The effect of methotrexate on the fallopian tubes of adult albino rats: a histological and immunohistochemical study
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Introduction
Tubal pregnancy is a common disease in women of reproductive age. It is also the leading cause of pregnancy-related death in the first trimester [1]. In recent years, earlier diagnosis of ectopic pregnancy has resulted in more opportunity for conservative treatment [2].

Methotrexate (Mtx) is used mainly as an anticancer drug [3]. It acts on cells in the S-phase of the cell cycle by inhibiting the synthesis of DNA precursors [4].

Mtx has been routinely used for the treatment of trophoblastic diseases since 1956 and more recently to deplete the proliferative activity of trophoblasts in nonmolar pregnancies [4]. Tanaka and colleagues [5,6] used Mtx in cases of ectopic pregnancy with implantation in the fallopian tube.

Besides these first clinical trials on patients with ectopic pregnancies, other studies have also shown that Mtx is efficient and safe when administered at various doses and by various methods [7–9].

Recently, Mtx was widely prescribed in association with misoprostol for the treatment of early abortion [10,11]. Morbidities such as tubal occlusion (reported incidence 0.9–18.6%) and recurrent ectopic pregnancies (reported incidence 9.1–22.0%) have been attributed to adverse effects of Mtx on the fallopian tube [12].

Background
Methotrexate (Mtx) (the anticancer drug) has been a prevalent drug in the conservative treatment for unruptured tubal pregnancy for many years. Unfortunately, current emphasis has been on its damaging effects on the ovaries and fallopian tubes.

Aim of the work
The aim of this study was to examine the acute and long-term toxic effects of different doses of Mtx on the fallopian tubes.

Materials and methods
The study was carried out on 60 female rats. The rats were divided into three groups: the control group (group I), comprising 20 rats; group II, comprising 20 rats given 2.5 mg/kg Mtx intraperitoneally for 10 days (acute study); and group III, comprising 20 rats given 2.5 mg/kg Mtx for 2 months (long-term study). Rats in each group were killed at each time point and the fallopian tubes were dissected and stained with H&E, following which estrogen receptor (ER) expression was detected by immunohistochemistry.

Results
Light microscopy (acute) study showed a decrease in the number of mucosal folds with fusions of some folds. Cellular infiltration was limited to the mucosa when Mtx was administered in small doses. With increasing dose of Mtx, cellular infiltration extended to the musculosa and serosal layer. In the chronic study some regions showed an improvement in epithelial folding and the muscle layer, together with a decrease in cellular infiltration, especially at low dose. The immunohistochemical study revealed a weak positive immunoreaction for ERs in all rats of the acute group and high-dose chronic group, whereas in the low-dose chronic study moderate positive reaction for ERs in epithelial cells was detected.

Conclusion
These results prove that Mtx (≥ 5 mg/kg) can induce long-term, irreversible damage to fallopian tubes and steroid hormone receptors (ER) in a dose-dependent manner. Therefore, Mtx should be used in a relatively small and safe range of dosage in order to avoid impairment and potential risk of subsequent tubal pregnancy or infertility.

Keywords:
fallopian tubes, immunohistochemistry, light microscope, methotrexate

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Tubal disease such as structural and functional impairment can lead to subsequent tubal pregnancy or infertility [13,14].

Bayram et al. [4] reported ultrastructural damage in the endosalpinx after 10 days of Mtx exposure. More recently, Çetin et al. [15] described reversible ultrastructural changes to epithelial cells of the endosalpinx after 1 month of local Mtx injection.

Estrogens are key regulators of fertility in both men and women [16]. Estrogen participates in the regulation of the structure and function of the oviduct. Estrogen actions in target tissues are mainly mediated by its interaction with the two intracellular estrogen receptor (ER) subtypes, ERα and ERβ [17,18].

In the oviduct of rats, mice, and rabbits, ERα is the predominant subtype and ERα gene regulation is under tissue-specific hormonal control; for example, in most mammals, cyclical estradiol (E2) concentrations regulate ERα gene expression in the uterus and oviduct [19–21]. Previous studies have reported the nuclear expression of ERα gene in epithelial, stromal, and smooth muscle cells of the rat oviduct [22]. During pregnancy, ERα immunostaining increases in all cell types of all rat oviduct regions [23].

The purpose of this study was to investigate the adverse effects of increasing levels of Mtx on the epithelium of fallopian tubes using light microscopy and simultaneously on the basis of steroid receptor (ER) changes in the rat endosalpinx.

Materials and methods

Animals and reagents

This study was carried out on 60 nonpregnant adult female albino rats weighing from 190 to 250 g, which were fed a standard rat diet and given tap water while being kept under normal laboratory conditions. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals [24]. The rats were randomly divided into three groups. The control group (group I): this group comprised 20 rats divided into subgroup Ia, which received injections of physiological saline for 10 days, and subgroup Ib, which received injections for 2 months. Group II: this group comprised 20 rats divided into subgroup IIa (10 rats), which received a daily intraperitoneal injection of 2 mg/kg of Mtx (methotrexate DBL flacon 50 mg, Orma, TR) for 10 days, and subgroup IIb (10 rats), which received an intraperitoneal injection of 5 mg/kg Mtx for 10 days. Group III: this group comprised 20 rats divided into subgroup IIIa (10 rats), which received a daily intraperitoneal injection of 2 mg/kg of Mtx for 2 months, and subgroup IIIb (10 rats), which received an intraperitoneal injection of 5 mg/kg Mtx for 2 months.

Histological examination

At the end of each experiment, the rats in estrus stage (diestrus phase) were determined by vaginal smear examination using a light microscope every morning [25] and sacrificed using a high dose of ether. The ampullae of both fallopian tubes were removed for investigation. One half of each specimen was fixed in 10% formalin, prepared for paraffin sections of 5 μm thickness, and stained with hematoxylin and eosin [26].

Immunohistochemical examination

To examine the cellular expression of ER in the fallopian tubes after Mtx treatment, 5-mm-thick sections were processed for immunohistochemical analysis. The primary antibody used was anti-ER [27]. The intensity of immunostaining was counted in 10 high-power random fields in one slide at ×400 magnification in a blinded manner. The intensity of immunostaining was subjectively characterized as negative (−) when less than 10% of cells, weakly positive (+) when 10–40% of cells, moderately positive (+++) when 41–70%, and intensely positive (++++) when 71–100% of cell nuclei and cytoplasm were positively stained [28].

Quantitative morphometric measurements

The main area of positive ER immunoreactive cells in the fallopian tubes was estimated using an OlympusBX40 image analyzer computer system in the Histology Department, Faculty of Medicine, Cairo University. Measurements were obtained within 10 nonoverlapping fields for each animal at ×400 magnification.

Statistical analysis

Data were analyzed using the Student t-test for comparison between samples and controls. Values of P less than 0.05 were considered to be statistically significant.

Results

During the entire experiment, Mtx treatment was well tolerated in all rats and no animal death occurred in any group. Tissue samples close to the ampulla segment of the fallopian tubes in estrus stage were chosen for the study as tubal ampulla accounts for ~78% of ectopic pregnancies [27] and the structure of the endosalpinx and the expression of estrogen and progesterone receptors in the fallopian tubes could be varied during an estrous cycle [28].

Histological results

Light microscopic results of the control sections of fallopian tubes stained with H&E showed a normal structure in the form of mucosa with columnar epithelium, lamina propria, musculara, and serosa (Fig. 1). The mucosa is formed of columnar ciliated and columnar nonciliated secretory cells with a core of connective tissue (Fig. 2). On the 10th day, inflammatory cell infiltration and interstitial edema were observed in the endosalpinx in rats of group IIa (Fig. 3). Findings in group IIb were similar to those of group IIa but were more intense with an increasing dose of Mtx; these findings presented as inflammatory cells (lymphocytes) scattered in the columnar epithelium with acidophilic material on the folia with fusion of the folia with each other and thinning of the epithelium (Fig. 4). The submucous membrane and muscular layer of the fallopian tubes were infiltrated with inflammatory cells.
These were mainly granular leukocytes with thinning and flattening of the epithelium (Figs 5 and 6). At the end of the second month in group IIIa the changes were fewer than the ones mentioned above; for example, infiltration of inflammatory cells and interstitial edema decreased in most layers (Fig. 7). However, in group IIIb the changes became more prominent (Fig. 8).

**Immunohistochemical results**

Positive reaction for ER protein was detected in the nuclei of epithelial cells of the control group (Fig. 9). On the 10th day in group IIa, weak positive ER staining was detected (Fig. 10). In group IIb as well weak positive ER staining was detected (Fig. 11). At the end of the second month, the positivity for ER staining increased in group IIIa but did not reach the control level (Fig. 12). Also in group IIIb a weak positive immunoreaction was detected (Fig. 13). Quantitative immunohistochemistry for estrogen receptor is shown in Tables 1 and 2.

**Figure 1.** A photomicrograph of a section of the fallopian tube of a control adult rat showing three layers: mucosa (Mu), musculosa (M), and serosa (S). The mucosa showed marked infoldings (F) and the fold consists of the core of Connective Tissue (CT) covered with simple columnar epithelium in the lumen (L).

**Figure 2.** A photomicrograph of a section of the fallopian tube of a control adult rat showing simple columnar epithelium mostly ciliated (CC) and secretory nonciliated (SC) covering the lamina propria (LP) of the mucosa.

**Figure 3.** A photomicrograph of a section of the fallopian tube of an adult rat of group IIa showing inflammatory cell infiltration (ICI), interstitial edema observed with dilated blood vessels (BV), and adhesion of the folds with epithelial shedding (arrow). White arrow for edema, but black arrow for adhesion and shedding.

**Figure 4.** A photomicrograph of a section of the fallopian tube of an adult rat of group IIa showing absence of plica or folds (arrow) with dilated and congested blood vessels (CBV).

**Figure 5.** A photomicrograph of a section of the fallopian tube of an adult rat of group IIb showing shallow low folds, no branching of the folds (arrow), and adhesion of some folds (A). Inflammatory cell infiltration in the muscle layer (ICI) and interstitial edema (IE).
Figure 6. A photomicrograph of a section of the fallopian tube of an adult rat of group IIb showing inflammatory cell infiltration in the lamina propria (ICI). The serosal and muscular layer shows cellular infiltration (arrow).

H&E, × 400.

Figure 7. A photomicrograph of a section of the fallopian tube of an adult rat of group IIIa showing fewer inflammatory cells (ICI) (white arrow), shedding of some surface epithelium (black arrow), and an increase in the thickness of the wall (T) relative to the above changes.

H&E, × 400.

Figure 8. A photomicrograph of a section of the fallopian tube of an adult rat of group IIIb showing a decrease in the height of some epithelial folds (arrow). Thin muscle layer (M) with dilated congested blood vessels of the serosa (cbv).

H&E, × 400.

Figure 9. A photomicrograph of a section of the fallopian tube of a control adult rat showing highly positive immunostaining for estrogen receptor (ER) (arrow) in the nuclei of the lining epithelium.

ER immunostaining, × 400.

Figure 10. A photomicrograph of a section of the fallopian tube of an adult rat of group IIA day showing weak positive estrogen receptor (ER) staining in the nuclei of epithelial cells. (arrow) For the weak reaction.

ER immunostaining, × 400.

Figure 11. A photomicrograph of a section of the fallopian tube of an adult rat of group IIb showing weak positive immunoreaction in the nuclei of epithelial cells for estrogen receptor (ER) staining. (arrow) For the weak reaction.

ER immunostaining, × 400.
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Figure 12. A photomicrograph of a section of the fallopian tube of an adult rat of group IIIa showing moderate positive immunoreaction in the nuclei of epithelial cells for estrogen receptor (ER) staining (arrow). ER immunostaining, × 400.

Figure 13. A photomicrograph of a section of the fallopian tube of an adult rat of group IIIb showing weak positive immunoreaction in the nuclei of epithelial cells for estrogen receptor (ER) staining. ER immunostaining, × 400.

Table 1. Estrogen receptor expression among groups as determined using immunohistochemistry

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>−</th>
<th>+</th>
<th>++</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group I</td>
<td>20</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Group II (a, b)</td>
<td>20</td>
<td>12</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>At the 10th day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (a, b)</td>
<td>20</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>At the end of 2nd month</td>
<td></td>
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</table>

−, negative; +, weakly positive; ++, moderately positive; ++++, intensely positive.

Table 2. Mean area (µm) of positive estrogen receptor immunostaining in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a (± SD)</td>
<td>b (± SD)</td>
<td>a (± SD)</td>
</tr>
<tr>
<td>Mean area</td>
<td>280 ± 15.98</td>
<td>294.76 ± 8.03</td>
<td>99.6 ± 4.38</td>
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<tr>
<td>P-value</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Significance</td>
<td>Highly significant</td>
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Discussion

Mtx, a folic acid antagonist, is now used successfully in conservative treatment for unruptured tubal pregnancy [4], with lower toxic side effects and fewer complications, and it does not affect tubal patency [29,30]. However, other reports have indicated the occurrence of Mtx-induced adverse effects on the fallopian tubes, such as recurrent ectopic pregnancy [4], possible tubal occlusion, and secondary infertility [15,31].

The results of the present study, revealed a reduction in the number of mucosal folds and cellular infiltration into the mucosa of the fallopian tubes at low-dose administration of Mtx for a short period. With increasing dose, cellular infiltration extended to the muscular layer and serosal layer, which was partly in agreement with previous studies in the fallopian tubes [4,15,28] and in other organs exposed to Mtx treatment [8,32].

In the chronic study at low dose, some improvement was seen to occur in the mucosal foldings and thickness of the muscle layer, together with a decrease in cellular infiltration, which was in agreement with the findings of [4,28].

Some investigators reported that normal structures in the healthy endosalpinx are essential for successfully picking up the egg and effectively transferring it to the uterine cavity [4,14]. Hence, severe chronic damage to the epithelial lining and musculature of the fallopian tubes can potentially lead to tubal dysfunction and eventually result in ectopic pregnancy or infertility [1,14,33]. The immunohistochemical study revealed weak positive immunoreaction for ERs in the epithelial cells of the mucosa of the fallopian tube in all groups. However, a moderately positive reaction for ERs was detected at low dose administered for 2 months. These results were in
accordance with an improvement in the histological profile of the fallopian tubes by Light Microscope (LM) as seen in the present study in the form of regions of mucosal folds and normal musculature of the tube together with a decrease in cellular infiltration. These results were in agreement with those of Yang et al. [28].

Some researchers stated that a balanced local hormone environment and coordination effects of estrogen and progesterone were essential for intratubal transfer of the fertilized egg into the uterine cavity [34,35]. Estrogen and progesterone in the fallopian tubes played an important role in regulating the reproductive process and were essential for tubal physiology and pathology. Similar to intratubal pregnancy, the fallopian tubes had similar mechanisms of blastocyst implantation in the endosalpinx. Uncoordinated expression of ER and PR in the endosalpinx has a close relation to tubal implantation and subsequent tubal pregnancy; declining local hormone levels will increase the risk of tubal implantation [13,27].

Some scientists further demonstrated that normal expression of ER in the endosalpinx acted in the pivotal role of the mucosal barrier against tubal implantation. Damaged steroid receptors constituted the reasons for the pathogenesis of tubal pregnancy [13]. More recent studies showed simultaneous bilateral tubal pregnancies occurring after ovulation induction with clomiphene citrate [34,35]; the underlying reason may be the antagonizing effects of clomiphene citrate, which acts against the role of steroid hormone receptors in regulating the reproductive function of the tubes.

Conclusions

From this study we have concluded that Mtx administered at a low dose for short period of time can induce reversible damage to fallopian tubes. However, in large doses (≥5 mg/kg) it can induce long-term, irreversible damage to fallopian tubes and steroid hormone receptors (ER) in a dose-dependent manner. Therefore, Mtx should be used in a relatively small and safe range of dosage in order to avoid impairment and potential risk for subsequent tubal pregnancy or infertility.

Acknowledgements

Conflicts of interest

There is no conflict of interest to declare.

References

تأثير عقار الميثوتركسات على انابيب فالوب في الفئران البيضاء البالغة دراسة هستولوجية وهستوكيميائية مناعية

شريفة عبد السلام مرسى وشيرين محمد صبحى
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عقار الميثوتركسات (المضاد للسرطان) دواء منتشر في العلاج الواقي من الحمل الوبقي (الحمل في قنوات فالوب) لسنوات عديدة. وللأسف في الوقت الحالي هناك تركيز على الآثار الضارة منه على المبيض وقنوات فالوب. هدفت هذه الدراسة إلى اختبار الآثار السمية الحادة وطويلة الأجل لجرعات مختلفة من الميثوتركسات على قنوات فالوب. تم إجراء الدراسة على ستون فأرا من إناث الفئران و تم تقسيم الفئران إلى ثلاثة مجموعات. المجموعة الأولى الضابطة وتشمل (20) من الفئران، المجموعة الثانية (20 فأرا) تم حقنها بجرعتين من الدواء 5 و2 مج / كجم لمدة عشرة أيام (دراسة التأثير الحاد) والمجموعة الثالثة (20 فأرا) تم حقنها بجرعتين من الدواء 5 و2 مج / كجم لمدة شهرين (دراسة الأثار طويلة الأجل). وقد تم قتل فئران كل مجموعة حسب الفترة المحددة وقد أخذت عينات من قنوات فالوب وصبغها بصبغة الهيماتوكسيلين ودراسة مستقبلات هرمون الاستروجين بواسطة الهيستوكيميائية المناعية للأنسجة.

أظهرت نتائج الدراسة (الحادة) من خلال المجروحة الصغيرة كان هناك نقص في عدد تفرعات الطبقة الطلائية مع تلاحم بعض هذه التفرعات وانتشار الخلايا الالتهابية لهذه الطبقة. ومع زيادة الجرعة كان هناك انتشار واسع للخلايا الالتهابية إلى كل من طبقة العضلات وطبقة الخلايا الخارجية للأنابيب. كما أظهرت الدراسة المزمنة تحسن في بعض مناطق الطبقة الطلائية مثل التفرعات وأيضاً طبقة العضلات مع انخفاض معدل انتشار الخلايا الالتهابية. كما أظهرت الدراسة الهيستوكيميائية المناعية أن التفاعل المناعي لمستقبلات الاستروجين الإيجابي كان ضعيف في كل المجموعات الحادة وأيضاً المجموعة المزمنة ذات الجرعة العالية بينما كان التفاعل متوسط مع الجرعة الصغيرة لدراسة المزمنة. من هذه الدراسة نستنتج أن عقار الميثوتركسات له تأثير ضار على المدى الطويل ولا رجعة فيه على قنوات فالوب ومستقبلات هرمون والاستروجين في شكل يعتمد على الجرعة ولذا يجب أن نستخدم الميثوتركسات بجرعات صغيرة نسبيا وأمانة لتجنب ضعف والمخاطر المحتملة من حمل الأنابيب اللاحق أو العقم.