNA binding) according to concentration, duration and type of drugs used or strength and duration of exposure to radiations (1). Methotrexate can inhibit DNA synthesis and replication due to it'-s antifolate activity and can also increase the production of different types of reactive species that causes general cytotoxic effects and particular gonadotoxic effect (5, 22).

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In the ovaries; there is a huge number of ovarian follicles at different stages of growth; beginning as a primordial one then primary, secondary and ending as atretic follicles (6,7). Many cells of reproductive system are known to have high proliferative activity during certain period of their lives that makes these cells affected by the action of the anti- proliferative agents like methotrexate (9).Anti-Mullerian hormone which produced by the granulosa cells in the ovary; can be used to evaluate female reproductive function since its levels can give information about the number of remained primordial follicles and hence the current ovarian reserve situation (8,23).In this study we counted both the number of primordial and atretic follicles during slide examination to obtain a brief idea about methotrexate effect on the number of ovarian follicles and compare the effect of two different concentration on the number of primordial follicles remained and the increment in the atretic follicles number due to methotrexate suggested acceleration of ovarian aging effect.

Materials and methods

Animals: The experimental rats which have been used in this study were bred in the animal house of Iraqi center of cancer and genetic research of Al-Mustansiriyah university, their weights ranged between 155 and 245 g, their ages were between 12 and 18 weeks and were kept at 23 to 27 C˚ under a 12-hr light/ dark cycle. The experimental animals were treated with humanity and had free access to water and food till the last moment of the experiment. Ethical approval: The study was carried out in accordance with protocol that was approved by Iraqi board review of the college of medicine of Baghdad University. Drugs: Methotrexate vials used in the experiment were provided by Mylan® France®, Ketamine 10% vial was provided by Alfosan®, Holland® and Xylazine vial that provided VMD®, Belgium®. Experimental groups and procedure: The thirty adult female rats were randomly divided in in to three groups each group included ten animals and were treated as follows: the first group, control Group A; n=10), were given 0.5- ml of normal saline by intraperitoneal injection every week for four weeks, second group, (Group B; n=10) in which the rats were treated with 1mg/ kg bw of intraperitoneal injection of methotrexate injection every week for four weeks(2), and the third group, (Group C; n=10) in which the animals were treated with weekly intraperitoneal injection of 2.5 mg/ kg bw of methotrexate for four weeks. Every week during the experiment the animals were weighted to correct the errors that might occur during the calculation of methotrexate dose that being administered weekly. After 48 hours of last methotrexate dose, the animals were anesthetized, blood samples were drawn from each animal in order to be utilized in determination of anti- Mullerian hormone levels after that the animals were sacrificed and their ovaries were extracted for further histopathological examination.

Histopathological analysis of ovarian tissues: After ovarian tissue extracting and preservation in formalin,5-micron sections were obtained for histopathological evaluation. These sections were stained with hematoxylin and eosin (H&E) stain. The ovarian tissues were evaluated under a light microscope, and the images were captured using a digital camera. Histopathological examination was carried out.

Statistical analysis

Statistical analysis of data was performed using SPSS software "(Statistical Packages for Social Sciences-version 24) and Microsoft Office Excel (2013)"; utilizing one-way ANOVA to assay differences among studied groups.The results were presented as mean ± standard deviation in which $P < 0.05$ is "considered statistically significant.

Results

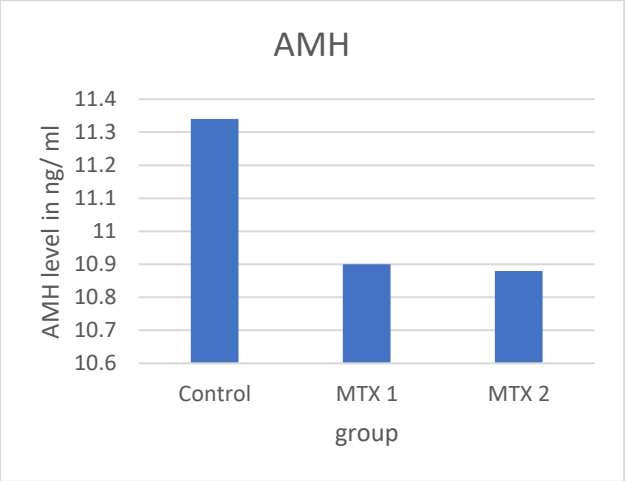
Effects of Methotrexate in the present study on level of anti- Mullerian hormone : The levels of anti-Mullerian hormone were not affected significantly by treatment with methotrexate in at1mg/ kg bw and 2.5mg/ kg bw doses in comparison to control group as shown in table (1).

Table (1) Effects of methotrexate 1mg/kg bw (Group B) and 2.5mg/kg bw (Group C) on level of anti-Mullerian hormone in comparison to controls (Group A).

Ovarian function parameter	Control (A) (Mean±SD)	MTX (B) (Mean±SD)	MTX (C) (Mean±SD)	P-value *
AMH	11.34±2.02	10.9±2.31	10.88±2.86	0.891

One– way ANOVA test , significant difference when P- value < 0.05.

Figure (1) Effect of treatment by 1mg/ kg bw of methotrexate (MTX1) and 2.5 mg/ kg bw dose of methotrexate (MTX2) compared to the first control group on the level of anti- Mullerian hormone (AMH).



Effects of Methotrexate on number of primordial follicles:

The number of primordial follicles was not affected significantly by treatment with methotrexate at both doses in comparison to control group as shown in table (2).

Table (2) Effects of methotrexate 1mg/kg bw (Group B) and 2.5mg/kg bw (Group C) on number of primordial follicles in comparison to controls (Group A).

Ovarian follicle type	Control (A) (Mean±SD)	MTX (B) (Mean±SD)	MTX (C) (Mean±SD)	P-value *
Primordial follicles	3.9±4.7	3.8±1.13	2.7±2	0.623

One– way ANOVA test , significant difference when P-value < 0.05.

Table (3) Effects of methotrexate 1mg/kg bw (Group B) and 2.5mg/kg bw (Group C) on number of atretic follicles in comparison to controls (Group A).

Ovarian follicle type	Control (A) (Mean±SD)	MTX (B) (Mean±SD)	MTX (C) (Mean±SD)	P-value*
Atretic follicle	2.4±1.5	4.1±1.6	6.1±5.1	0.056

one– way ANOVA test , significant difference when P- value < 0.05.

Effects of methotrexate on number of atretic follicles: The number of atretic follicles were not affected significantly by treatment with methotrexate in both 1mg/ kg bw and 2.5mg/ kg bw in compare to control group as shown in table (3).

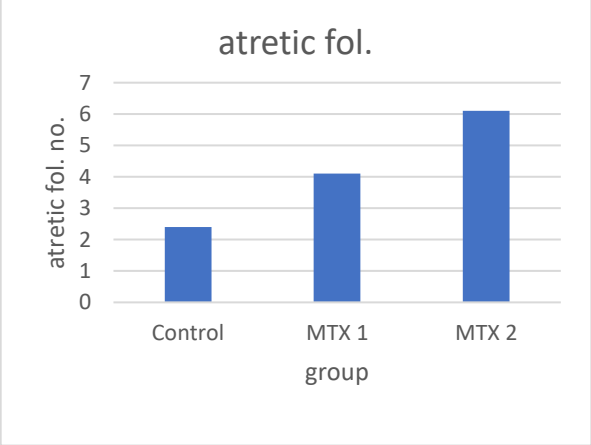


Figure (3) Effect of treatment by 1mg/ kg bw of methotrexate (MTX1) in the second group and the effect of 2.5 mg/ kg bw dose of methotrexate (MTX2) in third group in compared to the first control group on the -number of atretic follicle (atretic fol. no.).

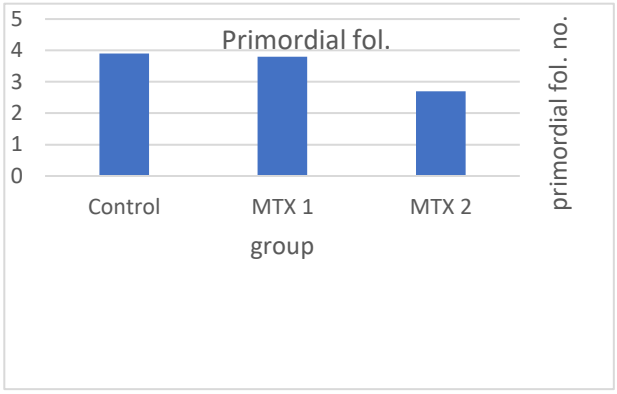
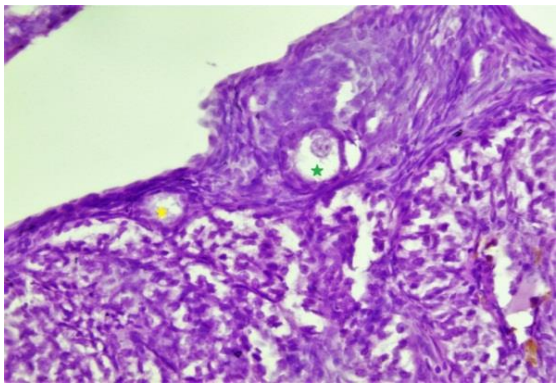
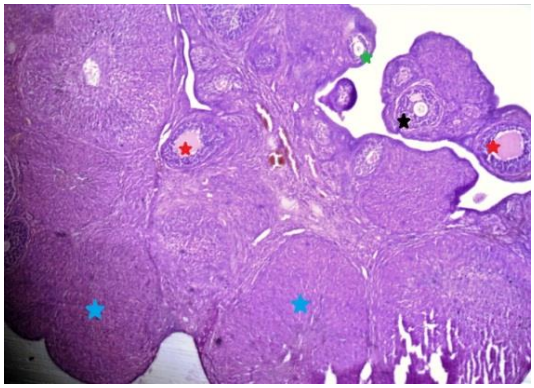


Figure (2) Effects of treatment by 1mg/ kg bw of methotrexate (MTX1) in the second group and the effect of 2.5 mg/ kg bw dose of methotrexate (MTX2) in third group in compared to the first control group on the number of primordial follicles (primordial fol. no.).



(A 2)



(A 1)

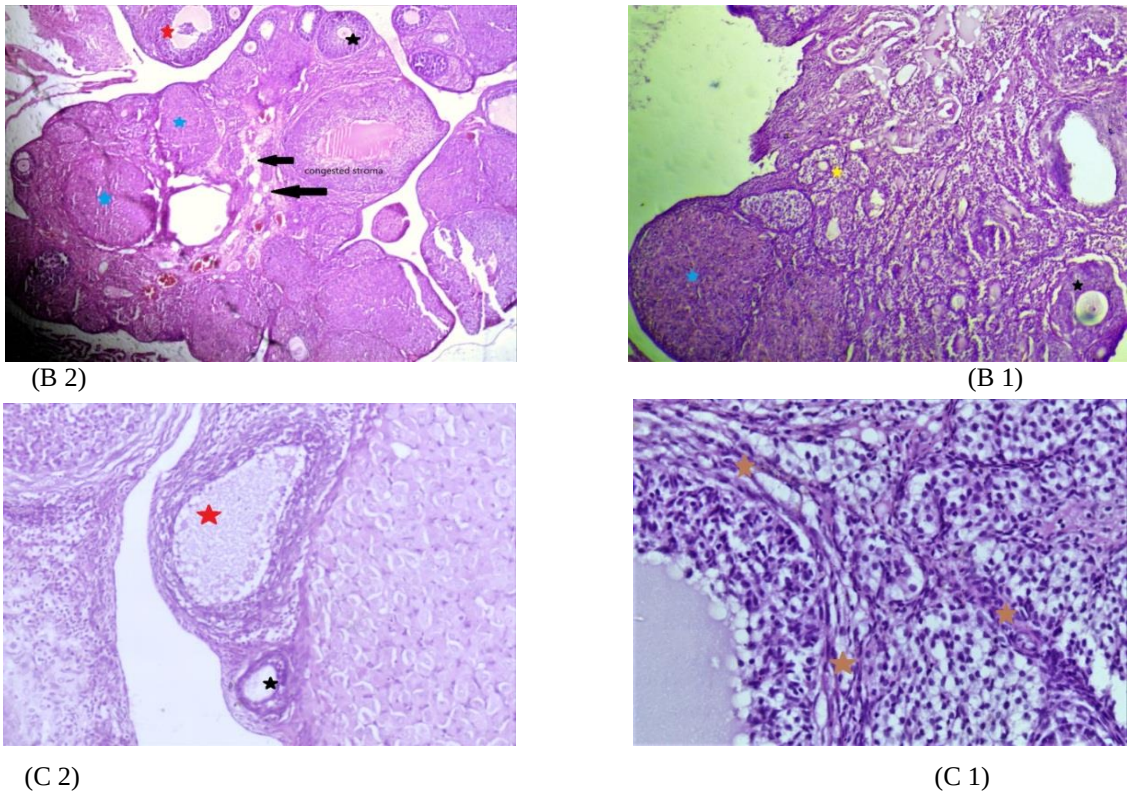


Figure (4) hematoxyline and eosine staining of ovarian tissues; (A1&2) control group, (B1&2) MTX1 group, (C1&2) MTX2 group

Black star= primary follicle. Blue star= corpus luteum.
Green star= primordial follicle. Red star= secondary follicle.
Yellow star= atretic follicle. Brown star= luteinized stroma.

Histopathological results

The histopathological investigation of the ovaries showed normal structure of ovarian tissues in case of control (Group A), while moderate damages were seen in case of MTX1 (Group C) which featured in form of cellular degeneration and increased luteinization of stroma. The MTX2 (Group B) showed only mild damage as there were some primary and secondary empty follicles seen in many slides.

Discussion:

It is very obvious that there is an increment in the number of patients who diagnosed with malignant diseases and due to the development of medicine that provides early diagnosis, new and effective ways of treatment discovering and increasing the awareness of people about these diseases' symptoms and necessity for the following up after the affliction, many patients recovered and survived may undergo some problems due to chemotherapy that may be used in their trip of treatment, one of these problems is the infertility or the early ovarian aging (5). Anti-Mullerian hormone is a novel ovarian reserve indicator (11), the level of this hormone was estimated and statistically analyzed for all samples in all groups in the present study and the results of the group (B) that was treated with 1mg/ kg bw methotrexate were only slightly lower than the results obtained from the control group. Such results were obtained in other studies (12- 15) and the same results were obtained in the group (C) as the decrement in AMH levels were insignificant compared to control (A) group. Although all the previously noticed studies used different doses of methotrexate, the results of anti-Mullerian levels obtained in the present study were confirmed by the statistical estimations of the number of primordial follicles and atretic follicles. The number of primordial follicles was counted and statistically analyzed to evaluate the extent of damage that produce by the use of weekly intraperitoneal injection of methotrexate in both doses inspected. The primordial follicle is an arrested oocyte at the meiotic prophase covered by a layer of granulosa cells, it remains in this prophase until time of folliculogenesis (16). It was found that the number of primordial follicles in the group treated with methotrexate in both group (B) and (C) was only slightly lower than that in the control (A) group and statistically insignificant, this result is contrasting with the other results of the other studies (17,18) which use different doses of methotrexate to induce ovarian damage and this might be a reason for such contrasting in addition to the difference in the time

interval and duration of the treatment used in each study (19,18). In the present study there were no important increment in the number of atretic follicles in the groups treated with methotrexate (whether in group (A) or (B)) in compared to the number of them in the control group , this result contrast the results of the previous studies (20), the contrast results might be related to the relatively low dose of methotrexate used in the present study and relatively long time interval between each dose (which gave enough time for the ovary to recover from the damaged effect of methotrexate) in compared to those utilized in other studies (19). There were no histopathological changes in the ovarian tissue noticed in the control group that has been treated with weekly injection of normal saline for four weeks, a normal stroma with no cellular degeneration or vascular congestion were found. The opposite was noticed in the group (C) which were treated with 2.5 mg/ kg bw of methotrexate that showed moderate histopathological damages of the ovaries represented by stromal luteinization, vascular congestion and follicular degeneration especially of many secondary and primary follicles which presented empty from their nuclei. This result is expected to be seen in ovaries treated with methotrexate since it is capable of inducing cell apoptosis or hindering the cell cycle by increasing the production of destructive reactive species (21). Histopathological examination of ovarian tissue in samples of group (B) showed only mild damaging effect of methotrexate characterized by the presence of empty follicles and a slight stromal congestion in many slides that have been examined. The result of histopathological examination of the slides in this group when compared to the (A) and (C) group's results gave us a hint about the effect of increasing methotrexate dose that led to increase the amount of damage and change the type of it.

Conclusion:

The treatment with methotrexate can be safe on the future reproductive function of ovaries only at low doses, but as the dose of methotrexate increases, it can be harmful to ovarian tissue and may affect ovarian reserve and may cause accelerated ovarian failure or aging.

Authors' Contributions:

The post graduate student the pharmacist Alaq Hussein Ali did the practical part of the research in addition to writing the research and following up the steps for its publication. Dr. Samara Mowaffaq Ali supervised the student's work during the research writing and reviewing period.

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مقارنة قصيرة المدى بين تأثير جرعتين مختلفتين من الميثوتريكسات على أنسجة ووظيفة المبيض في إناث الجرذان البيضاء

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الخلاصة:

الخلفية: الميثوتريكسات هو مضاد للفولات يستخدم على نطاق واسع في السرطانات والأمراض الالتهابية أو المناعة الذاتية ، ومن المعروف أيضًا أنه يمنع استخدامه أثناء الحمل والرضاعة ولا ينصح به للمرضى الذين يخططون لأن يكونوا آباء لأن له تأثيرًا ضارًا على الجنين والغدد التناسلية.
يمكن تقييم وظيفة المبيض من خلال معايير معينة مثل مستويات الهرمونات الأنثوية أو الهرمون المضاد للمولر والذي يعتبر مؤشراً جيداً لهذا الغرض أو الفحص التشريحي المرضي لحويصلات المبيض وخاصة الجريبات البدائية.

الهدف: الهدف من هذه الدراسة هو تحديد تأثير تركيزين مختلفين من الميثوتريكسات على سلامة أنسجة المبيض ووظيفتها.

المواد والطريقة: تم تقسيم إناث الفئران البيضاء البالغة (ن = 30) بشكل عشوائي إلى 3 مجموعات. المجموعة أ (ن = 10) والتي عولجت فيها الحيوانات بالحقن داخل الصفاق بمحلول ملحي طبيعي مرة واحدة أسبوعياً لمدة أربعة أسابيع ، المجموعة ب (ن = 10) حيث عولجت الحيوانات بالحقن داخل الصفاق بمقدار 1 مجم / كجم من وزن الجسم من الميثوتريكسات مرة واحدة أسبوعياً لمدة أربعة أسابيع ، وأخيرًا المجموعة C التي عولجت فيها الحيوانات بالحقن داخل الصفاق بمقدار 2.5 مجم / كجم من وزن الجسم من الميثوتريكسات مرة واحدة أسبوعياً لمدة أربعة أسابيع. ثم تم قياس مستوى الهرمون المضاد للمولر في الدم وفحص المبيضين النسيجي المرضي لتقييم إصابة المبيض الناتجة عن العلاج بالميثوتريكسات.

النتائج: أظهرت هذه الدراسة أن الميثوتريكسات في جرعات 1 مجم و 2.5 مجم / كجم من وزن الجسم لم تقلل بشكل كبير من مستويات الهرمون المضاد للمولر وعدد البصيلات البدائية والجريبات الرتقية لم تتأثر أيضًا بشكل كبير بعلاج الميثوتريكسات في كلتا الجرعتين. بينما كشف الفحص التشريحي المرضي عن تلف خفيف في المبايض في حالة العلاج ب 1 ملجم / كجم من وزن الجسم من الميثوتريكسات وضرر متوسط في حالة العلاج ب 2.5 ملجم / كجم من وزن الجسم من الميثوتريكسات.

الاستنتاجات: لا يوجد فرق مهم بين تأثيرات 1 و 2.5 ملغم / كغم من وزن الجسم من الميثوتريكسات على وظيفة المبيض ولكن هناك اختلاف طفيف في تأثير الجرعتين المختلفتين على سلامة نسيج المبيض.

الكلمات المفتاحية: الميثوتريكسات ، أنسجة المبيض ، الهرمون المضاد للمولر ، البصيلات البدائية ، الجريبات الرتقية ، احتياطي المبيض.