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Original Research Article

Testicular toxicity induced by T-2 toxin and protective effect of vitamin E in Wistar rats

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Abstract

Background and purpose. *Mycotoxicosis is a very common problem in stored feed and fodder. Ingestion of T-2 toxin through feedstuff leads to serious consequences and production losses in exposed populations. That may lead to reproductive organ system damage resulting in the reproductive problem. The study was planned to investigate the effect of T-2 toxin and vitamin E on testicular function in Wistar rats.*

Material and methods. *Male Wistar rats were fed with standard rat feed. T-2 toxin treatment groups received T-2 toxin (2.5 ppm and 5.0 ppm) mixed feed, while vitamin E (100 mg/kg) was given by oral gavage alone and in T-2 toxin fed groups. After completion of 28 days of oral feeding of text compounds reproductive toxicity parameters were evaluated.*

Results. *Total live sperm counts, sperm motility, and serum testosterone levels were reduced in T-2 toxin-treated groups. Antioxidant enzymes/non-enzymes and oxidative stress indicators were altered significantly and cellular damage to testis and epididymis in T-2 toxin groups was in a dose-dependent manner. However, vitamin E intervention caused a marked reversal effect in T-2 toxin-fed rats on testicular damage and male reproductive parameters. Oxidative stress and related events appear to be a potential mechanism involved in T-2 toxin-mediated testicular damage.*

Conclusion. *This study validated that the use of an optimum dose of vitamin E has the potential to protect against the testicular damage caused by the T-2 toxin.*

INTRODUCTION

Mycotoxicosis caused by mycotoxins is the most common problem affecting animal production and reproduction. Mycotoxins are secondary metabolites produced by various fungi and are the most common source of contamination in food grains and other agricultural-related products. They are responsible for more than 25% of the world's cereal crop contamination every year with variable levels of known origin (*1,2*). Direct consequences of consumption of mycotoxin-contaminated feed are reduced feed intake, feed refusal, poor feed conversion, decreased immunity, increased disease incidences, and poor reproductive performance that lead to significant economic losses to the farmers (*1*).

Trichothecene, mycotoxins form a family of a large number of structurally related secondary metabolites which include the T-2 toxin. The scientific community paid attention to T-2 toxin because it was one of the first trichothecenes discovered in grains produced by Fusarium spo-

rotrichioides and Fusarium poae (*3*). T-2 toxin production is greater in cereal-based food and feed and their concentration ranges from 0 to 40 ppm in all over the world. The prevalence of T-2 toxicosis has been reported as 1% in Poland and, 23.5% to 31.2% in Turkey (*4,5*). In India, exact data about T-2 toxin occurrence is still not available due to variations in environmental conditions and topographic diversification. Even though, few reports indicate that T-2 toxin contamination is one of the major problems in India responsible for the reduction in animal production. The level of T-2 toxin contamination in feed has been reported by some states of India, from 34.8% (Karnataka and Maharashtra) to 52% (Punjab) (*6,7*). The main problems associated with low-level T-2 toxin consumption are nutritional deficiency, dermal toxicity, neurotoxicity, cardiotoxicity, and immunoinhibition in different species of animals. Further, it has a direct toxic effect on vital organs such as the liver, spleen, thymus, and ovary. Its exposure altered the hormonal level and precipitates the problem of male and female infertility via oxidative stress (*8–10*).

Antioxidants are molecules, which interact with free radicals and terminate oxidation reactions; prevent damage to vital organs, and could be an appropriate means to minimize the toxic manifestation caused by environmental toxicants in the mammalian system (*11,12*). Natural endo-exo-antioxidant such as vitamin E, carotenoids, and ascorbic acid showed promising antioxidant activity against free radicals generated by environmental toxicants (*13*). The protective effect of vitamin E in oxidative damage caused by environmental toxicants on organs like the intestine, brain, liver, and hemopoietic system in animals has been documented (*14,15*).

The T-2 mycotoxin exposure real field problem affecting production and reproduction in a variety of animal species is a reality. However, the effect of T-2 mycotoxin on testicular toxicity through natural means of exposure with environmental persistence level and toxicosis may be prevented by using potential antioxidant vitamin E at the most commonly recommended dose level. This study has not been undertaken by the earlier co-workers. In light of the above, the present study was designed to evaluate the degree of oxidative stress and testicular toxicity of T-2 mycotoxin and the possible ameliorative potential of vitamin E in male rats.

MATERIALS AND METHODS

Chemicals

Vitamin E (α-tocopherol acetate) was purchased from Sigma Aldrich, USA. Freeze-dried culture of *Fusarium sporotrichioides* var. sporotrichioides (NRRL-3299; Peoria, USA, and MTCC-1894; IMTECH, Chandigarh, India) was used to produce T-2 mycotoxin in the laboratory. All other chemicals used in this study were of analytical grade and purchased from standard manufacturers.

T-2 toxin production and quantification

Freeze-dried culture of Fusarium sporotrichioides var. sporotrichioides: NRRL-3299 procured from Peoria, USA and MTCC-1894 procured from Institute of Microbial Technology (IMTC), Chandigarh, India, was used to produce T-2 toxin in the laboratory. The stock culture of *Fusarium sporotrichioides* var. sporotrichioides was subcultured in 'Sabouraud's dextrose agar' media (SDA; 1% peptone, 4% glucose, and 3.5% agar in distilled water). T-2 toxin production in the laboratory was done as per the standard method on maize and wheat. Maize and wheat were partially grounded and soaked with 4% broth (1% peptone, 4% glucose, and distilled water) for 2 hours, and intact wheat grains were soaked with 60% distilled water overnight. The grain mixtures were put in flasks (500 g in 2000 mL flask) and sterilized by autoclaving at 103.4 kPa (15 lbs) pressure for 15 minutes. Loop full inoculums containing freshly grown mycelia were added to each flask under proper sterile conditions and kept in a bio-oxygen demand incubator at 16 ± 1 °C. To avoid clumping and facilitate uniform growth, the flasks were shaken properly twice a day. After the adequate quantity of fungal growth (3 weeks post-incubation), the cultures in flasks were again autoclaved at 103.4 kPa (15 lbs) for 30 minutes to destroy the mycelia and spores, and dried at 80 °C for 10 hours and grounded to a fine powder for further use. T-2 mycotoxin produced in cultured wheat and maize was analyzed and quantified by HPTLC.

Sample extraction

Autoclaved and dried cultured wheat and maize were grounded to a fine powder. The ground sample (10 g) was placed in a 50 mL flask. 25 mL ethyl acetate: acetonitrile (4:1) and 10 mL water was added. The resulting mixture was kept on shaking on an orbital shaker for the next one hour. Finally, the solution was filtered through a filter paper (Whatman No. 1) and the filtrate was collected in a clean vessel. The filtered extract was diluted with purified water (20 mL) and filtered through the glass microfibre filter.

Sample clean-up

The resulting filtrate was evaporated to dryness in a sample concentrator. The dried sample was reconstituted with n-hexane, acetonitrile, and water (2:1.5:1, respectively) and shaken with a vortex mixer. The layers had separated, and the upper n-hexane layer was removed. The aqueous layer was accurately returned to the vial and 2 mL n-hexane was added and the procedure was repeated. The aqueous acetonitrile layer was evaporated to dryness on a sample concentrator at 45 °C. The residue was dissolved in 2 mL chloroform: methanol (90:10) and passed through a florisil solid phase column (500 mg), which was been previously activated with 2.5 mL methanol followed by 2 mL chloroform: methanol (90:10). The vial was

rinsed with 5 mL chloroform: methanol (90:10). The total eluant was collected in a cleaned vial and evaporated to dryness using a sample concentrator. Finally, the resulting precipitate was dissolved in 1mL acetonitrile.

HPTLC procedure

Chromatography was performed on mini silica HPTLC gel 60 plates (Merck). Standard and test solutions were applied to the HPTLC plate using a semi-automatic plate spotter (Camag, Nanomat III, Switzerland) set to dispense along a line 12 mm from the bottom edge of the plate. Samples (2 x 2µL) were applied at 5 mm intervals, with standards (2 µL; 0.25, 0.5, 1, 2, 4 µg mL^{-1}). Plates were placed in a TLC tank containing 25 mL chloroform: acetone (9:1) and developed for 25–30 min. Further, plates were air-dried in a hot air oven. The T-2 toxin was visualized by spraying the plates 1.5% sulphuric acid in methanol. The plate was then left in a fume cupboard and transferred to a hot air oven at 100 °C for 5 to 10 min. T-2 toxin, which appeared as blue spots under long-wave UV light. Spot was quantified by UV fluorescence using an HPTLC scanning fluorodensitometer (Camag Scanner III; Anchrom Enterprises (I) Pvt. Ltd, Mumbai, India) fitted with a 400 nm emission filter and controlled by CATS 3 software.

Precision of HPTLC

The extracts spiked to the equivalent of 100, 200, 400 and 800 µg/kg with T-2 toxin were analyzed and quantified. Recovery at each level was expressed as a percentage and the overall recovery was taken as the mean of the recoveries. The extracts were spiked with T-2 toxin over a concentration range equivalent to 250 to 2500 µg/kg. The response was linear over the whole range and fitted the linear regression value of 0.995. The mean recovery from spiked extracts of T-2 toxin was 95.0%. The limit of detection (LoD) of T-2 toxin was found to be 2 µg/kg and the limit of quantification (LoQ) was 10 µg/kg.

Preparation of T-2 toxin test diet

T-2 toxin levels in analysed samples were quantified and found to be 32.8 mg/kg (ppm) and 16.5 mg/kg (ppm) in wheat and maize samples, respectively (Figure 1). This cultured crushed wheat and maize were used as a known stock for further formulation of T-2 toxin animal diet to feed the experimental animal.

Stock finely ground cultured wheat and maize containing a known amount of T-2 toxin were thoroughly mixed with standard to achieve the resultant concentration of 2.5 and 5.0 ppm levels in the experimental diet. Representative samples were again drawn in duplicate for estimation of T-2 toxin content in experimental diets to confirm the concentration and were found to be approximately 2.5 and 5.0 ppm levels, respectively.

Experimental animals and experimental design

The experiment was carried out in adult male Wistar rats weighing between 110–120 g at about 8 weeks of age. These animals were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly (India). The rats were housed in polypropylene cages for 7 days for acclimatization before commencing the experiment. The temperature was maintained at 23 ± 1 °C and 57–60% relative humidity with 12 h dark and light cycle. They provided access to standard ration and water *ad libitum* throughout the experiment. The rats were handled and the study was conducted as per the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and

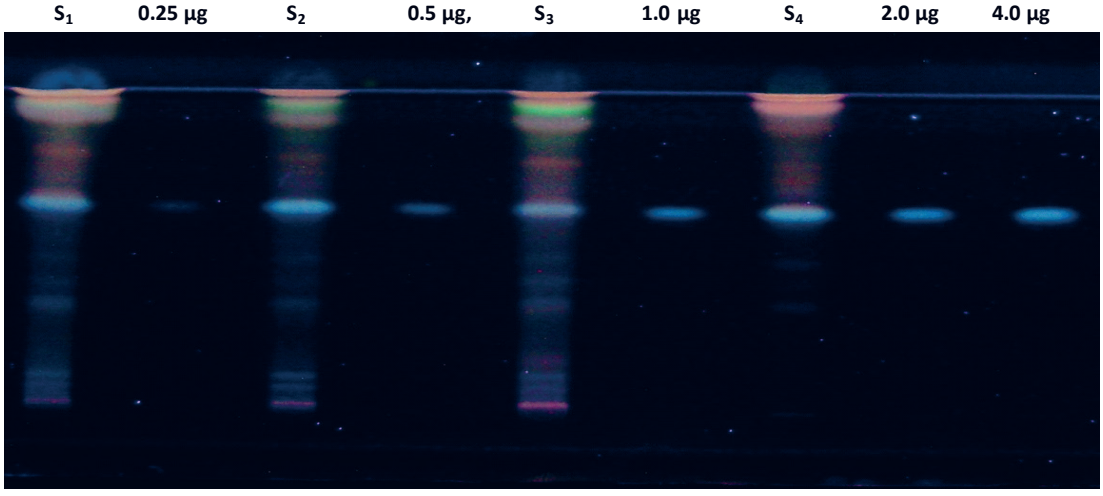

Figure 1. *Thin-layer chromatography (TLC) plate showing presence of T-2 toxin cultured ground wheat and maize samples (S1, S2, S3 & S4) against known T-2 standards (0.25, 0.50, 1.0, 2.0 and 4.0 µg).*

Supervision of Experiments on Animals (CPSCEA), Ministry of Environment, Forests and Climate Change, Government of India.

Adult male Wistar rats were divided into seven groups comprising of six animals each. Test animals received a diet consisting of T-2 toxin and vitamin E alone and in combination for 28 days. Group I served as normal control, while group II served as vehicle control (corn oil, 1 ml/kg, body weight, *p.o.*). Animals from groups III and IV were fed with the feed containing T-2 toxin @2.5 and 5 ppm, respectively. Group V received vitamin E (100 mg/kg, body weight) by oral gavage. Group VI and VII received T-2 toxin at 2.5 ppm and 5 ppm, respectively via feed plus vitamin E (100 mg/kg body weight) by oral gavage.

Blood collection

Blood was collected after 24 hours of the last dosing of drugs to obtain serum for hormonal estimation and preserved at –20 ºC until analysis. Rats were sacrificed on day 29 and organs were collected and washed with ice-cold saline. Absolute organ weights were recorded and relative organ weights were also calculated. One testis from each rat was preserved at –80 °C for oxidative stress-related parameters. The one testis and one epididymis were preserved in Bouins fixative for histopathological examination. One epididymis from each rat was used for the analysis of reproductive toxicity parameters.

Assessment of sperm parameters and serum testosterone level

Epididydimis were carefully dissected to eliminate adventitious tissues. Epididymal sperms obtained were incubated at 35 °C in Dulbecco's PBS. The spermatozoa were counted by hemocytometer using the improved Neubaur's chamber (Labart, Germany). To determine the percentage of morphologically abnormal spermatozoa, the slides were stained Eosin-Negrosin stain (1.67% eosin and 10% nigrosin in 0.1 M sodium citrate) and viewed under a light microscope at 100x magnification. A total of 200 spermatozoa were examined on each slide per animal and the head, tail, and total abnormality rates of spermatozoa were expressed as a percentage. Testosterone concentration in serum was quantified by using a commercial RIA kit (Immunotech, France).

Measurement of oxidative stress

Testis was collected after sacrifice and weighed and 10% tissue homogenate was prepared in 100 mM potassium phosphate buffer (pH 7.4). Reduced glutathione (GSH) estimation, separately tissue homogenate was prepared 0.02 M EDTA. The homogenates were centrifuged for 10 min at 3000 rpm at 4 °C. The supernatant was collected and stored at –28 °C for different biochemical estimations.

Lipid peroxidation (LPO) was measured in terms of malondialdehyde (MDA) production (*16*). Total reactive oxygen species (ROS) generation was measured in single-cell testicular cells using flow cytometry.

To measure ROS single-cell suspension was prepared as per the method described in the literature. The cell density was adjusted to $1.5x10^6$ cells/mL and the cell viability of the freshly isolated cells was determined by using 0.1% trypan blue dye exclusion test. For measurement of intracellular total ROS, the oxidation-sensitive probe 2,7-dichlorofluorescin diacetate (DCFHDA) was used, as described by previously (*17*). Finally, cells were suspended in 500 µL of 25 mM 3-amino 2,3,4-triazole in PBS and kept on ice for 15 min in the dark. Then diluted with 500 µL PBS and 50 µL DCFH-DA dye in 100 µM concentration was added and again kept for 15 min in the dark at room temperature. Flow cytometric analysis for DCF fluorescence in cells was recorded at an excitation of 485 nm and an emission setting of 530 nm. The assay was analyzed by FACScan (Becton, Dickinson and Company, India).

Antioxidant enzyme activity evaluation

The 10% testicular tissue homogenate was prepared in the ice-cold 100 mM potassium phosphate buffer (pH 7.4). The protein content was estimated by the Lowry protein assay method. Bovine serum albumin was used to prepare the standard curve. Catalase (CAT) activity was assayed as per the method described elsewhere (*18*), while, the activity of superoxide dismutase (SOD) was assayed by the method described by Madesh and Balasubramanian (*19*). Glutathione peroxidase (GPx) activity was determined using the method of Paglia and Valentine (*20*) and glutathione reductase (GR) activity was determined by a method described by earlier workers (*21*). Reduced glutathione (GSH) level was estimated in separate tissue homogenate samples prepared in a solution containing 0.02 M EDTA (*22*).

Histopathology

One testis and one epididymis from each rat were collected and fixed in Bouins fixative (saturated aqueous solution of picric acid-75 ml, formalin-25 ml and glacial acidic acid-5 ml) for initial 6 hours and then transferred into 70% alcoholic solution. Tissues were cut into 2–4 mm sizes after proper fixation and embedded in paraffin blocks. Final tissue sections were stained with hematoxylin and eosin by the standard method and examined under a light microscope.

Statistics

Statistical analysis of data was performed by using IBM SPSS software and Microsoft Excel. All the data were expressed as mean ± SE and compared by employing one-way ANOVA with Duncan multiple comparisons test. A $p<0.05$ was considered statistically significant.

RESULTS

Effect on in-life parameter

There was no mortality amongst the rats of any treatment groups throughout the exposure period. T-2 toxin alone fed groups and vitamin E co-treated groups showed variable degrees of clinical signs which included dullness, weakness, lethargy, rough hair coat, disinclination to move, and reduced feed intake, retardation in growth and body weight as compared to animals of the control group. Clinical signs of toxicity in animals treated with a higher dose of T-2 toxin appeared earlier than that in the lower dose of T-2 toxin animals.

Effect on body weight and organ weight

The mean body weight gain and percent weight gain of the rats were significantly ($p<0.05$) decreased in T-2 toxin-treated animals as compared to the control. The reduction in the body weights of the rats treated with a T-2 toxins was significantly greater ($p<0.05$) than that of the respective vitamin E co-treated groups (Figure 2).

Significant ($p<0.05$) changes were observed in absolute and relative organ weights of the liver, lungs, heart, and adrenals in rats fed T-2 toxin and vitamin E co-treated groups when compared with the control group. The absolute and relative organ weights of the spleen and thymus showed significant ($p<0.05$) changes in T-2 toxin alone treated groups in a dose-dependent manner (Tables 1 and 2) and that can be compared with that of vitamin E co-treated groups.

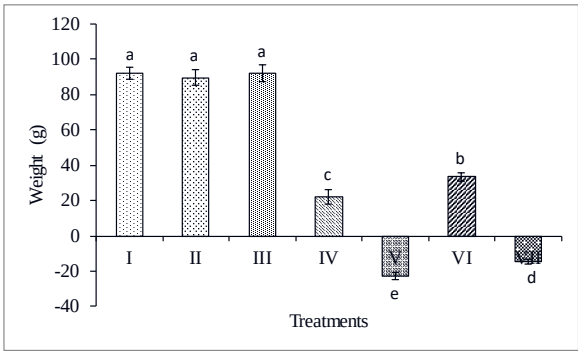

Figure 2. Body weight gain/loss (g) in rats, following exposure to T-2 toxin with and without vitamin E. *I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm); VI: T-2 (2.5 ppm)+vitamin (100 mg/kg b.w.); VII: T-2 (5.0 ppm) + vitamin E (100 mg/kg b.w.). Values are mean ± SEM (n=6). Bars bearing different superscripts vary significantly ($p<0.05$) from each other in one-way ANOVA and Duncan multiple comparison post hoc tests.*

Effect on total epididymal sperm count

The total epididymal sperm count (TESC) in rats treated with T-2 toxin alone was significantly ($p<0.05$) less as compared to the control. Vitamin E plus low dose T-2 toxin group showed significant improvement ($p<0.05$) when compared with the low dose T-2 toxin alone treated group. However, a higher dose T-2 toxin plus vitamin E co-treated groups did not show any significant increase ($p>0.05$) in TESC when compared with the higher dose T-2 toxin alone group (Table 3). The T-2 toxin alone and

Table 1. *Absolute organ weights and relative organ weights following 28-days exposure to T-2 toxin alone and in combination with vitamin-E in rats.*

Groups		Liver	Kidneys	Adrenal glands	Heart	Brain	Lungs	Spleen
I	AOW	10.80 ± 0.74^{a}	1.96 ± 0.10^{abc}	0.09 ± 0.01^{abc}	0.93 ± 0.03^{a}	1.78 ± 0.05	1.54 ± 0.05^{ab}	0.73 ± 0.04^{a}
	ROW	3.95 ± 0.19^{b}	0.72 ± 0.02^{c}	0.02 ± 0.00	0.34 ± 0.01^{c}	0.66 ± 0.02^{c}	0.57 ± 0.02^{b}	0.27 ± 0.01^{a}
II	AOW	11.00 ± 0.59^{a}	1.95 ± 0.09^{abc}	0.10 ± 0.02^{ab}	0.96 ± 0.01^{a}	1.75 ± 0.06	1.53 ± 0.07^{ab}	0.72 ± 0.05^{a}
	ROW	4.03 ± 0.17^{ab}	0.71 ± 0.02^{c}	0.02 ± 0.00	0.35 ± 0.01^{c}	0.64 ± 0.02^{c}	0.56 ± 0.02^{b}	0.26 ± 0.02^{a}
III	AOW	10.88 ± 0.65^{a}	1.97 ± 0.09^{ab}	0.10 ± 0.01^{a}	0.94 ± 0.03^{a}	1.81 ± 0.05	1.50 ± 0.05^{b}	0.74 ± 0.05^{a}
	ROW	3.99 ± 0.17^{b}	0.72 ± 0.02^{c}	0.02 ± 0.00	0.35 ± 0.01^{c}	0.67 ± 0.02^{c}	0.55 ± 0.02^{b}	0.27 ± 0.02^{a}
IV	AOW	10.58 ± 0.54^{a}	2.17 ± 0.15^{a}	0.06 ± 0.00^{cd}	0.95 ± 0.02^{a}	1.74 ± 0.02	1.70 ± 0.04^{a}	0.55 ± 0.02^{bc}
	ROW	4.78 ± 0.34^{a}	0.98 ± 0.10^{a}	0.01 ± 0.00	0.43 ± 0.01^{a}	0.78 ± 0.03^{b}	0.77 ± 0.03^{a}	0.24 ± 0.00^{ab}
V	AOW	7.80 ± 0.83^{b}	1.60 ± 0.14^{c}	0.05 ± 0.00^{d}	0.69 ± 0.06^{b}	1.64 ± 0.11	1.24 ± 0.06^{c}	0.39 ± 0.02^{d}
	ROW	4.45 ± 0.29^{ab}	0.92 ± 0.05^{ab}	0.02 ± 0.00	0.40 ± 0.01^{b}	0.94 ± 0.02^{a}	0.71 ± 0.02^{a}	0.22 ± 0.01^{b}
VI	AOW	9.30 ± 0.63^{ab}	1.86 ± 0.11^{abc}	0.07 ± 0.01^{bcd}	0.86 ± 0.02^{a}	1.69 ± 0.03	1.60 ± 0.06^{ab}	0.57 ± 0.04^{b}
	ROW	4.15 ± 0.25^{ab}	0.83 ± 0.04^{bc}	0.02 ± 0.00	0.38 ± 0.01^{b}	0.76 ± 0.01^{b}	0.72 ± 0.02^{a}	0.26 ± 0.02^{ab}
VII	AOW	8.07 ± 1.04^{b}	1.61 ± 0.11^{bc}	0.06 ± 0.01^{d}	0.70 ± 0.05^{b}	1.65 ± 0.09	1.28 ± 0.09^{c}	0.45 ± 0.04^{cd}
	ROW	4.15 ± 0.25^{ab}	0.90 ± 0.03^{ab}	0.02 ± 0.00	0.39 ± 0.01^{b}	0.93 ± 0.02^{a}	0.71 ± 0.01^{a}	0.25 ± 0.01^{ab}

Values (mean ± SE, n=6) bearing different superscript in the same column vary significantly ($p<0.05$). (One-way ANOVA and Duncan multiple comparison post hoc test). AOW: absolute organ weight (g); ROW: relative organ weight (g/100g b. wt.) I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm); VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg); VII: T-2 toxin (5.0 ppm) + vitamin (100 mg/kg).

Table 2. *Absolute organ weights and relative organ weights following 28-days exposure to T-2 toxin alone and in combination with vitamin-E in rats.*

Groups		Thymus	Testes	Epididymis	Seminal vesicles	Prostate
I	AOW	0.69±0.04^{a}	3.05±0.06^{a}	1.15±0.08^{a}	0.74±0.01^{ab}	0.29±0.01^{a}
	ROW	0.25±0.01^{a}	1.12±0.02^{a}	0.43±0.03	0.27±0.01^{b}	0.11±0.00^{b}
II	AOW	0.70±0.04^{a}	3.06±0.14^{a}	1.12±0.07^{a}	0.75±0.01^{a}	0.30±0.01^{a}
	ROW	0.26±0.01^{a}	1.12±0.04^{a}	0.41±0.03	0.27±0.01^{b}	0.11±0.01^{b}
III	AOW	0.66±0.03^{a}	3.09±0.13^{a}	1.12±0.03^{a}	0.74±0.01^{a}	0.30±0.01^{a}
	ROW	0.24±0.01^{a}	1.13±0.04^{a}	0.41±0.01	0.27±0.01^{b}	0.11±0.00^{b}
IV	AOW	0.41±0.01^{b}	2.43±0.10^{b}	0.98±0.06^{ab}	0.68±0.01^{b}	0.25±0.01^{b}
	ROW	0.18±0.00^{b}	1.09±0.03^{a}	0.44±0.03	0.30±0.01^{a}	0.11±0.00^{b}
V	AOW	0.25±0.02^{c}	1.80±0.20^{c}	0.82±0.13^{b}	0.56±0.03^{c}	0.22±0.01^{b}
	ROW	0.14±0.01^{c}	1.03±0.08^{ab}	0.45±0.05	0.33±0.02^{a}	0.13±0.00^{a}
VI	AOW	0.43±0.03^{b}	2.48±0.07^{b}	1.10±0.04^{a}	0.69±0.01^{ab}	0.29±0.01^{a}
	ROW	0.19±0.01^{b}	1.11±0.03^{a}	0.49±0.03	0.31±0.01^{a}	0.13±0.00^{a}
VII	AOW	0.27±0.03^{c}	1.62±0.25^{c}	0.81±0.06^{b}	0.59±0.03^{c}	0.23±0.02^{b}
	ROW	0.15±0.01^{c}	0.88±0.09^{b}	0.46±0.05	0.33±0.01^{a}	0.13±0.00^{a}

Values (mean ± SE, n=6) bearing different superscript in the same column vary significantly (p<0.05). (One-way ANOVA and Duncan multiple comparison post hoc test). AOW: absolute organ weight (g); ROW: relative organ weight (g/100g b. wt.) I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm); VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg); VII: T-2 toxin (5.0 ppm) + vitamin (100 mg/kg).

co-administered with vitamin E-treated groups TESC values remain significantly less (p<0.05) as compared to the control group.

Effect on sperm motility

The sperm motility was found to be significantly decreased (p<0.05) in a dose-dependent manner in T-2 toxin-alone fed groups as compared to the control group (Table 3). However, in T-2 toxin plus vitamin E groups, a significant improvement in sperm motility was observed when compared with respective alone treated groups.

Effect on live sperm and sperm morphology

Significant reduction (p<0.05) in live sperm counts in T-2 toxin alone groups in a dose-dependent manner was noticed as compared to the control group. The live sperm count was restored after vitamin E co-treatment in a lower dose of T-2 toxin-treated groups as compared to the control group (Table 3).

Sperm abnormalities (head and tail) were significantly increased (p<0.05) in dose in T-2 toxin alone treated groups when compared with the control group (Table 3).

Table 3. *Sperm related parameters in rats following 28-days exposure to T-2 toxin alone and in combination with vitamin E.*

Groups	TESC	S Motility	LSC	DSC	HA	TA	Total A
I	85.83±2.71^{b}	76.67±2.47^{a}	87.17±2.07^{a}	12.83±2.07^{c}	2.86±0.19^{d}	3.30±0.29^{d}	6.16±0.27^{d}
II	84.17±2.22^{b}	75.83±2.01^{a}	87.50±2.05^{a}	12.50±2.05^{c}	2.81±0.22^{d}	3.34±0.37^{d}	6.15±0.51^{d}
III	126.04±3.13^{a}	78.83±3.79^{a}	89.17±2.46^{a}	10.83±2.46^{c}	2.88±0.27^{d}	2.91±0.27^{d}	5.78±0.46^{d}
IV	52.92±1.69^{d}	62.33±2.96^{c}	65.83±2.71^{bc}	34.17±2.71^{ab}	6.38±0.38^{b}	8.34±0.48^{b}	14.72±0.76^{b}
V	8.17±0.30^{e}	49.50±3.30^{d}	56.50±3.18^{c}	43.50±3.18^{a}	8.19±0.54^{a}	10.58±0.56^{a}	18.77±0.81^{a}
VI	74.17±1.37^{c}	70.33±1.86^{ab}	72.50±2.42^{b}	27.50±2.42^{b}	4.57±0.38^{c}	5.56±0.22^{c}	9.96±0.40^{c}
VII	13.85±1.00^{e}	60.83±3.75^{c}	67.50±3.35^{b}	32.50±3.35^{ab}	6.37±0.37^{b}	7.61±0.35^{b}	13.98±0.31^{b}

Values (mean ± SE, n=6) bearing different superscripts in the same column vary significantly (p<0.05). (One-way ANOVA and Duncan multiple comparison post hoc test). TESC: total epididymal sperm count (in millions or x10^{6}), LSC: live sperm count (%), DSC: dead sperm count (%), S Motility: sperm motility (%), HA: head abnormality (%) and TA: tail abnormality (%); Total A: total abnormality (%). I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm); VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg); VII: T-2 toxin (5.0 ppm) + vitamin (100 mg/kg).

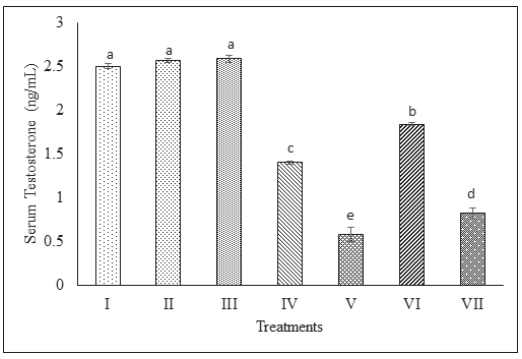

Figure 3. Serum testosterone levels in rats after exposure to T-2 toxin, vitamin E and their combination. *I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm) VI: T-2 (2.5 ppm) + vitamin E (100 mg/kg b.w.); VII: T-2 (5.0 ppm) + vitamin E (100 mg/kg b.w.). Values are mean ± SEM (n=6). Bars bearing different superscripts vary significantly (p<0.05) from each other in o-way ANOVA and Duncan multiple comparison post hoc tests.*

However, a significant reduction in head and tail abnormalities were noticed in T-2 toxin plus vitamin E co-treated groups.

Effect of serum testosterone level

Serum testosterone levels in rats treated with T-2 toxin alone were significantly reduced (p<0.05) in a dose-dependent manner when compared with the control group. However, testosterone levels were partially restored in T-2 toxin-vitamin E co-treated groups (Figure 3).

Effect on lipid peroxidation and ROS generation

The LPO levels of testes of T-2 toxin alone treated rats were significantly increased (p<0.05) when compared with the control rats, however, vitamin E co-treated rats showed significant inhibition in LPO as compared to T-2 toxin alone treated groups (Figure 4a).

Treatment with T-2 toxin alone resulted in a significant increase (p<0.05) in percent ROS positive cells in a dose-dependent pattern as compared to control. Co-administration of vitamin E in T-2 toxin-treated groups resulted in a significant (p<0.05) reduction of ROS-positive cells when compared with T-2 toxin-alone treated groups (Figures 4b and 5).

Effect on oxidative stress and antioxidant system

A significant reduction (p<0.05) in testicular protein and GSH levels in T-2 toxin-alone treatment groups was observed. However, significant restoration was noticed in vitamin E co-treatment groups in the protein level, while, GSH level restoration against a lower dose of T-2 toxin was noticed (Figure 6). T-2 toxin caused a decrease (p<0.05) in CAT, SOD, and GR activities in testes when compared with the control group. Vitamin E co-treatment in a lower dose of T-2 toxin-treated animals resulted in significant restoration (p<0.05) in CAT, SOD and GR activities as compared to T-2 toxin-alone treated groups.

Histopathological examination

Testes: Histomicrograph of testes showing pathological changes in different treatment groups are presented in Figure 7. Testes of rats exposed to 2.5 ppm T-2 toxin revealed moderate to severe changes like the atrophic lining of seminiferous tubules, mild interstitial edema, destruction of germinal epithelium, depletion of spermatozoa with few necrotic cells in the lumen, and arrested spermatogenesis with a moderate decrease of sperm cells in seminiferous tubules (Figures 7c and 7d). Testes of 5 ppm T-2 toxin-treated rats showed almost total atrophy of seminiferous tubules with increased intertubular space

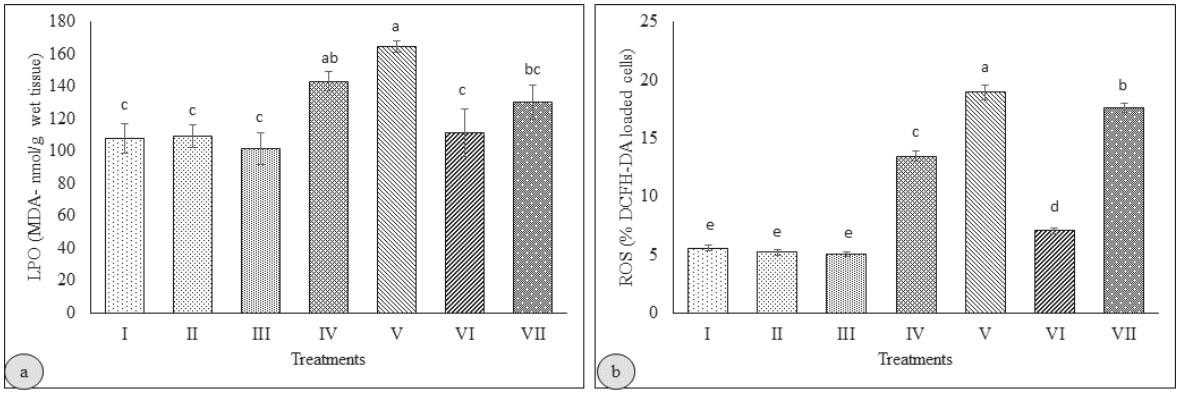

Figure 4. Effect of exposure of T-2 toxin, vitamin E alone, and with the combination on testicular cells reactive oxygen species (ROS) and lipid per-oxidation (LPO) production in Wistar rats. *I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm) VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg b.w.); VII: T-2 toxin (5.0 ppm) + vitamin E (100 mg/kg b.w.). Values are mean ± SEM (n=6). Bars bearing different superscripts vary significantly (p<0.05) from each other in one-way ANOVA and Duncan multiple comparison post hoc tests.*

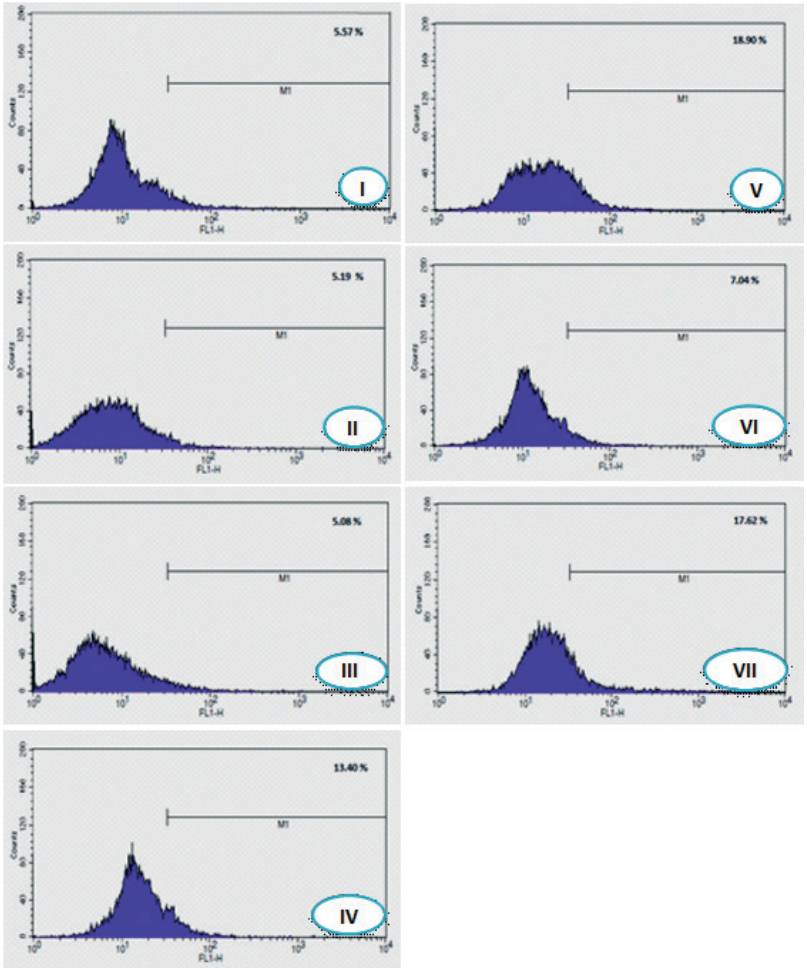

Figure 5. Effect of exposure of T-2 toxin, vitamin E alone, and with the combination on testicular cells reactive oxygen species (ROS) production in Wistar rats. *Representative histograms of FACS showing % testicular cells with ROS-positive cells. The fluorescence of DCFH-DA-loaded cells was measured by a flow cytometer. I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm) VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg b.w.); VII: T-2 toxin (5.0 ppm) + vitamin E (100 mg/kg).*

and mild to severe interstitial edema. There was complete destruction of the normal architecture of the seminiferous tubules with severe depletion of germinal epithelial cells. A remarkably total absence of sperm cells in the wide luminal space was observed due to the complete arrest of spermatogenesis in seminiferous tubules (Figures 7e and 7f). However, rats treated with 2.5 ppm T-2 toxin plus vitamin E showed restoration of testicular architecture and almost normal spermatogenesis (Figures 5g and 5h), while, 5.0 ppm T-2 toxin with vitamin E treatment rats did not provide appreciable changes (Figures 7i and 7j).

Epididymis: Histomicrograph of epididymis with pathological changes are presented in Figure 8. Epididymis of rats treated with 2.5ppm T-2 toxin revealed atrophic lining epithelium with some degenerative changes and at some places detachment from the basement membrane (Figures 8c and 8d). The lumen of the epididymis showed a slight decrease in sperm concentration with some degenerated and necrotic spermatozoa. Rat fed with 5 ppm T-2 toxin showed severe degenerative changes in the lining epithelium of epididymis with complete detachment from the basement membrane; a decrease in the sperm concentration with increased necrotic spermatozoa in the lumen (Figures 8e and 8f). Vitamin E co-treatment in 2.5 ppm T-2 toxin-treated group protected epididymis from toxic damage caused by T-2 toxin. Epididymis of this group showed mild to moderate restoration of lining epithelium towards normal (Figures 8g and 8h). Further, no increment in sperm concentration in the lumen in 5 ppm T-2 toxin plus vitamin E treated rat was noticed (Figures 8i and 8j).

DISCUSSION

The T-2 toxin exposure damage the liver, kidney, digestive tract, and other vital organs of the body. T-2 toxin resulted in the induction of apoptosis in the thymus and spleen and, atrophy of bursa of fabricus in animals. This is responsible for decreased feed intake and absorption, biosynthesis of essential proteins, and even loss of

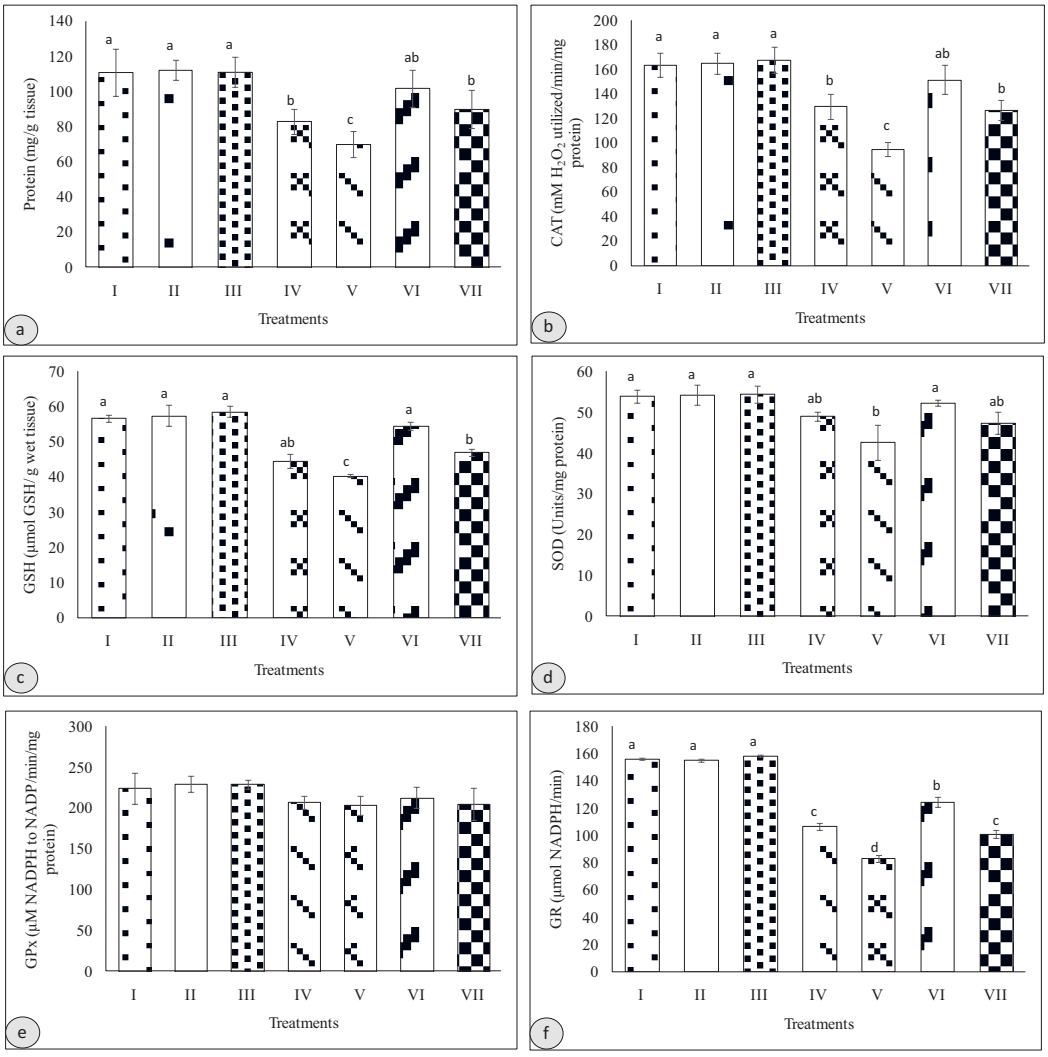

Figure 6. Oxidative stress-related parameters in testes of rats after exposure to T-2 toxin, vitamin E and their combination for 28-days. *I: control; II: corn oil (1 ml/kg b.w.); III: vitamin E (100 mg/kg b.w.); IV: T-2 toxin (2.5 ppm); V: T-2 toxin (5.0 ppm); VI: T-2 toxin (2.5 ppm) + vitamin E (100 mg/kg); VII: T-2 toxin (5.0 ppm) + vitamin (100 mg/kg). Values are mean ± SEM (n=6). Bars bearing different superscripts vary significantly (p<0.05) from each other in one-way ANOVA and Duncan multiple comparison post hoc tests. CAT – catalase; GSHR – reduced glutathione; SOD – superoxide dismutase; GPx – glutathione peroxidase; GR – glutathione reductase*

more protein in the urine. Ultimately leading to loss of body weight, growth retardation, and change in vital organ weights in animals (*9,23-25*). In the present study, T-2 toxin alone fed rats showed variable degrees of clinical signs such as dullness, weakness, disinclination, rough hair coat, and retardation in growth indicating that T-2 toxin has a direct effect on vital organs responsible for the digestion and energy metabolism.

In the present study total epididymal sperm counts, and sperm quality was decreased in rats treated with T-2 toxin. These findings indicate that feeding of T-2 toxin-contaminated feed caused severe damage to testes and depleted germinal cells responsible for altered spermatogenesis as evidenced by histopathological findings. Similarly, intra-peritoneal administration of T-2 toxin increased the abnormal spermatozoa, decrease the live spermatozoal count and low pregnancy rate with high foetal resorption in mice has been reported (*9*). An increase in sperm motility and live sperm counts in vitamin E plus T-2 toxin co-treated animals might be due to a decrease in the oxidative damage to the membrane phospholipid of sperm.

Testosterone is very much essential for normal spermatogenesis and maintenance of cell structure, morphology, and normal physiology of seminiferous tubules. It is also important for the attachment of germ cells in seminiferous tubules (*26*). The low-level may lead to the detachment of germ cells from the seminiferous epithelium and initiate germ cell apoptosis and degeneration. The low level of testosterone and detachment of the germinal layer was noticed in T-2 treated animal testis in the present study. The reduction in serum testosterone level is

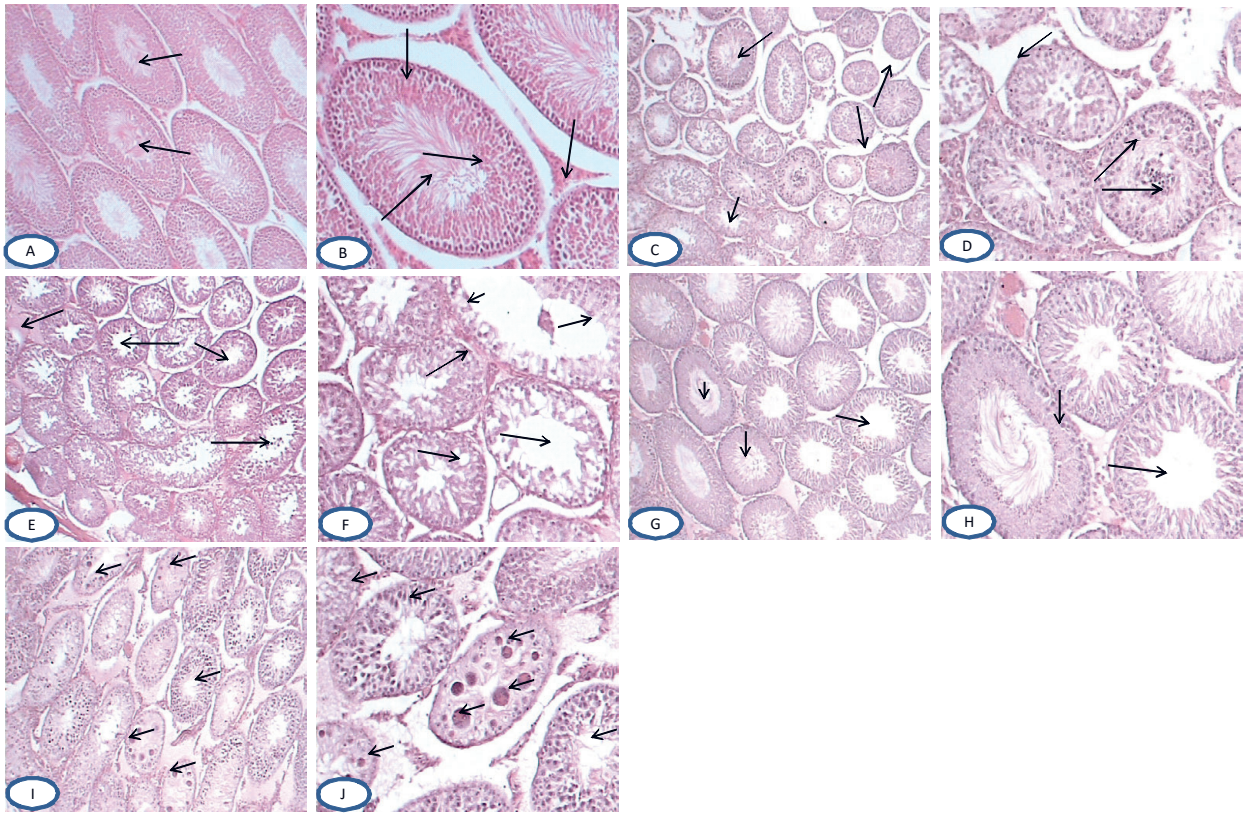

Figure 7. Photomicrograph of testis of rats at the end of 28 days of exposure. *a; b) Testicular section of control male rat showing seminiferous tubules with intact basement membranes, normal spermatogenesis, and normal interstitial cells. c; d) Testis of T-2 toxin 2.5 ppm treated rat showing moderate changes: atrophy of seminiferous tubules, mild interstitial oedema, destruction of germinal epithelium and arrested spermatogenesis. e; f) Testis of T-2 toxin 5 ppm treated rat showing severe changes: total atrophy of seminiferous tubules, interstitial oedema, complete destruction, severe depletion of germinal epithelium and widened lumen in seminiferous tubules. g; h) Testis of T-2 toxin 2.5 ppm plus vitamin E treated rat showing seminiferous tubules with restored spermatogenesis and intact germinal epithelium layer. i; j) Testis of T-2 toxin 5.0 ppm plus vitamin E treated rat showing partial improvement in spermatogenesis and restoration of geminal epithelium with few spermatid giant cells in seminiferous tubules. Magnification: a, c, e, g, i 40X, and b, d, f, h, j 100X. All changes in micrographs are marked by arrows.*

might due to T-2 toxin-induced cytotoxic damage to Leydig's cells in the testes. These findings are supported by earlier workers, who reported a reduction in serum testosterone levels in T-2 toxin-fed animals (*9*). Similar changes in testes have been reported in animals when they are fed with ochratoxin A and T-2 toxin (*10*). Germinal epithelium of T-2 toxin-treated rat showed 'spermatid giant cells' possibly due to the formation of the multinucleated cells as a result of the degeneration of spermatogonial cells with a fusion of degenerating spermatogonia. Improvement in testicular and epididymal cellular architecture is almost normal in a lower dose of T-2 toxin-vitamin E co-treated animal. This indicates that vitamin E plays a significant role in maintaining the spermatid cell's viability and allowing epididymal epithelial cells to acquire their full differentiation and structural normalization (*27,28*).

Spermatozoa particularly are susceptible to ROS-induced damage caused by a wide variety of endogenous and exogenous factors such as environmental toxicants which includes phthalate esters, sulphur-dioxide, sodium fluoride, estrogens, chemotherapeutic agents, and many of the toxins (*29*). They compromise male fertility through oxidative stress and ROS production in the testicular cells. Oxidative damage is one of the main manifestations of cellular damage associated with mycotoxin-mediated testicular toxicity. Critical biomolecules like nucleic acids, proteins, and lipids molecules are the main target for mycotoxicosis in the form of apoptosis and cell death (*10*). An increase in lipid peroxidation and, ROS production in testicular cells altered the level of the antioxidant system (SOD, CAT, GPx and GSH) in T-2 toxin-exposed animals indicating cellular damage and functional abnormality. Similarly, an increase in lipid peroxidation and hydroxyl radicals in the liver, spleen, kidney, thymus and bone marrow of rats with T-2 toxin treatment has been reported (*30*). Free radicals-mediated lipid peroxidation is suggested as the main mode of action for liver damage in trichothecene (T-2 toxin) toxicosis (*31,32*). The T-2 toxin is an amphophilic molecule thought to be taken up into the lipid bilayer membrane

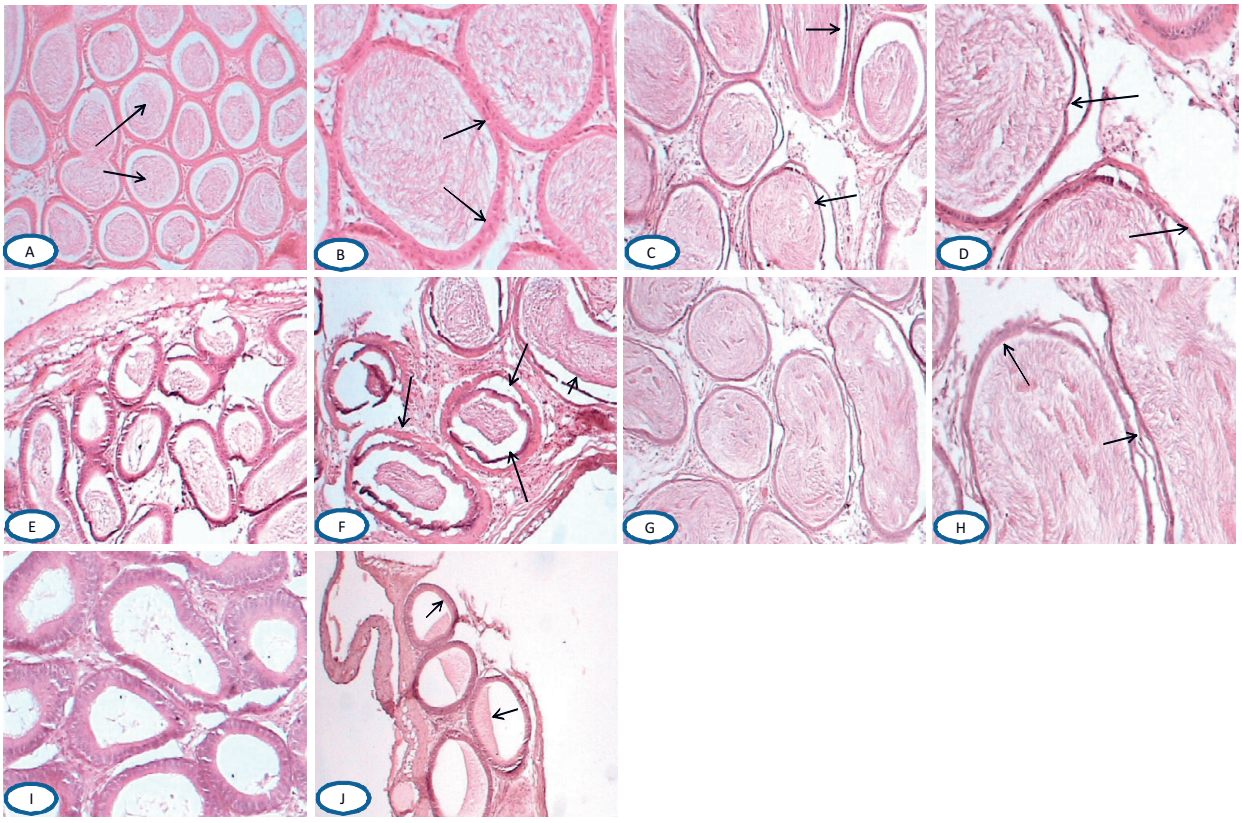

Figure 8. Photomicrograph of epididymis of rats at the end of 28 days of exposure. *a; b) Epididymis of control male rat showing normal epithelial lining, normal spermatozoa in lumen, and peripheral fat layer. c; d) Epididymis of T-2 toxin 2.5 ppm treated rat showing moderate changes: trophy of lining epithelium with degeneration and detachment from basement membrane. e; f) Epididymis of T-2 toxin 5 ppm treated rat showing severe changes: degenerated and necrotic spermatozoa in lumen and complete detachment of lining epithelium from basement membrane. g; h) Epididymis of T-2 toxin 2.5 ppm plus vitamin E treated rat showing restored epithelial lining with improved number of spermatozoa in lumen. i; j) Epididymis of T-2 toxin 5.0 ppm plus vitamin E treated rat showing improved epithelial lining with the absence of spermatozoa in lumen. Magnification: a, c, e, g, j 40X, and b, d, f, h, i 100X. All changes in micrographs are marked by arrows.*

of cells and initiated the generation of free radicals thereby damaging the lipid membrane and subsequently DNA. Testis contains a high concentration of polyunsaturated fatty acids and has low antioxidant capacity so male germ cells likely to be more susceptible to oxidative stress mediated by toxins.

Testicular damage is responsible for reproductive dysfunction mediated via T-2 toxin and amelioration in the presence of vitamin E. Complete protection was noticed against a lower dose rather than a higher dose of T-2 toxin. It has been reported that vitamin E improved sperm quality, testicular morphology, and level of luteinizing and follicle-stimulating hormone. Vitamin E prevented cellular structural damage in the brain, liver, and epididymis caused by environmental toxicants (*33,34*). Vitamin E was found to be effective in minimizing spermatogenic and, steroidogenic disorders and the sertoli cells damage resulting from exposure to lindane, aluminium chromium, and arsenic (*35,36*). It restored the altered level of glucocorticoids, delta(5)3beta-HSD and, testicular TNF-alpha, IL-6, LHRH, LH, and testosterone. It prevented adrenal gland hypertrophy, hypothalamic, pituitary and, testicular germ cell apoptotic in vanadium-exposed rats and contributed to its antioxidant activity (*37*). Vitamin E supplementation could effectively increase the activity of SOD, T-AOC, GSH-Px and, GSH, and protein contents along with the decrease in LPO of the liver and reproductive organs (*29,38*). It helps in the functional restoration of renal, myocardial, reproductive, and metabolic organ damage caused by ethanol, T-2 toxin, fluoride and nonylphenol. Vitamin E enhances spermatogenesis through the inhibition of lipid peroxidation, further it protected the sperm progenitor cell from oxidative damage caused by excessive ROS generation and improves infertility associated with toxins (*39,40*).

CONCLUSIONS

The present study validates that sub-acute exposure to T-2 toxin altered the activities of the testicular antioxidant system and increased oxidative stress owing to testicular

damage resulting in abnormal sperm count, abnormalities, and hormonal imbalance. Concurrent administration of vitamin E at 100 mg/kg body weight was found to be more effective in counteracting oxidative stress and, testicular damage caused by 2.5 ppm T-2 toxin. In conclusion, vitamin E may have the potential to ameliorate the T-2 toxin-induced testicular damage but that needs to be modulated in the dose of vitamin E where T-2 toxin exposure is higher.

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