SUBACUTE TOXICOLOGICAL EFFECTS OF EMAMECTINE BENZOATE ON WISTAR RAT TESTES: HISTOPATHOLOGICAL CHANGES, DETERMINATION OF EMAMECTIN BENZOATE RESIDUES BY UPLC-MS/MS AND PROTECTIVE EFFECT OF VITAMIN C

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Abstract

Emamectin benzoate (Proclaim®) is a biopesticide derivative of the avermectins developed for the control of insect pests. In the present study, the ameliorating effect of vitamin C was examined histologically and biochemically in testicle tissue of male Wistar rats, Rattus norvegicus, exposed to emamectin benzoate (EMB).

Male rats (n=21) were allocated into 3 groups of seven each: Group I served as control rats; Group II was given daily EMB at dose of 10 mg/kg b.w. by gavage. Group III received EMB (10 mg/kg b.w.) and were co administered with vitamin C intraperitoneally (200 mg/kg/day) during 28 days. Rats were followed-up for an additional 14 day-period to detect delayed occurrence or persistence of toxic effects.

At 28 and 42 days of experiment, absolute body weight, absolute and relative testis weights, histological examinations, and measurements of residual concentrations of EMB mixture B1a and B1b in testes by UPLC-MS/MS were recorded. A decrease of the body weight after 42 days of treatment for EMB-treated rats and a significant increase ($p<0.05$) for EMB+VitC-treated rats were observed. Treatment with EMB decreased the relative testis weight compared to that of the control and EMB +VitC groups. In the testis tissue, after administration of 10 mg/kg of EMB, residual concentrations of B1a and B1b were clearly detected after 28 and 42 days in all treated groups. A significant higher concentration of EMB residue was found in EMB-treated groups at 28 and 35 days (28 day; B1a: 4.00 ± 0.3 ng/mg and B1b: 0.48 ± 0.06 ng/mg, at 42 days B1a: 0.85 ± 0.2 ng/mg and B1b: 0.10 ± 0.03 ng/mg) compared with the EMB + Vit C group (28 day; B1a: 3.18 ± 0.9 ng/mg and B1b: 0.33 ± 0.12 ng/mg, at 42 days; B1a: 0.29 ± 0.07 ng/mg and B1b: 0.02 ± 0.00 ng/mg).

Histopathological examination showed damage in the tissue architecture of the testes from the EMB-treated group, i.e. oedema in intertubular spaces with vacuolation within tubules, damage involving both germinal and interstitial (Leydig) cells. Histopathological changes were more intense in rats from the EMB-treated group than in those from the EMB+VitC-treated group at 28 and 42 days.

In conclusion, vitamin C administered at moderate doses appears to reduce the adverse effects of EMB on the testis histopathology of Wistar rats, probably by reducing its accumulation in testes.

Key words: *emamectin benzoate, testes, vitamin c, histopathology, uplc-ms/ms, rat*

1. INTRODUCTION

Infertility is a worldwide reproductive health problem which affects approximately 15% of couples, with male factor infertility dominating nearly 50% of the affected population (Lee and Foo, 2014). Infertility in humans has been correlated with exposure to pesticides (Strohmer et *al*., 1993). It has been observed that the spermatocytogenesis and spermiogenesis are the spermatogenic stages most susceptible to environmental toxicants. Pesticides also produce endocrine alterations, leading to changes in diverse reproductive parameters.

Avermectin (AVM) products are macrocyclic lactone product derived from the soil microorganism *Streptomyces avermitilis*. They are primarily used in veterinary medicine for the treatment and prevention of parasitic diseases in animals and as plant protection agents in the agricultural sector (Castanha Zanoli et *al*., 2012). They are highly lipophilic substances and dissolve in most organic solvents (Roth et *al*., 1993).

Emamectin benzoate (EMB) is a mixture of avermectins containing about 90% avermectin B1a and 10% avermectin B1b. These two components, B1a and B1b, have similar biological and toxicological properties (Lankas and Gordon, 1989). Vitamin C (Vit C) is a water-soluble antioxidant that is thought to be important in protecting bio-membranes from oxidative stress (Harapanhalli et *al*., 1996). Studies have reported that Vit C can reduce lipid peroxidation caused by toxic substances. There are limited number of studies evaluating effects of EMB on male fertility and reproduction. Besides, levels of EMB residue in testicular tissue by UHPLC MS/MS have not been determined.

Because of the health problems induced by many environmental pollutants, much effort has been expended in evaluating the relative antioxidant potency of vitamin C.

Therefore, in the present study, the ameliorating effect of vitamin C was examined histologically and biochemically in testicle tissue of male Wistar rats, *Rattus norvegicus*, exposed to emamectin benzoate (EMB). Then we investigate the possible modulatory effects of vitamin C in accumulation of EMB residue B1a and B1b in the testes after subacute toxicological study

2. MATERIAL AND METHODS

The study was performed in accordance with the National Institutes of Health Guidelines for the Care and Handling of Animals; and according to the OECD guideline 407 for repeated dose 28-day oral toxicity studies in rodents (OCDE, 2003).

2.1. Chemicals

In this study, a commercial formulation of emamectin benzoate (MK244), named Proclaim®, 5% purity of active compound, was used. A pure reference standard of emamectin benzoate (> 98.1%, purity, Syngenta Agrochemicals, Greensboro, USA) was purchased from the Ministry of Agriculture and Rural Development (Direction de la Protection des Végétaux et Contrôles Techniques DPVCT, Algeria). Acetonitrile (ACN) and formic acid (HPLC grade) were purchased from Biosolve Chimie (Valkenswaard, the Netherlands). All biochemical reagents used were obtained from commercial sources (BIOLABO S.A., France).

2.2. Animals

Male adult Wistar rats weighing between 210 ± 10 g were used in the study. Animals were housed in cages with controlled temperature (22 °C) and 12 h light/dark cycles. The rats were acclimated to the laboratory conditions for 8 days before treatment and had free access to a standard commercial pellet diet (ONAB: Office National des Aliments de Bétail, Alger, Algerie) and water *ad libitum*.

2.3. Experimental design

Healthy adult rats (n = 21) were randomly allocated into 3 groups (n = 7/group) as follows: Group I served as control rats; Group II was given daily EMB at dose of 10 mg/kg b.w. by gavage. Group III received EMB (10 mg/kg b.w.) and were co administered with vitamin C intraperitoneally (200

mg/kg/day) during 28 days. EMB was prepared in distilled water. Doses of EMB were adjusted according to changes in animal weight. Body weights were recorded daily throughout the acclimatation, experimental, and post treatment periods.

Rats were followed-up for an additional 14 day-period to detect delayed occurrence or persistence of toxic effects and therefore expected better evaluation of effects of Vit C.

2.4. Relative testes weight

Rats were sacrificed, using cervical decapitation under light diethyl ether, at the end of the treatment period 28 d, (three rats/group) and 14 d after the last treatment day 42 (four rats/group). The testes were carefully dissected out and weighed in grams (absolute organ weight). The relative testes weight of each animal was then calculated as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Body weight of rat on sacrifice day (g)}}$$

2.5. Testes EMB concentrations

An ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method employing electrospray ionization (ESI) was developed for the determination of avermectin B1a and B1b in the testes of rats using methyl clonazepam as the internal standard (EI). The standard calibration curve samples of ABM at concentrations of 0, 1, 5, 10, 25, 50, 100, 250, 500 and 1 000 ng/mL were prepared by serial dilutions with plasma from stock solution of 20 μg/ mL.

Right testicular tissues of rats were cut into small pieces and placed in a glass tube; 1 000 μL of ACN were sequentially added to testes sample, then vortexed. The mixing and extraction steps were performed using a vortex for 2 min and ultrasonication for 15 min. After centrifugation at 10,800 rpm for 8 min, the supernatant was evaporated under nitrogen. The testes residues were reconstituted in 100 μL of the mobile phase [(A): (87%) ammonium formate solution and (B): (13%) ACN and formic acid]. After centrifugation at 4000 rpm for 10 min, 15 μL of the supernatant was injected into the UPLC—MS/MS.

2.6. Histopathological evaluation

Before euthanasia by cervical decapitation under light diethyl ether necropsy was performed on all animals. Left testicular tissues of rats were fixed in 10% formalin, routinely processed, paraffin embedded and then stained with hematoxylin and eosin (HE) to be interpreted by light microscopy.

2.7 Statistical analysis

Statistical analysis was performed using Statistica version 10.0 (StatSoft Inc., Tulsa, USA). Data were calculated using one-way analysis of variance (ANOVA) followed by the Duncan's post-hoc tests. Data were expressed as the mean ±SD. $P < 0.05$ was considered as the level of significance.

3. RESULTS

3.1. Evaluation of body and testes weights

No deaths occurred in any group throughout the experiment. Some signs of general toxicity were apparent in male rats treated with EMB 10 mg/Kg/d. Body weights of rats significantly change after EMB and EMB + Vit C exposure (Figure 1).

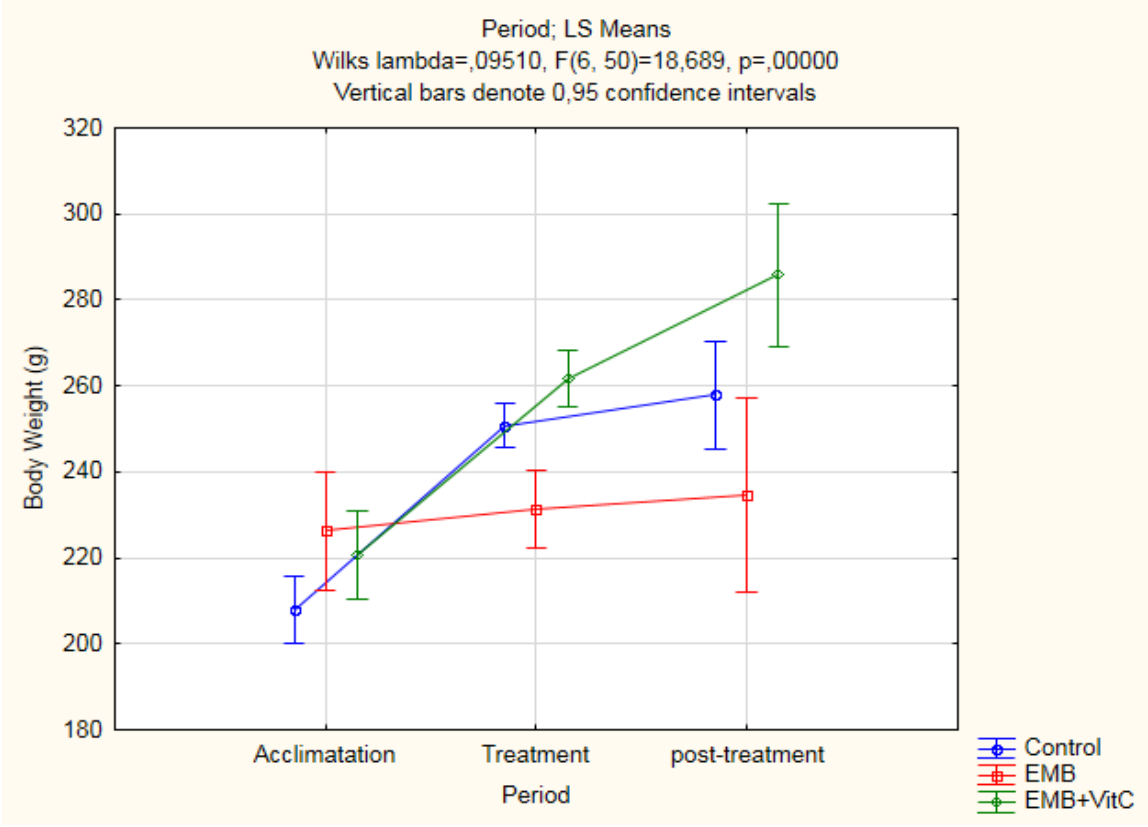


Figure 1: Effect of emamectin benzoate (EMB) on body weight of rats, throughout the acclimation (1-8 days) experimental (1-28 days) periods and post-treatment period (1-14 days).

Significant difference in the absolute and percentage of body weight gain at the end of the experimental and post treatment periods was observed in EMB-treated group when compared with the control group (Table 1).

A decrease in percentage body weight in EMB treated rats (6.82 %, 1.39 %) and a significant ($P < 0.05$) increase in EMB+VitC group (18.67 %, 9.15 %) after 28 and 42 days when compared with control rats (20.39 %, 3.11%).

Table 1: Absolute and percentage body weight gain, in rats from control, EMB and EMB +Vit C-treated groups.

Body Weight Gain (g)	**Control**[c]	**EMB** [a, c]	**EMB + VitC**[b, c]
Initial BW	219.04 ± 3.38	221.22 ± 8.14	220.62 ± 4.7
Experimentation BW (28 d)	264.76 ± 2.19	236.50 ± 5.28*	261.81 ± 3.05
% BWG after treatment	20,39 %	6,82 %*	18,67 %
Final BW(42 d)	273.01 ± 3.18	239.58 ± 9.41*	285.52 ± 6.31*
% BWG post-treatment	3,11 %	1,39 %*	9,15 %*

[a]10 mg of EMB/kg/day; [b]10 mg of EMB/kg/day+ 200 mg/Kg VitC; [c]Results are given as a mean ± SD (n=7). [*]Statistically different from the control group ($P < 0.05$).

Results in Table 2 indicate that administration of proclaim resulted in a significant increase in the relative weight of treated male rat's testes in comparison with that of control rats. Similarly, a significant increase in the absolute and relative weights of testes was observed in EMB group after 28 days. On the other hand, EMB + Vit C exhibited no significant alteration in the relative weight of treated rat's testes in comparison with that of control rats after 28 and 42 days. Also, as shown in Table 2, testes tissue relative weights were not significantly different between all groups after 42 days.

Table 2: Absolute and relative testes weights (g) in rats from control EMB and EMB- Vit C groups

	Control[c]		**EMB** [a, c]		**EMB + VitC**[b, c]	
	Absolute	**Relative**	**Absolute**	**Relative**	**Absolute**	**Relative**
First sacrifice day 28						
Left testis	1,09 ± 0,04	0,4 ± 0,01	1,23± 0,19*	0,5 ± 0,08*	1,23 ± 0,06*	0,43 ± 0,02
Right testis	1,09 ± 0,02	0,4 ± 0,02	1,31± 0,16*	0,56 ± 0,07*	1,27 ± 0,04*	0,44 ± 0,01
Seconde sacrifice day 42						
Left testis	1,42 ± 0,07	0,54 ± 0,02	1,29± 0,08*	0,58 ± 0,03	1,17 ± 0,04*	0,51 ± 0,02
Right testis	1,40 ± 0,01	0,53 ± 0,04	1,23± 0,11*	0,55 ± 0,05	1,20 ± 0,06*	0,52 ± 0,02

[a]10 mg of EMB/kg/day; [b]10 mg of EMB/kg/day+ 200 mg/Kg VitC; [c]Results are given as a mean ± SD (n=7).

[*]Statistically different from the control group ($P < 0.05$).

3.2 Testes emamectin benzoate concentrations

EMB residue B1a and B1b were found in all testes samples of male EMB and EMB-VitC-treated rats in a time-dependent manner at 28 and 42 days (figure 2).

In the testis tissue, after administration of 10 mg/kg/d of EMB, residual concentrations of B1a and B1b were clearly detected after 28 and 42 days. A significant higher concentration of EMB residue was found in EMB-treated groups at 28 and 35 days (28 day; B1a: 4.00 ± 0.3 ng/mg and B1b: 0.48 ± 0.06 ng/mg, at 42 days B1a: 0.85 ± 0.2 ng/mg and B1b: 0.10 ± 0.03 ng/mg) compared with the EMB + Vit C group (28 day; B1a: 3.18 ± 0.9 ng/mg and B1b: 0.33 ± 0.12 ng/mg, at 42 days; B1a: 0.29 ± 0.07 ng/mg and B1b: 0.02 ± 0.00 ng/mg).

Our results show that EMB residue B1a and B1b concentrations were significantly lower in EMB+VitC compared with EMB-treated rats at 24 days and after 14 days post-treatment period (figure 2).

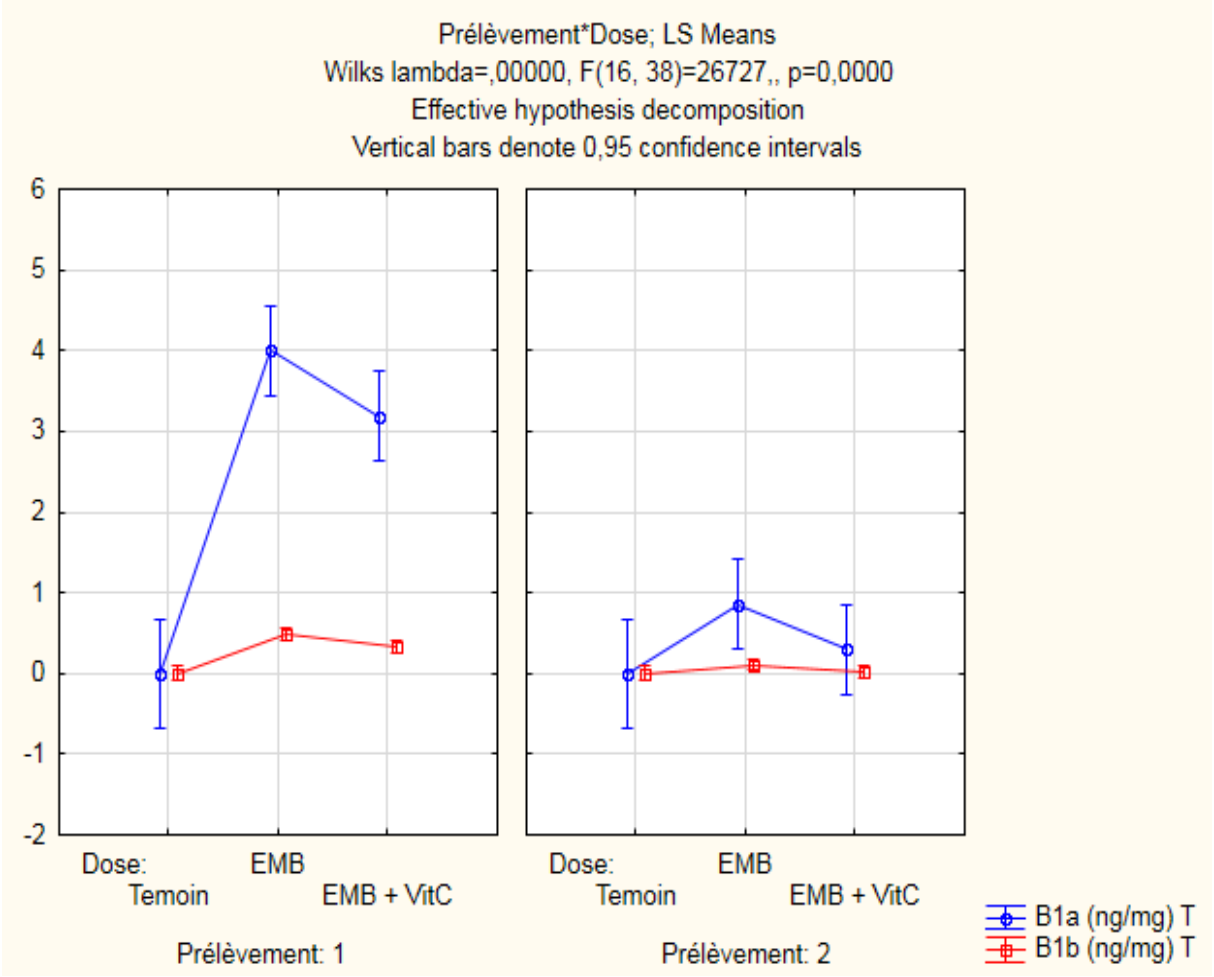


Figure 2: Testes emamectin benzoate [B1a and B1b (ng/mg)] concentrations at 28 and 42 days obtained after administration of 10 mg/Kg/day (EMB group) and co-administered with vitamin C (EMB+ Vit C group) daily for 28 days to male rats. [c]Results are given as a mean ± SD (n=7).

3.3. Histopathological evaluation of the testes

Control rats showed normal spermatogenesis, whereas rats exposed to EMB at 10 mg/kg/day show a significant testicular damage (Figure 3).

In the control group, histopathological examination of testes biopsies was in normal limits. Histopathological examination showed damage in the tissue architecture of the testes from the EMB-treated group, *i.e.* oedema in intertubular spaces with vacuolation within tubules, damage involving both germinal and interstitial (Leydig) cells. There were degenerative tubules with disrupted cellular organizations in EMB-exposed testes at 42 days. Histopathological changes were more intense in rats from the EMB-treated group than in those from the EMB+VitC-treated group at 28 and 42 days.

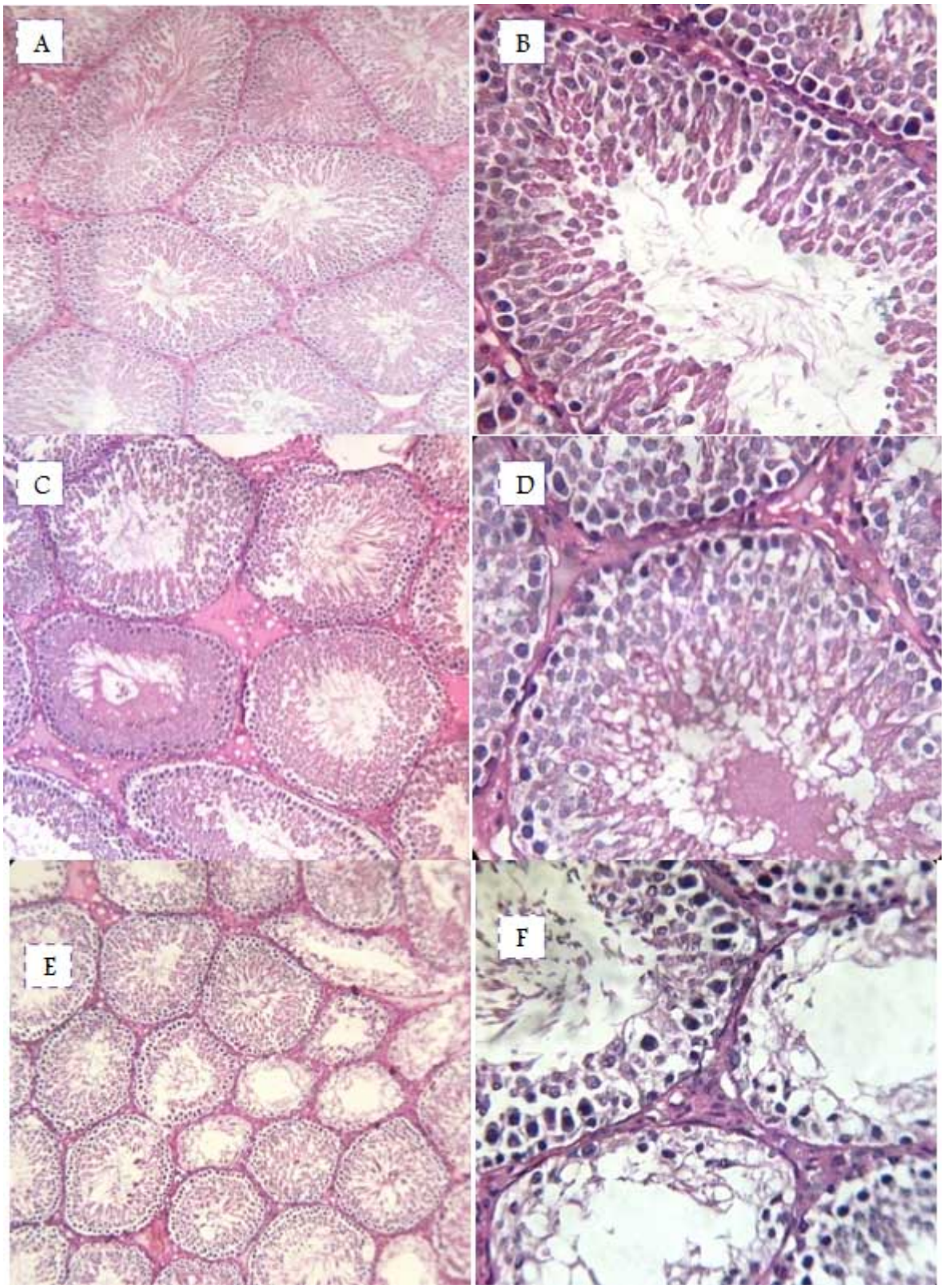


Figure 3: Photomicrographs of seminiferous tubule sections of rats from the control (A, B) showing the normal architecture of the seminiferous tubules. EMB-exposed testes at 28 days (C, D) showing oedema in intertubular spaces with vacuolation within tubules. EMB-exposed testes at 42 days (E, F) showing degenerative tubules with disrupted cellular organizations, completely absence of spermatozoon and decreased Sertoli cells . H & E x 200 (A, C) H & E x 400 (B, D).

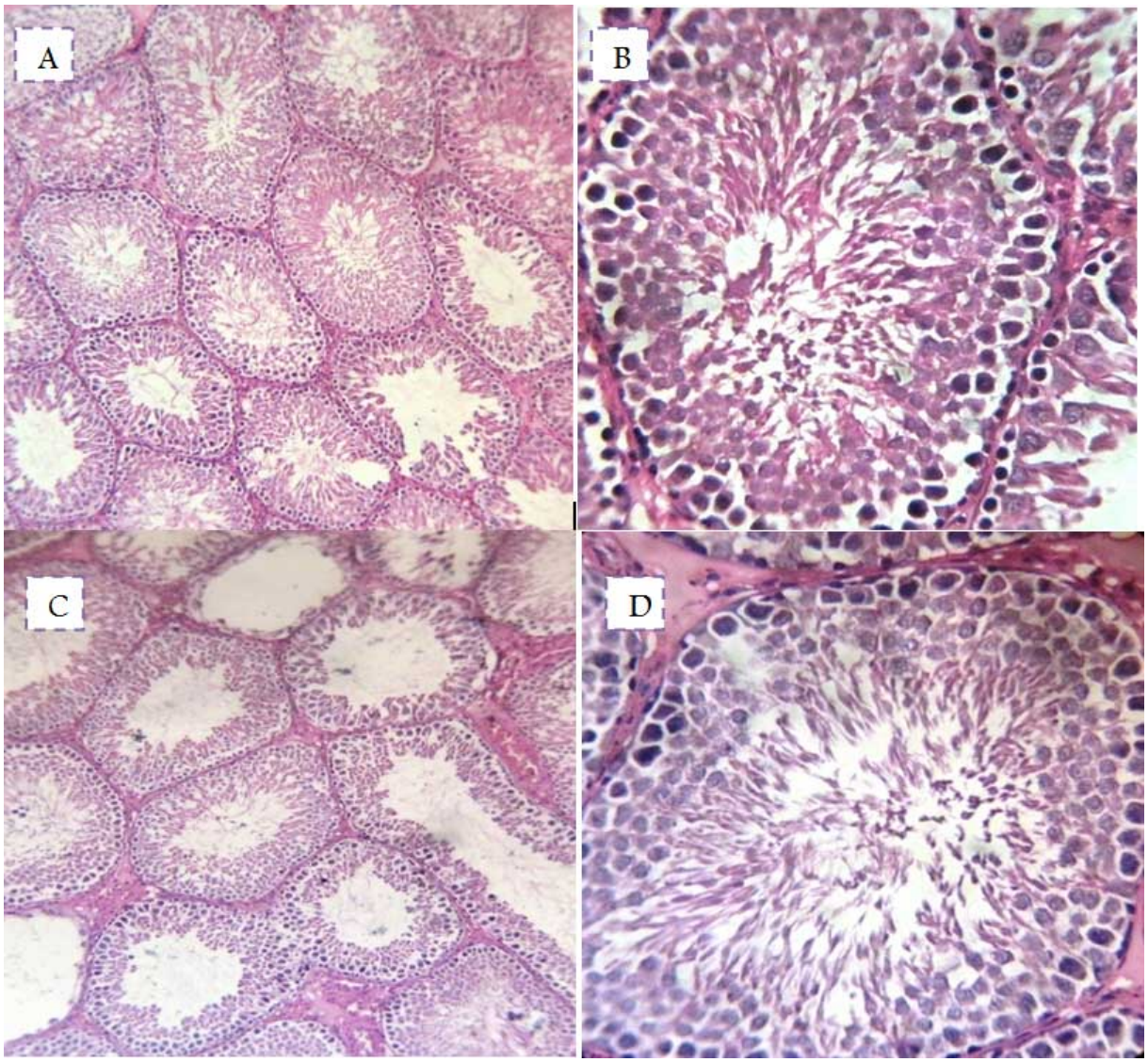


Figure 4: Photomicrographs of the testes of rat in the EMB+ Vit C- group at 28 days (A, B) showing oedema in intertubular spaces and mild degeneration in the seminiferous tubules. EMB + Vit C exposed testes at 42 days (C, D) showing mild changes in the cellular components of germinal epithelium H & E x 200 (A, C) H & E x 400 (B, D).

4. DISCUSSION

A number of pesticides have been shown to induce testicular dysfunction and impairment of spermatogenesis in animals (Zhang et *al*., 2007; Okamura et *al*., 2009). In general toxicity studies, it is well known that the changes in body and organ weights are important criteria for evaluation of organ toxicity (Bailey et *al*., 2004). Monitoring body weight provides information on the general health level of animals, which can be important to the interpretation of reproductive effects (Kavlock et *al*., 1996). In the present study, we observed a reduction of weight gain (%) and a decrease in absolute testes weights in EMB- treated group. The reduction in body weight is probably attributed to the reduction in food intake and toxic effects of EMB in treated rats. Similar results have been found in animals exposed to avermectin compounds (abamectin, emamectin benzoate) (Khaldoun et *al*., 2013; khaldoun et *al*., 2014). The results presented in this paper also show that the absolute and relative testes weights were significantly changed in males that ingested EMB insecticide and those co-administered with vitamin C. The increased weight of testes may be attributed to the accumulation of interstitial connective tissue around the seminiferous tubules. Elbetieha et Daas, (2003) reported that the weight of the testes significantly increased in males that ingested an avermectin insecticide (abamectin). In the present study, vitamin C significantly limited the effect of EMB on body and testes weights.

Histopathological finding showed damage in the tissue architecture of the testes from the EMB and EMB+ Vit C-treated groups. The structures of the seminiferous tubules were severely damaged; histopathological changes were more intense in rats from the EMB-treated group than in those from the EMB+VitC-treated group at 28 and 42 days, suggests that the pesticide specifically affects the testes structure. In the present subacute study, we show that the co-treatment with vitamin C after the administration of EMB led to a significant decrease in testes damage in the group EMB + VitC

Up to 80–98% of the initial administered concentrations of AVM can be excreted through animal feces (Herd, 1996). Residual AVM is difficult to degrade due to its physico-chemical properties of lipophilicity and water insolubility (Kolar et *al*., 2008). Several analytical methods have been developed to detect avermectin residues in various biological samples (e.g. food, fish, sheep; and liver, animal and human plasma, blood, urine and faeces) (Valenzuela et *al*., 2000 ; Turnipseed et *al*., 2005). Significant levels of EMB were detected in rat testes of EMB and EMB+VitC groups by a sensitive and rapid method using UPLC-MS/MS, in particular, after 28-day exposure to 10 mg EMB /kg/day. B1*a* and B1*b* were still detected in testes samples at 42 days.

5. CONCLUSION

In summary, our results strongly suggest that exposure to the pesticide emamectin benzoate would have adverse effects on testes in adult male rats. Testicular damage might be due to elevated concentrations of free oxygen radicals released upon EMB exposure, however, antioxidant pretreatment with vitamin C, administered at moderate doses, prevents or diminishes this damage, probably by reducing its accumulation in testes. The vitamin C supply is a putative protector against such effects, and should prevent farmers, agriculture workers and consumers intoxication by this product.

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