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Ethephon causes reproductive malfunction in adult male mice: Histological and biochemical evidence

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Abstract

Ethephon ($C_2H_6ClO_3P$; ETP), an organophosphorus pesticide regulating plant growth, is widely used for early ripening of fruits and vegetables in agriculture. The aim of this study was to investigate the effects of ETP on histomorphometrical and biochemical parameters in mouse testicular tissue. In this study, 90 adult male mice were randomly divided into six equal groups (n = 15). The ETP was administered orally at different doses (120, 240 and 480 mg kg^{-1}) daily for 35 days. Untreated control, sham (received only normal saline) and neostigmine bromide-treated (positive control; 0.10 mg kg^{-1} orally; once per week) groups were also considered. Following 35 days, animals were euthanized and testicle and serum samples were taken. Accordingly, blood and serum acetylcholinesterase (AChE), catalase, total antioxidant capacity (TAC) and malondialdehyde (MDA) levels as well as histomorphometrical changes of testicles were investigated. The ETP-administered animals represented a significant reduction in AChE, TAC and catalase levels and remarkable increment in MDA content. A marked reduction was also seen in the germinal epithelium height, connective tissue thickness, seminiferous tubules diameter and Leydig cell number as well as spermiogenesis and Sertoli cell indices in ETP-treated mice compared to control ones. Similar findings were found in neostigmine bromide-treated animals. In conclusion, the ETP significantly affects the serum and blood anti-oxidant statuses and results in severe histological damages both at germ and somatic cell levels, suggesting its hematotoxic and reprotoxic characteristic.

Introduction

Ethephon (2-chloroethyl phosphoric acid; ETP) is an organophosphorus pesticide widely used as a plant growth-regulator to accelerate plant growth process.[1-6] The ETP contains chloride and phosphate in its chemical structure and after being absorbed into the plant or animal tissues, it is gradually converted into ethylene gas and chloride; thereafter, the phosphorus molecules are released due to chemical bonds breaking.[5,7] As a phosphorylation agent, ETP inhibits acetyl cholinesterase (AChE) enzyme activity, especially in mice, rats, dogs and humans.[2,4,8] As a result of these changes, systemic disorders as well as male reproductive system impairment can be occurred. Accordingly, several previous reports have illustrated the organophosphorus pesticide-induced impairments in spermatogenesis, sperm characteristics and pre-implantation embryo development. Considering higher susceptibility of mouse plasma AChE *in vitro* and *in vivo* to ETP, this species has been used as an animal model in several studies.[4,5] Correspondingly, the occupational exposure of agricultural and industrial workers to pesticides has been shown to result in several systemic and genetic disorders.[5] In line with that and to inhibit the exposure and/or usage of organophosphorus pesticides in various fields, the United States Environmental Protection Agency has stated that plant growth regulators should be used at adequate times and quantities as prescribed, if required. Plant hormones including ETP could not be harmful for human health if applied in suitable doses and convenient time on plants.[5,9,10] However, in Western Asia and mostly in Iran, this statement is disregarded and ETP, similar to other organophosphorus chemicals, is used quite arbitrarily.[5]

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Reportedly, gastroenteritis, respiratory diseases, folded retina, microphthalmia, glomerulosclerosis, nephritis, heart fibrosis and brain mineralization have been found as common complications of ETP.[3,11-14] Additionally, the plant growth promoters such as ETP have been reported to induce chromosomal aberrations (structural and numerical) in both somatic and gametic cells.[5] Previous animal model-based studies have also been executed to evaluate the mutagenicity of ETP through detection of its capacity to inhibit enzymes activity (mainly AChE) and to change plasma protein content as well as the DNA and RNA concentrations determination.[4-7] It has also been revealed that ETP even at low non-cytotoxic doses has *in vitro* genotoxic and mutagenic effects.[12] Accordingly, the plant-growth regulators have been shown to negatively impact the reproduction system resulting in subfertility and/or infertility through oxidative stress induction and anti-oxidant defense system disturbance.[13-15] Consequently, the present study was undertaken to investigate the effects of ETP on male mice reproductive system via testicular tissue histomorphometrical analysis, biochemical parameters measurement and anti-oxidant machinery examination. Moreover, the neostigmine bromide (AChE blocker) was considered as a positive control for ETP and the data regarding ETP-induced possible changes were also compared with those from neostigmine bromide-treated animals.

Materials and Methods

Chemicals. The commercial 48.00% ETP was purchased from Aventis (Istanbul, Turkey) and neostigmine bromide was provided from Sigma-Aldrich Company (St. Louis, USA). Both ETP and neostigmine bromide were dissolved in distilled water.

Animals and housing. Ninety adult male mice (4-5 weeks old and weighting 30.00-35.00 g) were obtained from Animal Resource Center, Faculty of Veterinary Medicine, Urmia University (Urmia, Iran). The animals were maintained at 12 hr light and 12 hr dark cycle in propylene cages with free access to food and water. This study was approved by the Ethic Committee of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (IR-UU-AEC-437/DP3).

Experimental groups. The animals were randomly divided into main six groups (15 mice in each group) as follows: Group I: Untreated control group (not receiving any treatment); Group II: Sham group (receiving only normal saline); Group III: Low dose ETP (120 mg kg^{-1}; orally for 35 days); Group IV: Mid-dose ETP (240 mg kg^{-1}; orally for 35 days); Group V: High dose ETP (480 mg kg^{-1}; orally for 35 days) and Group VI: Neostigmine bromide (0.10 mg kg^{-1}; orally for 35 days as an AChE blocker). The administration dosages were calculated based on International Program on Chemical Safety instruction.[16]

Samples preparation. Following animal euthanasia with intraperitoneal injection of 150 mg kg^{-1} ketamine (Alfasan, Woerden, Holland) and 10.00 mg kg^{-1} xylazine (Rotexmedia, Trittau, Germany), testicular tissue samples were collected and fixed in 10.00% formalin solution. The tissue samples were cut, stained with Hematoxylin and Eosin (H&E) and examined under a light microscope (Olympus, Tokyo, Japan). The micro-scopic data were recorded using latticed and graded objective lens (Ernst Leitz Wetzlar GmbH, Germany). Also, the blood samples were collected from heart and slowly transferred to test tubes. Afterwards, the samples were centrifuged for serum separation at 3,000 rpm for 15 min.

Histological parameters. Thirty seminiferous tubules were randomly evaluated per section to record spermiogenesis index (SPI) as the ratio of the number of seminiferous tubules having spermatozoa to the depleted seminiferous tubules. In order to examine Sertoli cell index (SCI) and meiotic index (MI), the ratio of the number of germ cells to the number of Sertoli cells and the ratio of the number of round spermatids/primary spermatocytes were randomly computed in 20 seminiferous tubules per section, respectively. The number of Leydig cells per 1.00 × 16^{-1} mm^2 of the interstitial connective testicular tissue was morphometrically determined using lens device at 100× magnification in at least 20 different fields and the mean values were computed. To measure germinal epithelium height and seminiferous tubules diameter as well as testicular capsule and interstitial tissue thicknesses, 200 round or nearly-round cross-sections of the seminiferous tubules from each mouse were randomly analyzed using an ocular micrometer with light microscopy.[17,18]

Acetylcholinesterase enzyme activity. To evaluate AChE activity in erythrocytes and serum, Ellman and colorimetry methods were used, respectively. For this purpose, after blood sampling and half centrifugation by hemolysis, 3.00 mL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), (Elabscience, Houston, USA) reagent and 100 µL of substrate solution acetyl thiocholine iodide (Elabscience) were placed in a glass tube for 10 sec in an aqueous bath at 37.00 ˚C and then 100 µL of hemolysis solution was added to initiate the reaction. Finally, 1.00 mL of stopper reagent was added and recorded at 440 nm wavelength.[19]

Testicular total antioxidant capacity (TAC) measurement. To evaluate TAC level, 0.20 g of the testicular tissue sample was transferred to 0.05 M phosphate buffer with pH = 7.40 (10.00% w/v) and ground by mortar and pestle. To assess the serum TAC level, 30.00 µL of serum was considered. Total antioxidant capacity of testicular tissue was measured using ferric reducing antioxidant power method. This method is based on the strength of anti-oxidants in restoring Fe^{+3} to Fe^{+2} in the presence of a substance called tripyridyl triazine. The regeneration rate of each sample was measured by increasing the concentration of Fe^{+2} complexes with

tripyridyl triazine at 593 nm wavelength through spectrophotometry (Bio-Rad Laboratories, Hercules, USA).[20]

Testicular malondialdehyde (MDA) measurement. To evaluate MDA concentration, 0.20 g of the testicular tissue was transferred to 0.05 M phosphate buffer with pH = 7.40 (10.00% w/v) and ground by mortar and pestle. To assess the serum MDA level, 50.00 µL of serum was considered. Then, the resulting solution was centrifuged at 1,000 rpm. Afterwards, 150 µg of the supernatant of the centrifuged sample was removed, 300 µg of 10.00% trichloroacetic acid was added and it was centrifuged at 1,000 rpm for 10 min. Then, 300 µL of the supernatant was transferred to the test tube and incubated with 300 µL of 0.67% thiobarbituric acid (TBA) at 100 ˚C for 25 min. After 5 min, the pink color resulted from reaction between MDA and TBA was evaluated with a spectrophotometer at 535 nm wavelength. The MDA concentration was calculated using MDA absorption coefficient and expressed in terms of nmol per g tissue.[21]

Catalase activity assessment. The enzyme activity in testicular tissue was assessed based on its ability to decompose H_2O_2 in homogenized tissue via Aebi method.[22]

Serum testosterone level analysis. The serum concentration of testosterone was measured by an enzyme-linked immunosorbent assay method using instructions provided by the kit manufacturer (Sigma-Aldrich).

Statistical analysis. The Statistical Package for Social Sciences (SPSS®, version 22.0; IBM Corp., Armonk, USA) was used for data analysis and statistical analysis was performed using one-way ANOVA followed by Tukey comparisons test as a post hoc test to determine the significant differences between the means. Moreover, numerical values were reported as mean ± Standard Deviation. A *p*-value less than 0.05 was considered as statistically significant.

Results

Histological findings. As shown in Table 1, reductions were observed in the germinal epithelium height, inter-stitial tissue thickness, seminiferous tubules diameter and Leydig cell number as well as SPI and SCI in ETP-received mice versus control ones. However, no significant changes were seen regarding MI among mice treated with mid-dose and high dose of ETP and control ones. Moreover, testicular capsule thickness was increased in ETP-received groups compared to control group. Testicular tissue morphological examination also revealed germinal epithelium dissociation as well as mild to severe tubular depletion in ETP-received groups in comparison with control group exhibiting normal spermatogenesis and spermiogenesis (Fig. 1).

Table 1. Histomorphometrical parameters in all experimental groups.

Parameter	Control	Sham	LD-ETP	MD-ETP	HD-ETP	Neostigmine
Testicular capsule thickness (µm)	9.79 ± 0.23^{b}	10.72 ± 0.21^{a}	11.14 ± 0.16^{a}	11.04 ± 0.15^{a}	10.31 ± 0.21^{ab}	9.82 ± 0.31^{b}
Seminiferous tubules diameter (µm)	200.80 ± 2.14^{a}	193.60 ± 2.27^{ab}	194.40 ± 3.45^{ab}	185.10 ± 3.69^{bc}	173.80 ± 3.30^{c}	185.60 ± 3.94^{bc}
Germinal epithelium height (µm)	57.56 ± 0.56^{a}	48.60 ± 0.46^{b}	46.10 ± 0.73^{b}	47.80 ± 0.77^{b}	47.20 ± 0.86^{b}	48.10 ± 0.68^{b}
Sertoli cell index	26.78 ± 0.84^{a}	23.50 ± 0.51^{bc}	24.75 ± 0.81^{ab}	24.69 ± 0.49^{ab}	20.90 ± 0.72^{c}	24.00 ± 0.51^{b}
Number of Leydig cell per 0.06 mm^2	4.40 ± 0.28^{a}	4.67 ± 0.25^{a}	3.39 ± 0.20^{b}	3.18 ± 0.23^{b}	3.04 ± 0.20^{b}	2.96 ± 0.20^{b}
Interstitial tissue thickness (µm)	52.00 ± 5.20^{a}	47.50 ± 1.50^{ab}	31.50 ± 1.50^{ab}	29.00 ± 2.00^{b}	26.50 ± 3.50^{b}	31.00 ± 4.00^{ab}
Spermiogenesis index (%)	81.70 ± 1.32^{a}	72.00 ± 1.83^{b}	57.10 ± 2.39^{c}	55.50 ± 3.14^{c}	50.90 ± 2.56^{c}	79.60 ± 1.58^{ab}
Meiotic index	0.53 ± 0.03^{b}	0.39 ± 0.03^{b}	0.73 ± 0.05^{a}	0.46 ± 0.05^{b}	0.47 ± 0.05^{b}	0.37 ± 0.04^{b}

LD-ETP: Low dose ethephon (120 mg kg^{-1}); MD-ETP: Mid-dose ethephon (240 mg kg^{-1}); HD-ETP: High dose ethephon (480 mg kg^{-1}).
^{abc} Different letters indicate significant difference among groups ($p < 0.05$).

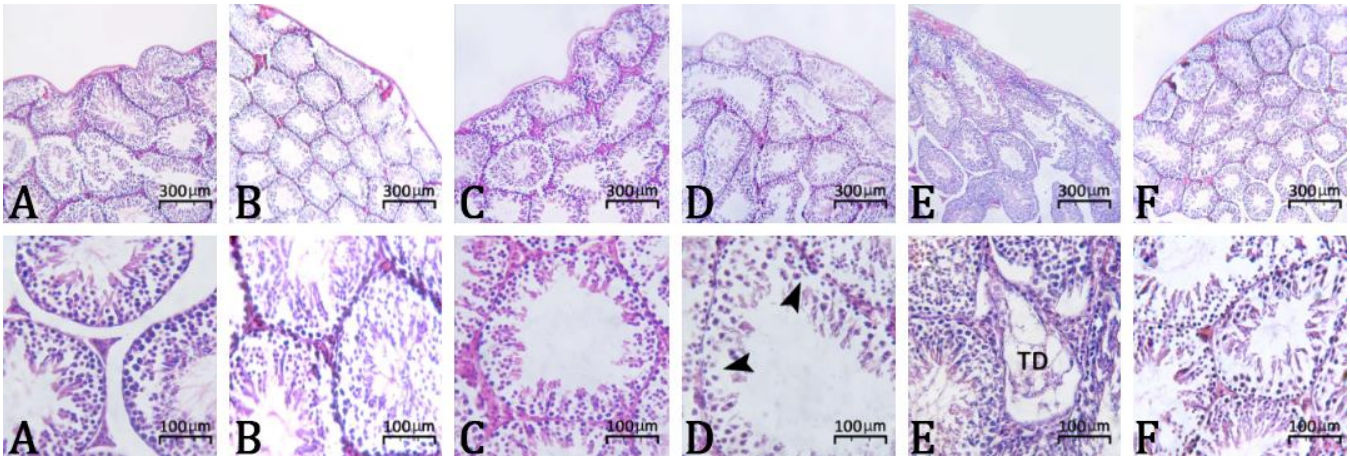

Fig. 1. Cross sections of mice testis in **A)** Control; **B)** Sham; **C)** Low dose ethephon; **D)** Mid-dose ethephon; **E)** High dose ethephon; **F)** Neostigmine bromide groups. Germinal epithelium dissociation (arrowheads) and tubular depletion (TD) can be observed in mid-dose and high dose ethephon groups, respectively, (H&E).

Acetylcholinesterase enzyme activity. The AChE activity levels in erythrocytes and serum were reduced in ETP groups in a dose-dependent manner compared to control group (Table 2).

Catalase enzyme activity. Catalase enzyme activity in testicular tissue was declined significantly ($p < 0.05$) in all ETP-received animals compared to control ones (Table 2).

Total antioxidant capacity. In ETP-treated groups, the serum and testicular tissue TAC levels were decreased significantly in a dose-dependent manner *versus* the control group (Table 2).

Malondialdehyde concentration. The MDA concentration in testicular tissue of ETP groups was increased significantly compared to the control group. However, its concentration in serum was significantly higher in mid-dose and high dose ETP groups compared to the control group (Table 2).

Serum testosterone concentration. There was no significant difference regarding serum testosterone level among all experimental groups (Table 2).

Discussion

The ETP is a plant growth regulator commonly used in agricultural systems for fruits and vegetables ripening and its excessive use may cause organopathies.[1-3] In the present study, ETP treatment resulted in testiculopathies through oxidative stress induction and anti-oxidant defense system weakening. These results are in agreement with previous studies reporting that ETP can cause androgen deprivation having adverse effects on male reproductive system through sperm abnormalities and testicular tissue damage inductions.[23] Further, role of oxidative stress in ETP toxicity has been demonstrated previously.[10] In the current study, spermatogenic indices reduction was also observed in ETP-treated animals. Our findings are consistent with former reports indicating that organophosphorus compounds can induce testicular toxicities.[24] Confirming our findings, previous reports have also implied that plant growth regulators induce spermatogenic arrest, testicular degeneration, Leydig cells destruction and seminiferous tubules disorganization associated with lipid peroxidation elevation and free radicals over-generation.[14] Accordingly, it has been revealed that plant growth regulators may act as repro-toxic agents as well as metabolic deregulators and spermatogenesis inhibitors in testicular tissue.[15,25]

Recently, ultra-structural studies have also showed marked smooth endoplasmic reticulum dilatation in Sertoli cells and dilated perinuclear cisternae formation in spermatogonia and primary spermatocytes as indicators of lipid peroxidation following plant growth regulator administration in rats, confirming our results regarding the role of free radicals overproduction and oxidant/anti-oxidant imbalance in ETP-induced testicular damages.[26] Correspondingly, observed testicular germ cell hypoplasia in ETP-treated animals in this study could be attributed to the pro-apoptotic effects of ETP leading to cell death.[27] Considering the role of free radicals scavenging enzymes including catalase in cellular apoptosis regulation, it can be suggested that ETP triggers apoptotic cell death through scavenger enzymes downregulation.[28] Our findings exhibited that AChE activity was diminished in mice red blood cells and serum following ETP administration. Accordingly, it has been suggested that AChE activity inhibition is considered as a marker of ETP toxicity.[9] Joshi *et al*. have demonstrated that treatment of rats with organophosphorus compound, chlorpyrifos, induces testicular toxicity. Marked reduction in testosterone production in association with highly reduced circulating levels of it confirmed the change in the reproductive physiology of rats.[29] In a parallel manner, it was also found that treatment of ETP-intoxicated rats with costus root extract prevented/reversed the ETP induced negative impacts on fertility parameters, protected the Leydig and Sertoli cells from ETP-induced damages and normalized serum levels of reproductive hormones altered by ETP administration. Moreover, it has also been reported that anti-oxidants improve steroidogenesis through promoting Leydig cell function and consequently increase testosterone production leading to spermatogenesis enforcement.[30]

Taken together, our results disclosed ETP repro-toxicities at histological and biochemical levels in male mice due to its ability to induce oxidative stress, to disturb anti-oxidant machinery and to inhibit AChE activity.

Table 2. Biochemical parameters in all experimental groups.

Parameter	Control	Sham	LD-ETP	MD-ETP	HD-ETP	Neostigmine
Serum AChE (mU mL^{-1})	0.34 ± 0.00^{a}	0.32 ± 0.00^{a}	0.25 ± 0.01^{b}	0.22 ± 0.00^{bc}	0.18 ± 0.00^{c}	0.25 ± 0.01^{b}
Blood AChE (mU mL^{-1})	0.67 ± 0.01^{ab}	0.69 ± 0.01^{a}	0.62 ± 0.01^{b}	0.35 ± 0.01^{d}	0.21 ± 0.00^{e}	0.50 ± 0.01^{c}
Catalase activity (U mg^{-1})	502.20 ± 1.12^{a}	503.90 ± 1.45^{a}	405.60 ± 1.65^{b}	370.50 ± 3.10^{b}	303.30 ± 1.01^{c}	404.10 ± 2.22^{b}
Testicular TAC (mmol g^{-1})	2.32 ± 0.00^{a}	2.47 ± 0.07^{a}	1.88 ± 0.05^{b}	1.38 ± 0.03^{c}	0.92 ± 0.02^{d}	1.91 ± 0.03^{b}
Testicular MDA (nmol g^{-1})	13.42 ± 0.10^{d}	13.52 ± 0.09^{d}	13.86 ± 0.06^{c}	20.76 ± 0.06^{b}	23.05 ± 0.01^{a}	14.03 ± 0.01^{c}
Serum TAC (µmol L^{-1})	4.03 ± 0.01^{a}	3.87 ± 0.06^{a}	3.22 ± 0.06^{b}	2.50 ± 0.10^{c}	1.70 ± 0.14^{d}	2.78 ± 0.10^{bc}
Serum MDA (µmol L^{-1})	5.54 ± 0.12^{c}	5.23 ± 0.09^{c}	5.52 ± 0.22^{c}	6.44 ± 0.18^{b}	7.46 ± 0.26^{a}	5.56 ± 0.14^{c}
Testosterone (ng mL^{-1})	0.29 ± 0.07^{a}	0.43 ± 0.11^{a}	0.31 ± 0.01^{a}	0.23 ± 0.01^{a}	0.19 ± 0.01^{a}	0.32 ± 0.01^{a}

LD-ETP: Low dose ethephon (120 mg kg^{-1}); MD-ETP: Mid-dose ethephon (240 mg kg^{-1}); HD-ETP: High dose ethephon (480 mg kg^{-1}); AChE: Acetylcholinesterase; TAC: Total antioxidant capacity; MDA: Malondialdehyde.

^{abcde} Different letters indicate significant difference among groups ($p < 0.05$).

Therefore, due to possible public health hazards, general awareness regarding the use of plant growth regulators should be promoted. Further studies are needed to fully understand the ETP effects on fertility at molecular levels.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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