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Vitamin D: An effective way to combat methotrexate-induced testis injury

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Abstract

Methotrexate (MTX) is a frequently used anticancer drug in the treatment of several diseases. However, MTX therapy causes significant cytotoxicities in testicular tissue. In this experimental study, the therapeutic utility of vitamin D (VD) on MTX induced testicular injury was investigated. Twenty-eight Wistar rats were randomly divided into four equal groups; Control, VD, MTX, and MTX+VD. Following the treatment, the rats were sacrificed and testicular tissues were removed. Testicular tissues were analyzed for routine histopathology and apoptosis. The activities of glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were evaluated by enzyme-linked immunosorbent assay and malondialdehyde (MDA) levels were measured spectrophotometrically. MTX-treatment group exhibited degeneration of spermatogenic cells, desquamation of the epithelial cells into the lumen of the tubules, lack of spermatozoa in tubule lumen, edema at interstitial area, a statistically significant decrease in both height and diameter of the tubules ($p < 0.05$). These values were also concordant with the Johnsen's testicular biopsy score (JTBS). In addition, a significantly increased caspase-3 immunoreactivity was observed. Serum and testicular tissue SOD, and GSH-Px enzyme activities were found to be decreased while MDA increased in the MTX group compared to control group ($p < 0.05$). In the MTX + VD group, the histological injury was reduced, the caspase-3 positivity and MDA level decreased whereas activities of SOD, and GP-x enzymes were significantly increased compared to those in MTX group ($p < 0.05$). VD supplement may have a therapeutic utility in reducing the MTX-induced cytotoxicities in testicular tissue.

Keywords: Methotrexate, testicular injury, vitamin D, caspase-3, oxidative stress

Introduction

Methotrexate (MTX) is a widely used chemotherapeutic drug that is used for the treatments of a wide variety of malignancies as well as autoimmune diseases [1]. However, MTX causes toxic side effects in some tissues including the gastrointestinal mucosa, bone marrow, hair roots, and spermatogenic cells in testis which are highly proliferative [2]. Oxidative stress is an underlying cause in the pathogenesis of MTX-induced testis injury [3] as it causes damage in seminiferous tubules of testicular tissue [4] which may often cause infertility in affected individuals [5]. MTX decreases the cellular antioxidant capacity while elevating reactive oxygen species (ROS) levels [3], which in turn leads to cytotoxicities in germ cells of the testicular tissue [1, 2]. Studies by Armagan et al. [3] and Nouri et al. [5] reported that seminiferous tubules atrophy and apoptosis observed in spermatocytes is related with elevated ROS levels. Therefore, antioxidants may be utilized as protective agents against the oxidative stress induced cytotoxicity in the testis [6].

Vitamin D (VD), a potent antioxidant has been shown to exhibit beneficial effects in some pathological conditions and is important for overall human health [7]. It is a lipid soluble vitamin that contains Vitamin D2 (ergocalciferol) and Vitamin D3

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(cholecalciferol). VD is metabolized to 1, 25 (OH) 2 derivative, the hormonal active form of cholecalciferol. It has many biological activities such as the control of both phosphorus, and calcium metabolism [8]. 1, 25 (OH) 2 is reported to reduce oxidative stress, cell and tissue injury [8] and protects cell membrane against oxidative damage caused by free radicals [9]. It was previously reported that higher concentrations of these lipophilic compounds may diminish peroxided lipids that accumulate in membranes [10]. Consistent with these, Liu et al. [11] notified that 1, 25 (OH) 2D3 deficiency may affect the progression of diabetes mellitus□driven hypogonadism by converting tissue microenvironment that leads to cellular senescence. Other important functions of VD have also been proposed to reduce chronic inflammation, suppress oxidative stress, and sustaining mitochondrial respiratory function [7]. Furthermore, VD deficiency is also implicated in the pathology of number of diseases via increasing cellular oxidative damage [12]. As VD is fundamental for health, VD deficiency is an important public health issue that affects all ages and ethnic groups [13].

This study was devised to determine whether VD could exert a therapeutic potential against MTX-induced testicular injury in a rat model.

Material and Methods

The current study was approved by the Kahramanmaras Sutcu Imam University Faculty of Medicine Animal Experiments Local Ethics Committee (2019/04). To analyze the effect of VD on MTX-induced testicular cytotoxicity, a total of 28 healthy Wistar albino male rats weighing 200-220 g at 10-11 weeks old were used in the study. The rats were randomly divided into four groups, each group containing seven animals and were not treated for the initial 7 d to facilitate cage adaptation. The rats were housed at 22 ± 2 ºC room temperature with alternating light and dark periods of 12 h and given access to standard feed (Korkutelim Yem Gıda San. Tic,, Antalya, Turkey) and water ad libitum. The duration of the study was 15 days [14]. Experimental groups were as follows:

Control group: No treatment was performed. MTX group: A single 20mg/kg dose of MTX (Methotrexate ®, Kocak Farma, Turkey) was treated intraperitoneally (i.p.) on day 1 [15]. VD group: 200 IU/day VD (DEVİT-3® Deva, Turkey) was applied by oral gavage (o.g.) [16] daily for 15 days. MTX + VD group: A single 20mg/kg dose of MTX was treated i.p. on day 1. Then a dose of 200 IU/day VD was applied by o.g. daily for 15 days.

After 15 days, animals were anaesthetized (Rompun®, Bayer Turk Chemistry Industry. Ltd. Corp., Istanbul, Turkey) and 75 mg/kg ketamine HCl (Ketalar®, Eczacıbası; Istanbul, Turkey), blood specimens were collected from hearts, allowed to clot in laboratory temperature for 20 min and then centrifuged at 4000 rpm for 10 min to obtain serum. The testes of both sides were rapidly extracted. The rats were sacrificed in accordance to the ethical international guidelines for the care and use of laboratory animals.

Histopathological analysis

Following 10% formalin fixation, testes were isolated and subjected to increasing ethanol series dehydration and xylene clarification, and embedded in paraffin blocks. For histopathological examinations and histomorphometric analysis, 5 µm thickness sections were taken from paraffin blocks and stained with hematoxylin-eosin (HE) (Abcam, Cambridge, United States). Sections were blindly examined and photographed under a light microscope (Leica DM500 attached Leica DFC295 Digital Image Analyze System, Leica Biosystems, Nussloch, Germany).

To measure the diameter and the height of the germinal epithelium of the tubules, two vertical diameters (small and long) of each cross-section were used under 10x magnification image analyzer (Lecia Qwin 500 image analyzer computer system, Cambridge, England). A total of three serial sections were evaluated for each rat. Measurements of diameters and heights of tubules were made by selecting totally 45 round or round tubules, fifteen from each section (Figure 1A). For determining the diameter of tubule, diameter (small and long) of the seminiferous tubules in each section were measured, summed up and divided into two. For determining the height of the germinal epithelium, the length between tunica albugina and the last spermatozoid or spermatid of each tubule was measured in 4 different points. Next, measured data were summed up and divided into four [5].

Johnsen testicular biopsy score (JTBS)

To determine the degree of injury in spermatogenetic cells, Johnsen testicular biopsy score (JTBS) was performed. According to JTBS, the tubular cells of the testis gradually disappear in a certain order following any testicular injury. More than 20 seminiferous tubules for every testis was examined and given a JTBS [17]. All of the tubular sections in each sample of the testicular biopsy are analysed systematically and each tubule is given a score from 1 to 10. No cells in the tubule section is evaluated as score 1. Complete spermatogenesis with many mature sperm cells is considered as score 10.

Immunohistochemical analysis

Caspase-3 protein expression was evaluated to determine apoptosis within testicular tissue sections. For this purpose 5 µm thick tissue sections obtained from the paraffin blocks were stained and evaluated with caspase-3 antibody (Caspase-3, Rabbit polyclonal IgG, ab2302, Abcam, London, UK) according to the previous study [18]. Histopathological scores for immunoreactivity were based on the prevalence interval, as follows: < 25%, 0.1; 26–50%, 0.4; 51–75%, 0.6; 76–100%, 0.9. Staining intensity was classified as absent (0), very low (+ 0.5), low (+1), moderate (+ 2), and severe (+ 3). The histopathological score was assessed as the prevalence × staining intensity.

Measurement of MDA level

Testes tissue samples were homogenized and analyzed for the malondialdehyde (MDA) level by using thiobarbituric acid described by Esterbauer and Cheeseman [19]. Butanol phase was taken and read at 532 nm wavelength against butanol as blank. The results were expressed as nmoles/gm tissue.

Measurement of GSH-Px and SOD activities

Enzyme activities of Glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were determined using a rat GSH-Px ELISA kit (Rel Assay Diagnostics, Gaziantep, TURKEY, LOT No: 201903 and a rat SOD ELISA kit (Rel Assay Diagnostics,

Gaziantep, TURKEY, LOT No: 201902, respectively, as described by manufacturer on an ELISA reader Bio-Tek ELX800 ELISA (BioTek Instruments, USA) at 450 nm wavelength. Results were expressed as ng/ml.

Statistical analysis

Statistical analyses were performed using the SPSS 15.0 version (SPSS Inc., Chicago, IL). The normal distributions of clinical findings were evaluated by the Kolmogorov-Smirnov test. Levene's statistic was used for the homogeneity test of variances. The one-way analysis of variance (ANOVA) was used for group comparisons of clinical findings (initial body weight and final body weight), biochemical findings (MDA, SOD, GSH-Px) and histopathological findings (tubules diameter, epithelium height, JTBS values and caspase-3 immunoreactivity). Then, Tukey's pair-wise multiple comparison test was used to determine the intergroup differences between the significant variables. The results were presented as mean ± SD. The level of significance was accepted to be at least $p < 0.05$.

Results

Clinical findings

Body weight values which were taken before and after the treatment are shown in Table 1. Although there were no deaths in any group at the end of 15 days, when compared the initial and final body weights of rats in all groups, the final body weights of the VD group was higher than the control group in a significant manner ($p < 0.05$). On the other hand, MTX group revealed significantly decreased final body weight compared to the control group ($p < 0.05$). VD, when combined with DOX, nonsignificantly reduced final body weight ($p > 0.05$).

Table 1. Data of investigated body weights in experimental groups

GROUPS (n = 7)	Initial body weight (gr)	Final body weight (gr)
CONTROL	215.1 ± 10.3	215.1b ± 11.6
MTX	217.6 ± 9.5	194.6c ± 12.5
MTX+VD	217.4 ± 10.7	203.4c ± 16.9
VD	219.5 ± 13.4	235.5a ± 18.1
P* Value	0.932	< 0.0001

abc Means within the same column with differing superscripts are significantly different ($p < 0.05$, Tukey's test). *One Way Anova

Histopathological findings

On histopathological evaluation (Figure 1), comparison between the control and VD groups revealed no changes in the testicular tissue ($p > 0.05$). Testicular tissues from MTX treatment group exhibited degeneration of spermatogenic cells, desquamation of the epithelial cells into the lumen of the tubules, lack of spermatozoa in tubule lumen, and edema at interstitial area compared with the control group (Figure 1). In addition, the MTX group had significantly lower tubule height and diameter values along with lower JTBS compared with the control group ($p < 0.05$) (Figure 2). In comparison with the MTX group, all of the histopathological findings reversed including tubule diameter, tubule height and JTBS values in the MTX+VD group ($p < 0.05$) (Figure 2).

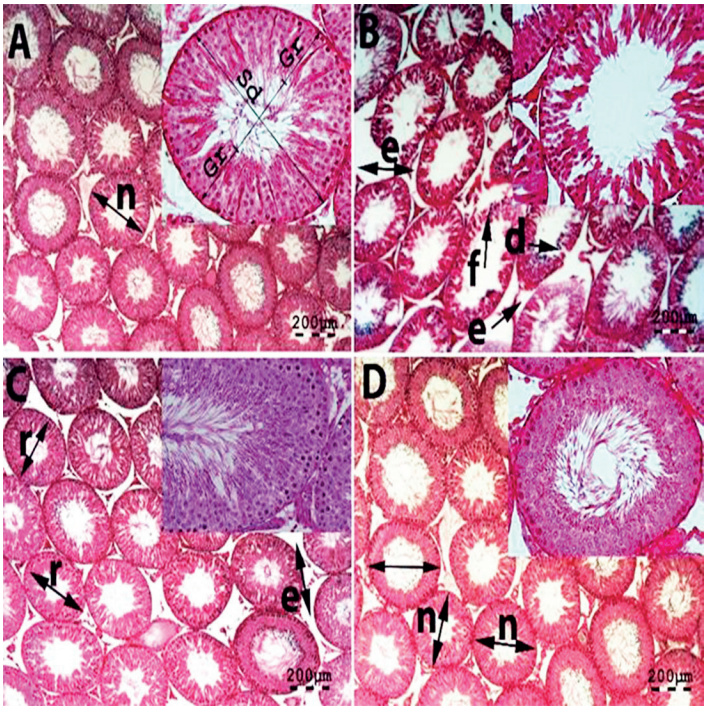

Figure 1. HE stained testes tissue. Arrows pointing events; n: normal tubulus seminiferus contortus, e: edema at interstitial area, d: degenerated spermatogenic cells, f: desquamation, r: recovered tubulus seminiferus contortus, Gr: germinal epithelium height, Sd: Tubules diameter. A-Control Group: normal histological view of testis. B-MTX Group: obvious degeneration and edema, C-MTX+VD Group: diminished degeneration and edema, D-VD group: Normal testicular appearance. Scale bar represents 200 µm.

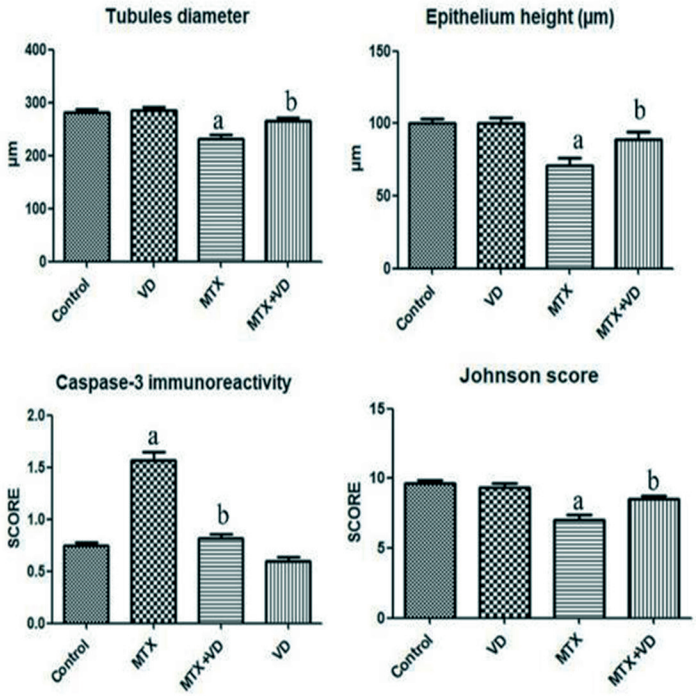

Figure 2. The tubular diameter, epithelium height, caspase-3 level and JTBS of experimental groups. (n = 7 for each group, ap < 0.05 compared with Control; bp < 0.05 compared with MTX group)

Immunohistochemical findings for cleaved caspase-3

Caspase-3 immunoreactivity was observed in the seminiferous tubule epithelium as shown in Figure 3. Upon intergroup comparison, similar caspase-3 positivity was observed at testicular tissues from the control and the VD groups ($p > 0.05$). On the other hand, the statistically significant increase in caspase-3 positivity in the MTX group was remarkable compared with the control group ($p < 0.05$). In contrast to MTX-treated group, positivity of cleaved caspase-3 decreased in the MTX+VD group in a significant manner ($p < 0.05$) (Figure 2).

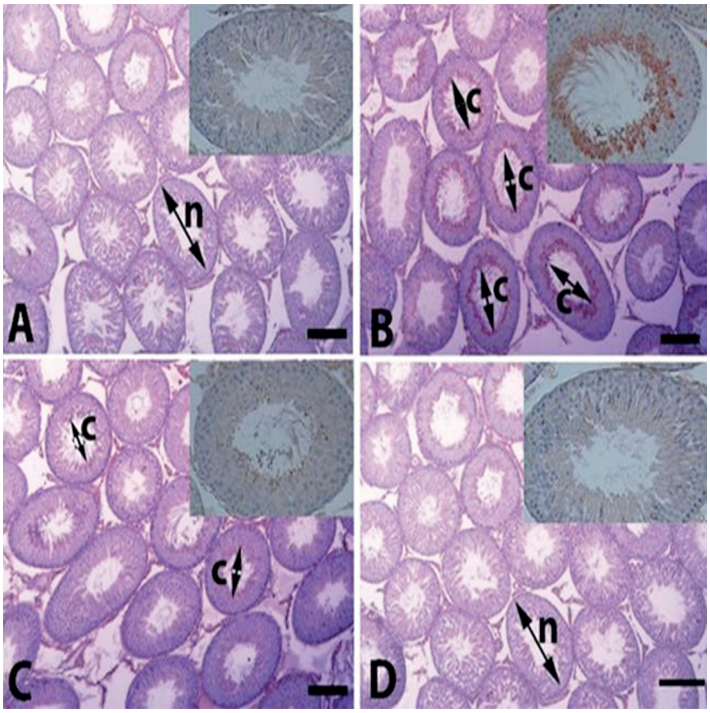

Figure 3. Immunohistochemical staining for caspase-3. Arrows pointing events; n: normal tubulus seminifereus contortus, c: caspase-3 positivity in the spermatogenic cells. A-Control Group: minimum or no positivity in the testis. B-MTX Group: severe positivity in the testis, C-MTX+VD Group: diminished positivity in the testis, D-VD group: Normal testicular appearance. Scale bar was 200 µm.

Biochemical Findings

Effects of MTX and VD on MDA level

The control and the VD groups exhibited similar MDA activities ($p > 0.05$) while significant increase in MDA level was observed in MTX-treated group versus the control group ($p < 0.05$). On the other hand, MDA level decreased significantly in the MTX+VD group as compared with that in the MTX-treated group ($p < 0.05$) (Table 2). These data was consistent with the histopathological and immunohistochemical findings.

Effects of MTX and VD on SOD activity

SOD activity was observed to be lower in control group than VD group in serum, but observed to be higher in testes tissue. Activity of SOD significantly decreased in MTX group compared to control group both in serum and testes tissues ($p < 0.05$) while significantly increased in MTX+VD group in comparison with the MTX group ($p < 0.05$) (Table 2).

Effects of MTX and VD on GSH-Px activity

It was demonstrated that GSH-Px exhibited similar activity both in control and VD groups ($p > 0.05$) while significantly decreased in MTX group compared to the control in serum and testes tissues ($p < 0.05$). On the other hand, in MTX+VD group, GSH-Px enzyme activity significantly increased when compared to the serum and testicular tissues from MTX group ($p < 0.05$) (Table 2).

Discussion

MTX is commonly used for chemotherapy [20]. Despite its usage in the treatment of cancer, its side effects limit the use of this anti-neoplastic agent [6]. MTX has been reported the most commonly studied chemotherapeutic drug in terms of gonadal toxicity in laboratory animals [21]. In the present study a single 20mg/kg dose of MTX administration caused oxidative stress in testicular tissues in accordance with the previous data [3, 6, 22]

Table 2. Statistical results on testicular tissue and serum activities of SOD, and GSH-Px and testicular tissue of MDA levels of experimental groups

GROUPS (n = 7)	Serum SOD (ng/ml)	Serum GSH-Px (ng/ml)	Testis SOD (umol/g)	Testis GSH-Px (umol/g)	Tissue MDA
CONTROL	$4.951^{b}\pm0.320$	$0.149^{a}\pm0.013$	$247.91^{b}\pm32.0$	$180.9^{b}\pm25.3$	$11.520^{b}\pm0.560$
MTX	$2.837^{d}\pm0.490$	$0.071^{c}\pm0.020$	$229.0^{a}\pm19.4$	$97.3^{a}\pm12.8$	$18.761^{a}\pm1.236$
MTX+VD	$3.890^{c}\pm0.220$	$0.104^{b}\pm0.018$	$249.8^{b}\pm15.2$	$176.2^{b}\pm28.1$	$12.097^{b}\pm0.909$
VD	$5.927^{a}\pm0.206$	$0.160^{a}\pm0.012$	$244.1^{a}\pm20.6$	$182.6^{b}\pm10.1$	$11.897^{b}\pm1.170$
P* value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

^{abc} Means within the same column with differing superscripts are significantly different ($p < 0.05$, Tukey's test). *One Way ANOVA

defined as degeneration of the seminiferous tubular epithelium, desquamation of the epithelial cells in to the lumen of the tubules, lack of spermatozoa in tubule lumen, and edema at interstitial area which are similar with the findings of the previous studies [6, 21]. In addition, a statistically significant decrease in height and diameter of the tubules along with JTBS values were observed in line with the previous reports [14, 23, 24]. It has been noted that exposure to MTX causes reduction of spermatogenesis [5]. This may be due to high mitotic index of the germinal epithelium in testicular tissue and thus vulnerable to destructive effects of

cytotoxic drugs. Based on the data presented here, when combined with MTX, VD treatment reduced the MTX-induced cytotoxicity. Therefore, findings of this study provide an evidence for the therapeutic utility of VD in MTX-induced cytotoxicity.

It is well known that ROS and oxidative stress play a key role in apoptosis [25]. Anticancer agents such as MTX [1] induces apoptosis in germ cells, which may result in a significant reduction of spermatogenic cells and also cause germ cell toxicity in testicular tissue. Caspase-3 is known as an important means of apoptosis in mammalian cells [26]. In the current study, testicular tissues in MTX group revealed significantly increased cleaved caspase-3 activity via oxidative stress in agreement with the previous literatures [27, 28].

The end product of lipid peroxidation, MDA, is a highly toxic substance. It represents an oxidative stress marker and is an indicator of tissue injury [20]. In the present study MTX treatment significantly increased MDA levels indicating the presence of oxidative stress as supported by previous publications [20, 27]. Whereas, MTX+VD group exhibited significantly lower MDA levels compared to MTX group as VD is a potent anti-oxidant that has a role in balancing mitochondrial activities, preventing oxidative stress, lipid peroxidation and DNA damage [7].

Excessive levels of reactive oxygen radicals cause abnormal sperm formation and infertility [27]. Therefore, protection of germinal cells against MTX is a significant clinical problem. Testicular tissue possesses different antioxidant enzymes, and free radical scavengers for protecting itself against cytotoxicities [24] and sperm against ROS [29]. SOD, one of the major antioxidant enzymes, protects male reproductive organs against the damaging effects of ROS [30] and has an important role in spermatogenesis. Changes in SOD activity may cause testicular dysfunction [31]. Similarly, GSH and GPx play a crucial role in the scavenging of free radicals in oxidative stress [32]. MTX treatment reduces SOD, CAT and GP-x activities [27] in testis. These findings support the notion that MTX accumulation in testis causes damage by oxidative stress as MTX decreases effectiveness of antioxidant enzyme system [22]. In line with these findings, in the current study MTX administration significantly decreased both SOD, and GSH-Px enzyme activities that had an important value in evaluation of oxidant/antioxidant balance. Reduced antioxidant enzymatic activities might lead to oxidative stress in the cells as resynthesize mechanism is impaired [27]. If the efficiency of the antioxidant enzyme defense system is significantly reduced, cells become vulnerable to ROS-induced damage [33] as observed in this study. On the other hand, when combined with MTX, VD upregulated the activities of SOD and GSH -Px significantly compared to MTX group.

Protection of germinal cells is an important clinical problem in patients receiving chemotherapeutic treatment [6]. Meanwhile, it was reported that VD has antioxidant potential that has an important role in protecting normal cell membranes against oxidative damage induced free radicals as this lipophilic compound place in the cell membranes to prevent lipid peroxidation [10]. Luong et al. reported that 1, 25 (OH) 2D3 appears to play a role in the prevention of diabetes in early age and/or healing of the disease rather than treating [34]. Vitamin D-deficient rats have deficient spermatogenesis and degenerative changes in testicular tissue [35]. 1, 25 (OH) 2D3 has been addressed to mediate increasing of cell cycle regulators in vitro and in vivo [36]. The endogenous VD has been shown to regulate vital functions in testis via suppression of inflammation and oxidative stress [37]. Normal semen conventional values (morphology, sperm count, and motility) have a positive association with VD status. Furthermore, it has been reported that VD deficiency causes important gonadal insufficiency that leads reproductive dysfunction such as deteriorated spermatogenesis, diminished sperm count and motility [38] and increased apoptosis of spermatogenic cells [25].

Previously ameliorative, anti-oxidative and anti-inflammatory activities of VD on lead-induced toxicity model in rats were reported [39]. In addition VD was shown to exert restorative and anti-apoptotic effects on diabetic rat testicular tissue [11], also a protective effect on alloxan-induced testis injury via suppressing oxidative stress, cellular toxicity and maintaining the spermatozoids count and motility [40]. In the current study in accordance with the previous data, in comparison with the MTX group, testis restoration of VD was observed with significant increases in anti-oxidative markers in MTX+VD group. Moreover, the VD when combined with MTX significantly down regulated apoptotic marker caspase-3 and MDA, also improved histopathology of testis.

Conclusion

In conclusion, VD supplement significantly reverses the MTX-induced cytotoxicities and might be utilized in the clinical setting to protect testicular tissue against chemotherapeutic toxicities primarily caused by MTX therapy

Conflict of interests
The authors declare that they have no competing interests.

Financial Disclosure
All authors declare no financial support.

Ethical approval
The current study was approved by the Kahramanmaras Sutcu Imam University Faculty of Medicine Animal Experiments Local Ethics Committee (2019/04).

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