

Effects of aqueous extract of *Salvia* on some parameters of sperms and histopathological changes in testes of mice treated with Methotrexate (MTX)

تأثير مستخلص المريمية المائي على بعض معايير الحيامن والتغيرات النسيجية الامراضية لخصى الفئران المعاملة بعقار الميثوتركسيت

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Abstract

Methotrexate commonly used to treat cancer and causes reproductive damage in mice. The aim of this research is to study the effects of *salvia* aqueous extract on some parameters of sperms and histopathological changes in testis of mice treated with methotrexate. Twenty-four adult albino male mice were divided into three groups, (8 mice in each group). First group was negative control received normal saline intraperitoneally, while second group was treated with 50 µg/kg, for 35 days intraperitoneally (ip). The third group was treated with methotrexate (50 µg/kg) also (ip) and extract of *salvia* 85 mg/kg for 35 days. The results showed significant ($P<0.05$) decrease in sperms motility , increased percentage of dead sperms and abnormalities of sperms in mice treated with MTX, there was significant ($P<0.05$) decrease in diameters of seminiferous tubules, primary spermatocytes and spermatids, increased interstitial spaces as compared with control, while in the third group result showed significant ($P<0.05$) increase in sperms motility , decreased percentage of dead and abnormalities of sperms and significant ($P<0.05$) increase in diameters of seminiferous tubules, primary spermatocytes and spermatids and decrease interstitial spaces compared with first and second groups.

المستخلص

ان الاستخدام الشائع لعقار الميثوتركسيت هو لعلاج الامراض السرطانية ، الهدف من هذا البحث هو دراسة تأثير المستخلص المائي للمريمية على بعض قياسات الحيامن والتغيرات النسيجية المرضية لخصى الفئران المعاملة بالميثوتركسيت ، 24 ذكر ناضج من الفئران البيض قسمت الى ثلاث مجاميع كل مجموعة تحتوي على 8 ذكور . المجموعة الاولى السيطرة السالبة تم اعطائها في الخلب البريتوني المحلول الملحي الفسيولوجي ، بينما المجموعة الثانية عولمت بعقار الميثوتركسيت بجرعة 50 مايكروغرام/كغم لمدة 35 يوما عن طريق الخلب البريتوني . المجموعة الثالثة فعولمت بعقار الميثوتركسيت بجرعة 50 مايكروغرام / كغم عن طريق الخلب البريتوني وعولجت بالمستخلص المائي لنبات المريمية لمدة 35 يوما. اظهرت النتائج انخفاضاً معنوياً في حيوية الحيامن ، وزيادة معنوية في نسب الحيامن الميتة والمشوهة وكذلك انخفاض معنوي في اقطار النبببات المنوية ، وخلايا النطاف الاولى والنطاف ، وزيادة في المسافات البينية للنبببات المنوية لخصى الذكور المعاملة بالميثوتركسيت مقارنة مع مجموعة السيطرة السالبة ، بينما اظهرت النتائج زيادة معنوية في حيوية الحيامن ، وانخفاضاً معنوياً في نسب الحيامن الميتة والمشوهة وكذلك انخفاضاً معنوياً

في اقطار النبيبات المنوية ، وخلايا النطاف الاولية والنطاف ، ونقصان في المسافات البينية للنبيبات المنوية لخصى الذكور في الفئران المعالجة بالمستخلص المائي للمريميا مقارنة مع المجموعة الاولى والثانية .

Introduction

Numerous species of the genus *Salvia* have been employed since ancient times in folkloric medicine and subjected to an extensive pharmacognostic research to identify their biologically active compounds. Sage has a very long history of effective medicinal uses and is an important domestic herbal remedy for disorders of the digestive system. Its antiseptic qualities make it an effective gargle for the mouth where it can heal sore throats and ulcers. The leaves applied to an aching tooth will often relieve the pain. The whole herb is antihydrotic, antiseptic, antispasmodic, astringent, carminative, stimulant, tonic and vasodilator [1]. Sage is also used internally in the treatment of excessive lactation, night sweats, excessive salivation (as in Parkinson's disease), profuse perspiration (as in tuberculosis), anxiety, depression, female sterility and menopausal problems [2]. The essential oil from the plant is used in small doses to remove heavy collections of mucous from the respiratory organs and mixed in embrocations for treating rheumatism. In larger doses, however, it can cause epileptic fits and giddiness. The leaves make excellent tooth cleaners, have antiseptic properties and can heal diseased gums [3]. Leaves extract appeared with antimicrobial against bacteria and fungi including dermatophyte [4].

The leaf extracts have antioxidant activity exhibit strong antioxidant activity, largely attributable to various phenolic constituents including phenolic diterpenes such as carnosol and hydroxycinnamic acid derivatives, notably rosmarinic acid [5].

Methotrexate is a mild immunosuppressant that also exhibits anti-inflammatory activity. Methotrexate is commonly used for the treatment of certain cancers including but not limited to leukemia, Hodgkin's disease and head and neck cancers. In these illnesses, methotrexate is used in very large doses so that it interferes with the reproduction of the cancer cells. Methotrexate is used in much smaller doses for the treatment of rheumatoid arthritis, Crohn's disease and psoriasis.[6]

Methotrexate is known to be teratogenic in women, but few data are available on the effects of methotrexate on male reproductive capability [7]. Studies in animals have shown altered spermatogenesis, cytotoxicity and degeneration of spermatocytes, Sertoli cells, and Leydig cells [8]. In patients with psoriasis treated with methotrexate, there were case reports of oligospermia that resolved when the methotrexate was stopped [9].The effects of MTX also documented in reproductive system. It induces cellular alteration in male gonads (testes) in human as well as in animals.[10]But there is little documentation in literature regarding mode of action and mechanism of cell death on testes during proliferative stage of reproductive system after long term continuous exposure of MTX on an animal model. The aim of this work is to study the effects of salvia extract on some parameters of sperms and some histopathological changes on mice testes treated with methotrexate.

Materials and Methods

Animals and Treatment

Twenty-four adult albino male mice (30-36 gm) were purchased from Biotechnology Research Center and maintained on a 14:10-hour light dark cycle in the animal

house, control and treated mice were provided with food and water *ad libitum*. One week after arrival, males were randomly divided into three groups, containing 8 mice in each group, first group negative control intraperitoneally received normal saline. While the second group was treated with methotrexate in dose 50 µg/kg, for 35 days administration intraperitoneally (ip). And the third group was treated (ip) with methotrexate in dose 50 µg/kg, and extract of salvia in dose 85 mg/kg for 35 days. Animals in each group were killed by cervical vertebrate dislocation. Testes fixed with Bouin fluid (BDH Inc, Toronto, Canada). Spermatozoa were obtained from the two tails of epididymides by mincing in 500 µl TCM-199, and maintained at 37°C in 5% CO₂ incubator and the percentages of motility and dead and abnormalities of spermatozoa were measured.

Histological Examinations

The perfuse-fixed testes placed in Bouin fluid overnight, and processed for routine paraffin embedding. The testes were cut into 5-µm sections. Three serial sections per testes were mounted on slides, deparaffinized, rehydrated, and stained with hematoxyline - eosin stain. Sections of the testes were examined by light microscope. Seminiferous tubules, interstitial spaces, primary spermatocytes and spermatids diameters were assessed in each testis using a previously calibrated Micrometer (Ocular micrometer, Stage micrometer).

The diameter of 25 seminiferous tubules was measured in 5 fields (5 seminiferous tubules per field). In similar manner diameter of primary spermatocytes, spermatids, leydig cells were measured in 5 fields and the mean value of each was calculated. The interstitial space observed between seminiferous tubules measured by using the ocular micrometer.

Microscopical examination

Spermatozoa were assessed according to WHO laboratory manual [11] for viability, percentage dead/live spermatozoa, motility and abnormalities.

Statistical analysis

Statistical analysis was performed to compare two different groups by using ANOVA-test. Statistical significance was determined at $P < 0.05$ [12].

Results and Discussion

Methotrexate is a well-known anti-cancer agent used for the treatment of malignant and non-malignant conditions. In recent years, large number of reports has been published on potential gonadal damage. In this study the results showed significant decreased of motility, and significant increase in percentage of dead sperms and abnormalities of sperms Figure (1), in the groups treated with MTX compared with control group which showed normal sperms Figure (2), while there was significant increase in motility, and significant decrease in percentage of dead sperms and abnormalities of sperms Table (1) in the group treated with salvia extract compared with first and second groups.

Table (1): percentage of sperms motility, dead sperms, and sperms abnormalities in treated and control groups.

Groups	Motility of sperms (%) (mean+SD)	Dead sperms (%) (mean+SD)	Abnormalities of sperms (%) (mean+SD)
Control	A 80.00+9.28	A 18.36+4.58	A 14.69+5.21
Treated with 50 µg of MTX	B 55.00+8.44	B 32.47+4.81	B 31.31+4.77
Treated with 50 µg of MTX + Salvia extract	A 75.00+6.52	A 21.12+5.31	A 18.93+4.30

Differences A, B are significant ($P < 0.05$) to comparison rows



Fig (1): Abnormal sperms from treated groups with MTX (E X40)



Fig (2): Normal sperms from control group treated with normal saline (E X40)

Administration of MTX is known to cause reproductive damage, including decreased epididymal and testicular weights, and reduced epididymal sperm counts and fertility [13]. Histopathology of the testis is characterized by vacuolization of Sertoli cells, and sloughing of elongating spermatids and spermatocytes when damage is severe [14]. A corresponding decrease in the number of microtubules in Sertoli cells after MTX treatment has been observed using electron microscopy and immunohistological techniques [15].

Several case reports and series have documented reversible sterility in men using methotrexate. [16] They reported a decrease in sperm count and quality with the use the agent. When the medication was discontinued, the sperm returned to normal levels and quality. Van Scott and Reinertson[17] reported a decrease in sperm count 12 to 14

days after a single intravenous injection of methotrexate. [18] Also observed an age-dependent effect in terms of reversibility of the altered spermatozoa; men younger than 40 years were more likely to experience recovery. Azoospermia was confirmed in men treated with MTX.

Methotrexate treatment caused abnormal sperms morphology, in male reproductive function which is under hormonal control, spermatogenic process is under control of follicular stimulating hormone (FSH) and testosterone, while the formation of type A spermatogonia and conversion of primary spermatocyte into secondary spermatocyte (Meiosis I) are dependent on testosterone and the final step of maturation of spermatids are dependent on FSH, so the abnormal sperm morphology may reflect an abnormal intratesticular maturation as a result of drug treatment, also the drug induce an alteration in androgen secretion that usually produce changes in the reproductive system, such changes might include the production of abnormal sperms ,also any effect on spermatogenesis lead to production of abnormal sperms[19].

The decrease in the abnormalities of sperms (head and tail)especially after 35days of treatment with plant extract occurs when the sperms were in the spermatogonia stage and before mitotic division which represents a source of sperms, and this will be in a agreement with [20,21] which revealed that this extract does not contain any mutagenic agents,[22] pointed that these mutagenic agent can induce the abnormalities, in sperms head and tail while non-mutagenic agents do not induce these abnormalities. Active compounds which detected in salvia extract may play a role as antioxidant protecting against sperm damage. Decreasing in the sperms head and tail abnormalities may be returned to that these extracts which have protective effects in germ stem cells (spermatogonia) which act as a source of all sperms [23].

The results also showed significant decrease in diameters of seminiferous tubules Table (2) and Figure (4) , primary spermatocytes, spermatids Table (3) and increase in interstitial space Table (2) Figure (4) when treated with MTX compared with control group Figure (3), certainly this is due to antimitoic activity of the drug, The diameter of leading cells Table (3) in the present study did not reveal any alteration after MTX administration with respect to controls. Figure (5), while the results of mice treated with salvia extract showed significant increase in diameters of seminiferous tubules Table (2), primary spermatocytes, spermatids Table (3) and decrease in interstitial space Table (2) when compared with first and second groups.

Table (2): Diameters of seminiferous tubules and interstitial space in both treated and control groups

Groups	Diameter of seminiferous tubules (μm) (mean \pm SD)	Interstitial space (μm) (mean \pm SD)
Control	A 214.51+16.55	A 19.81+2.15
Treated with 50 μg of MTX	B 182.11+14.24	B 43.09+6.16
Treated with 50 μg of MTX + Salvia extract	A 9.81 \pm 202.03	A 24.12+3.61

Differences A, B are significant ($P < 0.05$) to compression rows

Table (3): Diameter of primary spermatocytes, spermatids and leydig cells in treated and control groups.

Groups	Primary spermatocytes (µm) (mean±SD)	Spermatids(µm) (mean±SD)	Leydig cells(µm) (mean±SD)
Control	A 5.30±0.84	A 3.70±0.79	A 4.51±0.85
Treated with 50 µg of MTX	B 3.75±0.72	B 1.98±0.63	A 3.77±0.69
Treated with 50 µg of MTX + Salvia extract	A 5.24±0.64	A 3.26±0.44	A 0.31 ± 4.04

Differences A, B are significant (P<0.05) to compression rows

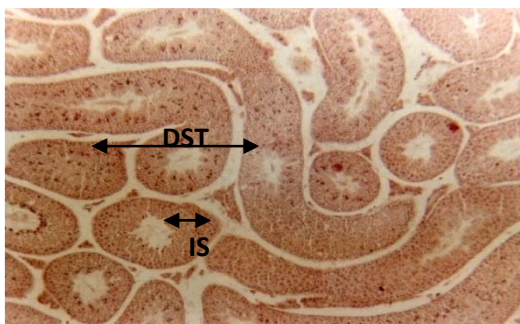


Fig (3):Photomicrograph of testes of mice (control group) showing normal structure of seminiferous tubules. (DST- Diameter of Seminiferous tubules, IS- Interstitial space) (H and E X 10).

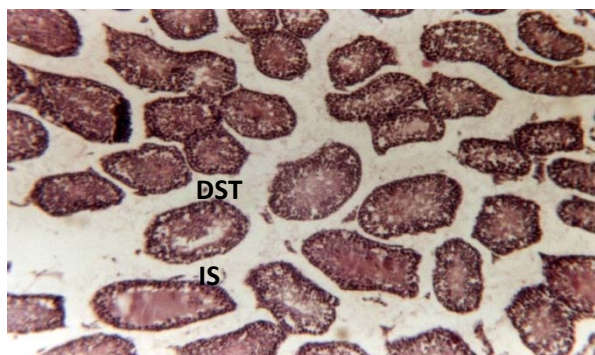
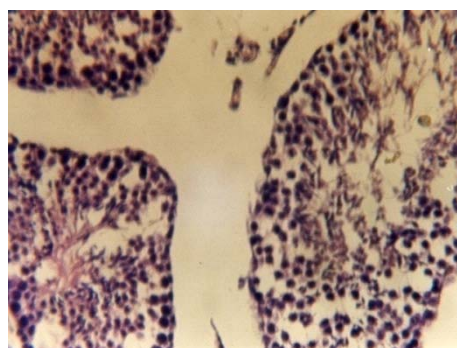
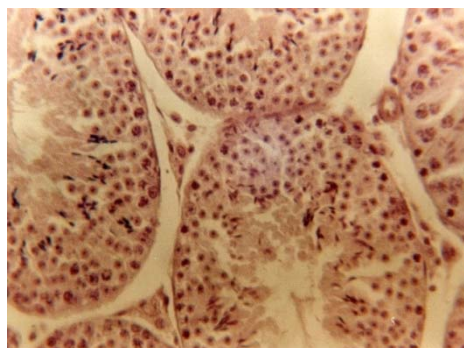


Fig (4): Photomicrograph of testes of mice (treated group with MTX) showing decrease in diameter of seminiferous tubules (DST) and increase of interstitial space (IS) (H and E X 10).



Methotrexate, an immunosuppressive drug used to treat cancer, psoriasis, and rheumatic diseases; it is a folic acid antagonist that binds to the enzyme dihydrofolate reductase. This inhibits synthesis of thymidylate, serine, and methionine, which disrupts synthesis of DNA, RNA, and protein and leads to cell death. In the present study, the size of cellular contents of seminiferous tubules were altered significantly, this may be because primary spermatocytes and spermatids failed to replicate DNA due to inhibition of an essential enzyme dihydrofolate reductase required for normal DNA synthesis, therefore, it can be concluded that these qualitative and quantitative changes in male gonads may alter the reproductive performance of animals. However, further study is required at ultra-structural and molecular level to explore the mechanism of action of Methotrexate [24].

A number of classes of compounds possess estrogenic activity. All isoflavones are estrogenic to animals. Others are coumarins and diterpenoids, steroid. *Salvia* is one of these plants containing steroidal saponins. Also ethanolic extract with essential oil of *salvia* was shown to have certain effect on fertility and that's why many parameters under study gave a positive result for *salvia* effects [25].

The extract of *salvia* significantly restores the glutathione level in the liver, and LPO; acid phosphatase and alkaline phosphates in testis of mice. Furthermore we observed that the extract of *salvia* reduced the stickiness of chromosomes and the chromosomal aberration. Improved results after treatment with *salvia* could be due to the antioxidant property of *salvia* as evidenced by restoration of glutathione and LPO levels. Restoration of acid phosphatase level pointed out the role of extract of *salvia* in promoting the stability of cellular, nuclear and organelle membranes [24].

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