



The histopathological evaluation of healing effects of vitamin C administered before methotrexate therapy on testicular injury induced by methotrexate

Aysun Sayılmaz¹, Yasemin Yuyucu Karabulut², Aydan Özgörgülü¹

ABSTRACT

Objective: Methotrexate (MTX) leads to acute toxic side effects in tissues or organs containing rapidly dividing cells such as seminiferous tubules. In this study, we investigated the protective effects of vitamin C against MTX-induced injury in rat testis.

Material and methods: A total of 31 rats were divided into 4 groups, including the control group. The study was completed within 4 weeks and the rats received daily doses of 2 mL/kg SF, 100 mg/kg vitamin C and 10 mg/kg/day MTX i.p according to their groups. The mean seminiferous tubular diameter, germinal epithelial cell thickness, and mean testicular biopsy score were determined by histologic examination of each group.

Results: The vitamin C + MTX group showed more similarity with the control group. Statistically significant results were achieved between groups as for mean seminiferous tubular diameter, germinal epithelial cell thickness, and mean testicular biopsy score. When compared with the group which received vitamin C after MTX therapy, values for mean seminiferous tubular diameter, germinal epithelial cell thickness, and mean testicular biopsy score were significantly higher in the group which received vitamin C before initiation of MTX therapy.

Conclusion: Vitamin C decreased MTX-induced testicular histological injuries, especially when used before MTX therapy.

Keywords: Vitamin C; methotrexate; testis.

Introduction

Folic acid antagonist methotrexate (MTX) is an agent used for the chemotherapy of malignant tumors.^[1-4] MTX which is used for a long time exerts cytotoxic effects on 'S phase' of the cell cycle, and inhibits cell division. Rapidly dividing cells as germinative epithelial cells of testis, and hair follicle cells etc. are susceptible to the effects of MTX. MTX is used in the treatment of various neoplasias as acute lymphoblastic leukemia, osteosarcoma, choriosarcoma, non-Hodgkin's lymphoma, and lymphoma, breast cancer, head and neck cancer, and non-neoplastic diseases as rheumatoid arthritis, and psoriasis.^[5,6] Harmful effects of MTX on testicular seminiferous tubuli, and sperm deoxyribonucleic acid (DNA) have been demonstrated related to the use of MTX.^[6,7]

Studies performed have shown that MTX exerts its effects on cells by decreasing cellular antioxidant activity, and exposing cells to the unfavourable effects of reactive oxygen species (ROS), eventually inducing detrimental alterations in testicular tissue, and germ cells. Atrophy of seminiferous tubuli, and apoptosis in germinal cells have been seen with the use of MTX.^[4-8] The effects of antioxidant materials in relieving toxic effects of MTX have been intensively investigated.^[4] Vitamin C is an important agent in exposing antioxidant capacity of seminal plasma. Vitamin C reinforces spermatogenesis.^[9,10]

In this study we aimed to investigate histopathologically whether vitamin C ameliorates deleterious effects of MTX on testicular tissue.

¹Necmettin Erbakan University
Meram School of Medicine,
Institute of Health Sciences,
Konya, Turkey

²Department of Pathology,
Mersin University School of
Medicine, Mersin, Turkey

Submitted:
03.03.2016

Accepted:
09.04.2016

Correspondence:
Yasemin Yuyucu Karabulut
E-mail:
yykarabulut@yahoo.com.tr

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Available online at
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Material and methods

Ethics committee approval was obtained from Ethics Committee of Necmettin Erbakan University, Meram Faculty of Medicine (protocol #: 2014-065).

Thirty-one 10-12-week-old male rats weighing between 300-550 gr obtained from our center were used in our study. The study was performed under $22\pm 2^{\circ}\text{C}$ room temperature with 60% humidity. The rats were kept under 12 hours of light, and 12 hours of dark conditions.

Groups of experimental animals

Rats were allocated into 4 groups as Group I (n=7): control, Group II (n=8): MTX-treatment group; Group III (n=8): MTX followed by vitamin C therapy; Group IV (n=8): Vitamin C followed by MTX treatment.

Group I (control group): Rats received isotonic saline solution through intraperitoneal (i.p.) route for 24 days at daily doses of 2 mg/kg.

Group II (MTX group): Rats received single weekly doses of 10 mg/kg MTX through (i.p.) route for 2 weeks.^[6]

Group III (MTX + vitamin C group): Rats priorly received single weekly doses of 10 mg/kg MTX for 2 weeks, followed by single daily doses of 100 mg/kg vitamin C for 10 days both delivered through (ip.) route.

Group IV (vitamin C + MTX group): Rats received single daily doses 100 mg/kg vitamin C for the first 10 days, followed by single weekly doses of 10 mg/kg MTX for 2 weeks both delivered through (ip.) route.

At the end of the experiment 10% ketamine (20 mg/kg), and xylazine (3 mg/kg) were injected via ip route. Rats were orchidectomized under anesthesia.

Histopathological preparation, and examination

Testicles were fixated in 10% formaldehyde solution. Then appropriate tissue specimens were rinsed with running tap water. The rinsed tissue specimens were passed through increasing grades of alcohol (70, 80, 90, and 96%) to dehydrate them, and made them transparent. Then these testicular tissues were awaited in paraffin, embedded in paraffin under room temperature, and paraffin blocks of the tissues were prepared. From paraffin blocks a 4-5 μ -thick sections were cut using HM 325 mikron brand microtome Thermo scientific, and cut sections were placed on a slide. The sections obtained were stained with Hemotoxylin & Eosin (H&E), and Periodic Acid- Schiff (PAS) dyes. From prepared slides 10 seminiferous tubuli were randomly selected. Then under

under 400X magnification of Olympus CX31 brand microscope, mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy scores (MTBS) were determined.^[7] In each group randomly selected 20 seminiferous tubuli were scored separately. Using calculated means of these parametres, MTBS were determined.^[11] MSTD was calculated with the aid of microscope-adapted micrometre. Thickness of germinal epithelial layer was estimated by counting epithelial cells from basement membrane towards the lumen.^[7]

Differences between estimated means for experimental groups were compared between groups using one-way analysis of variance (ANOVA). The groups which caused the intergroup differences were determined using Tukey's comparison test.

Results

Based on histological evaluation, MSTD, GECT, and MTBS values of the groups are shown in Table 1. (Figure 1-4). In intergroup comparisons (control group vs MTX ($p<0.001$) group; MTX + vitamin C group vs MTX ($p<0.05$) group, and vitamin C +MTX group vs MTX group) ($p<0.01$), statistically significant values regarding MSTD, GECT, and MTBS were obtained. When compared with only MTX applied group, MSTD, GECT, and MTBS values were significantly higher in MTX plus subsequent vitamin C group. At the same time, comparisons between vitamin C applications during pre-, and post-MTX treatment periods revealed prominently higher MSTD, GECT, and MTBS values in vitamin C +MTX group.

Discussion

Methotrexate has important testicular adverse effects which can explain infertility. Cellular, and molecular toxic effects of this antitumoral drug on gonads during spermatogenesis have been evaluated in comprehensive studies.^[11-14] Oligospermia was observed in many adult men even after successful treatment with MTX.^[6,14-17] Changes in dimensions of primary, and secondary spermatocytes alter the size of spermatids, and Leydig cells.^[18] As indicated in available studies MTX damages seminiferous tubuli of testis with resultant decrease in sperm counts, and harmful effects on sperm DNA. Studies performed have demonstrated that MTX exerts its cellular effects by decreasing antioxidative effectiveness of cells, and exposes them to deleterious effects of reactive oxygen species (ROS) leading to destructive changes in testicular tissue, and germ cells.^[14-17] As demonstrated in many studies, MTX causes atrophic changes in seminiferous tubuli, and apoptosis of germinal cells.^[8] In studies performed in mice, and rats, MTX had decreased testicular weight, and sperm counts, increased number of sperm head abnormalities, and damaged DNAs of seminiferous tubuli, and spermatozoa.^[19-21] In a study by Vardi et al.^[8] the authors demonstrated that MTX

Table 1. MSTD, GECT, and MTBS values of the groups

Group	MSTD±SD (µm)	GECT (Mean±SD)	MTBS±SD
Control	222.25±12.82	6.05±1.31	9.71±0.45
MTX ^a	180.75±10.79	3.45±1.05	3.74±1.44
MTX + vitamin C ^b	186.25±9.11	4.41±1.09	5.60±1.89
Vitamin C + MTX ^c	198.25±7.82	5.11±1.25	7.17±1.68

^aIn comparison with the control group $p < 0.001$. ^bIn comparison with the MTX group $p < 0.05$. ^cIn comparison with the MTX group $p < 0.01$. MSTD: mean seminiferous tubular diameter; MTX: methotrexate; GECT: germinal epithelial cell thickness; MTBS: mean testicular biopsy score; SD: standard deviation

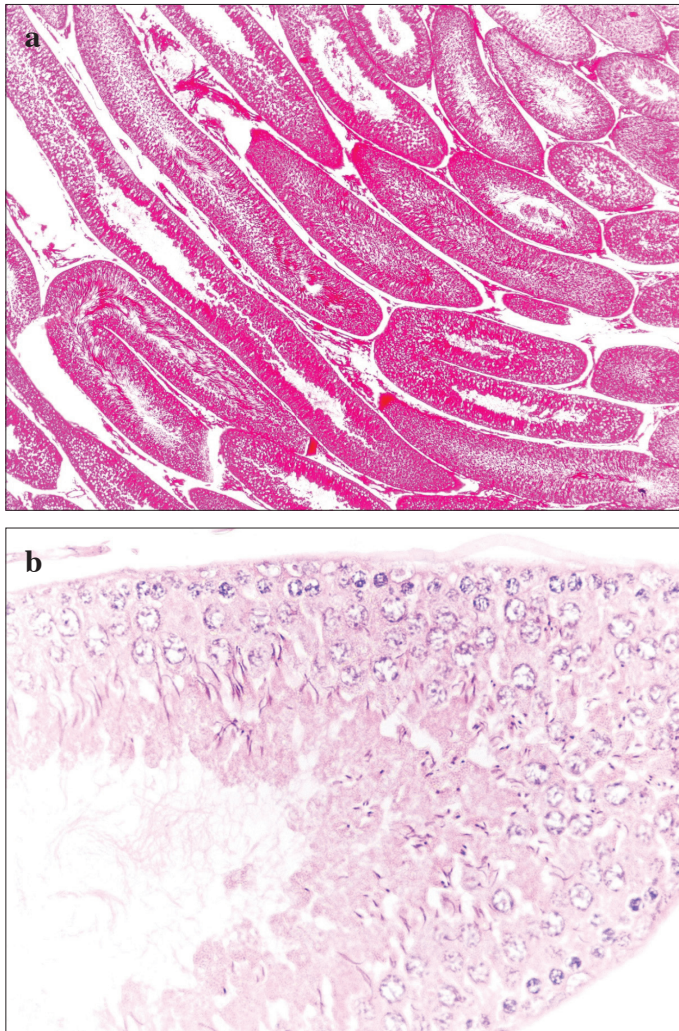


Figure 1. a, b. H&E stained seminiferous tubuli of the control group (x40) (a). x200 (b)

decreases effectiveness of antioxidant enzyme system, and renders cells vulnerable to harmful effects of ROS leading to destructive changes in testis. Oxidative stress had caused de-

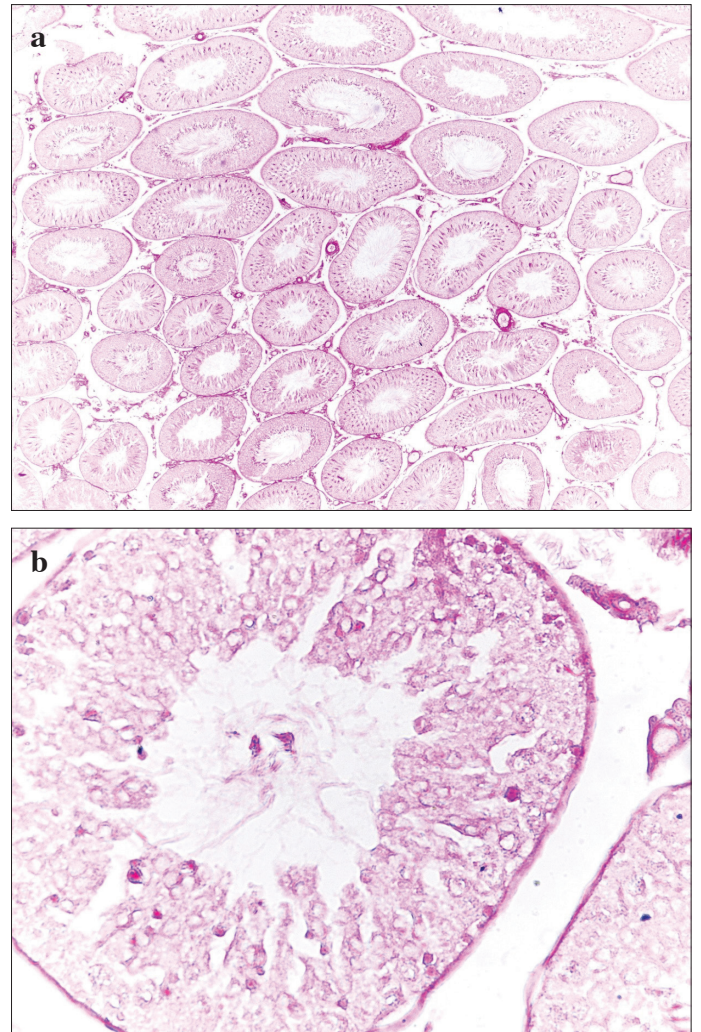


Figure 2. a, b. (a) H&E stained seminiferous tubuli of the MTX group (x40). (b) Leydig cells, and edematous spaces interposed between tubuli (x200)

structive changes in testicular tissue, and ameliorating effects of an antioxidant (chlorogenic acid) on testicular damage had been demonstrated. Chlorogenic acid also protected seminiferous tubuli against deleterious effects of oxidative stress. Ahmed et al.^[22] evaluated antioxidant activity of vitamin C, DPPD, and L-cysteine against oxidative injury related to cisplatin use. They observed a significant drop in activities of antioxidants as superoxide dismutase and Glutathion S transferase, contrary to marked increases in lipid peroxidation, and levels of total peroxidase, and superoxide anion. Testicular tissues of rats treated with cisplatin, and the control group were compared, and a significant drop in the levels of glutathione, vitamin E, and C was observed. Administration of cisplatin together with vitamin C, L-cysteine, had weakened the effects of cisplatin on superoxide anion, and antioxidant contents. Still, administration of vitamin C, DPPD or L-cysteine before cisplatin injection had improved histologi-

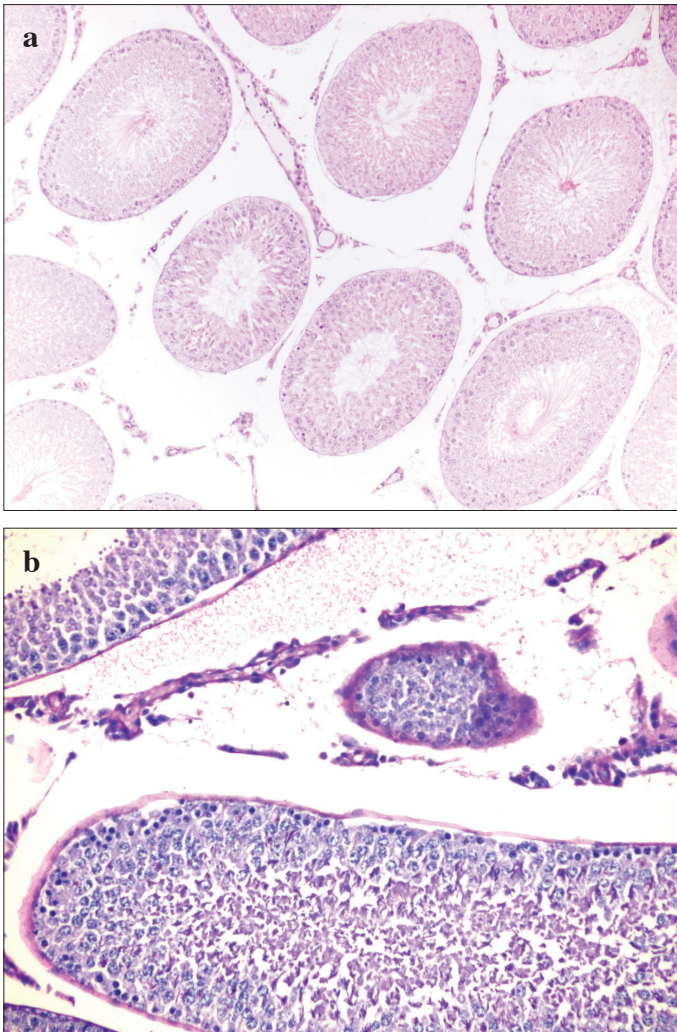


Figure 3. a, b. H&E (x100) (a). and PAS stained (x200) (b) seminiferous tubuli of MTX + vitamin C Groups

cal manifestations, and decreased number of apoptotic cells.^[22] In our study, as an antioxidant, vitamin C was used before, and after administration of MTX, and similar results were obtained. Use of vitamin C before administration of MTX decreased MTX induced testicular injury which is a good example of protective effects of antioxidants. Spermatogenesis has a capacity to produce 1000 spermatozoa per second, and it is an actively recurrent process which is exposed to deleterious effects of oxidative stress. Testis contains various antioxidant enzymes, and free radical scavengers so as to be able to protect itself against injurious effects. Vitamin C is an important agent which activates antioxidant capacity, and also supports spermatogenesis.^[9] Also in our study, based on the results of Johnsen testicular biopsy scoring system higher values were detected in vitamin C + MTX group when compared with MTX+vitamin C group. When compared with the rats which received an antioxidant, in rats which did not receive any antioxidant before administration of MTX,

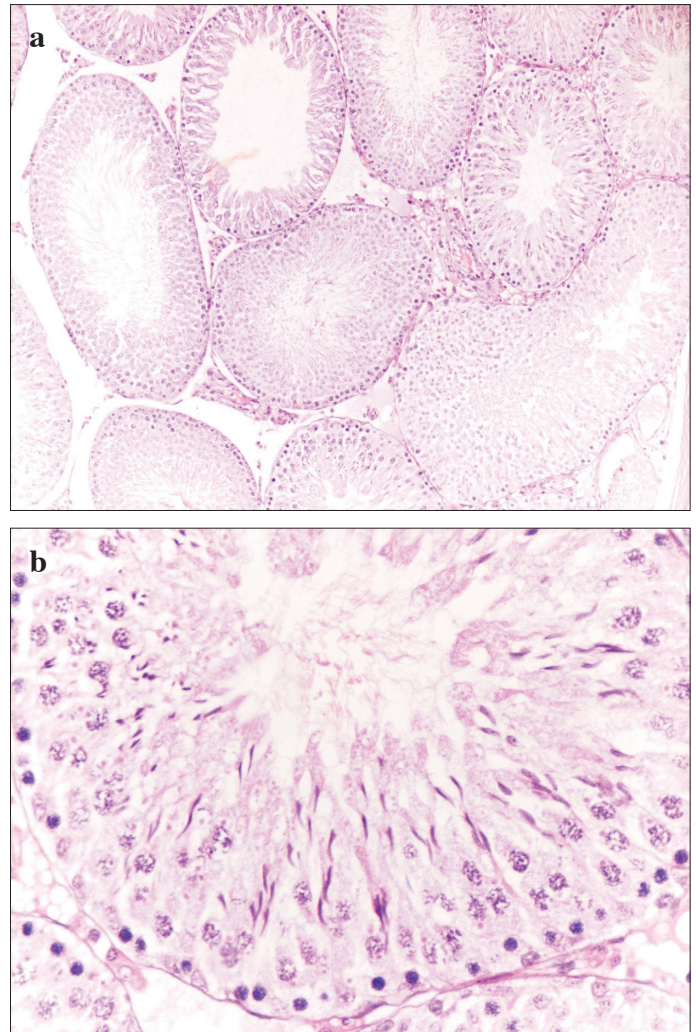


Figure 4. a, b. H&E stained seminiferous tubuli of MTX + vitamin C Groups (x100) (a). (x400) (b)

most of the seminiferous tubuli cells detached from their basement membrane, and shed into lumen, and also decrease in the number of cells of the spermatogenic series was observed. When we compared these two groups, vitamin C minimized MTX-induced testicular injury, and protected seminal plasma against harmful effects. With available information, cost-effective, and practical use, and easy applicability of vitamin C in clinical practice before MTX therapy has been proved.

In another study Aitken et al.^[23] demonstrated that antioxidant intake minimizes testicular damage induced by oxidative stress, and exerts protective effects. Prasad et al.^[10] emphasized induction of oxidative stress by vitamins C, and E insufficiency, and demonstrated that vitamin C decreases oxidative stress via preserving integrity of membranes, and cellular functions. In a similar study protective effects of resveratrol against testicular injury induced by MTX on biochemical, histopathological, and

apoptotic levels have been indicated.^[4] In that study rats were allocated into 4 groups as control, MTX, Resveratrol, and MTX+resveratrol groups. In the MTX group the rats received daily dose of 30 mg/kg MTX via ip route for 7 days, while resveratrol group received resveratrol at daily i.p. doses of 20 mg/kg for 10 days. The MTX+resveratrol group received daily i.p. MTX doses of 30 mg/kg for 3 days, followed by resveratrol given at daily i.p. doses of 20 mg/kg for 7 days. As an outcome of this experiment significantly higher MTBS were detected in the MTX + MTBS resveratrol group relative to MTX group.^[4] In our study, contrary to other studies which also used antioxidants, in addition to MTBS, MSTD the values of MSTD and GECT were also evaluated. Strikingly, study results displayed statistically significant intergroup differences.

In our study starting from all these literature information, it has been histologically demonstrated that MTX-induced testicular damage in rats can be prevented to a certain extent using vitamin C as an antioxidant before chemotherapy.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Necmettin Erbakan University Meram School of Medicine.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - A.S., A.Ö.; Design - Y.Y.K.; Supervision - A.Ö., Y.Y.K.; Resources - A.S., Y.Y.K.; Materials - A.S., Y.Y.K.; Data Collection and/or Processing - A.S.; Analysis and/or Interpretation - Y.Y.K., A.Ö.; Literature Search - A.S., Y.Y.K.; Writing Manuscript - Y.Y.K., A.S.; Critical Review - A.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

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