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Physiological and Histological Changes of the Testes of Laboratory Mice Treated with Methotrexate

Cambios Fisiológicos e Histológicos de los Testículos de Ratones de Laboratorio Tratados con Metotrexato

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SUMMARY: The current study aimed to demonstrate the physiological and histological effects of methotrexate (MTX) in laboratory mice's testes . 24 male laboratory mice, *Mus musculus* L., were used. The experimental mice were divided into three groups of equal number ,with eight mice for each group . The first group of mice was injected with 0.1 ml of distilled water and adopted (as a control group) , the second group was also injected with a low concentration of methotrexate (2 mg/kg), while the third group was injected with a high concentration of the drug (4 mg/kg) . The mice were injected into the Intraperitoneal daily for 16 days. The results of the current study showed a significant decrease in sperm count at both doses (2,4 mg/kg) with a probability level of $P < 0.05$ compared to the control group . The drug also caused a significant decrease in the number of normal sperm in mice treated with both doses, and an increase in the number of abnormalities of the sperm's head and tail . In addition, injecting males with the drug caused a significant reduction in the concentration of testosterone at both doses. The drug also had a clear effect on testicular tissue in general and the process spermatogenesis in particular. The results of the histological examination showed that the testicular tissue was affected in the treatment group compared with the control group , to varying degrees between the group treated with the low concentration and the high concentration . It has been observed that there are vacuoles in some seminiferous tubules, the appearance of degeneration and necrosis processes of germ cells, a decrease in the thickness of the germ mass, and a narrowing of the lumen of some seminiferous tubules. It was also observed that there was exfoliated , cytolysis , loss of germ cells , infiltration of inflammatory cells in some seminiferous tubules, and the appearance of amyloid between the seminiferous tubules , significantly in the group treated with the high concentration . The current study concluded that methotrexate causes severe damage to the testicles through a decrease in the number of sperm, distortions in their external appearance, a decrease in the level of testosterone, and a significant decrease in the process of spermatogenesis.

KEY WORDS: Methotrexate; Testosterone; Sperm; Spermatogenesis.

INTRODUCTION

Methotrexate (MTX), one of the commonly used chemotherapy agents, is an antimetabolite that inhibits the replication, repair and synthesis of DNA, it has anti-inflammatory and immunosuppressive properties (Cronstein, 1996). MTX was first used in 1951 to treat patients with rheumatoid arthritis and psoriasis (Gubner *et al*., 1951). The treatment began to receive great interest and was approved as an effective treatment by the Food and Drug Administration in 1988 (Cutolo *et al*., 2001). Despite the great benefit of this treatment, it has many side effects that appear in patients, and these effects range from mild to severe, including hepatotoxicity, gastrointestinal disorders, pneumonia, blood disorders, kidney toxicity, dermatitis and others (Bannwarth *et al*., 1994; Wang *et al*., 2018a).

Methotrexate is an anti-folate antagonist, as it works to disrupt the metabolic pathway of folic acid when it enters cells through reduced folate carriers and remains in the cells for long periods (Hess & Khasawneh, 2015). Folic acid plays an important role in cellular replication and repair of brain functions, and its deficiency is associated with high levels of the compound Homocysteine in the blood (hyperhomocysteine), and the latter plays a role in increasing the risk of arterial disease, blood vessel damage, Alzheimer's disease, and dementia (Malouf *et al*., 2003).

The testicles may be target organs exposed to harm caused by chemotherapy, as many chemotherapeutic drugs often cause clear abnormalities in the process of

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spermatogenesis and semen quality (Bahadur *et al*., 2005). Poor sperm formation is one of the early signs of a reversal of the effect of chemotherapy on testicular functions, as sperm counts decrease in patients after starting chemotherapy (Gandini *et al*., 2006). Moreover, MTX has side effects on the reproductive system, especially on the processes of oogenesis and spermatogenesis, which leads to infertility in both sexes (Gökçe *et al*., 2011).

The current study aims to investigate the physiological and histological effects of methotrexate treatment on the testes of male laboratory mice.

MATERIAL AND METHOD

Laboratory mice. 24 male laboratory mice *Mus musculus* L. were used as a model in the current research, their weight ranged between 22-24 g and they were 10 to 12 weeks old. They were raised and fed under optimal conditions of temperature (25 ± 2°C) and standard food (Jawad, 1996; Kata, 2008). And conducting laboratory experiments in the animal house of the Department of biology / College of Education for Pure Sciences / University of Basrah.

Methotrexate drug. The trade name for the chemotherapy drug METHOTREXATE KOCAK, which is used in the current study, is in the form of a 20 ml vial with a concentration of 500 mg and was supplied by Pharma World Ecza Deposu A.S. (KOCAK FARMA TURKY).

Experiment design. Male laboratory mice were injected with methotrexate into the intraperitoneal area daily for 16 days. The experimental mice were divided into three groups of equal number, eight mice for each group. The first group of male mice was injected with 0.1 ml of distilled water and was considered as a control group. The second group was also injected with a low concentration of methotrexate (2 mg/kg), and the third group was injected with a high concentration of methotrexate (4 mg/kg).

Estimating the fertility of male laboratory mice. The method of Vega *et al*. (1988), was adopted in calculating the total number of sperm after the end of the period of treatment of males with methotrexate on day 17 by anesthetizing them with chloroform. The mice were dissected and the right epididymis was isolated, then cut and mashed in 1 ml of Phosphate buffered saline (PBS) solution (PH=7.0). Then the pureed sperm was placed in a small test tube with a capacity of 2 ml, with the addition of one to two drops of aqueous eosin dye to stain the sperm while counting it. After a period of 15 minutes had passed, the pipette for counting white blood cells was used and the sperm was withdrawn to the mark of 0.5, then the volume was completed to the mark of 11. In the pipette of the PBS solution only, one drop was placed in the middle groove of the hematocytometer slide, and the slide was left for 5 minutes to ensure that the sperm remained stable and did not move during the count. The number of sperm is calculated in four 1 mm^2 squares for calculating WBC, multiplied by a factor of 5 x 104.

To calculate the percentage of sperm abnormalities, the method of Wyrobek & Bruce (1975) was adopted. If the left epididymis is cut into small pieces in a petri dish by adding 5 ml of PBS solution, then the sperm fluid is dripped using a dropper at an appropriate height on five clean glass slides lined up together. After finishing spreading the sperm on the slides, they are left to dry. Then the sperm is dyed by placing the slides in a coplin jar containing 1 % eosin dye for 5 minutes. Then they are left to dry and the percentage for each mouse is calculated. For five replicates, 100 sperm per slide were calculated.

Hormonal examination. Drawing blood directly from the heart from the heart puncture on day 17 from experimental animals treated with methotrexate after anesthetizing them with chloroform and placing the serum in special tubes for preservation after isolating it using a centrifuge. The concentration of testosterone in the serum of mice was measured using the principle of competitive reaction through the enzyme-linked absorbance titration method (ELISA) at a wavelength of 450 nm and using a measuring kit prepared by the German company Human.

Histological preparation. Based on the method of Humason (1972), testicle samples from the control and treated groups were fixed with 10 % formalin for 24 hours. The samples were then washed with water for 24 hours to remove formalin and then preserved in 70 % ethyl alcohol. In order to bury the samples in paraffin wax, the samples were passed through a series of alcohol concentrations: 90 % ethyl alcohol for one hour and 100 % ethyl alcohol for three hours. Then the samples were soaked in xylene for 7-10 minutes. The samples were then impregnated with molten paraffin wax at a temperature of 60 °C for 4 hours, with the wax replaced every hour. The samples were embedded using special molds, then cut to a thickness of 7 mm, then flattened and placed on glass slides, then stained with hematoxylin and eosin, then examined and photographed with a Leica microscope.

Statistical analysis. The research results were analysed statistically using the Analysis of Variance (ANOVA) test, and the significance between the averages was assessed through the Least Significant Difference (LSD) test at $p < 0.05$ and using the SPSS Ver 21.

RESULTS

The effect of methotrexate on the fertility of laboratory mice. The chemotherapy methotrexate has a high potential for toxicity to the testicles when treating male laboratory mice. Table I showed a significant decrease in sperm counts at both doses (2.4 mg/kg) with a probability level of P?0.05 when compared to the control group. The treatment also caused an increase in the number of deformed sperm. The statistical analysis showed a significant decrease in the numbers of normal sperm in the mice treated with the two doses and an increase in the number of deformed sperm heads and tails (B, C, D, E, F, G, H, I and J) when compared with the healthy sperm in the control group(A) Figure (1).

The effect of methotrexate on the level of testosterone in the serum of male laboratory mice. Injecting male laboratory mice with the chemotherapy methotrexate caused a significant reduction in the concentration of testosterone at both doses at the probability level of P?0.05 when comparing its level in the control group (Table II).

Table I. Impact of methotrexate on fertility of mice (Mean ± S D, N=8)

Treatments	Sperm count ($m \times 10^6$)	Normal sperms %	Deformed sperms	
			Sperm head %	Sperm Tail %
Control group	18.66 [a]	88.06 [a]	2.12 [a]	9.93 [a]
0.1ml normal saline	±1.07	± 9.00	± 0.69	± 2.35
Low Dose	6.67 [b]	57.90 [b]	7.26 [b]	34.80 [b]
2 mg/kg Methotrexate	±1.36	± 6.17	± 2.35	± 8.77
High Dose	3.27 [c]	49.00 [b]	11.00 [c]	39.80 [b]
4 mg/kg Methotrexate	±0.81	±10.31	± 2.26	6.33

a,b,c Different letters indicate significance at the 0.05 probability level.

Table II. Impact of methotrexate on testosterone hormone concentration of mice (Mean ± SD, N=8)

Treatments	Testosterone hormone ng/ml
Control group 0.1 ml normal saline	3.61 [a] ±0.87
Low Dose 2 mg/kg Methotrexate	1.06 [b] ±0.30
High Dose 4 mg/kg Methotrexate	0.28 [c] ±0.09

a,b,c Different letters indicate significance at the 0.05 probability level.

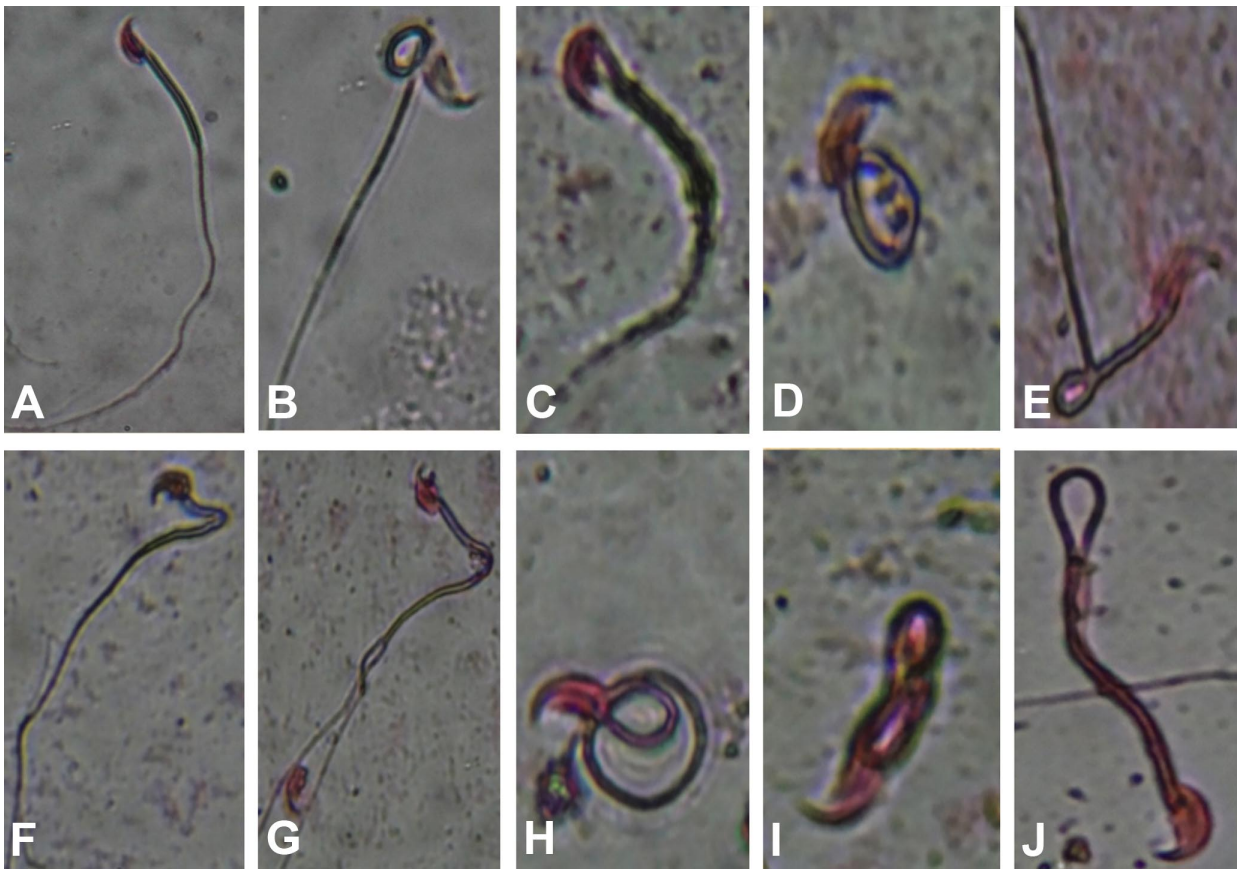

Fig 1 . Effect of methotrexate on the abnormalities of mice sperms.

The effect of methotrexate on testicular tissue and spermatogenesis in male laboratory mice The results of a study of histological sections of laboratory mice treated with methotrexate showed a clear effect on testicular tissue in general and the spermatogenesis process in particular, to varying degrees between low and high doses compared to the control group. The results of the histological examination of the group treated with low concentration showed the presence of vacuoles in some seminiferous tubules, the number of which does not exceed at least one vacuole in one seminiferous tubules, to a lesser extent compared to the group treated with high concentration. It was noted that there were vacuoles in many seminiferous tubules, and that one seminal tubule contained more than one vacuole compared to the control group (Figs. 2 to 4).

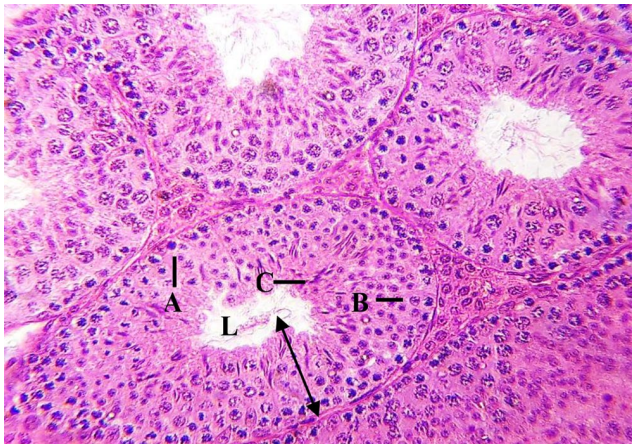

Fig. 2. A cross-section of the testicle of a control group mice showing the thickness of the germinal mass (⇔), A. spermatogonia, B. primary spermatocyte, C. spermatids, L, seminiferous tubule lumen, (H&E), 400X.

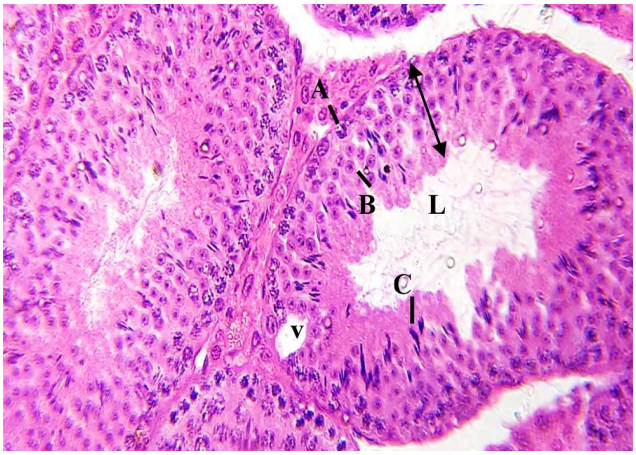

Fig. 3. A cross-section of the testicle of a low-concentration mouse showing the thickness of the germ mass (⇔), A. spermatogonia, B. primary spermatocyte, C. spermatids, L. seminiferous tubule lumen, (v) vacuole, (H&E), 400X.

As for the thickness of the germ mass in the seminiferous tubules of the group treated with a low concentration, it appeared similar to what was found in the control group. The process of spermatogenesis appears in almost all its stages in the presence of spermatogonia, primary spermatocytes, and spermatids (Figs. 2 and 3). While the germ mass appeared in some seminiferous tubules of the group treated with high concentration, with a thickness less than in the group treated with low concentration, and the process of sperm formation stopped in some seminiferous tubules at the stage of formation of primary spermatocytes (Fig. 5).

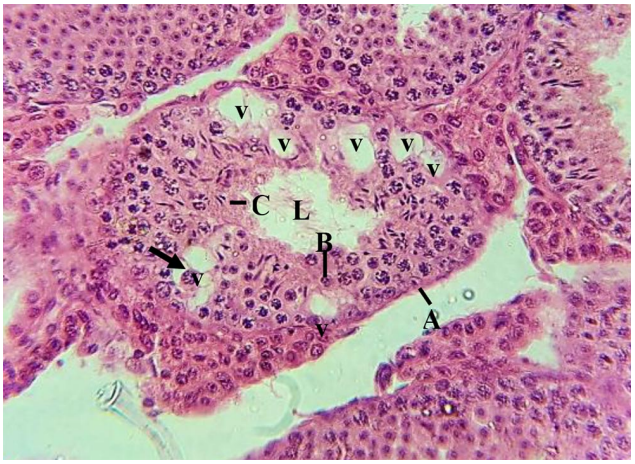

Fig. 4. A cross-section of a high-concentration mouse testicle showing the presence of many vacuoles in the seminiferous tubule (v), degeneration of the primary spermatocyte (arrow), A. spermatogonia, B. primary spermatocyte, C. spermatids, L. seminiferous tubule lumen, (H&E), 400X.

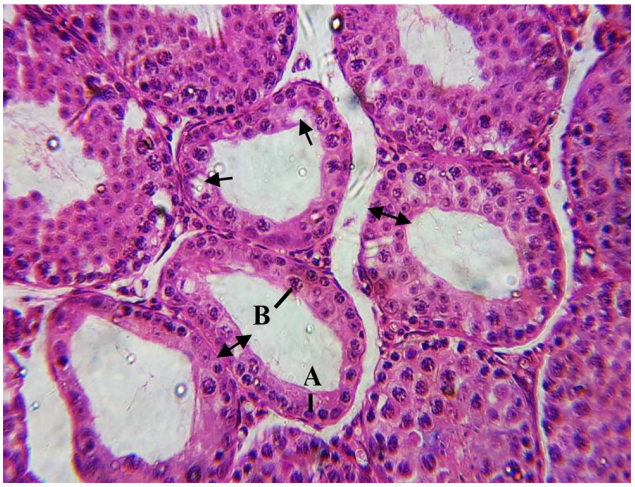

Fig. 5. A cross-section of a high concentration mouse testicle showing the thickness of the germ mass (⇔), degeneration of the primary spermatocyte (arrows), A. spermatogonia, B. primary spermatocyte, (H&E), 400X.

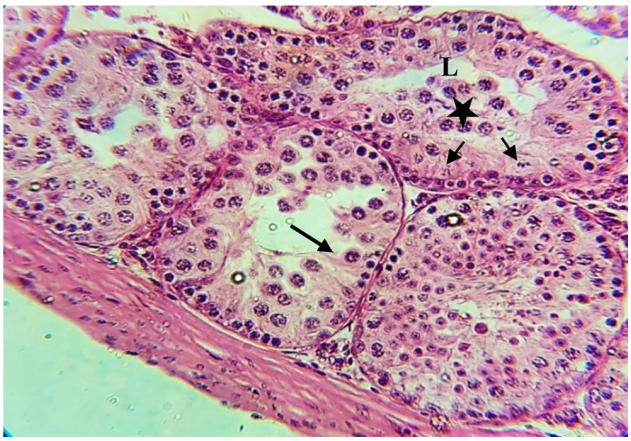

Fig. 6. Cross-section of a high concentration mouse testicle showing necrosis of the primary spermatocyte (small arrows), degeneration of the primary spermatocyte (large arrow), note the irregular arrangement of the germ cells (star), L. seminiferous tubule lumen, 400X (H&E).

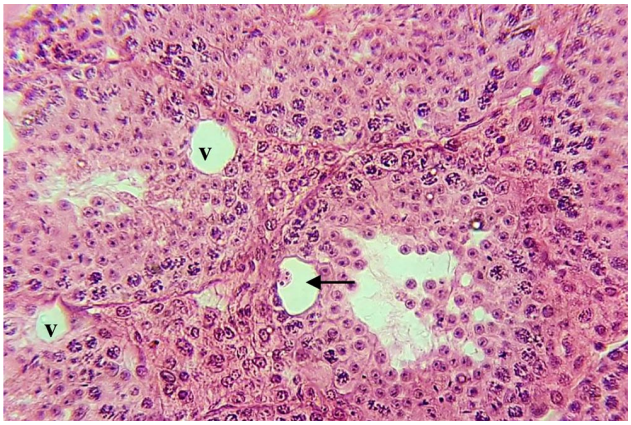

Fig. 7. Cross-section of a high concentration mouse testicle showing Sustentacular cells (Sertoli cell) degeneration (arrow), vacuole (v), 400X (H&E).

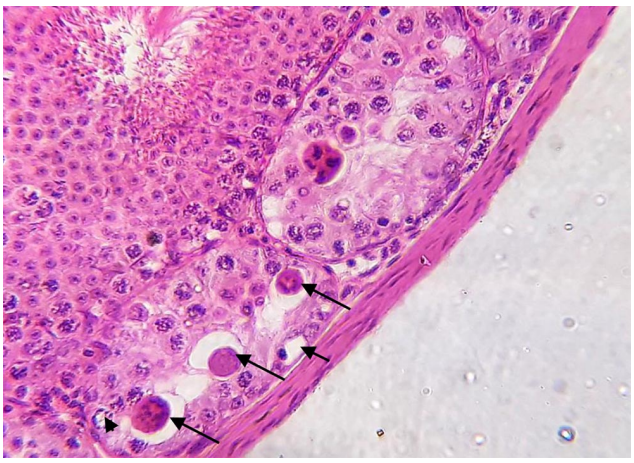

Fig. 8. A cross-section of a high-concentration mouse testicle showing degeneration of the primary spermatocyte (large arrows), degeneration of the spermatogonia (small arrow), and Sertoli cell necrosis (arrowhead), 400X (H&E).

The results of the histological study also showed that the germ cells and even somatic cells within the seminal tubule of the group treated with a high concentration were more affected compared to the group treated with a low concentration. Degeneration and necrosis processes appeared clearly in the germ cells, even in the Sertoli cell. It was also observed that there was an irregularity in the arrangement of the germ cells in some seminiferous tubules, leading to a narrowing of the lumen of the seminiferous tubule (Figs. 6 to 8), in addition to the appearance of a Giant cell multinucleate (Fig. 9).

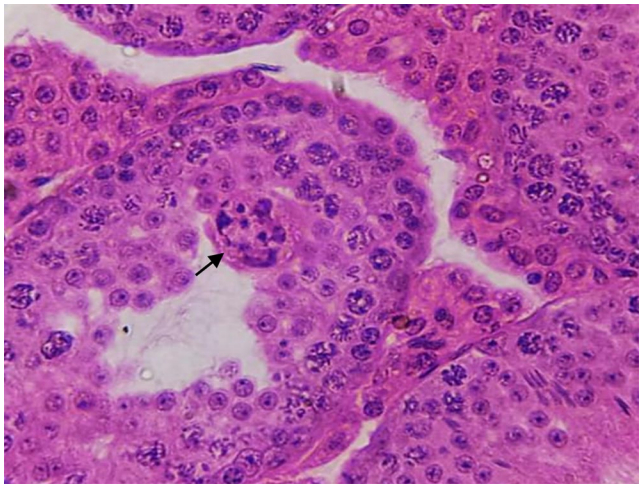

Fig. 9. A cross-section of a high-concentration mouse testicle showing the appearance of a multinucleated giant cell (arrow), 400X (H&E).

Histological sections showed desquamation or exfoliated of primary spermatocytes toward the seminiferous tubule (Fig. 10). Cytolysis and loss of germ cells occurred in some seminiferous tubules in the group treated with high

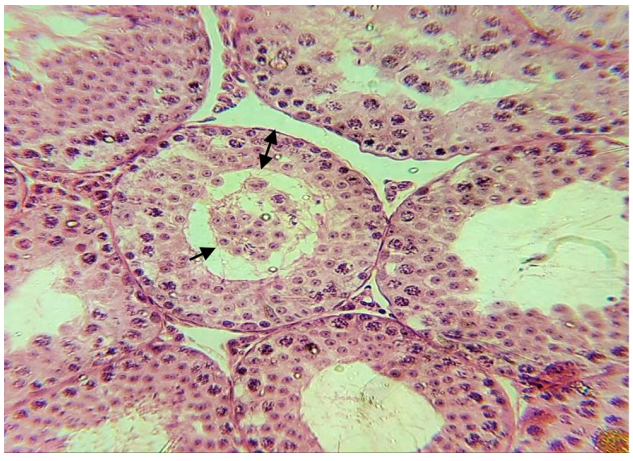

Fig. 10. A cross-section of a high-concentration mouse testicle showing exfoliated of primary spermatocyte (arrow) towards the seminiferous tubule, thickness of the seminiferous tubule (⇔), 400X (H&E).

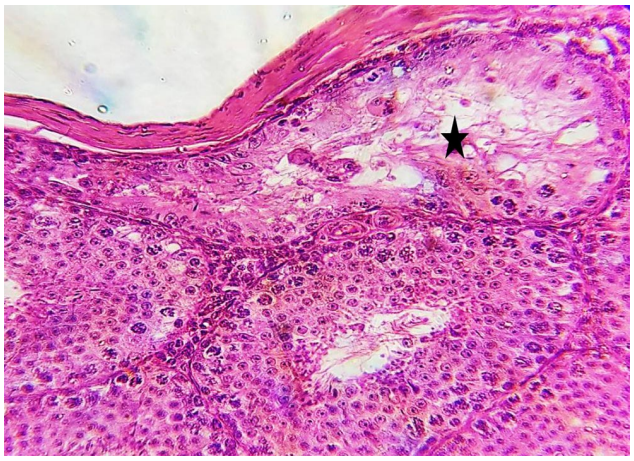

Fig. 11. A cross-section of a high-concentration mouse testicle showing the lysis and loss of germ cells in the seminiferous tubule (star), 400X (H&E).

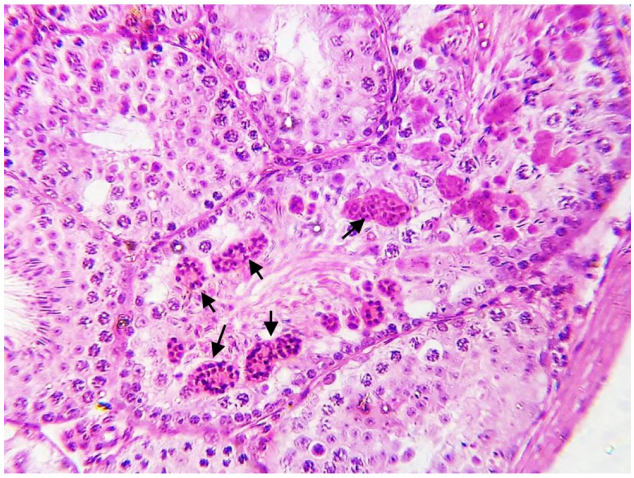

Fig. 12. A cross-section of a high-concentration mouse testicle showing infiltration of inflammatory cells in the seminiferous tubule (arrows), 400X (H&E).

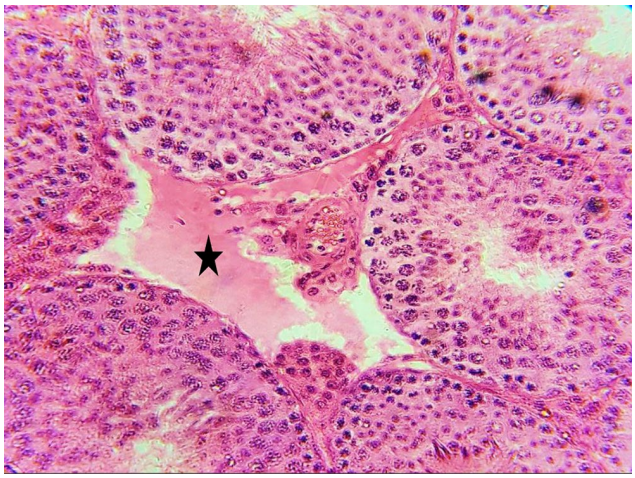

Fig. 13. A cross-section of a high-concentration mouse testicle showing the appearance of a pink-colored substance (amyloid) between the seminiferous tubules (star), 400X (H&E).

concentration (Fig. 11). With infiltration of inflammatory cells in some seminiferous tubules (Fig. 12). It was also noted through histological examination that a pink-colored substance (amyloid) appeared between the seminiferous tubules, significantly in the group treated with the high concentration (Fig. 13).

DISCUSSION

An increase in the number of sperm produced and their normal appearance is an indicator of fertility and reproductive ability, sperm abnormalities and the increased appearance of abnormal forms are a measure of inability to conceive (Vasan, 2011). The results of the current study showed a significant decrease in the sperm counts of male laboratory mice treated with MTX at both doses through its negative role in suppressing the sperm generation process in the gonads of laboratory animals due to damage to the germ cells as a result of the cessation of their divisions and their loss through necrosis or programmed cell death, which results in it causes a reduction in the rate of sperm production (Marcon *et al*., 2008). The decrease in the number of sperm is often due to the interference between the process of sperm formation and the decrease in sperm cells in the different stages of sperm development (Abdel Aziz *et al*., 1997). The decrease in sperm numbers may be due to damage and destruction of sustentacular cells (Sertoli cells), which are responsible for the development of sperm and the nourishment, incubation, and protection of germ cells, and are a basic component of testicular tissue (Sakr *et al*., 2018).

The results showed a significant increase in the number of deformed sperm and an increase in the number of head and tail deformities of sperm in mice treated with MTX at both high and low concentration doses. The reason for the occurrence of these abnormalities may be that the toxicity of the drug increases chromosomal abnormalities within the germ cells, leading to genetic and physiological changes in the DNA, which results in an increase in the number of deformed sperm and the loss of their functions (Jha & Kumar, 2006; Alam *et al*., 2011). Also, the reason for the bending, twisting, or splitting of the sperm tail may be due to damage to the microtubules that make up it, their sensitivity to free radicals resulting from the use of the drug, and the absence of enzymes that oppose or inhibit these radicals, therefore, sperm are exposed to major deformities and appear in abnormal shapes (Pınar *et al*., 2018). The results of the current study are consistent with the results of Zarei *et al*. (2014) and Güvenç & Aksakal (2018).

The results of the study showed a significant decrease in the level of testosterone in the serum of male mice treated with the drug (MTX). The reason for this may be that most

chemical treatments cause functional and histological damage to the Leydig cells in the testes and thus negatively affect the production of testosterone in the serum of laboratory animals (Cherry *et al*., 2004). Or the reason for this may be attributed to the effect of the MTX drug on the hypothalamic-pituitary-genital axis (HPG axis), which reduces the production of luteinizing hormone secreted from the pituitary gland, in addition to changes in the gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus, resulting in It has a direct toxic effect on the gonads and thus reduces the production of sex hormones (Padmanabhan *et al*., 2009). The results of the current study agreed with the results of (Atashfaraz *et al*., 2013; Abdelhameed *et al*., 2022), as they found that MTX has a toxic potential for sperm fertility and a reduction in testosterone levels in the serum of laboratory mice treated with MTX compared to control.

The results also showed the effect of MTX on the spermatogenesis process and a significant decrease in the spermatogenesis process. This may be due to the drug's effect on testicular tissue and causing an increase in oxidative stress through an increase in the products of oxidative lysis of fats and the production of ROS and a decrease in antioxidant enzymes in testicular tissue (Gutierrez & Hwang, 2017). The reason for the decrease in the process of sperm formation may be attributed to the loss of the germ mass in the seminiferous tubules and the degeneration, dissolution and necrosis of most of spermatogonia, with the occurrence of shedding of the germ cell mass lining the seminiferous tubules. These damages led to a sharp decrease in the number of sperm and inhibited the development of the germ cells in the seminiferous tubules (Yekeen *et al*., 2016). The results of the current study agreed with the results of Abdelhameed *et al*. (2022), and Howard *et al*. (2016), indicated that the mechanism of methotrexate in testicular injury is through the process of programmed cell death and the release of many pro-inflammatory cytokines and free radicals, which causes damage to the morphology of the testicles and germ cells and causes chromosomal changes, all of which lead to a disturbance in the process of sperm formation.

The results of a study of histological sections of the testicles of laboratory mice treated with methotrexate showed a clear effect on the tissue of testes in general and the spermatogenesis process in particular, to varying degrees between the high and low dose compared with the control group. The results of the histological examination of both treatment groups (low and high) showed that germ cells and sustentacular cell were significantly affected, especially in the group treated with the high dose, sustentacular cell appear as vacuoles in the seminiferous tubules. Degeneration, necrosis, and dissolution processes also appeared in various germ cells. The reason for this is that these cells are affected by free radicals resulting from the effect of treatment on testicular tissue (Snezhkina *et al*., 2019), these radicals oxidize phospholipids and protein components of the cell membrane, leading to morphological changes, cell destruction, or cytolysis (Juan *et al*., 2021). This was observed in the results of a study (Vardi *et al*., 2010), which showed that free radicals damage testicular tissue and germ cells and cause their cellular death in the lumen of the seminiferous tubules.

The study of histological sections also showed irregularity in the arrangement of germ cells and exfoliated of some of them toward the lumen of the seminiferous tubule, the reason for this may be due to the degeneration processes that occur in the germ and somatic cells within the seminiferous tubule and lead to changes in their shape from the normal shape, which causes a defect in the arrangement and regularity of the cells in the seminal tubule. Degeneration processes also cause the cell to separate from its communication sites with other cells, which causes it to exfoliate or shedding toward the lumen of the seminiferous tubule. Both the irregularity of the cells and their shedding towards the lumen of the tubule cause a narrowing of the lumen of the seminiferous tubule , this result agreed with the results of both, Saxena *et al*. (2004) and Spears *et al*. (2019), which showed a clear decrease in the lumen of the seminiferous tubules, an irregularity in the basement membrane, a difference in the sizes of primary and secondary spermatocytes and sperm, and a significant decrease in the size of sustentacular cell and Leydig cells in laboratory mice testes.

The thickness of the germ mass in the seminiferous tubules in the group treated with the high dose appeared less than that of the group treated with the low dose and the control group, the histological examination study showed that the process of germ cell development stops in some seminiferous tubules at the stage of the primary spermatocytes, and this confirms that the drug methotrexate has an inhibitory effect on the process spermatogenesis, which in turn leads to a weakness in sperm fertility (Khayat Nouri *et al*., 2010; Yaman *et al*., 2018).

Through histological examination, it was noted that giant cells appeared within the cells of the seminiferous tubule, this may be due to cellular fusion of immature primary spermatocytes, with hyperchromasia in the nuclei of primary germ cells (Yaman *et al*., 2018).

Inflammatory cells appeared clearly in some seminiferous tubules whose cells suffer from degeneration and necrosis processes, the presence of these cells is a natural reaction to degenerated and necrotic tissue, as these cells work to dissolution and lysis the necrotic tissue in preparation for

its removal (Wang, 2018b). Perhaps the reason why some germ and somatic cells within the seminiferous tubule are affected, as well as some of the entire seminiferous tubules, especially at the high dose, is due to the direct effect of methotrexate on the seminiferous tubules through the drug's delivery through the blood to the sustentacular cell and from there to the rest of the germ cells,sustentacular cell are a source of nutrition for seminiferous tubule cells (Griswold, 1998). Or indirectly, through increased secretion of amyloid (one of the extracellular degenerative changes) between the seminiferous tubules, which leads to pressure of that substance on the blood vessels, thus reducing the access of nutritional supplies to the germ cells, leading to their death and degeneration.

The current study concluded that treating male mice with methotrexate causes severe damage to the testicles, and this was evident through a decrease in the sperm counts, deformities in their external appearance, a decrease in the level of testosterone, a significant decrease in the process of sperm formation, and various histological damages in the testicular tissue. Therefore, this treatment can be considered one of the factors that cause infertility, especially when used for long periods.

ADLAN, M. M.; IBRAHIM, M. A. y KATA, F. S. Cambios fisiológicos e histológicos de los testículos de ratones de laboratorio tratados con metotrexato. *Int. J. Morphol., 42(6)*:1730-1738, 2024.

RESUMEN: El estudio tuvo como objetivo demostrar los efectos fisiológicos e histológicos del metotrexato (MTX) en los testículos de ratones de laboratorio. Se utilizaron 24 ratones macho, *Mus musculus* L. Los ratones se dividieron en tres grupos de ocho ratones. Al primer grupo de ratones - grupo control- se le inyectó 0,1 ml de agua destilada; al segundo grupo se le inyectó una concentración baja de metotrexato (2 mg/kg), mientras que al tercer grupo se le inyectó una concentración alta del fármaco (4 mg/kg). Los ratones fueron inyectados por vía intraperitoneal diariamente durante 16 días. Los resultados del estudio mostraron una disminución significativa en el recuento de espermatozoides en ambas dosis (2,4 mg/kg) con un nivel de probabilidad de $P<0,05$ en comparación con el grupo control. El fármaco provocó una disminución significativa en el número de espermatozoides normales en ratones tratados con ambas dosis, y un aumento en el número de anomalías de la cabeza y de la cola de los espermatozoides. Además, la inyección del fármaco provocó una reducción significativa en la concentración de testosterona en ambas dosis. El metotrexato también tuvo un claro efecto sobre el tejido testicular en general y en el proceso de espermatogénesis en particular. Los resultados del examen histológico mostraron que el tejido testicular se vio afectado en el grupo con tratamiento en comparación con el grupo control, en diversos grados entre el grupo tratado con la concentración baja y la concentración alta. Se observó que existían vacuolas en algunos túbulos seminíferos, la aparición de procesos de degeneración y necrosis de las células germinales, además de una disminución del grosor de la masa germinal y un estrechamiento del lumen de algunos túbulos seminíferos. También se observó que hubo exfoliación, citólisis, pérdida de células germinales, infiltración de células inflamatorias en algunos túbulos seminíferos y aparición de amiloide entre los túbulos seminíferos, significativamente mayor en el grupo tratado con concentración alta de metotrexato. Concluimos que el metotrexato causa daño severo a los testículos a través de una disminución en el número de espermatozoides, distorsiones en su apariencia externa, disminución en el nivel de testosterona y disminución significativa en el proceso de espermatogénesis.

PALABRAS CLAVE: Metotrexato; Testosterona; Espermatozoides; Espermatogénesis.

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