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·*Original Article*·

Protective effects of vitamin E on ethane dimethane sulfonate-induced testicular toxicity in rats

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Abstract

Aim: To evaluate the protective/ameliorative effects of vitamin E (vit E) on ethane dimethane sulfonate (EDS)-induced testicular toxicity in rats. **Methods:** The rats were assigned to eight groups, seven rats in each, and were injected intraperitoneally with vehicle, a single dose of ethane dimethane sulfonate (EDS) (75 mg/kg bodyweight), vit E (100 mg/kg bodyweight) or EDS + vit E for 3–7 days. Thereafter, the rats were weighed, anaesthetized with ether and killed by cervical dislocation. The left testis weights were recorded and the relative testis weights were calculated. The left testes were processed for routine paraffin embedding. Three right testes from each group were taken randomly and then processed for routine electron microscopy. Tissue sections were examined using light and electron microscopy, and were scored for histopathological changes. **Results:** Vit E coadministration did not prevent the bodyweight loss on days 3 and 7. However, vit E administration prevented the EDS-induced testicular-weight loss in rats that received vit E for 3 days but not 7 days. The relative testis weight was higher on day 3 (instead of on day 7) than other groups. Nevertheless, the testis histology was not markedly protected by vit E in the EDS-treated rats. Detailed microscopic assessment showed few Leydig cells and abundant fibroblast-like cells indicating only some protection. **Conclusion:** Vit E cotreatment showed partial protective effects on the testicular weight and testicular histology in rats that received EDS. *(Asian J Androl 2007 Jan; 9: 117–124)*

Keywords: vitamin E; ethane dimethane sulfonate; toxicity protection; testis; testicular toxicity; rats

1 Introduction

Ethane dimethane sulfonate (EDS) is a well-known alkylating agent used as a model to understand selective Leydig cell toxicity and testicular dysfunction in various animal species [1]. EDS is not commercially available but can be synthesized according to the method used by Jackson and Jackson [2]. Several studies have shown that EDS specifically and temporarily destroys Leydig cells during the first week of its administration in the adult testes, reduces serum testosterone levels, elevates pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), impairs spermatogenesis, and causes severe morphologic alterations of the testicular germinal and interstitial compartments [3, 4]. All these occur after only a single dose of EDS injection. The effect of EDS on spermatogenesis appears within

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the first week after its injection and it leads to a stage-dependent degeneration of germ cells within the seminiferous epithelium [5].

Vitamin E (vit E), especially important for normal reproduction, was originally considered a dietary factor for animal nutrition [6]. The requirement for vit E for normal testicular function is well established [7]. The function of vit E has been attributed to its capacity to protect the organism against free-radical attacks by acting as a lipid-based radical chain-breaking molecule. Studies have shown the protective/ameliorative effects of vit E against the reproductive toxicity of various toxicants. For instance, vit E treatment is shown to ameliorate aflatoxin-induced changes in the testis of mice [8] and the supplementation of vit E in CrO_3-injected mice partially prevents the incidence of abnormal sperm production and increases the sperm count [9]. In addition, vit E co-treatment has a protective role against mercury-induced reproductive toxicity in male mice [10]. Moreover, vit E has anti-alkylating properties and protects cells against peroxynitrite-induced lipid oxidation [11]. These data support the concept that the administration of vit E would also have a beneficial effect on EDS-induced testicular toxicity in rats.

To our knowledge, there is no information regarding the effect of vit E on the testicular toxicity of EDS in rats or other mammals. Furthermore, precise action of vit E is not fully elucidated and the interaction between vit E and testicular cells still requires further study. We therefore designed the present study to investigate the possibility that the administration of vit E would also have a beneficial effect on EDS-induced testicular toxicity, particularly in Leydig cells in adult male rats.

2 Materials and methods

2.1 Animals

A total of 56 adult male Sprague–Dawley rats (7–9 weeks of age, weighing 220–300 g at the end of the experiment) were obtained from the Medical and Surgical Research Center (TICAM) of Eskisehir Osmangazi University, Eskisehir, Turkey. The animals were housed one per cage under standard temperature (24 ± 2°C), humidity (55 ± 5%) and lighting (12 h:12h Light: Dark) conditions. Food (Oguzlar Yem Fb., Eskisehir, Turkey) and water were supplied *ad libitum*. The health status of the rats was monitored daily and following a 2-week acclimatization period to the room, the animals were randomly divided into eight groups with seven animals in each. The handling and treatment of the rats were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the National Academy Press, Washington D.C., 1996.

2.2 Experimental design

Animal groups and their treatments are summarized in Table 1. In brief, the effect of the vit E injection was studied for 3 and 7 days. Control groups received dimethyl sulphoxide (DMSO); EDS groups received EDS on day 0 for 3- and 7-day studies. Vit E groups received daily vit E injection for 3 or 7 days. EDS + vit E groups received a single dose of EDS on day 0 and a daily vit E injection for 3 or 7 days. The animals in all groups were killed on day 3 or 7.

2.3 Chemicals

Vit E in the form of dl-α-tocopherol acetate (Evigen) was obtained from Aksu Farma, Istanbul, Turkey. Of the raw materials required for EDS synthesis, methanesulphonyl chloride and ethylene glycol were purchased from Merck-Schuchardt Chemical Co. (Darmstadt, Germany); pyri-

Table 1. Animal groups and their treatment design (n = 7 rats per group). DMSO, dimethyl sulphoxide; EDS, ethane dimethane sulfonate; vit E, vitamin E; X, the day when the rats were killed.

Groups	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control-3	DMSO			X				
EDS-3	EDS			X				
Vit E-3	Vit E	vit E	vit E	X				
(EDS + vit E)-3	EDS + vit E	vit E	vit E	X				
Control-7	DMSO							X
EDS-7	EDS							X
Vit E-7	Vit E	vit E	vit E	vit E	vit E	vit E	vit E	X
(EDS + vit E)-7	EDS + vit E	vit E	vit E	vit E	vit E	vit E	vit E	X

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dine and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4 Vehicle treatment

DMSO diluted with water at 1:3 ratios was used as vehicle in the present experiment.

2.5 EDS treatment

EDS purity was checked using a Bruker DPX-400, 400 MHz High Performance Digital FT-NMR nuclear magnetic resonance spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) and was found to be over 99%. The rats received a single intraperitoneal injection of EDS at the dose of 75 mg/kg bodyweight, based on a previous report [4].

2.6 Vit E treatment

The effective dose of vit E was based on a previous report [12]. The rats were injected intraperitoneally with vit E dose at 100 mg/kg bodyweight. The rats in the (EDS + vit E)-treated groups received the first dose of vit E, 30 min before EDS injection.

2.7 Testis and body weights

At the end of the experimental period, having taken the bodyweights of the rats, the animals were deeply anaesthetized with ether and killed by cervical dislocation. The left testes were then removed and weighed. Finally, the relative testis weights were calculated by dividing left testis weight by body weight and then multiplying it by 100 (left testis weight/bodyweight × 100).

2.8 Tissue processing for light microscopy

After removal, the left testes were fixed in Bouin's solution and processed for routine paraffin embedding. A total of 20 cross-sections (5 μm thick) were cut from different depths of each testis, and the sections were stained with hematoxylin and eosin (HE).

2.9 Electron microscopy

Three right testes from each group were taken randomly and processed for routine araldite embedding. Semithin sections (1 μm thick) were cut, stained with 1% toluidine blue (TB) and examined using light microscopy. Thereafter, thin sections were cut and stained with lead citrate and uranyl acetate, and examined using a JEOL JEM 1220 transmission electron microscope (Jeol, Tokyo, Japan).

2.10 Assessment of the histopathology

Histological evaluations were carried out for sections stained with HE or TB using an Olympus BH-2 laboratory microscope (Olympus Corp., Tokyo, Japan). All of the light-microscopic images were captured using a high resolution Olympus DP 70 digital camera (Olympus, Tokyo, Japan). We used a modified scoring system for a semi-quantitative measurement of histopathological changes in the testis. Our criteria for assessing testis histopathology were the level of Leydig cell and seminiferous tubule destruction, and the increase in the number of macrophages and fibroblast-like cells (regarded as a source of new Leydig cells). Double-blind analysis was carried out by two independent observers on all samples to determine the degree of histological changes. All the interstitial areas and seminiferous tubules in each cross-section (20 sections in total) were scored under 40 × objective. A score point for each criterion was given as following: 0, absent; 1, mild; 2, moderate; and 3, severe. Furthermore, the mean score points for each criterion of each animal were calculated from 20 score points (data not showed). The fractional numbers were rounded to the nearest exact number. Finally, all mean score points for each criterion were added to obtain the total histopathological score (THS) points for individual rats.

2.11 Statistical analysis

The calculations and statistical analyses were carried out using with the Statistical Package for Social Sciences (SPSS) for Windows version 11.5 software (SPSS, Chicago, IL, USA). The data were expressed as mean ± SEM. $P < 0.05$ was considered statistically significant. In the first place, the normality for the data on weights was tested using one sample Kolmogorov–Smirnov test. Thereafter, the homogeneity of variances for the data on weights was tested using Levene's test. It was found that body and relative testis weight values were not fit to the normal distribution. Thus, the bodyweight data were transformed using log method and relative testis weight data were transformed using arcsine method. Because the normal distribution was not achieved after the data transformations, the non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) test was used for analysis of the body and relative testis weights. When $P < 0.05$, we tested all pairs of the groups using two-tailed Mann–Whitney test. Because the values of the testis weight fitted a normal distribution, the parametric ANOVA test was carried out,

and then all groups were compared using post-hoc Tukey's honest significant difference (HSD) test. Data of the THS were analyzed using the Kruskal–Wallis ANOVA test followed by two group comparisons using the two-tailed Mann–Whitney test.

3 Results

3.1 Body and testis weights

The comparisons of the body, testis and relative testis weights (representing the ratio between the mean body and testicular weights) are summarized in Table 2. In brief, EDS treatment for 3 days caused a significant loss in the mean body ($P < 0.05$) and testicular ($P < 0.05$) weights compared with the control groups; however, EDS treatment for 7 days did not decrease the mean bodyweight ($P > 0.05$) but lowered the testicular weight only ($P < 0.05$). Because the body and testicular weights decreased together, the relative testis weight did not change. Therefore, the relative testis weight was not different from the control groups for 3- and 7- day studies ($P > 0.05$). Nonetheless, the lowest relative testis weights were seen in EDS-treated groups.

The mean values of the body, testis and relative testis weights in the vit E-treated groups were not significantly different from those in the control groups on days 3 and 7 ($P < 0.05$).

In the (EDS + vit E)-treated groups, the body weights were reduced significantly on days 3 and 7 ($P < 0.05$). The testicular weight in this group was preserved and similar to the control group on day 3 ($P > 0.05$), whereas the testicular weight was reduced significantly on day 7 ($P < 0.05$). On day 3, the mean relative testis weight increased significantly ($P < 0.05$) in the (EDS + vit E)-treated group compared with that in the control group. Because the vit E co-administered with EDS for 7 days lowered the body and testicular weights together, the mean relative testis weight did not differ from the control group ($P > 0.05$).

When the EDS and vit E-treated groups were compared on day 3, there was no significant difference among the body, testis and relative testis weights ($P > 0.05$). By contrast, the mean testis weight ($P < 0.05$) and relative testis weight ($P < 0.05$) were significantly higher in the vit E-treated group on day 7.

On day 3, the mean body, testis and relative testis weights were significantly higher in the vit E coadministered group ($P < 0.05$). Interestingly, although an increase in the testis and relative testis weights in the vit E-coadministered group occurred on day 7, it was not statistically significant ($P > 0.05$) compared with the EDS-treated group.

Moreover, when vit E- and (EDS + vit E)-treated groups were compared on day 3, the (EDS + vit E)-treated group showed a significant ($P < 0.05$) increase in the mean relative testis weight. In contrast, even though the mean relative testis weight did not change on day 7, the body and testis weights decreased significantly ($P < 0.05$) in the (EDS + vit E)-treated group.

3.2 THS points

A comparison of the mean THS is summarized in Table 2. The EDS treatment caused a marked increase on the mean THS, compared with the controls and vit E-treated rats on days 3 and 7 ($P < 0.05$). However, although a reduction of the mean THS in the (EDS + vit E)-treated rats occurred (particularly on day 3), this reduction was

Table 2. The weights and total histopathology score points of control and experimental rats. Data are mean ± SEM, n=7 rats per group. EDS, ethane dimethane sulfonate; NP, no pathology was observed; vit E, vitamin E. [a]$P < 0.05$ *vs.* control-3, [b]$P < 0.05$ *vs.* EDS-3, [c]$P < 0.05$ *vs.* control-7, [d]$P < 0.05$ *vs.* vit E-7, [e]$P < 0.05$ *vs.* vit E-3, [f]$P < 0.05$ *vs.* (EDS + vit E)-3 groups.

Groups	Body weights (g)	Testis weights (g)	Relative testis weights (%)	Total histopathology score points
Control-3	267.14 ± 2.86	1.458 ± 0.028	0.547 ± 0.006	NP
EDS-3	240.00 ± 8.73[a]	1.249 ± 0.024[a]	0.523 ± 0.015	8.29 ± 0.36[a,e]
Vit E-3	262.86 ± 10.17	1.386 ± 0.046[d]	0.530 ± 0.024	NP
(EDS + vit E)-3	248.57 ± 2.61[a,b]	1.526 ± 0.044[b]	0.611 ± 0.016[a,b,e]	7.43 ± 0.57[a,e]
Control-7	285.71 ± 2.97[a]	1.572 ± 0.027	0.551 ± 0.010	NP
EDS-7	264.29 ± 11.92	1.343 ± 0.068[c,d]	0.509 ± 0.016[d]	10.86 ± 0.34[b,c,d]
Vit E-7	280.00 ± 3.78	1.622 ± 0.046	0.580 ± 0.020	NP
(EDS + vit E)-7	242.86 ± 5.65[c,d]	1.353 ± 0.035[c,d]	0.560 ± 0.023	10.29 ± 0.61[c,d,f]

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not significantly different from the EDS-treated rats ($P > 0.05$). In addition, the THS of the EDS and (EDS + vit E)-treated groups on day 3 were significantly lower than that of the same groups on day 7 ($P < 0.05$).

Overall, vit E coadministration did not prevent or ameliorate the EDS-induced degenerations in testicular cells according to our histopathological scoring system.

3.3 Detailed microscopic examination

The vehicle (DMSO)-treated control rats showed the typical morphological organization of the adult rat testis. The testicular interstitium contained Leydig cells, macrophages and fibroblast-like cells. In addition, analysis of the epithelium of the seminiferous tubules revealed the normal architecture seen in adult rats.

The morphology of the testicular interstitium and seminiferous tubules after EDS treatment has been extensively described elsewhere [3, 4] and our observations in the present study were consistent with their results and further supported their findings (Figures 1A and 1B). Since the impact of the vit E co-treatment on the testicular histology has not been studied previously, we focused here on the effect of the vit E co-treatment on the testicular histology.

In the vit E-treated rats, the morphology of the interstitial tissue (Figure 1C) and seminiferous tubules (Figure 1D) was not evidently different from the vehicle-treated control rat testis. The most obvious morphologic feature of the vit E-treated rats was the presence of large numbers of adult-type Leydig cells that filled the interstitial areas and showed mitosis. In addition, few apoptotic cells were seen in some of the seminiferous tubules.

In the (EDS + vit E)-treated rats, the seminiferous tubules (Figure 2A) and Leydig cells (Figure 2B) were not markedly preserved. Some dead Leydig cells and few normal Leydig cells were still present in the perivascular and peritubular regions of the interstitium (Figure 2C). However, the spermatogonia, primary spermatocytes and round/late spermatids were degenerated. Macrophages and, particularly, fusiform fibroblast-like cells

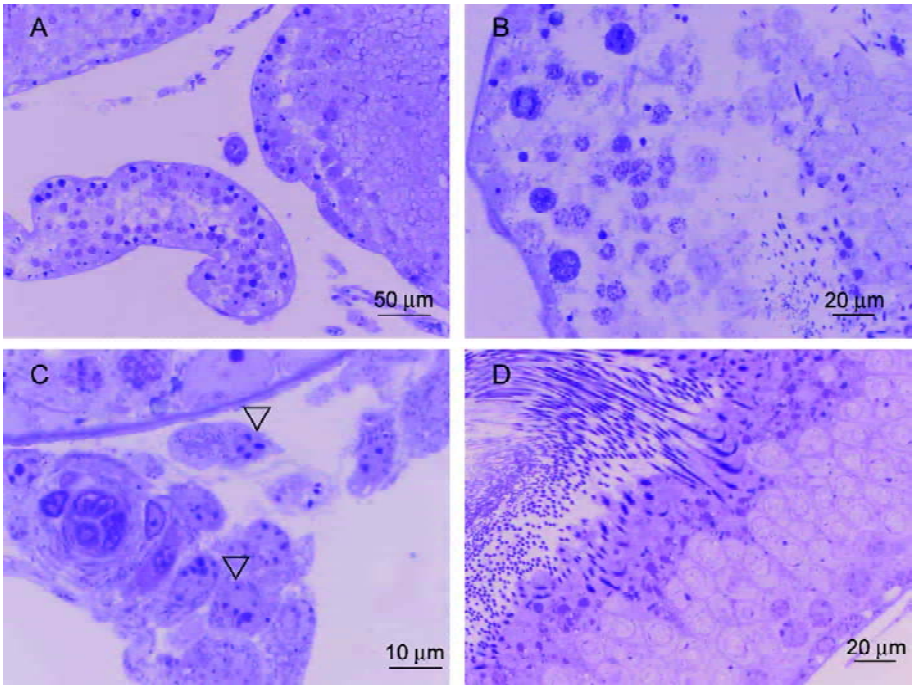

Figure 1. Representative light micrographs showing testicular histology. (A): Testis taken from the rats 3 days after EDS-treatment, showing marked destruction of the germ cells and a reduction in the diameter of the seminiferous tubule. Note that the interstitial areas contain no Leydig cells. (B): A seminiferous tubule of an EDS-treated rat after 7 days, showing complete degeneration and disorganization of the germ cells. (C): An interstitial area of a vitamin E-treated rat after 3 days, showing Leydig cells with mitotic figures in their nuclei (▽). (D): A seminiferous tubule of a vitamin E-treated rat after 7 days, showing normal epithelium with a regular arrangement of germinal cells. All sections were stained with toluidine blue.

were abundant (Figure 2D).

Our electron microscopic observations were consistent with our light-microscopic examinations.

4 Discussion

In the present study, we examined the effects of vit E treatment on testicular histology in rats that received a single dose of EDS. In our model, we treated rats with EDS because EDS destroys the Leydig cells and seminiferous tubules of the testis [3, 4].

EDS not only destroys the Leydig cells and seminiferous tubules [3, 4] but also reduces body [13] and testis weights [14] after its administration. Present results showed that vit E injection had only a partial protective effect on the testicular weight and histology of the rats after EDS treatment. Our data on body and testis weights were generally consistent with previous observations [13, 14]. The bodyweight reducing effect of EDS might be to the result of its Leydig cell destroying effect leading to the generation of little or no testosterone that has anabolic effects and stimulates bodyweight gain. However, the major factor responsible for the decreased testicular weight in the EDS-treated rats is the germ cell loss due to testosterone withdrawal and a part of this weight loss might represent a decrease in seminiferous tubular fluid production [15].

The data about the testis weight loss was interesting because while vit E administered for 3 days prevented the EDS-induced testis weightloss, its administration for 7 days failed to prevent the EDS-induced testis weight loss. Antioxidant molecules such as vit E can exhibit damaging pro-oxidant effects under certain conditions [16]. The difference in the effects of vit E on days 3 and 7 might have derived from vit E's potent antioxidant effect that prevails by day 7. Zini and Schlegel [14] re-

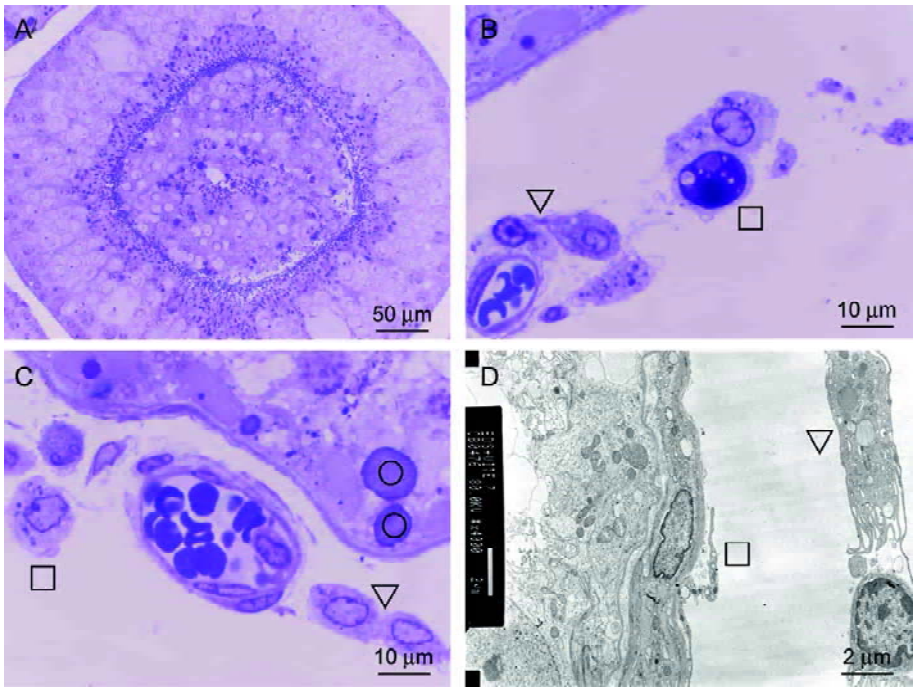

Figure 2. Representative light and electron micrographs of the testicular histology. (A): The testis of an EDS + vitamin E-treated rat after 3 days, showing sloughing and displacement of germ cells and numerous extra cellular vesicles. Note that there is less damage to the seminiferous tubule compared with EDS-treated rats. (B): An interstitial area of an EDS + vitamin E-treated rat after 7 days, showing a macrophage elongating its processes to a degenerated Leydig cell (▽) and a macrophage containing dense material in its cytoplasm (□). (C): The testis taken 7 days after EDS + vitamin E treatment, showing lipid inclusions (○) in the seminiferous tubule, and few Leydig cells (▽) and a macrophage (□) in the interstitial area. (D): Electron micrograph of the testis taken 7 days after EDS + vitamin E treatment showing a fibroblast-like cell (▽), which was numerous in this group of rats, with long processes and a myoid cell (□). All light microscopic sections were stained with toluidine blue.

ported that testicular lipid-peroxide levels in rats at 7, 14 and 21 days after EDS treatment elevate significantly; however, they did not find a significant increase in testicular lipid-peroxide level at day 3. In addition, seminiferous tubule destruction becomes progressively worse during the first weeks after EDS administration [4]. Our microscopic observations were similar to that of previous reports [4, 14]. The difference in the capacity of vit E to prevent EDS-induced testis weight loss on day 3 and 7 might result from the difference in the amount of free radicals generated by day 3 and 7. That is to say, more free radicals can occur in the testes of animals by day 7 than by day 3 and this stimulates testis weight loss. However, the precise mechanism involved in the preservation of testis weight at day 3 could not be clarified completely in our present study because we did not look at relative hormonal and biochemical factors. Furthermore, vit E can recover the reduction of testis and body weights caused by various toxic agents. For example, vitamin C (vit C) or vit E supplementation can restore decreased testicular and body weight in Aroclor 1254-treated rats [17]. Alpha-tocopherol coadministration can also recover cyclophosphamide, an alkylating agent similar to EDS, induced weight loss [18].

One of the widely used methods for the assessment of the toxic agents on the male reproductive system is microscopic examination. Our microscopic observations suggested that vit E coadministered with EDS had no marked protective/ameliorative effects on testicular histology. However, we observed some limited positive effects of vit E in the testes of the EDS-treated rats. Although no Leydig cells were observed in the EDS-treated groups, there were still some dead Leydig cells and few normal Leydig cells in the (EDS + vit E)-treated groups indicating some form of protection. Furthermore, fibroblast-like cells that are believed to be the progenitors of Leydig cells were evident in the testicular interstitiums of the (EDS + vit E)-treated rats [5]. These observations implied that vit E might have played some roles in the survival and repopulation of Leydig cells.

The effectiveness of alpha-tocopherol as a potent antioxidant has led to increasing interest in the potential use of this vitamin to prevent testicular cell damage in several pathological conditions. A previous study reported that the addition of vit E to cultures of porcine Leydig cells had little or no effect on basal testosterone production, but there was a synergistic increase in human chorionic gonadotropin hormone (hCG)-stimulated testosterone secretion [19]. The production of testosterone was threefold higher in the presence of vit E than in the absence of vit E by day 2 in the culture [19]. These data suggest that vit E can act differently under different conditions. Furthermore, in addition to Leydig cells, vit E can affect macrophage function as well. For example, vit E downregulates scavenger-receptor activity in macrophages in a dose-dependent manner [20]. Vit E also produces a significant decrease in monocyte superoxide-anion release, lipid oxidation, interleukin-1β release and adhesion to endothelium [6]. Resident macrophages in the testicular interstitium play an important role in the recovery of Leydig cells after EDS administration [21]. Furthermore, 3–10 days after EDS treatment an increase in testicular interstitial macrophage occurs and this stimulates the Leydig cell development [22]. In the present study, the elimination of dead Leydig cells from the interstitium was delayed. This might be the result of the mentioned inhibitory effect of vit E on macrophages so that dead cells remained in the testicular interstitium by day 7.

In conclusion, vit E, under our study conditions, did not completely protect the Leydig cells and seminiferous epithelium against EDS-induced toxicity; nonetheless, it did have some form of positive effects on testis. Further studies investigating biochemical and hormonal parameters and using various doses of vit E will be needed to elucidate the precise nature of the interaction between EDS and vit E.

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