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The effect of vitamin E and L-carnitine against methotrexate-induced injury in rat testis

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Background/aim: Methotrexate (MTX), used commonly as an antimetabolite drug in cancer therapy, leads to acute toxic side effects in tissues or organs containing rapidly dividing cells, such as bone marrow, gastrointestinal mucosa, and seminiferous tubules. In this study, we investigated the protective effects of vitamin E and L-carnitine against MTX-induced injury in rat testis.

Materials and methods: Rats were divided into 4 groups, including the control group. The study took 17 days and the animals received daily doses of 0.5 mL/kg saline, 250 mg/kg vitamin E, or 500 mg/kg L-carnitine intraperitoneally. The experimental groups received 20 mg/kg methotrexate intraperitoneally on days 3 and 10.

Results: Weight loss, testicular weight loss and marked histological injuries, increased malondialdehyde levels, and decreased superoxide dismutase levels were only observed in the MTX-treated groups. Vitamin E and L-carnitine treatments did not affect MTX-induced weight loss or testicular weight loss, but they inhibited MTX-induced testicular histological injuries. Vitamin E and L-carnitine treatments suppressed the increases in malondialdehyde levels and the decreases in superoxide dismutase levels.

Conclusion: Vitamin E and L-carnitine treatments decreased MTX-induced testicular histological injuries, and these results were supported by biochemical measurements.

Key words: Testes, methotrexate, vitamin E, L-carnitine, rat

1. Introduction

Methotrexate (MTX), a folic acid antagonist, is frequently used at low doses to treat inflammatory diseases, such as psoriasis and rheumatoid arthritis, and to treat leukemia and other malignancies as a cytotoxic agent (1,2). It is known that MTX used in cancer treatment has acute toxic side effects on tissues with high proliferation. The cells that are especially susceptible to these side effects are the ones that divide quickly, such as bone marrow, gastrointestinal mucosa, hair roots, and spermatogenic cells. Although gonadal injury is encountered frequently in MTX treatment, this side effect has been investigated less than the others (3,4).

The conducted studies revealed that MTX decreases the efficiency of the antioxidant enzyme system and therefore causes injury, sensitizing the cells against reactive oxygen particles (2). Oxidative stress causes injury in testis seminiferous tubules and a decrease in germ cells (5). Therefore, antioxidants can help protect testis tissue against oxidative stress.

MTX restricts enzyme binding to the active site of the dihydrofolate reductase enzyme. As a result, tetrahydrofolate synthesis through this enzyme from dihydrofolate acid is inhibited, and this reduces the purine bases (adenine and guanine) necessary for DNA, RNA, and ATP synthesis (6).

Like other antioxidants, vitamin E protects cell membrane lipids against the oxidative reaction started by oxygen radicals through their antioxidant effects and plays a protective role in the cell (7).

L-carnitine is a vitamin-type molecule structurally resembling amino acids. In the body, 75% of L-carnitine is taken up through diet and 25% is synthesized endogenously from lysine and methionine, which are the essential amino acids in tissues such as skeletal muscle, heart, kidney, liver, and brain (8). L-carnitine, used mainly for weight loss due

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to its fat-burning ability, was reported to increase sperm quality (9,10). Carnitine, which is present in males at a high concentration in preejaculatory fluid and produced mainly in the epididymis, increases the chance of fertilization by affecting sperm motility and other sperm parameters both qualitatively and quantitatively (11,12). In this study, we investigated the protective effects of vitamin E and L-carnitine against MTX-induced injury in rat testis.

2. Materials and methods

2.1. Animals

All the animal experimental protocols were approved by the Animal Experiments Local Ethics Committee of Gaziantep University (decision number: 01.2011-01) and conformed to the principles outlined in the Declaration of Helsinki. In this study, 26 young adult Wistar Albino male rats of about 5–6 months old obtained from the Gaziantep University Faculty of Medicine's Experimental Animals Research Center were used. The animals were isolated from the others 1 week before the experiment to acclimate them to the environment. They were taken into wire cages in rooms with an ambient temperature of 21 ± 1 °C and a 12/12 photoperiod. During the experiment, all animals were fed with standard feed and tap water.

2.2. Experimental design

The rats were randomly divided into four groups, consisting of the control group (Group 1, n = 5) and three treatment groups (n = 7): Group 2 (MTX), Group 3 (MTX and vitamin E, VEMTX), and Group 4 (MTX and L-carnitine, LCMTX). The weights of all animals were recorded at the beginning of the study and before they were sacrificed. The experimental period was 17 days. MTX at 20 mg/kg was administered intraperitoneally to the experimental groups on days 3 and 10, while the same volume (0.5 mL) of intraperitoneal physiological saline solution was injected to the control group.

From the beginning of the study until the end, vitamin E (250 mg/kg i.p.) was administered to the VEMTX group and L-carnitine (500 mg/kg i.p.) was administered to the LCMTX group every day. On day 17, all animals were anesthetized with 50 mg/kg i.p. ketamine and sacrificed with decapitation.

The taken testes were purged from the adjacent tissues and then weighed. Some of the obtained tissue samples were separated for light and electron microscopic analyses, and the rest of the testis tissue samples were used for determination of malondialdehyde (MDA) and superoxide dismutase (SOD).

2.3. Histopathologic procedures

For the light microscopic analysis, Bouin's fixation was performed on testis tissue samples. Paraffin blocks were prepared and sections of 6–7 μm in thickness were taken with a Shandon AS325 microtome (Thermo Scientific, USA). After trimming the blocks, slides of 4 different levels were prepared from each block, skipping 10 in each section. The slides were stained with hematoxylin and eosin and Masson's trichromatic staining, and photos were taken after evaluation with an Olympus BX50 light microscope (Olympus, Japan). Slides were scored according to the degree of intensity of the most frequently observed histopathological changes (0 = no injury, 4 = very severe lesion). The average scores were taken and assessed statistically for each group.

For the electron microscopic analysis, Millonig's phosphate buffer was prepared, and the testis tissue samples were fixed with 5% glutaraldehyde and 1% osmium tetroxide, dehydrated, and blocked via ethanol and propylene oxide after flushing. Semithin sections taken from the blocks through the Reichert Ultracut S ultramicrotome (Leica, Germany) were stained with toluidine blue. Thin sections taken with the same ultramicrotome were then stained with Reynolds' lead citrate solutions and uranyl acetate satiated in 70% ethyl alcohol. The stained sections were analyzed and photographed with a JEM 1400 (JEOL, Japan) transmission electron microscope.

2.4. Biochemical analysis

Testis tissue samples taken for the biochemical analysis were washed with cold (4 °C) 0.15 M KCl and dried with blotting paper. The tissues were then homogenized using an Ultra Turrax Type T25-B homogenizer (IKA Labortechnic, Germany) for 3 min at 16,000 rpm within a 0.15 M KCl solution. Supernatant was obtained by centrifuging at 4 °C for 1 h at 5000 × *g* and was kept for 1 week until the analysis. SOD values among the antioxidant enzymes and MDA values as an indicator of the oxidative injury pertaining to the obtained supernatant were determined spectrophotometrically.

2.4.1. SOD determination

SOD enzyme values were determined using the method modified by Sun et al. (13). The principle of this method depends on the basis of nitroblue tetrazolium reductase by the xanthine-xanthine oxidase system as the superoxide producer. In our study, SOD activity was expressed as unit per gram of tissue protein (U/g).

2.4.2. MDA determination

MDA determination was conducted by the Esterbauer method (14). MDA reacting with thiobarbituric acid at 90–95 °C creates a pink chromogen. Fifteen minutes later, the absorbance of the rapidly cooled samples was read spectrophotometrically at 532 nm. The results were expressed as nanomole per gram of tissue protein (nmol/g).

2.5. Statistical analysis

The Kolmogorov–Smirnov test was used in conformity control of continuous variables to the normal distribution. For the comparison of more than 2 independent groups with normal distribution, ANOVA and LSD multiple

comparison tests were used. Kruskal–Wallis and Dunnet multiple comparison tests were used for the variables that did not have normal distribution. For the comparison of 2 dependent measurements, a matched-pairs t-test was used. For the descriptive statistics, frequency, percentage, and the average ± standard deviation and median (25%–75%) values were given. For statistical analysis, SPSS 11.5 for Windows was used, and $P \leq 0.05$ was accepted as statistically significant.

3. Results

3.1. Body and testis weights

During the experiment, a weight increase (0.45%) was observed in the control group animals, while in all other groups a decrease in weight was observed: 15.49% in MTX, 25.22% in VEMTX, and 24.43% in LCMTX ($P = 0.001$). No significant difference was observed when the weight losses in these groups were compared to each other ($P = 0.763$) (Figure 1). At the end of the experiment, the

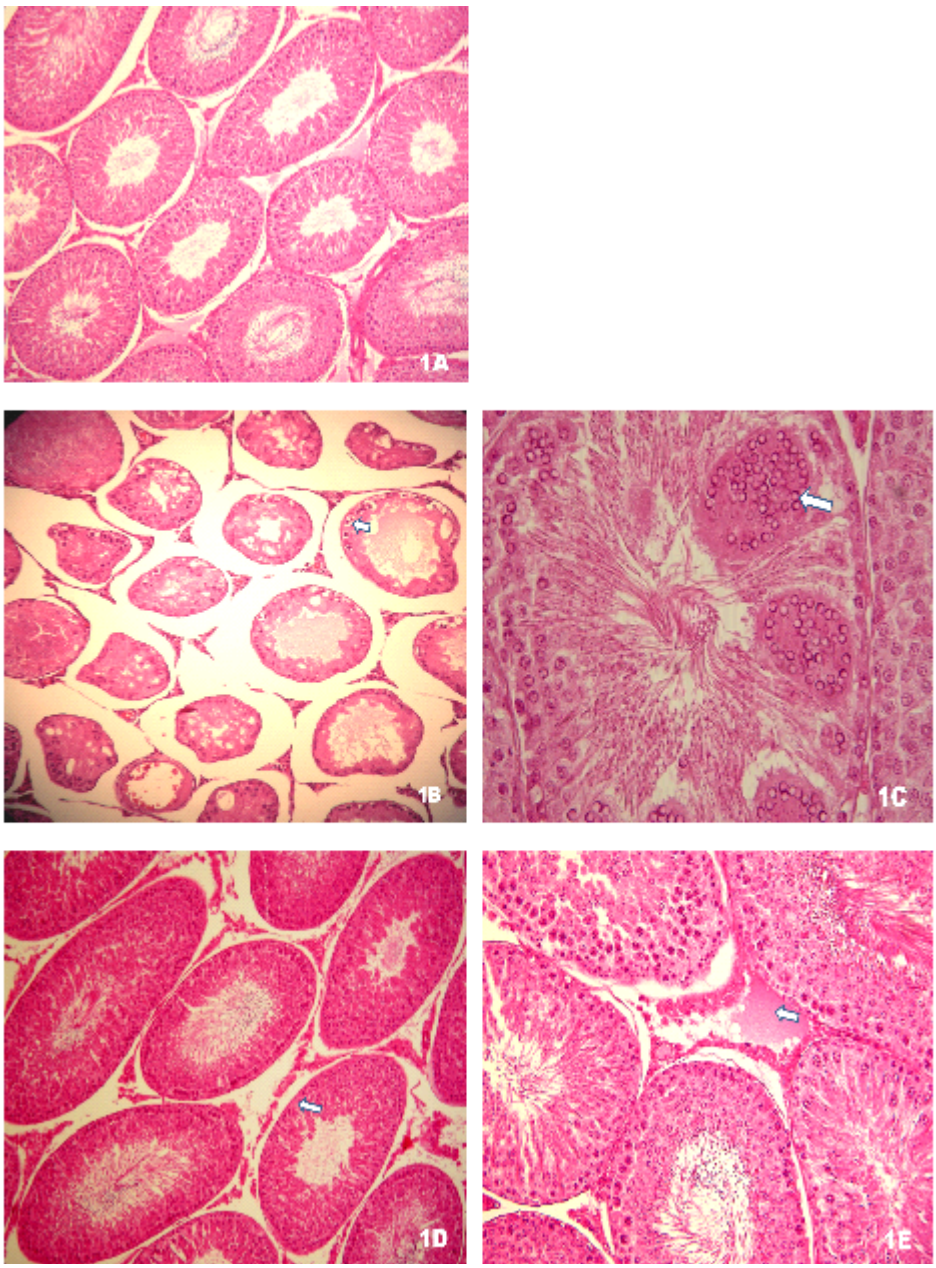

Figure 1. A) Control group, normal seminiferous tubular, H&E, 200×. **B)** MTX group, decrease in germ cells and vacuolization, H&E, 200×. **C)** MTX group, multinucleated giant spermatogenic cells (arrow), H&E, 400×. **D)** LCMTX group, decrease in germ cells and vacuolization, H&E, 200×. **E)** VEMTX group, interstitial edema, H&E, 400×.

testis weights of the animals were observed to be lower in all the experimental groups than in the control group (P = 0.001). However, the testis weight differences among the three experimental groups were not statistically significant (Table 1).

3.2. Biochemical findings

In all experimental groups, MDA levels increased compared to the control group (P = 0.001). However, when the experimental groups were compared to each other, the MDA values of the MTX groups were higher than those of the two other experimental groups (P = 0.001). Moreover, the MDA levels in the LCMTX group were higher than in the VEMTX group, but the difference was not statistically significant (P = 0.059) (Table 2).

SOD values were significantly lower in the MTX and LCMTX groups than in the control group (P < 0.001). The low SOD value in the VEMTX group was statistically insignificant (P < 0.063). However, the SOD value in VEMTX group was significantly different from the SOD value in the MTX group (P < 0.001). Another interesting finding here was the difference between the VEMTX group and the LCMTX group (P = 0.048). Vitamin E protected low SOD levels, rather than L-carnitine, in the MTX group.

Table 1. Testis weights after the experiment.

Groups	n	Weight of testes (g) Mean ± SD
Control	5	2.6280 ± 0.1785*
MTX	7	2.0243 ± 0.2533
VEMTX	7	1.8986 ± 0.3036
LCMTX	7	1.7714 ± 0.1190

* P < 0.001

Table 2. MDA and SOD levels in the groups.

Groups	MDA (nmol/g) Mean ± SD	SOD (U/g) Mean ± SD
Control[1]	9.45 ± 0.947	131.20 ± 8.843
MTX[2]	14.08 ± 1.712	96.29 ± 10.144
VEMTX[3]	10.73 ± 1.684	123.00 ± 2.646
LCMTX[4]	12.17 ± 0.592	115.00 ± 5.066

[1–2, 1–3, 1–4, 2–3, 2–4]: P < 0.001
[3–4]: P < 0.059
[1–2, 2–3, 2–4]: P < 0.001
[1–3]: P < 0.063
[3–4]: P < 0.048

3.3. Histological findings

3.3.1. Control group

In the control group, normal seminiferous tubules were observed (Figure 1A). In experimental groups, various histopathologic findings at different levels were encountered. The observed histopathologic changes were categorized into 6 parameters, and the severity of the findings was graded between 0 and 4.

3.3.2. Decrease in germ cells

The level of injury in the MTX group was significantly higher than in the control group (P < 0.0001). It was observed that the injury in the VEMTX group decreased significantly compared to the MTX group (P = 0.0294). In the LCMTX group, the decrease induced by MTX was not statistically significant (P = 0.1208). However, there was no difference between the VEMTX group and the LCMTX group concerning injury level (P > 0.5) (Figures 1B and 1C).

3.3.3. Edema in the interstitial site

In this parameter, as well, MTX application caused injury at a significant level (P = 0.0018). The level of injury in the two other experimental groups was lower than in the MTX group, but there was no statistical significance (P > 0.5). The level of injury in the VEMTX group was not different from that of the control group (P = 0.188). The LCMTX group had a higher level of injury than the control group (P = 0.0190). In other words, vitamin E prevented the interstitial edema induced by MTX (Figure 1D).

3.3.4. Congestion in interstitial site

In this parameter, there were more intense findings (congestion) in the MTX group than in the control group (P = 0.0022). Although vitamin E and L-carnitine decreased the severity of congestion, this was not statistically significant. However, even though the VEMTX group was different (P = 0.0299) from the control, the LCMTX group had more approximate injury than the control, and there was no statistically significant difference from the control group (P = 0.114). Even though there was no statistical significance, the animals administered with LCMTX were better protected in terms of this parameter.

3.3.5. Sertoli cell vacuolization

Significant vacuolization occurred in the MTX group (P = 0.0140). The severity of vacuolization in the VEMTX and LCMTX groups was not significantly different from the control group (P > 0.5 for both) (Figures 1B and 1C)

3.3.6. Separation in the basal lamina

Separation in the basal lamina was only encountered in the 3 experimental groups.

3.3.7. Multinucleated giant spermatogenic cells

When the multinucleated giant spermatogenic cells in the MTX group were compared with those of the control group, there was a significantly greater number of them

(P = 0.0139). In other groups, these cells were few in number, not significantly different from the control group (P = 0.9345 for VEMTX, P = 0.1804 for LCMTX) (Figure 1E). When the total injury, including evaluation of all the parameters, was analyzed for each group, it was observed that MTX caused specific injury (P < 0.0001). The total injury index in the VEMTX (P < 0.001) and LCMTX (P < 0.0001) groups was different from the control group, but vitamin E (P = 0.0049) and L-carnitine (P = 0.0 278) decreased the injury induced by MTX (Table 3).

3.4. Electron microscopic findings

3.4.1. Control group

Normal testis structures were observed in tissue samples of the control group. Normal structures were observed in the membrane propria, Sertoli cells, spermatogenesis cells, myoid cells, and Leydig cells (Figure 2A).

3.4.2. MTX group

There was thickening in the membrane propria and an increase in the amount of collagen fiber in testis tissue samples of the animals administered MTX (Figures 2B and 2C). It was also observed that spermatogonia located in the basal lamina and intercellular spaces among the Sertoli cells were extended and the cells were separated from each other.

Cytoplasmic density, lysosomes, and lipid droplets increased in Sertoli cells; spaces with irregular membranous whorl structures occurred; agranular endoplasmic reticulum cisternae grew larger; and vacuoles of different sizes were formed. Within the Sertoli cell cytoplasm, the presence of lytic regions observed in the form of dispersed electron dense structures and peripherally located heterochromatin aggregations was also observed (Figure 2C). Leydig cells in the interstitial region, macrophages, and capillaries were visualized with similar characteristics as in the control group.

Table 3. The total histopathological damage index values.

Groups	n	Total damage index Mean ± SD
Control[1]	5	0.0183 ± 0.04251
MTX[2]	7	0.9736 ± 0.6183
VEMTX[3]	7	0.4921 ± 0.4308
LCMTX[4]	7	0.5648 ± 0.4228

[1–2, 1–4]: P < 0.0001

[1–3]: P < 0.001

[2–3]: P < 0.0049

[3–4]: P < 0.02778

3.4.3. LCMTX group

This group also had characteristics similar to those of the membrane propria of the control group. Tight conjugation complexes among the Sertoli cells and Sertoli cell nucleus appeared normal, and enlargements in agranular endoplasmic reticulum cisternae and some increase in lipid droplets could be observed (Figure 2D). Spermatogonia, spermatocytes, and spermatozoa located among the Sertoli cells had similar characteristics to the control group (Figure 2D). Leydig cells and capillaries in the interstitial region were not different from the control group (Figure 2E).

3.4.4. VEMTX group

In the VEMTX group, the membrane propria was also similar to the control group (Figure 2F). Sertoli cells were locally separated from the basal lamina, the intracellular spaces among the Sertoli cells were increased, spaces of different sizes occurred, and whorl membranous structures were present in these spaces (Figures 2F and 2G). There were peripheral heterochromatin clusters within the cell nucleus. Enlargements in agranular endoplasmic reticulum cisternae in Sertoli cell cytoplasm and increases of lysosomes and lipid droplets were observed. Furthermore, the presence of the lytic regions in the form of dispersed electron-dense structures within the cytoplasm of these cells was also observed (Figure 2F and 2G). Leydig cells, macrophages, and the capillaries within the interstitial region had a normal appearance.

4. Discussion

Cancer is one of the leading health problems of today's world, and it is encountered more frequently day by day. While alternative treatment methods have been investigated, intensive studies have also been carried out on the mechanisms that will eliminate the side effects and make more efficient use of the antineoplastic drugs already available. MTX, an antagonist of folic acid, is an antineoplastic agent that is used often. However, its side effects limit its use. A way of surmounting this limitation is to add a drug or drugs to a treatment regimen that will reduce or prevent the side effects of the treatment. In the literature, there have been several studies carried out with a great number of antioxidant substances, such as caffeic acid, erdosteine, vitamin E, and simvastatin, with the intent of reducing MTX toxicity (7,15,16). In our study, as well, we aimed to research the protective effect of vitamin E and L-carnitine administered externally against MTX-induced testicular tissue injury.

It is known that MTX triggers leukocyte (especially neutrophil) infiltration in tissues, increasing the total antioxidant capacity and myeloperoxidase activity (17,18). MTX-induced toxicity is characterized by the formation of the inflammatory response due to the increased production

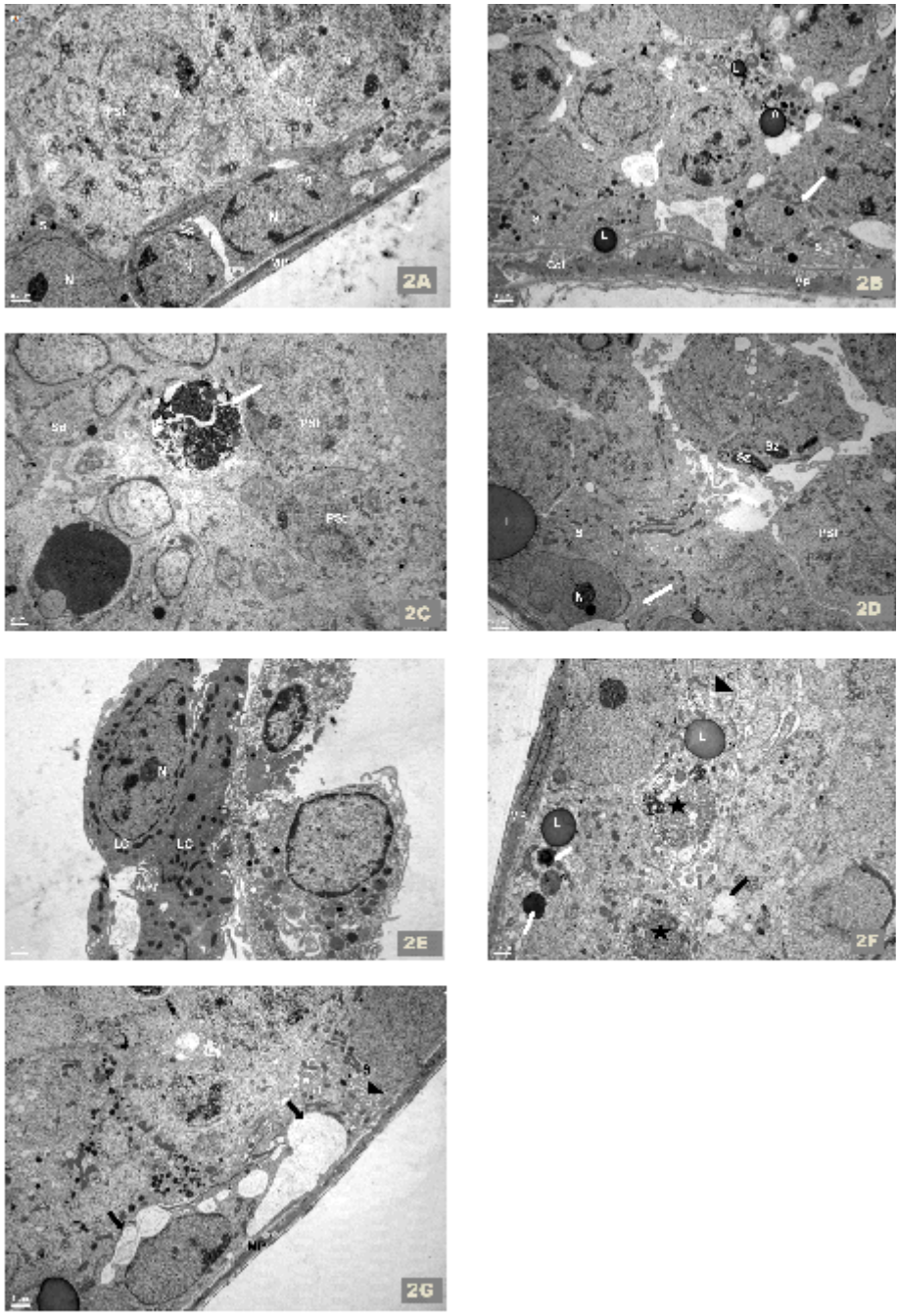

Figure 2. A) Control group: the membrane propria (MP) surrounding the seminiferous tubules had a normal structure. In the membrane propria, Sertoli cells (S), spermatogonia (Sg), and primary spermatocytes (PSt) located in Sertoli cells were visualized. Tight connection complexes among the Sertoli cells were visualized as normal. N = Nucleus. **B)** MTX group: thickened membrane propria (MP) surrounding the seminiferous tubules, increased collagen fiber (Col), thickened basal lamina (BL), increased spaces between Sertoli cells (S), spaces of different sizes that emerged and membranous whorl bodies that developed in some of these spaces (black arrow), peripherally located heterochromatin clustering within the nucleus of Sertoli cells (white arrow), expended agranular endoplasmic reticulum cisterns (arrow head), and increased lysosomes and lipid droplets (L) were visualized. **C)** MTX group: within the cytoplasms of Sertoli cells, lytic regions were observed in the form of dispersed electron-dense structures (white arrow). PSt = Primary spermatocyte, Sd = spermatid. **D)** LCMTX group: tight connection complexes (arrow) and nuclei (N) between the Sertoli cells located on the top of membrane propria (MP) were visualized as normal. Increased lipid droplets (L) were seen within the cytoplasm of Sertoli cells. PSt = Primary spermatocyte, Sz = spermatozoon. **E)** LCMTX group: Leydig cells had normal appearance (LC) and nuclei (N). **F)** VEMTX group: membrane propria (MP) with normal appearance, enlargements between the Sertoli cells (S) (black arrow), enlargements in straight endoplasmic reticulum cisterns between cytoplasms (arrow head), increased lysosomes (white arrow), and lipid droplets (L) were visualized. Within the cytoplasms of Sertoli cells, lytic areas (star) were noticed in the form of dispersed electron-dense structures. **G)** VEMTX group: membrane propria (MP) with normal structure, in some areas Sertoli cells (S) were separated from the basal lamina, spaces including membranous whorl structures within intracellular spaces (arrow), enlarged straight endoplasmic reticulum cisterns visualized within the cytoplasms (arrow head).

of procytokines (1,18). Gao et al. revealed that MTX caused xenobiotic and endotoxins to pass to the intestine; this started macrophage and neutrophil infiltration and finally caused an increase in the production of reactive oxygen types (free radical production) (19). Similar to the mechanism here, Oktar et al. showed that MTX increased oxidative stress through the release of macrophages and neutrophils into the testis (20).

Vitamin E protects membranes against lipid peroxidation, decreasing the activities of superoxide and lipid peroxides in membranes (21). There have been several studies reporting that vitamin E protects the testis against various toxic agents (22–25). Marin-Guzman et al. reported that selenium and vitamin E taken up through diet had a curative effect upon testis tissue, semen quality, and fertilization (24). Mauer and Mason observed a decrease in testis weights and advanced degeneration in seminiferous tubules of rats fed diets lacking vitamin E. These parameters improved when vitamin E was administered to these rats (26).

L-carnitine is a cofactor necessary for transferring long-chain fatty acids to the mitochondria, where they are broken down for cellular energy generation. L-carnitine's ability to protect against various toxic factors stems from its antioxidant and antiperoxidative effects (27,28). L-carnitine is an endogenous substance that is commonly present in male genital organs (29). It has been established that L-carnitine can prevent the organ injuries caused by MTX and leukocyte death (30) and that it has an antiapoptotic effect (31). In a study carried out on rat testes, L-carnitine decreased the number of apoptotic germ cells due to increased radiation (32).

In our study, there was no significant difference among animal body weights prior to the experiments ($P = 0.763$). However, weight loss was observed after the experiment in all of the groups except the control group ($P = 0.001$). When testis weights were evaluated at the end of the experiment, the total weight of both testes of the animals in the control group was significantly high ($P = 0.001$). However, the weight difference among the other groups was not statistically significant. Animals in all experimental groups were subjected to losses in body and testis weight compared to the animals in the control group. In other words, vitamin E and L-carnitine did not affect the MTX-induced decreases in both body and testis weights.

In several previous studies, it was reported that being exposed to MTX at doses of 20 mg/kg or more increased the level of MDA in the blood, kidneys, and testes (1,33). In some studies in which lower doses (10 mg/kg) were administered, it was emphasized that MDA level was not affected and, moreover, not performing anesthesia could be an important factor for this, besides the lower doses (20). In fact, the substances used in anesthesia can increase lipid peroxidation (34). In our study, in which anesthesia was administered with ethical concerns and MTX was administered at relatively higher doses (20 mg/kg), the significant increase in MDA levels was compatible with the previous studies. When the SOD values of our experimental animals were analyzed, it was observed that the MTX group had significantly lower levels than the other groups. This decrease in SOD values in experimental groups was compatible with the increase in MDA values. While vitamin E and L-carnitine decreased the MDA values increased by MTX, they also increased the SOD values decreased by MTX. MTX decreased sperm count and caused an increase in the rate of sperm-head abnormalities (35,36). Moreover, it has also been reported that MTX, administered in different doses, can cause diameter thinning in seminiferous tubular, decreases in spermatogenic cell content, vacuolization, and edema in the interstitial region (35,37). Similar histologic findings were observed in our study as well. Although administering vitamin E and L-carnitine alongside MTX could not prevent all these injuries, it caused a significant decrease. Samples were subjected to electron microscopic analysis, and normal testis structure was observed in the control group. In tissues of the MTX group, thickening in the lamina propria was observed around the seminiferous tubular. An increase in collagen fiber amount, thickening and disorders at the basal lamina, and an increase in intracellular spaces between the Sertoli cells and spermatogonia were also observed. These findings indicated a cell distortion in general. Although some of the findings (such as extending in agranular endoplasmic reticulum cisterns of Sertoli cells, increase in lysosome and lipid droplets, and spaces including lytic regions and whorl membranous structures) observed in the MTX group were also observed in the VEMTX and LCMTX groups, the number and severity of these findings was not as great as in the MTX group.

Vitamin E is a potent antioxidant that has the capacity to directly quench free radicals (21). Like many other antioxidants, L-carnitine has been reported to inhibit free radical generation, thereby preventing the impairment of fatty acid beta-oxidation in mitochondria and protecting tissues from damage by repairing oxidized membrane lipids (38).

In our study, when MDA and SOD values were considered, vitamin E had a better protective effect than L-carnitine. Moreover, vitamin E performed better when it came to decreasing interstitial edema severity and germ cell loss.

In summary, when the tissues in the MTX group were analyzed histologically after the experiment, a decrease in

germ cells in seminiferous tubular, edema and congestion in interstitial region, vacuolization in seminiferous tubular, separation in basal lamina, and multinucleated giant spermatogenic cells emerging in the epithelium were observed. Electron microscopic findings also confirmed these. When considering the entire injury, both vitamin E and L-carnitine prevented the histologic injuries induced by MTX. Furthermore, MDA and SOD values among the biochemical parameters supported this protective effect.

Consequently, we concluded in this study that vitamin E and L-carnitine partially prevented MTX-induced injury in the testis and significantly decreased the severity of injury.

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