

Protective effect of vitamin E on methyl methanesulfonate-induced teratozoospermia in adult Sprague-Dawley rats

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Abstract. The protective effect of vitamin E (VE, α -tocopherol) on methyl methanesulfonate (MMS)-induced teratozoospermia was investigated in adult rats. Rats (n=6 per group) were divided into three groups: i) Control group, treated with distilled water from days 1 to 5; ii) the MMS group, treated with MMS at a dose of 40 mg·kg⁻¹ from days 1-5; or iii) the VE+MMS group, treated with MMS at a dose of 40 mg·kg⁻¹ from days 1-5, followed by VE at a dose of 150 mg·kg⁻¹ from day 6 for 6 weeks. Sperm count, motility and morphology were examined following treatment with VE. The serum testosterone level and antioxidant enzyme activity were measured, and the localization of Vasa, promyelocytic leukemia zinc finger protein (Plzf) and synaptonemal complex protein 3 (Scp3) were also examined. MMS treatment decreased sperm count and motility, and the levels of immunoreactive serum testosterone and endogenous antioxidants. In addition, MMS increased the percentage of abnormal sperm and the levels of free radicals. After MMS and VE treatment, sperm count and motility were significantly higher in rats from the VE+MMS group than in the MMS group. In addition, the serum testosterone concentration, as well as the levels of Vasa and free radicals and the percentage of abnormal sperm, decreased. The results indicated that VE has protective effects against MMS-induced teratozoospermia in adult rats.

Introduction

Methyl methanesulfonate (MMS) exerts serious genotoxic effects, including induction of DNA damage, and micronuclei and cell cycle alterations. MMS, a potent alkylating agent, is a testicular toxicant that damages germ cell DNA, thereby affecting sperm morphology in mice (1) and rats (2,3). MMS also disrupts the differentiation of germ cells into sperm cells (4,5). Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological system's ability to detoxify reactive intermediates or to repair the resulting damage efficiently (6). The testis contains a high level of polyunsaturated membrane lipids; thus, it is a target of oxidative stress (7). Nevertheless, organisms have developed numerous defense mechanisms to protect themselves from damage caused by ROS. For example, antioxidants, such as VE can scavenge free radicals (8,9) and arrest the lipid peroxidation of lipoproteins that are present within biological membranes (10). VE is relatively abundant in the testis, where it protects sperm from ROS and inhibits lipid peroxidation (11). Any deficiency in VE may result in testicular damage, degenerated seminiferous tubules, and defective germ cells (12). By contrast, an increase in the level of VE has been shown to have an opposite effect, that is, to increase total sperm output in rabbits (13) and sheep (14). A previous study has shown VE to have a protective effect on pesticide-induced oxidative stress (13). Thus, the goal of this study was to investigate the protective role of VE on MMS-induced teratozoospermia in adult rats. Therefore, the effects of VE in adult rats treated with MMS was compared with the effects of treatment with MMS alone.

Materials and methods

Experimental animals. Eighteen adult male Sprague-Dawley (SD) outbred rats at 8 weeks of age and weighing 210-230 g were obtained from a closed random bred colony at the Animal Center of Nanjing Medical University. Animals were housed under standard conditions of 21±2°C and a 12 h light/dark cycle with access to food and water *ad libitum*. Animals received humane care in compliance with the guidelines of

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the National Institutes of Health. This study was approved by the Institutional Animal Care and Use Committee of Jiangsu University (Yixing, China).

Treatment of animals with MMS. Rats were randomly divided into three groups ($n=6$ per group) as follows: i) The control group, treated with distilled water from days 1 to 5; ii) The MMS group, treated with MMS at a dose of $40 \text{ mg}\cdot\text{kg}^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) from day 1-5; and iii) the VE+MMS group, treated with MMS at a dose of $40 \text{ mg}\cdot\text{kg}^{-1}$ from days 1-5, followed by VE (Sigma-Aldrich, St. Louis, MO, USA) at a dose of $150 \text{ mg}\cdot\text{kg}^{-1}$ from day 6 for 6 weeks. All experimental agents were delivered by gavage using a non-flexible stainless steel feeding tube (Sangon Biotech Co., Ltd., Shanghai, China). Thereafter, rats were anesthetized with 5% chloral hydrate (1.5 ml), and blood was collected by cardiac puncture into standard test tubes after the rats had been anaesthetized. Samples were centrifuged at $500 \times g$ for 15 min at 4°C to obtain serum and then stored at -40°C . Testes and epididymis were quickly excised and suspended in ice-cold phosphate buffered saline (PBS). One testis from one rat was fixed in 10% formaldehyde (w/v) and the other testis was stored at -80°C . Sperm count, motility and morphology of the three groups were examined following treatment with VE.

Counting of epididymal sperm. The epididymis was weighed prior to sperm counting. Epididymal spermatozooids were counted as previously described with minor modifications to the protocol (15). In brief, one of the epididymis was minced in 5 ml PBS (pH 7.4), placed on an orbital shaker (Sangon Biotech Co., Ltd.) for 10 min, and incubated at room temperature for 2 min. Semen ($25 \mu\text{l}$) was transferred to 1 ml fixative, and spermatozooids were counted under a light microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan) and using a hemocytometer (Sangon Biotech Co., Ltd.). The other epididymis was stored at -80°C .

Sperm motility. Approximately 200 spermatozooids with progressive motility from each epididymis were evaluated under a light microscope within 2-4 min after they were isolated as previously described (16).

Detection of sperm abnormalities. Fixed spermatozooids were smeared onto a slide glass, air-dried overnight and stained with the Diff-Quick kit (Baso Diagnostics, Inc., Zhuhai, China) (17). Approximately 300 spermatozooids from each treatment group were counted under a light microscope, and the percentages of morphologically abnormal spermatozooids (detached head and/or coiled tail) were recorded as previously described (1).

Measurement of the serum testosterone level. The serum testosterone level was measured as previously described (18) using a commercially available chemiluminescence-linked immunoassay (Beckman-Coulter UniCel DxI 800, Beckman-Coulter, Brea, CA, USA).

Antioxidant enzyme activity and oxidative stress assays. Different oxidative stress indicators, including free radicals

Table I. Immunohistochemical staining by comparison of the average IOD values.

Group	Vasa	Plzf	Scp3
Control	0.18 ± 0.04	0.18 ± 0.05	0.18 ± 0.06
MMS	0.12 ± 0.03^a	0.20 ± 0.04	0.17 ± 0.07
MMS+VE	0.21 ± 0.04^b	0.22 ± 0.02	0.19 ± 0.05

Data are presented as the mean \pm standard error of the mean, $n=15$. ^a $P<0.05$, compared with the control group and ^b $P<0.05$, compared with the MMS group. One way analysis of variance test. IOD, integrated optical density; Plzf, promyelocytic leukemia zinc finger protein; Scp3, synaptonemal complex protein 3; MMS, methyl methanesulfonate; VE, vitamin E.

(e.g., superoxide anion and hydroxyl radical) and antioxidants [e.g., superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)], were measured in sera from rats in each treatment group. All assays were performed according to the instructions provided in the kits (Jiancheng Bioengineering Research Institute, Nanjing, China).

Immunohistochemistry. Testes were fixed in 10% formaldehyde (w/v) for at least 24 h and then processed for paraffin embedding (19). Rabbit polyclonal anti-Vasa (cat. no. ab13840), rabbit polyclonal anti-promyelocytic leukemia zinc finger protein (Plzf; cat. no. ab39354), and rabbit polyclonal anti-synaptonemal complex protein 3 (Sycp3; cat. no. ab150292) antibodies were purchased from Abcam (Cambridge, UK). Anti-mouse IgG (H+L, alkaline conjugate) was purchased from Promega Corporation (Madison, WI, USA). Sections ($5 \mu\text{m}$) were deparaffinized in xylene, rehydrated in a gradient series of alcohol, and boiled in antigen retrieval solution (Sangon Biotech Co., Ltd.) at 95°C for 10 min. The sections were treated with 3% hydrogen peroxide (v/v) for 10 min, blocked with 3% bovine serum albumin (v/v) (Sangon Biotech Co., Ltd.) and 10% normal donkey serum (v/v) (Sangon Biotech Co., Ltd.) in Tris-buffered saline (TBS), and then incubated with antibodies overnight at 4°C . Thereafter, sections were washed with TBS and incubated with a biotinylated and streptomycin-labeled goat anti-mouse antibody (cat. no. KIT-5010; Maixin Bio, Ltd., Fujian, China) for 15 min at room temperature. Immunoreactive proteins were visualized with 3,3'-diaminobenzidine tetrahydrochloride. Sections were examined under a light microscope (Eclipse 80i; Nikon, Tokyo, Japan). The expression levels of protein were measured and demonstrated by the Integrated Optical Density (IOD) values using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. SPSS 14.0 (SPSS Inc., Chicago, IL, USA) statistical software for Windows was used. Results are presented as the mean \pm standard deviation. One-way analysis of variance (followed by Tukey's post hoc test) or Mann-Whitney U-test was used to compare the means across different treatment groups. $P<0.05$ was considered to indicate a statistically significant difference.

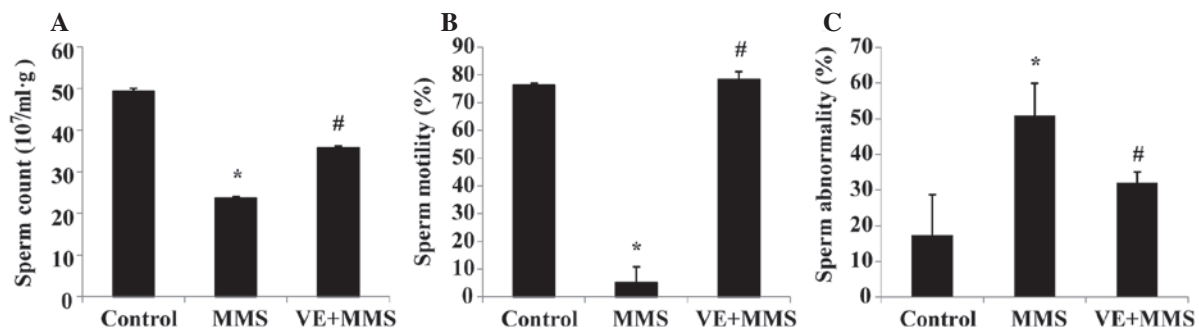


Figure 1. Effects of MMS and VE on sperm characteristics in male rats. (A) Sperm count. (B) Sperm motility. (C) Sperm abnormalities. * $P < 0.05$, compared with the control group and # $P < 0.05$, compared with the MMS group. A one way analysis of variance test was used for the statistical analysis of A, and a Mann-Whitney U test for B and C. MMS, methyl methanesulfonate; VE, vitamin E.

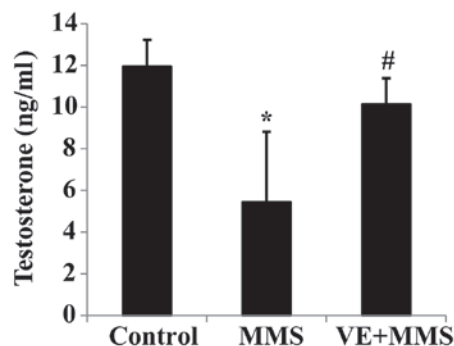


Figure 2. Effects of MMS and VE on the serum testosterone level in male rats. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the MMS group, as determined by a one way analysis of variance test. MMS, methyl methanesulfonate; VE, vitamin E.

Results

Sperm characteristics. Sperm count ($2.36 \pm 0.04 \times 10^8$) and motility ($5.0 \pm 5.8\%$) decreased significantly ($P < 0.05$) in rats from the MMS group compared with those from the control group ($4.92 \pm 0.07 \times 10^8$ and $76.3 \pm 0.7\%$, respectively). However, there was an increase in the percentage of abnormal sperm ($50.9 \pm 9.0\%$) compared with the control group ($17.3 \pm 11.4\%$). After MMS and VE treatment, sperm count ($3.57 \pm 0.04 \times 10^8$) and motility ($78.3 \pm 2.9\%$) were significantly higher ($P < 0.05$) in rats from the VE+MMS group than in the MMS group (Fig. 1A and B). However, there was a significant decrease ($P < 0.05$) in the percentage of abnormal sperm in rats from the VE+MMS group ($32.0 \pm 3.0\%$) compared with the MMS group (Fig. 1C).

Levels of serum testosterone. There was a significant decrease ($P < 0.05$) in the serum testosterone level in the MMS group (5.4 ± 3.4 ng/ml) compared with the control (11.9 ± 1.3 ng/ml) and a significant increase in the VE+MMS group (10.1 ± 1.3 ng/ml) compared with the MMS group (Fig. 2).

Serum oxidative stress indicators. The levels of hydroxyl and superoxide free radicals were significantly higher in the MMS group, as compared with the control group; and lower in the VE+MMS group, as compared with the control group

(Fig. 3A and B). By contrast, the levels of antioxidants SOD and GSH-Px, were lower in the MMS group than in the control and VE+MMS groups (Fig. 3C and D).

Immunohistochemical findings. The histo-architecture of the testes from control rats was normal, consisting of uniform, well-organized seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Fig. 4). By contrast, testes from rats treated with MMS showed seminiferous tubule degeneration manifested by shrunken, disorganized tubules with irregular, buckled basement membranes and incomplete spermatogenesis (Fig. 4). Moreover, the seminiferous tubules were virtually devoid of spermatids and sperm. After MMS+VE treatment, there was an improvement in spermatogenesis, demonstrated by the presence of elongated spermatids and sperm in the majority of the seminiferous tubules (Fig. 4).

As observed using immunohistochemistry, the level of Vasa, a biomarker of germ cells, decreased in the testis of rats treated with MMS; however, its level increased in rats treated with VE+MMS compared with those treated with MMS alone (Fig. 5). There were no changes in Plzf and Sycp3, biomarkers of spermatogonial stem cells and spermatocytes, respectively, following MMS and MMS+VE treatment (Table I).

Discussion

Previous studies have shown MMS to decrease sperm number and affect sperm head morphology in mice and rats (1-3). Although VE can protect cells from damage caused by free radicals and oxidative products, there are few studies on the protective effect of VE in animals previously exposed to MMS (8,10,12-14). The present study demonstrated that VE partially alleviated the damage caused by MMS in the testis.

Ozawa *et al* (2) demonstrated that body, testis and epididymis weights, as well as food consumption decrease following administration of MMS. Germ cell exfoliation, Sertoli cell vacuolization, and epididymal duct cell debris were also observed (2). Although VE does not protect the testes from acrylamide toxicity, treatment with VE following cessation of acrylamide treatment improves recovery (20). In the present study, MMS treatment resulted in seminiferous tubule degeneration, and VE treatment improved the recovery of spermatogenesis.

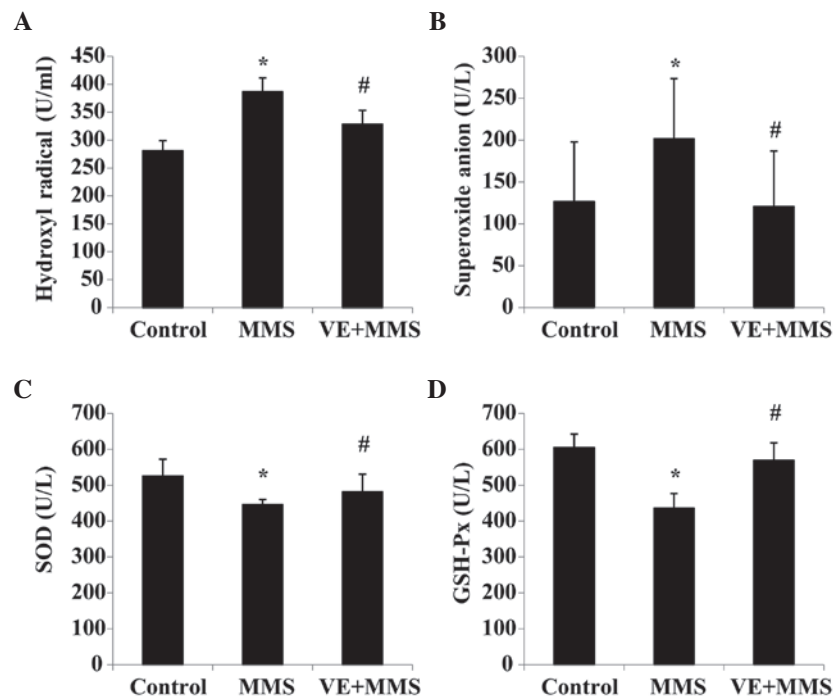


Figure 3. Effects of MMS and VE on antioxidant enzyme activities in male rats. (A) Level of the hydroxyl radical. (B) Level of the superoxide anion. (C) Level of SOD. (D) Level of GSH-Px. * $P < 0.05$, compared with the control group; # $P < 0.05$ compared with the MMS group. One way analysis of variance test. MMS, methyl methanesulfonate; VE, vitamin E; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

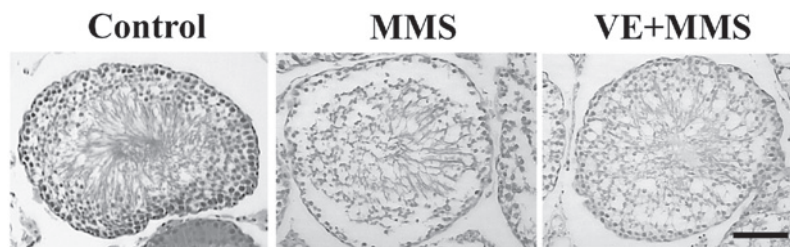


Figure 4. Structure of the testis of different groups. Scale bar, 50 μm . MMS, methyl methanesulfonate; VE, vitamin E.

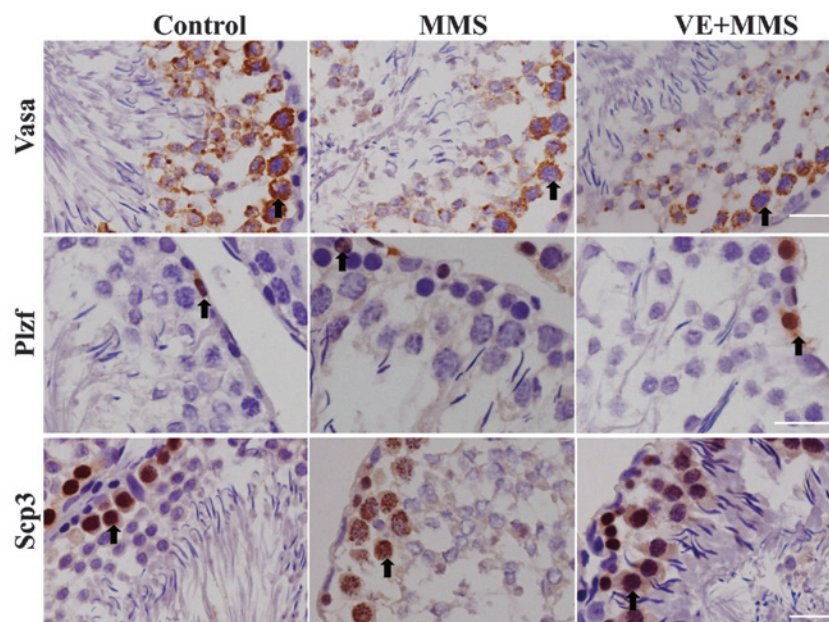


Figure 5. Immunolocalization of Vasa, Plzf, and Scp3 in the three treatment groups. Arrows point to protein localization. Scale bar, 50 μm . MMS, methyl methanesulfonate; VE, vitamin E; Plzf, promyelocytic leukemia zinc finger protein; Scp3, synaptonemal complex protein 3.

VE can protect critical cellular structures from free radical and oxidative damage (21). As a soluble, lipid-based antioxidant, VE is protective against oxidative stress by preventing the production of lipid peroxides and scavenging free radicals (11). VE is also important in maintaining the function of the testis, epididymis and accessory glands (22), possibly by improving sperm quality and quantity. For example, VE improved semen quality and quantity in humans (11), sheep (12), and chickens (22). VE has also been demonstrated to protect rat testis against experimental cryptorchidism (23,24). Furthermore, the increased free radicals generated by acrylamide exposure in the testes may have been scavenged in the testes during recovery period (20). MMS at doses of 20-40 mg/kg for two consecutive weeks increased heme oxygenase-1 (HO-1) mRNA and protein in the rat testis (25). In this study, MMS increased free radical formation, which decreased following VE treatment, while the levels of two endogenous antioxidants indicators (SOD and GSH-Px) showed an opposite tendency. These results indicate that MMS induces oxidative stress in adult rats, and VE has the capability of scavenge them.

MMS damages germ cell DNA by creating lesions within the DNA, which can disrupt the differentiation of germ cells into spermatozoa (5). This is in agreement with the results of the present study, which showed an increase in the percentage of defective spermatozooids. Moreover, the level of Vasa decreased after MMS treatment, while the levels of Plzf and Sycp3 remained unchanged, suggesting that MMS has little influence on spermatogonial stem cells and spermatocytes. Additional experiments are required to address the mechanism of MMS action in the adult testis.

In conclusion, MMS treatment of adult rats affects germ cells in the testis, and VE may aid in repairing the damage caused by oxidative stress.

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