



Ameliorating Effects of Green Tea on Ethephon-Induced Immunotoxicity and Oxidative Stress In Mice

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Abstract

The immunotoxic effects of the organophosphorus plant growth regulator ethephon and the probable protective effects of green tea were investigated in mice. Animals received ethephon in the diet at a dose level equivalent to 1/10th oral LD50 for 8 weeks. Red blood cell count, hemoglobin level and hematocrit value were reduced by ethephon treatment, while leucocytosis, neutrophilia, monocytosis and lymphocytopenia were recorded. Significant elevations were observed in activities of serum transaminases and levels of total bilirubin, urea and creatinine. Serum total protein, albumin and albumin/globulin ratio were reduced. Serum hemolyzing antibody titer against sheep red blood cells (SRBC) and levels of serum immunoglobulins were decreased indicating inhibition of humoral immunity. The delayed-type hypersensitivity response to SRBC and the phagocytic activity of polymorphonuclear cells towards candida albicans were suppressed. Levels of malondialdehyde in spleen and thymus were elevated while glutathione levels were reduced. The activities of antioxidant enzymes catalase and glutathione peroxidase were inhibited in both spleen and thymus. Histopathological alterations were recorded in liver, kidney and spleen. Co-administered green tea extract with ethephon ameliorated most of the toxic effects of ethephon.

Keywords: Ethephon, Immunity, Hematology, Oxidative stress, Green tea, Mice

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Introduction

Many plant growth regulators are being used in agriculture. Excessive use of these chemicals would certainly be associated with many health hazards. Ethephon (2-chloroethyl phosphonic acid) is a plant growth regulator, being used for artificial ripening acting by release of ethylene, directly influencing several physiological processes, such as ripening and maturation, and stimulating the production of endogenous ethylene. It is used on a variety of crops including fruits, vegetables, cereals and oilseed crops⁽¹⁾.

Ethephon was found to inhibit the activity of plasma cholinesterase⁽¹⁾ and produce hematological changes in intoxicated animals⁽²⁾. Hepato- and nephrotoxicity of ethephon were also reported^(3,4,5). Reproductive toxicity and oxidative stress⁽⁶⁾ and genotoxicity of ethephon were demonstrated in mice⁽⁴⁾.

The available data concerning the impact of ethephon on the immune system are scarce. However, the phosphonic acid derivative trichlorophon was reported to be immunotoxic. It induced dose-dependent suppressive effects on lymphocyte proliferation and myeloid cell respiratory burst activities in carp fish in vitro⁽⁷⁾ and reduced the phagocytic ability of neutrophils and phagocytic index in carp in vivo⁽⁸⁾. In addition, other organophosphates such as malathion, parathion, diazinon and dimethoate were previously reported to induce immunotoxic effects⁽⁹⁾.

Tea is a popular drink all over the world. Tea extracted from plant *Camellia sinensis* is used as green, black or Oolong tea. Of all these types, green tea has the most significant effects on health. Green tea was reported to prevent many diseases including diabetes, cancer, inflammation and obesity. The active principles in tea are polyphenols which have many useful effects including anti-inflammatory, anti-estrogenic, anti-mutagenic and anti-carcinogenic effects⁽¹⁰⁾.

The present study was conducted to assess how safe is this widely used ethephon on the humoral and cell mediated immune responses of mice. Hematology, blood chemistry and oxidative stress in lymphoid organs were also studied. Moreover, the possible protective effect of green tea extract was evaluated.

Materials and methods

Chemicals

Ethrel, the commercial form containing 48% ethephon (2-chloroethyl phosphonic acid) was purchased from Chema Industries. Test kits for total protein and bilirubin were obtained from Sigma Aldrich and Spectrum, respectively. Diagnostic kits for all other serum biochemical parameters and oxidative stress were purchased from Biodiagnostic, Egypt

Green tea extract

The green tea was purchased in the form of dried leaves by Rabea Co, Saudi Arabia. Tea extract was made by soaking 2 grams of tea leaves in 100 ml of hot water maintained at $85 \pm 3^\circ\text{C}$ for 5 min⁽¹¹⁾. The aqueous extract was filtered with muslin cloth, allowed to cool to room temperature and given to mice as their sole source of drinking water. Fresh tea extract was prepared every second day.

Animals

This study was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals approved by the Committee on the Ethics of Animal Experiments of University of Sadat City, Egypt. Male mice, 4 weeks of age, were obtained from Vaccera Pharmaceutical Company, Agouza, Egypt. Animals were housed in plastic cages and offered water and balanced diet ad libitum and ac-

climated to laboratory conditions (a 12-h light/dark cycle; temperature maintained $23 \pm 2^\circ\text{C}$) for 2 weeks before start of experimental work.

Experimental design

Experiment I

Forty-five mice were used for studying the hematological changes, blood chemistry, humoral immune response and oxidative stress in spleen and thymus. Animals were allocated into 3 equal groups, 15 animals each. **Group I** received no chemical treatment and served as control. **Group II** received ethephon in the diet at a dose rate of 1995 ppm (equivalent to 1/10th of the oral LD50 (1) for 2 months. **Group III** received both ethephon as mentioned above in addition to green tea extract.

All animals were immunized by I/P injection of 0.5 ml of a 20% SRBC suspension at the 40th day of chemical exposure. A second (booster) dose was given 15 days after the first immunization. Blood samples were collected five days after the booster dose from inner venous canthus of the eye of each mouse under diethyl ether anesthesia. Part of blood was taken on EDTA for hemogram evaluation. The other part was used for separation of serum which was stored at -20°C until assayed for biochemical changes, antibody titer and immunoglobulin levels. Animals were then sacrificed, and spleen and thymus were rapidly removed and stored at -20°C to be used for study of oxidative stress.

Hematological and Biochemical changes

Red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (Hct), total and differential leucocytic counts were estimated according to Feldman et al.⁽¹²⁾. Activities of serum ALT and AST⁽¹³⁾, and levels of total protein⁽¹⁴⁾, albumin⁽¹⁵⁾, total bilirubin⁽¹⁶⁾, urea⁽¹⁷⁾ and creatinine⁽¹⁸⁾ were assessed according to manufacturer instructions. Total globulin was determined by subtracting albumin from total protein and then albumin-globulin ratio (A/G) was estimated.

Humoral Immunity

Serum antibody titer

The hemolysin antibody titer was measured according to Seinen et al.⁽¹⁹⁾. Serial twofold dilutions (100 μl) of decomplexed sera (56°C for 30 min) were prepared in the microtitration plates. To which 50 μl of a 1% SRBC suspension and 50 μl of guinea pig complement diluted 1:10 were added. The hemolysin titers were read after 1-hr incubation at 37°C . Titers represent the reciprocal of the highest dilution giving total hemolysis.

Serum immunoglobulins

Serum levels of immunoglobulins M and G were determined using single radial immunodiffusion methods derived primarily from the works of Fahey and McKelvey⁽²⁰⁾ and Mancini et al.⁽²¹⁾.

Experiment II

Thirty animals were used to measure the delayed - type hypersensitivity (DTH) response to SRBC and the phagocytic activity of polymorphonuclear cells. Animals are randomly classified into 3 equal groups, ten animals each. Group I was kept as control without chemical treatment. Group II received ethephon in the diet at 1995 ppm for 2 months. Group III received both ethephon in diet at 1995 ppm and green tea extract. All animals were inoculated on day 55 with an I/P injection of SRBC (1×10^8 in saline). After five days, animals were challenged S/C with SRBC suspension (1×10^8) in the left hind paw and with the same volume of saline in the right hind paw.

The Delayed- type hypersensitivity (DTH) response

The thickness of edema was measured 24 and 48 hours post-chal-

lenge, with a digital microcaliper. The degree of edema was calculated by subtracting the thickness of the saline-injected hind paw from that of the SRBC-injected hind paw⁽²²⁾.

Phagocytic activity

Blood from mice of all groups was collected on heparin (20 IU/ml) according to the method described by Wilkinson⁽²³⁾ for studying the phagocytic activity of polymorphonuclear cells towards candida albicans. The index of phagocytosis was calculated by dividing the number of ingested Candida albicans on the phagocytic activity.

Oxidative stress

Spleen and thymus of mice of Experiment I were used for measuring the levels of malondialdehyde, MDA⁽²⁴⁾ and reduced glutathione, GSH⁽²⁵⁾ and activities of catalase, CAT⁽²⁶⁾, glutathione peroxidase, GPx⁽²⁷⁾ and superoxide dismutase, SOD⁽²⁸⁾ following the manufacturer's instructions.

Histopathological study. Liver, kidney and spleen of mice from Exper-

iment II were fixed in 10% neutral buffered formalin and prepared for histopathological examination according to Bancroft et al.⁽²⁹⁾.

Statistical analysis.

The data were presented as mean \pm standard error (SE) and were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by a multiple comparison Duncan test. Differences at $p \leq 0.05$ were considered significant.

Results

Hematological changes

Data shown in Table 1 revealed that administration of ethephon to mice significantly reduced ($P < 0.05$) RBC count, hemoglobin level and hematocrit value. Total WBC count and percentages of neutrophils and monocytes were significantly elevated ($P \leq 0.05$), while lymphocyte percentage was significantly reduced ($P \leq 0.05$). Co-administration of green tea extract ameliorated the toxic effects of ethephon on hematological parameters.

Parameters	Control	Ethephon	Ethephon + Green tea
RBCs ($\times 10^6/\mu\text{l}$)	9.75 \pm 0.15 ^a	8.42 \pm 0.29 ^b	9.49 \pm 0.10 ^a
Hb (g/dl)	14.79 \pm 0.09 ^a	12.78 \pm 0.39 ^b	14.31 \pm 0.08 ^a
Hct (%)	51.60 \pm 0.50 ^a	44.40 \pm 1.12 ^c	48.00 \pm 0.31 ^b
WBCs ($\times 10^3/\mu\text{l}$)	6.37 \pm 0.43 ^c	8.73 \pm 0.39 ^a	7.62 \pm 0.16 ^b
Neutrophils (%)	56.20 \pm 0.37 ^c	60.80 \pm 0.37 ^a	58.20 \pm 0.37 ^b
Lymphocytes (%)	38.20 \pm 0.48 ^a	32.40 \pm 0.50 ^c	35.60 \pm 0.24 ^b
Monocytes (%)	5.20 \pm 0.20 ^b	6.60 \pm 0.24 ^a	5.80 \pm 0.20 ^b
Eosinophils (%)	0.40 \pm 0.02	0.20 \pm 0.01	0.40 \pm 0.02
Basophils (%)	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00

Table (1): Hemogram in different groups of mice.

Values are presented as mean \pm SE, n= 5

Different letter superscripts (a,b,c) in the same row are significantly different at ($P \leq 0.05$).

Biochemical changes

Serum activities of ALT and AST, and levels of total bilirubin, globulin, urea and creatinine were significantly elevated ($P \leq 0.05$) in animals intoxicated with ethephon (Table 2). On the contrast, serum total protein, albumin and albumin/globulin ra-

tio were significantly reduced ($P \leq 0.05$). Concurrent administration of green tea extract neutralized most of the toxic effects of ethephon and shifted the values of most parameters in the direction of control values

Parameters	Control	Ethephon	Ethephon + Green tea
ALT (U/L)	45.94 \pm 0.68 ^c	62.69 \pm 0.72 ^a	57.13 \pm 0.18 ^b
AST (U/L)	91.85 \pm 0.73 ^c	156.59 \pm 0.64 ^a	116.08 \pm 0.52 ^b
Total protein (g/dl)	5.93 \pm 0.02 ^a	5.59 \pm 0.01 ^c	5.76 \pm 0.02 ^b
Albumin (g/dl)	3.45 \pm 0.07 ^a	2.83 \pm 0.06 ^c	3.14 \pm 0.04 ^b
Globulin (g/dl)	2.48 \pm 0.06 ^b	2.75 \pm 0.06 ^a	2.62 \pm 0.02 ^{ab}
A /G ratio	1.39 \pm 0.06 ^a	1.03 \pm 0.04 ^c	1.20 \pm 0.02 ^b
Total bilirubin (mg/dl)	1.52 \pm 0.00 ^c	1.63 \pm 0.00 ^a	1.60 \pm 0.00 ^b
Urea (mg/dl)	36.86 \pm 0.81 ^c	54.65 \pm 1.37 ^a	46.50 \pm 0.64 ^b
Creatinine (mg/dl)	1.25 \pm 0.01 ^c	1.53 \pm 0.03 ^a	1.40 \pm 0.02 ^b

Table (2): Blood biochemical parameters in different groups of mice

Values are presented as mean \pm SE, n= 5

Different letter superscripts (a,b,c) in the same row are significantly different at ($P \leq 0.05$).

Immune responses

Administration of ethephon to mice for 8 weeks significantly suppressed ($P \leq 0.05$) hemolysin antibody titer and the levels of serum immunoglobulins (Table 3). In addition, the DTH reaction to SRBC was inhibited ($P \leq 0.05$) at 24 hours (Fig.1). The administration of green tea extract improved the inhibition of humoral immune response and completely recovered the suppression of DTH reaction.

	Control	Ethephon	Ethephon + Green tea
Antibody titer (\log_2)	6.41 ± 0.198^a	4.57 ± 0.310^c	5.60 ± 0.189^b
IgM (mg/dl)	30.81 ± 0.746^a	25.38 ± 0.798^b	26.17 ± 0.974^b
IgG (mg/dl)	81.82 ± 0.831^a	24.78 ± 0.955^c	27.42 ± 0.653^b

Table (3): Mean values of serum hemolysin antibody titer and IgM and IgG in control and treated rats.

Values are presented as mean \pm SE, n= 5

Different letter superscripts (a,b,c) in the same row are significantly different at ($P \leq 0.05$).

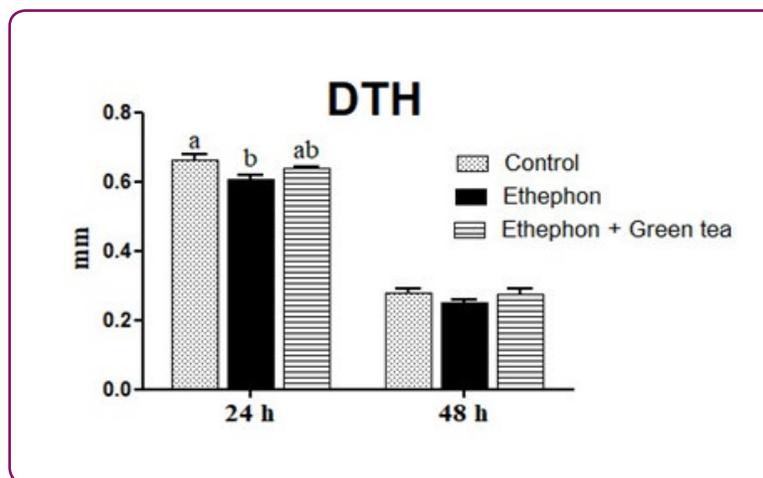


Fig. (1): Delayed-type hypersensitivity reaction in control and treated mice.

Values are presented as mean \pm SE, n= 5

Different letters (a,b) are significantly different at ($P \leq 0.05$).

Phagocytic activity

The phagocytic activity and phagocytic index were significantly decreased ($P \leq 0.05$) in mice intoxicated with ethephon (Table

4). Coadministration of green tea extract partially ameliorated the toxic effect of ethephon on neutrophil phagocytic activity.

Parameters	Control	Ethephon	Ethephon + Green tea
Phagocytic activity (%)	63.20 ± 0.58^a	56.20 ± 0.96^c	59.60 ± 0.50^b
Phagocytic index	2.96 ± 0.00^a	2.41 ± 0.02^c	2.61 ± 0.01^b

Table (4): Phagocytic activity of neutrophils in control and treated mice

Values are presented as mean \pm SE. n= 5

Different letter superscripts (a,b,c) in the same row are significantly different at ($P \leq 0.05$).

Oxidant/antioxidant markers

Fig. 2 reveals that administration of ethephon to mice caused oxidative stress in spleen and thymus as evidenced by elevated MDA concentration, reduced GSH level and inhibited activities of CAT and GPx,

while SOD activity showed no change. Marked improvement of all parameters was observed in both spleen and thymus of mice received green tea extract with ethephon.

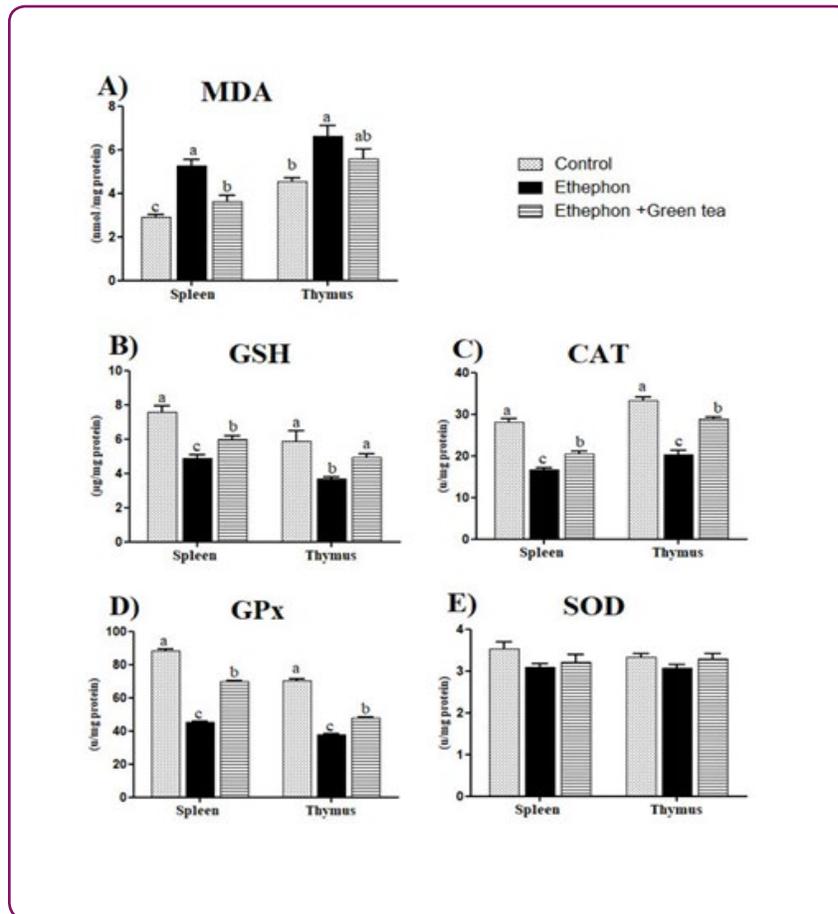


Fig (2): Oxidant and antioxidant markers in spleen and thymus of mice of different groups. A) Malondialdehyde (MDA), B) Reduced glutathione (GSH), C) Catalase (CAT), D) glutathione peroxidase (GPx) and E) Superoxide dismutase (SOD).

Values are presented as mean \pm SE, n= 5

Different letters (a,b,c) are significantly different at ($P \leq 0.05$).

Histopathological findings

Liver of control mice showed normal histology (Fig 3a). Mice treated with ethephon showed congestion of central vein, necrosis of hepatocytes, dissociation of hepatic cords and multifocal mononuclear cell infiltration in the hepatic parenchyma (Fig. 3b). Co-administration of green tea extract protected the liver so that only mild mononuclear cells infiltration in the hepatic parenchyma was noted (Fig. 3c). Kidney of ethephon treated mice revealed vacuolation and degeneration of renal tubular epithelium, interstitial hemorrhage and multifocal

mononuclear cells infiltration between the renal tubules (Fig. 4b). Animals treated with both ethephon and green tea extract showed only interstitial hemorrhage between the renal tubules (Fig. 4c).

Spleen of ethephon treated group showed lymphoid depletion and some tingible body macrophages in the germinal center imparting a starry sky appearance of the white pulp (Fig. 5b). Spleen of animals treated with both ethephon and green tea extract showed normal histology of spleen white and red pulps (Fig. 5c).

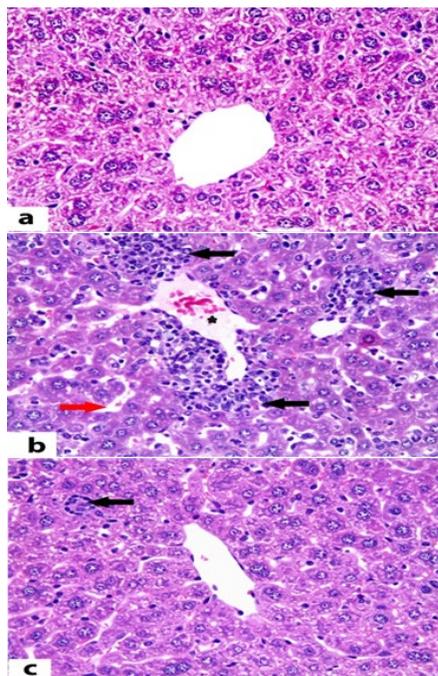


Fig.3: a) Liver of control mouse showing normal histology and arrangement of hepatocytes (hepatic cords) around the central vein. b) Liver of ethephon treated mouse showing congestion of central vein (star), necrosis of hepatocytes, dissociation of hepatic cords (red arrow) and multifocal mononuclear cells infiltration in the hepatic parenchyma (black arrows). c) Liver of mouse treated with both ethephon and green tea extract showing only mild mononuclear cells infiltration in the hepatic parenchyma (arrow). H&E, X20.

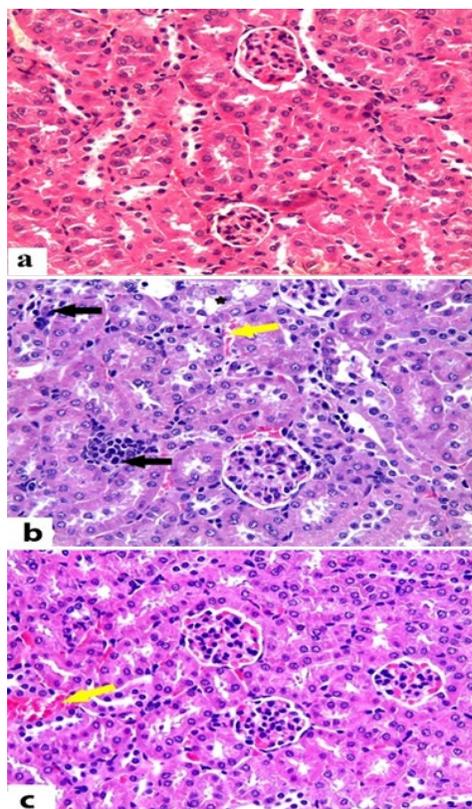


Fig. 4: a) Kidney of control group showing normal histology of renal tubules and glomeruli. b) Kidney of ethephon-treated group showing vacuolation and degeneration of renal tubular epithelium (star), interstitial hemorrhage (yellow arrow) and multifocal mononuclear cells infiltration between the renal tubules (black arrows). c) Kidney of mouse received both ethephon and green tea extract showing only interstitial hemorrhage between the renal tubules (arrow). H&E, X20.

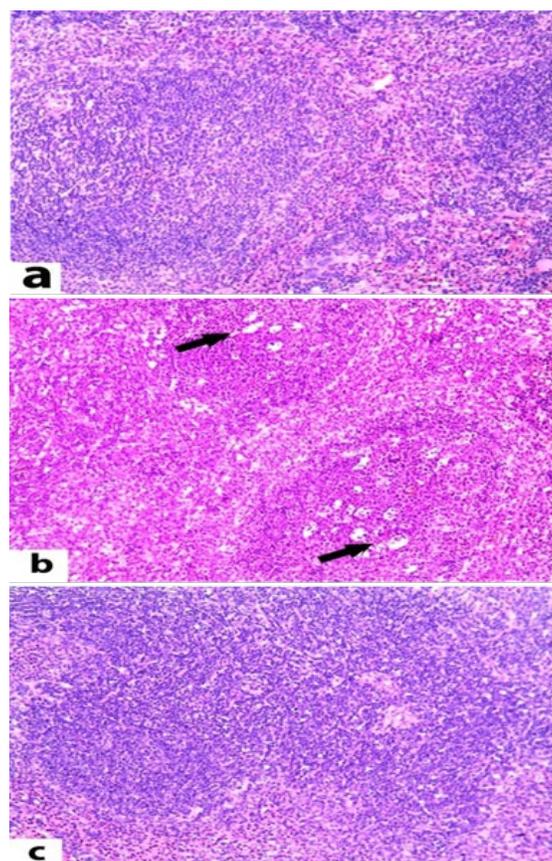


Fig. 5: a) Spleen of control group showing normal histological pattern of the spleen white and red pulp. b) Spleen of ethephon treated group showing lymphoid depletion and some tingible body macrophages (arrows) in the germinal center imparting a starry sky appearance of the white pulp. c) Spleen of group with dual treatment showing normal histology of spleen white and red pulp. H&E, X10.

Discussion

Ethephon (2-chloroethylphosphonic acid) is a plant growth regulator of the organophosphorus group of pesticides. The current investigation was conducted to explore its toxic effects on humoral and cell-mediated immune responses of mice and relating them to oxidative stress in spleen and thymus. Hematology, blood chemistry and histopathological alterations in liver, kidney and spleen were also investigated. Furthermore, the protective effects of green tea against ethephon-induced toxic effects were studied.

Our findings revealed that ethephon intoxicated mice showed anemia evidenced by significant decreases of RBC count, Hb level and Hct value. These findings are consistent with those reported by Abd El Raouf and Girgis⁽⁴⁾ who observed significant decrease of Hb concentration in mice intoxicated with ethephon. In addition, our results revealed leucocytosis, neutrophilia and lymphopenia. In agreement with our results, Wang et al.⁽³⁰⁾ reported that ethephon reduced the number peripheral blood lymphocytes in mice. Stress leukogram may be the result of oxidative stress induced by ethephon on lymphoid organs. Acute stress in animals has been widely reported to be associated with leucocytosis and occasionally with neutrophilia and increase of neutrophil/lymphocyte ratio⁽³¹⁾. Oxidative stress has been implicated in the mechanism of damage of RBCs⁽³²⁾ and abnormalities in the shape, function and metabolism of different types of blood cells⁽³³⁾.

Regarding serum biochemical parameters in mice intoxicated with ethephon, there were significant elevations in serum ALT and AST activities, and serum levels of total bilirubin, globulin, urea and creat-

inine. On the contrary, total protein, albumin and albumin/globulin ratio were significantly reduced. These results are consistent with our histopathological findings in liver. Our findings agree with those of Abd El Raouf and Girgis⁽⁴⁾ who reported that ethephon reduced total protein while elevated transaminases, urea and creatinine values in mice. Ethephon caused inflammatory and degenerative changes in the liver associated with cholestasis, probably suggestive of toxic hepatitis⁽⁵⁾. The elevation of serum bilirubin and decrease of total protein and albumin indicate compromised liver excretory and synthetic functions⁽³⁴⁾. However, hypoproteinemia may be associated with chronic renal disease, malnutrition, malabsorption or chronic blood loss⁽³⁵⁾. The elevated serum globulin level may be a part of general response to inflammation causing elevated release of the acute-phase proteins⁽³⁶⁾. Moreover, the elevation of serum urea and creatinine indicates kidney affection as reported in our histopathological findings.

Ethephon treatment caused suppression of humoral immune response evidenced by reduced serum hemolyzing antibody titer against the SRBC (T-dependent antigen). In addition, the serum levels of IgM and IgG were reduced. The toxic effect of ethephon on humoral immunity may be attributed to the effect on B cells and/or T helper cells^(37,38). Cellular immunity measured by the delayed-type hypersensitivity reaction to SRBC and neutrophil phagocytosis was suppressed in ethephon-intoxicated mice. This suggests affection of T effector cells⁽³⁹⁾. These cells are responsible for release of lymphokines after the interaction with a specific antigen. In the delayed hypersensitivity reaction, lymphokines are responsible for accumulation of mononuclear cells and their interaction with subsequent increased vascular permeability

occurring around the stimulus⁽⁴⁰⁾.

In agreement with our results, it has been documented that administration of ethephon to mice reduced the hemolytic plaque logarithm, footpad swelling and phagocytic index⁽⁴¹⁾. Also, Wang et al.⁽³⁰⁾ reported that administration of ethephon to pregnant mice from the 6th day of gestation to weaning resulted in inhibition of both humoral and cell-mediated immunity in pups at 4 weeks of age as indicated by suppressed hemolytic plaque assay and DTH response against SRBCs.

The phosphonic acid derivative trichlorphon was also reported to have an immunotoxic potential in carp fish^(7,8). Other organophosphates such as malathion, parathion and diazinon were also previously reported to induce immunotoxic effects in animals⁽⁹⁾.

The immunotoxic potential of ethephon may be attributed to oxidative stress as indicated by increased levels of MDA and decreased level of GSH with inhibition of GPx and CAT activities in spleen and thymus. In accordance with our findings, Dutta⁽⁶⁾ mentioned that ethephon evoked oxidative stress in the testis of intoxicated rats. Ethephon may act, like other organophosphates, by suppression of esterases such as those in lymphocyte membrane, inhibition of non-target serine hydrolases, such as thrombin system and complement and suppression of serine hydrolases which in turn modulates signal transduction resulting in interference with immune cell proliferation and differentiation. Indirect effect on immune system may occur through affection of the nervous system by irreversible suppression of cholinesterases⁽⁹⁾.

Our findings revealed that co-administration of green tea extract with ethephon ameliorated the toxic effects of ethephon on mice hematology and blood biochemistry. Our results are consistent with the findings of Albokhadaim⁽⁴²⁾ who reported that green tea extract improved the hematological and biochemical alterations induced by carbon tetrachloride toxicity.

The humoral immune response was improved while cellular immune response returned to control value in mice co-administered green tea extract with ethephon. Also, green tea exerted reasonable protection against the oxidative stress and the histopathological alterations in liver, kidney and spleen evoked by ethephon. Similarly, a stimulatory effect of green tea on humoral immune response of mice was recorded⁽⁴³⁾. Green tea may have exerted its protective effect through reduction of ROS production, improvement of the redox status and reduction of pro-inflammatory cytokine production by lymphocytes⁽⁴⁴⁾.

The protective effect of green tea against the toxic effect of ethephon may be attributed to the major components of green tea specially catechin, epicatechin, epicatechin gallate, gallic acid, epigallocatechin, epigallocatechin gallate which were found to protect against toxic effects of many environmental toxicants including bacterial toxins, some mycotoxins, snake venoms, pesticides and metals. The protective effects of green tea extract are due to the free radical scavenging, antioxidant anti-apoptotic and chelating properties of green tea components^(45,46,47).

Conclusion

The present study demonstrated that subchronic administration of ethephon to mice suppressed both the humoral and cell-mediated immunity and produced oxidative stress in lymphoid organs. Other toxic effects included alterations in hematology and blood chemistry and histopathological changes. Green tea extract coadministration ameliorated the immunotoxic potential of ethephon, improved the redox status and protected against other toxic effects. Therefore, it is recommended to use green tea to protect from the toxic effects of ethephon and other organophosphates.

Authors' contributions

Shimaa M. Abou-Zeid performed the experimental design and assessed oxidative stress, humoral immune response and DTH. Tamer S. Allam assessed the hematological and biochemical parameters and phagocytic activity. Mostafa A. Mohamed and Amanallah El-Bahrawy performed histopathological examination. All authors have contributed to writing this article and analyzing data. All authors critically read and revised the manuscript, and approved its submission for publication.

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