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An Evaluation of the Protective Effects of Coenzyme Q10 on Fertility Parameters of Male Mice Treated with Methotrexate

M. Babaei (PhD)[*1], A. Kalantari-Hesari (PhD)[2], M. Yavari (PhD)[1], M. Hallaj (PhD)[3], H. Morovvati (PhD)[4]

1.Department of Clinical Sciences, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, I.R.Iran.

2.Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, I.R.Iran.

3.Faculty of Veterinary Medicine, Urmia University, Urmia, I.R.Iran.

4.Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, I.R.Iran.

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ABSTRACT

Background and Objective: Methotrexate (MTX) is a chemotherapy drug that has destructive effects on body tissues, including the male reproductive system. Since coenzyme Q10 is an antioxidant that can reduce its destructive effects to some extent, this study was conducted to investigate the effects of the simultaneous use of methotrexate and coenzyme Q10 on testis tissue structure and sperm parameters in male mice.

Methods: In this experimental study, 24 male NMRI mice were divided into four groups of six: the control group received 0.2 ml of distilled water intraperitoneally, the MTX group received 20 mg/kg methotrexate once a week intraperitoneally, the CoEnQ10 group received coenzyme Q10 orally at a dose of 200 mg/kg three times a week, and the MTX+CoEnQ10 group, in addition to receiving methotrexate 20 mg/kg intraperitoneally once a week, also received coenzyme Q10 orally at a dose of 200 mg/kg three times a week. All groups were treated for five weeks. After sacrificing the animals, epididymal cauda and testis tissue were sampled and analyzed respectively in order to check sperm parameters and histomorphometry changes and the process of spermatogenesis.

Findings: After treatment with MTX, viability, motility, tubule differentiation index, spermatic index, sperm repopulation index, mean seminiferous tubules diameter, seminiferous tubules lumen diameter, and germinal epithelium height decreased significantly compared to the control group ($p<0.05$). Furthermore, the treatment with MTX significantly led to DNA damage, an increase in the mean diameter of the testicular capsule and the percentage of tubular degeneration, and an increase in the number of abnormal sperms compared to the control group ($p<0.05$). Simultaneous use of coenzyme Q10 with MTX resulted in significant improvement of the mentioned parameters.

Conclusion: The results of the present study showed that MTX has a negative effect on the metabolic activity of sperm, integrity of membrane and DNA, causing a decrease in sperm parameters and changes in testicular tissue and the process of spermatogenesis. Significant improvement of coenzyme Q10 and MTX co-administration enhances the protective effect of coenzyme Q10.

Keywords: *Methotrexate, Coenzyme Q10, Testicular Tissue, Sperm Parameters, Mice*.

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[*]Corresponding Author: M. Babaei (PhD)
Address: Department of Clinical Sciences, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, I.R.Iran.
Tel: +98 (81) 34227350. **E-mail:** mohammad.babaei@basu.ac.ir

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Introduction

In recent decades, advances in cancer treatment have increased the long-term survival of cancer patients (1, 2). The future fertility of cancer patients after treatment is a serious concern among cancer patients (3, 4). In chemotherapy, one or more anticancer drugs (chemotherapy agents) are used as part of a standard chemotherapy regimen for treatment. Most chemotherapy drugs cause short-term and long-term toxicities (1, 5). Methotrexate (MTX) is a chemotherapy drug with antagonistic feature of folic acid, which is successfully prescribed in the treatment of various malignancies (such as acute lymphoblastic leukemia, non-Hodgkin lymphoma, osteosarcoma, and breast cancer) and autoimmune disorders (such as rheumatoid arthritis and psoriasis) (6, 7). Considering the therapeutic importance of MTX, its toxicity has been investigated in various body systems, including hematopoietic system, digestive system and central nervous system (8). MTX inhibits folate metabolism, interferes with nucleic acid synthesis, and in this way, exerts its cytotoxic effects on cells with a high proliferation rate (1, 9). MTX, with the aforementioned destructive effects, can cause long-term or permanent gonadal toxicity in men treated with this drug (10). The genotoxic effects of MTX in somatic cells have been proven using the micronucleus and chromosome aberration test (8, 11). MTX increases the production of sperm with an abnormal trend and decreases the sperm count (8). Oxidative stress is one of the main mechanisms of MTX in testicular tissue damage. In general, antioxidants protect various tissues against the destructive effects of oxidative stress. In the last decade, several studies have investigated the use of natural antioxidant agents to improve the side effects caused by MTX consumption (1).

In human body, coenzyme Q10 is naturally synthesized from tyrosine. Coenzyme Q10 is considered a vital component of the mitochondrial inner membrane and plays an important and key role in inhibiting lipid peroxidation and DNA oxidation (12). Coenzyme Q10 also plays an essential role in electron transport in the mitochondrial respiratory chain and oxidative phosphorylation and acts as a fat-soluble antioxidant in cell membranes and lipoproteins (13, 14). Coenzyme Q10 also participates in the production of adenosine triphosphate in aerobic respiration (15). Coenzyme Q10 has been used as a medicinal aid in the control of various pathological disorders such as diabetes, cancer, Parkinson's disease, Huntington's disease, heart disorders and infertility (16).

Several studies have investigated and proved the effectiveness of coenzyme Q10 supplementation in increasing male fertility and cardiovascular function (17). Coenzyme Q10 acts as an antioxidant by inhibiting the lipid peroxidation of the sperm membrane. Coenzyme Q10 is present in the mitochondria of the middle part of the sperm and participates in all the processes of regulating the cellular energy of the sperm (18).

In a clinical study that examined the effects of oral administration of coenzyme Q10 on semen concentration, sperm concentration and sperm motility, the results showed that coenzyme Q10 supplementation led to a significant increase in all three parameters of semen concentration, sperm concentration and sperm motility (19).

Since no study has been conducted on the effects of coenzyme Q10 on testicular dysfunction caused by MTX drug, the present study was conducted to determine the healing effect of coenzyme Q10 against testicular damage caused by MTX.

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Methods

This study was carried out after approval by the ethics committee of Bu-Ali Sina University with code IR.BASU.REC.1399.002.

Chemical reagents: MTX ampoule was purchased from (PubChem, EBEWE Pharma Ges.m.b.H) and coenzyme Q10 (PubChem).

Animals: Male NMRI mice (weight: 20-25 grams, age: 8-10 weeks) were used for the experiment. Mice were kept in the Animal Breeding Center of Bu-Ali Sina University under standard conditions (temperature 20-25°C, relative humidity 55-60% and 12-hour light/dark cycle). Water and standard pellet diet were freely available to the animals.

Experimental protocol: 24 male mice were randomly divided into 4 groups of 6:

1. Control group receiving 0.2 ml distilled water intraperitoneally, 2. MTX group receiving methotrexate 20 mg/kg intraperitoneally once a week, 3. CoEnQ10 group receiving oral coenzyme Q10 at a dose of 200 mg/kg three times a week, 4. The MTX+CoEnQ10 group, in addition to receiving methotrexate 20 mg/kg intraperitoneally once a week, also received coenzyme Q10 orally at a dose of 200 mg/kg three times a week.

One day after the last treatment, the mice were euthanized by cerebrospinal displacement and after weighing, the tail of the epididymis was removed and placed in 1 cc of HTF (Human Tubular Fluid) culture medium. Then, sperm parameters were evaluated. The tissue of the right testicle was transferred to 10% buffered formalin for tissue fixation, and the gonadosomatic index (GSI) was calculated based on the formula: GSI= 100 × (body weight / weight of both testicles).

Investigation of sperm parameters: In order to study the number, viability, motility and morphology of sperm, epididymal tails were cut and transferred to a microtube containing 1 ml of pre-heated HTF medium. Sperm suspension was used to analyze different sperm parameters. The carried-out investigations include counting the mean number of sperms per unit volume and constant dilution using hemocytometer slides, determining the percentage of sperm motility, the percentage of sperm viability (Eosin-Nigrosin staining), the amount of DNA damage (using acridine orange staining method) and nucleus maturation rate (using aniline blue staining method).

Histological evaluations: After tissue passage and preparation of tissue sections (7 μm thickness), testicular tissue samples fixed in 10% buffered formalin were stained with hematoxylin-eosin, and all samples were evaluated in terms of tissue and morphometric changes in multiple magnifications (400x and 1000x) (20). The mean seminiferous tubules diameter (STsD), seminiferous tubules lumen diameter (STsLD), germinal epithelium height (GEH), testicular capsule diameter (TCD) in micrometers were analyzed by Dino-Lite microscope and DinoCapture software (version 2). In order to check the tubular differentiation index (TDI), the percentage of seminiferous tubules with more than three layers of differentiated germ cells from type A spermatogonia were counted and TDI was considered positive. Moreover, to calculate the sperm repopulation index (RI), the ratio of active spermatogonia (type B spermatogonia with dark nuclei) to inactive spermatogonia (type A spermatogonia with light nuclei) was calculated in seminiferous tubules. In order to determine the spermatic index (SI), the percentage of seminiferous tubules with normal spermatogenesis was considered as positive SI. In addition, the tubular degeneration percentage (TDP) was calculated in different experimental groups.

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The results of this study were statistically evaluated using SPSS version 19 and the results were expressed as mean±standard deviation. To compare between groups, one-way analysis of variance followed by Tukey's supplementary test was used and $p<0.05$ was considered significant.

Results

Biometric parameters: Comparison of GSI index and testicle width between control group and experimental groups showed no significant difference. However, in the examination of the testis length of the MTX and MTX+CoEnQ10 groups, it showed a significant decrease compared to the control group ($p<0.05$) (Table 1).

Table 1. Comparison of the mean testicular biometric parameters, the number, viability and motility of sperm, the percentage of mature sperm, the percentage of sperm with damaged DNA and the percentage of abnormal sperm in the experimental groups

Groups / Parameters	Control Mean±SD	MTX Mean±SD	CoEnQ10 Mean±SD	MTX+CoEnQ10 Mean±SD
Gonadosomatic Index (GSI)	0.72±0.06[a]	0.69±0.1[a]	0.68±0.14[a]	0.72±0.06[a]
Right testicle length (mm)	8.54±1.51[a]	6.28±0.47[b]	7.11±0.65[ab]	6.36±0.31[b]
Right testicle width (mm)	6.71±2.41[a]	4.89±0.35[a]	5.28±0.77[a]	5.11±0.47[a]
Sperm count (10^6/ml)	14.41±2.49[a]	3.96±1.07[b]	15.88±1.62[a]	8.74±1.46[c]
Sperm viability (%)	78.17±5.57[a]	35.33±4.63[b]	80.17±5.11[a]	55.67±4.46[c]
Sperm motility (%)	83.5±5.17[a]	34.33±4.72[b]	85.17±4.58[a]	57.5±6.72[c]
Sperm maturation (%)	96.67±1.75[ac]	87.67±3.08[b]	98.33±1.63[a]	92±4.82[cb]
Sperm with damaged DNA (%)	8.67±2.73[a]	29.33±3.14[b]	10.33±2.73[a]	18.67±3.2[c]
Abnormal sperm (%)	9.68±2.66[a]	30.67±5.17[b]	8.6±2.16[a]	15.33±1.86[c]

Non-similar English letters indicate significant differences in each column ($p<0.05$).

Sperm parameters: MTX injection significantly decreased the number of sperms compared to the control group ($p<0.05$). Moreover, in this group, sperm motility and viability decreased significantly compared to the control group, and the percentage of abnormal sperm increased ($p<0.05$). Compared to the MTX group, treatment with coenzyme Q10 prevented adverse changes in sperm parameters (number, motility and viability of sperm, abnormal sperm) in the (MTX+CoEnQ10) group. MTX injection, once a week during a period of 5 weeks, led to a significant increase in the DNA damage of sperm cell nuclei in the groups receiving MTX ($p<0.05$) (in the MTX group, the level of DNA damage was 3 times more and in the MTX+CoEnQ10 group, 2 times more than the control group). However, coenzyme Q10 consumption significantly reduced the DNA damage of sperm cells following MTX consumption ($p<0.05$). Furthermore, MTX injection decreased the maturation of the nucleus of sperm cells compared to all experimental groups ($p<0.05$). The nuclear maturation of sperm cells in the groups receiving coenzyme Q10 was not significantly different from the control group and improved the effects of MTX ($p<0.05$) (Table 1).

Histomorphometry: The results of this study showed that the mean thickness of germinal epithelium height (GEH) in the MTX group was significantly reduced compared to the control group ($p<0.05$). In the MTX+CoEnQ10 group, the use of coenzyme Q10 along with the administration of MTX caused a

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significant improvement in the average thickness of the germinal epithelium height compared to the MTX group ($p<0.05$). In the MTX group, the measurement of the thickness of the testicular capsule (TCD) showed an increase in thickness compared to other experimental groups. Examination of the seminiferous tubules diameter (STsD) and seminiferous tubules lumen diameter (STsLD) showed that the mean value of both parameters in the MTX group showed a significant decrease compared to the control group and other groups ($p<0.05$). In addition, the results of this study showed that the simultaneous consumption of coenzyme Q10 along with MTX improves the mean level of the mentioned parameters ($p<0.05$). Moreover, no significant difference was observed in the mentioned parameters between the control group and the CoEnQ10 group that received coenzyme Q10 alone (Table 2). MTX significantly reduced the tubular differentiation index (TDI) compared to the control and coenzyme Q10 groups ($p<0.05$). In the MTX group, the indices of sperm repopulation index (RI), and spermatic index (SI) also showed a significant decrease compared to the control group and coenzyme Q10 ($p<0.05$). Administration of coenzyme Q10 along with MTX drug significantly improved the adverse effects of MTX ($p<0.05$) (Table 2).

Table 2. Comparison of the mean seminiferous tubules diameter, the seminiferous tubules lumen diameter, the germinal epithelium height, the diameter of the testicular capsule, the percentage of tubular degeneration, the tubular differentiation index, the sperm repopulation index and the spermatic index in the experimental groups

Group / Parameters	Control Mean±SD	MTX Mean±SD	CoEnQ10 Mean±SD	MTX+CoEnQ10 Mean±SD
Seminiferous tubules diameter (STsD) (µm)	211.33±15.24[a]	134.82±6.74[b]	206.98±5.98[a]	173.25±8.68[c]
Seminiferous tubules lumen diameter (STsLD) (µm)	135.85±4.66[a]	85.93±6.16[b]	140.94±6.44[a]	115.51±5.17[c]
Germinal epithelium height (GEH) (µm)	62.68±3.47[a]	32.95±5.06[b]	65.85±2.285[a]	45.51±4.95[c]
Testicular capsule diameter (TCD) (µm)	9.58±0.69[a]	12.55±1.11[b]	10.01±0.49[a]	11.42±0.58[b]
Tubular degeneration percentage (TDP) (%)	3.6±2.07[a]	16±2.24[b]	2.4±1.14[a]	7.4±2.07[c]
Tubular differentiation index (TDI) (%)	88±4.95[a]	60.2±5.97[b]	90±4.06[a]	75.4±4.04[c]
Sperm repopulation index (RI) (%)	92±3.39[a]	56.8±3.49[b]	93.2±3.56[a]	73.4±5.59[c]
Spermatic Index (SI) (%)	91.2±3.89[a]	54.6±4.78[b]	93.8±3.11[a]	74.8±4.38[c]

Non-similar English letters indicate significant differences in each column ($p<0.05$).

The results of hematoxylin and eosin staining: In the examination of tissue sections of the testis in the control group, the structure of the testis capsule, seminiferous tubules and Leydig cells showed a normal state. However, the number of tubules in the MTX group was greatly reduced. Moreover, a large number of degenerated spermatogenic tubules were also observed in this group. Nevertheless, the tissue structure was normal in both CoEnQ10 and MTX+CoEnQ10 groups (Figure 1).

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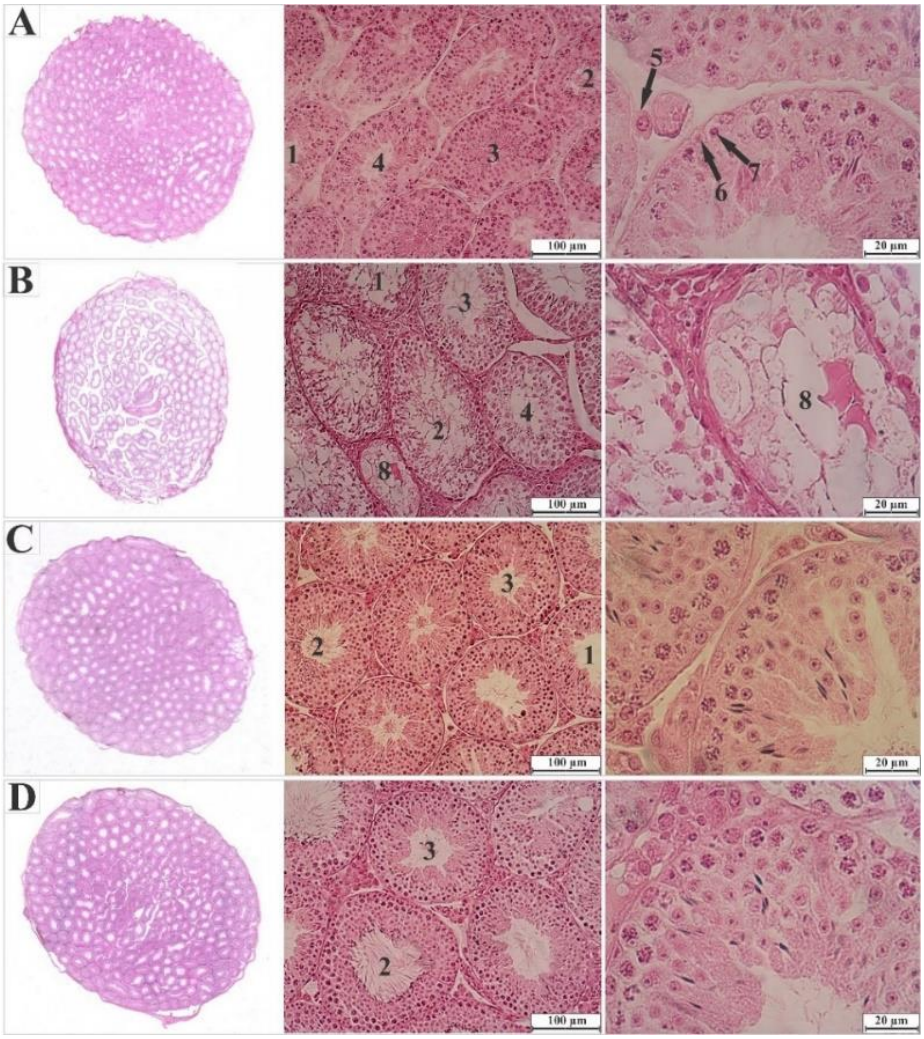

Figure 1. Sections of testicular tissue in the control, sham and experimental groups. Hematoxylin-eosin staining (400x and 100x magnification). **A: control group, B: MTX group, C: CoEnQ10 group, D: MTX+CoEnQ10 group. Numbers 1: negative SI tubule, 2: positive SI tubule, 3: positive TDI tubule, 4: negative TDI tubule, 5: Leydig cell, 6: type A spermatogonia, 7: type B spermatogonia, 8: degenerated tubule.**

Results of PAS staining: The study of the presence of carbohydrate or sugar-containing substances inside the cytoplasm of testicular tissue cells was done by PAS method. These observations showed that the PAS reaction inside the cytoplasm of Leydig cells and the connective tissue surrounding the wall of the seminiferous tubules along with the basement membrane showed a positive response to this reaction with the presence of a bright red color. In the control group, PAS-positive grains were observed inside the cytoplasm of the cells of the spermatogenesis series in the rows adjacent to the basal layer and rarely in the higher rows. This was despite the fact that the use of MTX caused the presence of PAS-positive grains to decrease in Sertoli cells. Also, these seeds were observed with greater density in the series of spermatogenesis cells in the rows adjacent to the middle cavity. Also, the administration of CoEnQ10 in the two groups that received it clearly caused a decrease in the PAS-positive grains in the upper levels of the spermatogenesis series (Figure 2).

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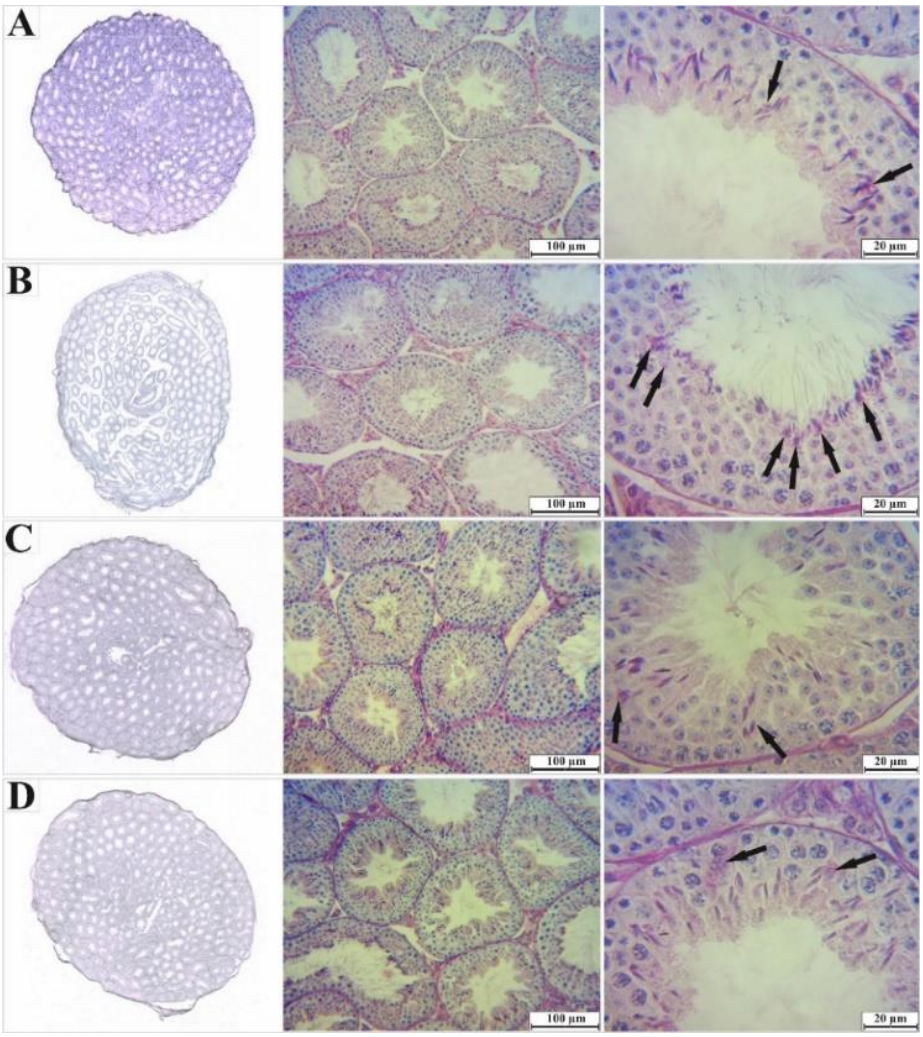

Figure 2. Sections of testicular tissue in the control, sham and experimental groups. PAS staining (400× and 100× magnification). A: control group, B: MTX group, C: CoEnQ10 group, D: MTX+CoEnQ10 group. Areas that were positive for PAS staining are indicated by arrows.

Discussion

In the present study, the results showed that coenzyme Q10 has protective effects and prevents the damage of fertility parameters of male mice such as sperm quality (number, viability, morphology and motility, maturity and DNA), histomorphometry of testicular tissue (mean seminiferous tubules diameter, seminiferous tubules lumen diameter, germinal epithelium height and testicular capsule diameter) as well as spermatogenesis indices (tubular differentiation index, sperm repopulation index and spermatic index). MTX increases the production of free oxygen species (ROS) and lipid peroxidation, resulting in cell membrane damage (1). Therefore, the use of antioxidant supplements against reproductive toxicity caused by MTX has been the focus of researchers in recent years. MTX inhibits folate metabolism, interferes with nucleic acid synthesis, and thereby exerts its cytotoxic effects in rapidly dividing cells (9, 21). Therefore, it can cause long-term or permanent gonadal toxicity in male patients (10). The results of the study by Heidari

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Khoei et al. show that the consumption of MTX causes a significant decrease in sperm parameters and a change in the oxidant/peroxidative profile of the testicular tissue, resulting in an increase in lipid peroxidation. The increase in lipid peroxidation levels in patients treated with MTX causes the inefficiency of the antioxidant defense and ultimately increases the oxidative stress in the testicular tissue (1). However, the incidence of cancer has increased with the development of human society, the death rate of cancer patients has decreased due to the use of anti-cancer agents (22, 23). Fertility after treatment is important for young cancer patients. In addition to prescribing MTX as a chemotherapy drug, this drug is also used in low doses to treat many other diseases, and in addition to its beneficial effects, toxicity of MTX has been reported in various body systems (22, 24). Reproductive toxicity is one of the important side effects of MTX in young patients, which causes harmful effects such as a decrease in sperm count, normal morphology, and motility (8, 25-27). Therefore, protection against testicular toxicity caused by MTX during the period of taking this drug is of particular importance.

Although low levels of ROS have some physiological functions in sperm maturation and capacitation, the imbalance between ROS and seminal fluid antioxidants impairs male reproductive performance (28). Similarly, the acrosome reaction and capacitation are also enhanced by superoxide anion radicals (29). However, excessive production of ROS will lead to oxidative stress and reduce the antioxidant capacity of sperm (15, 30). Enzymatic antioxidants in semen include catalase, superoxide dismutase, and glutathione peroxidase, and non-enzymatic antioxidants include coenzyme Q10, glutathione peroxidase, vitamins A, B complex, C, and E, L-carnitine, and minerals (chromium, selenium, zinc, and copper) are included (15, 17, 31). The imbalance between ROS production and antioxidant capacity leads to increased exposure of sperm to oxidative stress, which plays an important role in the pathogenesis of male infertility and impairs sperm function (32). Coenzyme Q10 is among the antioxidants studied and approved for the treatment of male infertility (17).

In the present study, the injection of MTX at a dose of 20 mg per kilogram of body weight, once a week during a period of 35 days, led to a significant increase in the DNA damage of the nucleus of sperm cells in the groups receiving MTX. Also, sperm fertility parameters such as number, viability, morphology, motility and maturity decreased significantly in MTX groups. Excessive production of ROS is associated with DNA damage through the induction of DNA strand breaks, chromatin cross-linking, and base changes and low mitochondrial membrane potential (15, 30, 33). The sperm plasma membrane is composed of lipids, and high levels of polyunsaturated fatty acids and excessive levels of ROS make the membrane susceptible to lipid peroxidation damage (34). In sperm, motility and reduced membrane fluidity due to lipid peroxidation are associated with lower fertilization capacity (35). On the other hand, MTX is a strong antimetabolic drug. This drug is an analog of folic acid that inhibits the synthesis of purine and pyrimidine, and its therapeutic and toxic effects are related to this function. Folic acid is necessary for the synthesis of thymidylic acid and purine nucleotides and finally for the production of DNA. MTX competes with folic acid to occupy the active site of the dihydrofolate reductase enzyme and prevents the conversion of dihydrofolate to tetrahydrofolate, and as a result, methyl donors are not made. The affinity of methotrexate for this site is a hundred thousand times higher than that of folic acid, and its binding to the enzyme is permanent. By disrupting this step, methotrexate prevents the synthesis of nuclear materials and ultimately leads to cell death (36, 37). The significant decrease in the number of sperms in the MTX group in the present study also confirms this.

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The results of a study by Coleshowers et al. showed that MTX induces oxidative stress by reducing the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (38). In another study, Yuluğ et al. stated that MTX increased MDA levels and significantly decreased SOD and CAT activities, thereby leading to testicular tissue damage. Also, previous studies have reported that several antioxidant agents such as thymoquinone, resveratrol, and beta-carotene reduce MTX-induced oxidative stress by reducing MDA concentration and increasing enzymatic and non-enzymatic antioxidant activity in different tissues (26, 27, 39). In the present study, the simultaneous use of coenzyme Q10 with MTX, causing a protective effect, prevents the damage of histomorphometric parameters, including the mean seminiferous tubules diameter, the seminiferous tubules lumen diameter, the germinal epithelium height and the diameter of the testicular capsule, as well as spermatogenesis indices (tubular differentiation index, sperm repopulation index and spermatic index).

A significant improvement in sperm parameters and total antioxidant capacity (TAC) has been reported in men treated with coenzyme Q10 at a dose of 100 mg/day for 3 months (40). In general, the results of the present study show that coenzyme Q10 supplementation has an effect on spermatogenesis indices such as tubular differentiation index, sperm repopulation index and spermatic index, and by having a protective effect, it prevents damage to parameters in toxicity caused by MTX drug consumption.

Sperm DNA fragmentation is one of the main disorders at the molecular level of sperm cells. Oxidative stress is considered as the key mechanism of DNA fragmentation. Excessive ROS cause breaks in DNA that require repair and regeneration. Greater chromatin density makes sperm DNA more resistant to fragmentation during transport in the male and female reproductive tract. However, DNA fragmentation may be induced by exposure of sperm to ROS in the epididymis or by abnormal chromatin condensation (41-43).

DNA fragmentation can lead to infertility by changing sperm function. Men with high rates of DNA fragmentation are less likely to conceive naturally. Based on this, patients with a high percentage of sperms affected by DNA fragmentation have high levels of seminal ROS and reduced antioxidant capacity (44). Studies have reported that coenzyme Q10 deficiency is associated with increased sperm DNA damage and low sperm count and motility (13, 19, 45). Seminal coenzyme Q10, with its antioxidant and metabolic properties, plays a major role in mitochondrial bioenergetics and maintaining a stable seminal state. In the present study, sperm DNA fragmentation was investigated using acridine orange staining, and the results show a significant improvement in DNA fragmentation in the MTX and coenzyme Q10 co-administration group compared to the control group.

In a healthy state, cells of the spermatogenesis series located on the basement membrane of the seminiferous tubules use carbohydrate sources and cells of the higher series use fats for metabolism. But in pathological conditions and disruption of metabolic cycles, changes in the type of cellular metabolism occur. One of the effects of changing metabolic cycles is the cell's use of other food sources in the environment or the failure to store substances in the expected cells (46). As shown in this study, the cells of the spermatogenesis line in the MTX group, the carbohydrate particles identified by PAS staining were observed in the cell line near the center of the seminiferous tubule with greater density, which indicated the change in the metabolic cycles of these cells. Here, CoEnQ10 was also able to partially compensate for the adverse effects caused by MTX administration.

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The results of the present study showed that treatment with coenzyme Q10 prevents the toxic effects of MTX in the reproductive system of male rats. It also prevents the reduction of fertility parameters of male mice, including sperm quality parameters (number, viability, morphology and motility, maturity and DNA), histomorphometry of testicular tissue (mean seminiferous tubules diameter (STsD), seminiferous tubules lumen diameter (STsLD), germinal epithelium height (GEH) and testicular capsule diameter (TCD) as well as spermatogenesis indices (tubular differentiation index (TDI), sperm repopulation index (RI) and spermatic index (SI)). According to the present study, the results of coenzyme Q10 were the same as the control group and did not have extra effect. MTX disrupts male fertility through oxidative damage. On the other hand, coenzyme Q10 treatment prevents fertility disorders caused by oxidative stress caused by MTX and by causing a protective effect, it prevents the damage of male reproductive parameters.

Conflict of interest: The authors declare that there is no conflict of interest in publishing this article.

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