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Original Article

Humoral and Cellular Immunomodulation Induced by Propoxure in C57-bl/6 Mice

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Abstract

Propoxure (PPX) is a well-known carbamate insecticide, which has been used for several decades in the world and Iran in agriculture and public health programs. However, there is no clear investigation toward its immunotoxicity as yet. In this study, we examined the effects of subchronic i.p. exposure of PPX on humoral (PFC & HA) and cellular (DTH) responses, and also monitored T-Cell subtypes using FACS technique. Briefly, female C57b1/6 inbred mice were administered PPX (0.2, 2 and 10 mg/kg/day i.p. [5 inj/wk] for 28 days) or positive and negative controls. On the day 28, mice were examined for DTH, PFC and HA responses to SRBC. Splenocyte single cell suspension was used for measuring the spleen CD4/CD8 percentage and absolute number. In vitro lymphocyte proliferation response to non-specific antigen (PHA) was also measured using MTT method.

Results showed that PPX at 10 mg/kg/day could suppress DTH response and could increase the spleen CD4-/CD8+ T-cell percentage. On the other hand, PPX at medium dose (2 mg/kg) could increase the antibody formation response against SRBC as determined by PFC and HA. Subchronic PPX at low dose (0.2 mg/kg/day) could not show any significant effects on humoral or cellular responses. It could be concluded that, subchronic PPX at high dose (10 mg/kg), possessed cellular immunosuppressive effect. However, PPX at 2 mg/kg does not change cellular response to antigen but can stimulate humoral responses. It seems that PPX has no adverse effects on mice immune system at low doses as 0.2 mg/kg, which is 10 fold greater than PPX Allowed Daily Intake limit.

Keywords: Immunomodulation; Propoxure; Carbamate insecticide; Immunotoxicity.

Introduction

Propoxure (2-Isopropoxy Phenyl N-Methyl Carbamate) (PPX) is a well-known carbamate insecticide, which has been used for several decades in the world and Iran in agriculture and public health programs (1). During recent years, immunotoxicity has been increasingly recognized as an important endpoint in rodent short-time studies, which has been documented by United States Federal Insecticide, Rodenticide, Fungicide Act (FIFRA), FDA and United States Environmental Protection Agency (EPA) guidelines (2, 3).

There are some limited and contradictory reports in the literature about adverse alteration of the immune system after exposure to carbamate pesticides such as carbaryl, aminocarb, aldicarb ... but there is no clear investigation on PPX immunotoxicity potential as yet (4, 5). At the present time, exposure of the general population to PPX may occur through the consumption of foodstuff treated with this insecticide and/or harvested before

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residues have been declined to acceptable levels. PPX is readily and completely absorbed from gastrointestinal tract and metabolized in a great ratio by the liver. The main feature of PPX toxicity in mammals is correlated to its cholinesterase inhibition effect (1). While some carbamates could induce suppression in immune responses, some others could stimulate those responses (5). Propoxure (i.p.) LD_{50} in mice is about 14 mg/kg and its Allowed Daily Intake (ADI) is 0.02 mg/kg/day (1).

Experimental

Animals

Female C57b1/6 inbred mice (4 weeks old) were purchased from Pasteur Institute (Karaj, Breeding Center). The mice weights were in 19-21 g range at the beginning of the test and after one week (for acclimatization), 5 groups of mice (5-7 mice per group), were treated by different doses of PPX (3 different doses) or positive and negative (vehicle) controls. Mice were housed in polystyrene cages (6-7 mice per cage) with free access to food and water with a 12/12 light-darkness cycles and at ambient temperature of 20-25°C.

Materials

PPX technical grade (Bayer, Germany) (>99% purity) was offered by Iran-Bayer Corporation (Tehran-Iran) and dissolved in normal saline at appropriate concentrations to prepare 1, 0.2 or 0.02 mg/ml of PPX in saline. These solutions were prepared freshly before the injection. Dexamethasone (DXMN), Cyclophosphamide (CPS), 3-(4, 5-diamethyl-2-thiazolyl) 2, 5 diphenyl-2H-tetrazolium) (MTT) and RPMI-1640 were purchased from Sigma Chemical Company and freshly solutions (0.4 mg/ml DXMN and 1 mg/ml CPS) were prepared in normal saline. Freund Complete (and Incomplete) Adjuvant (FCA & FICA), Sheep Red Blood Cell (SRBC) and Guinea Pig Complement (GPC) were purchased from Iran Pasteur Institute (Karaj, Iran)

Methods

Doses and Exposure Schedules

The mice were administered appropriate volume of PPX solutions intraperitoneally (i.p., 10 ml/kg) in order to receive 10, 2 and 0.2 mg/kg PPX for 28 days (5 inj/wk). The negative control group received vehicle (Saline, 10 ml/kg), for 28 consecutive days (5 inj/wk) and positive control groups were administered CPS (10 mg/kg, i.p.) or DXMN (4 mg/kg, i.p.) for 5 days.

Serum SRBC antibody titer: Hemmagglutination (HA) titer

Four days before the treatment period ended, the mice were immunized by injecting i.p. $5x10^8$ SRBC in saline. After preparing sera from peripheral blood, aliquots (25 µl) of two-fold diluted sera in saline were challenged with 25 µl of 1 % v/v SRBC suspension in microtiter plates. The plates were incubated at 37°C for 1 h and then observed for hemmagglutination. The highest dilution hemmagglutination was taken as the antibody titer (6)

IgM-Plaque Forming Cells (IgM-PFC)

The mice were immunized by SRBC ($5x10^8$ SRBCs in saline, i.p.) as for HA test and the number of Plaque Forming Cells was determined (7). Briefly, spleen single cell suspension (2×10^5 cells/ml) in RPMI-1640 was prepared and efficient amounts of SRBC and guinea pig complement (GPC) were added to achieve final concentrations of 7% SRBC and 10% GPC was (which would be then incubated in humid atmosphere at 37°C for 3 hrs.). The number of lyses plaques produced by 10^5 splenocyte was counted by 10X objective light microscope. The results were reported as the number of PFC in 10^6 splenocytes or after correcting for spleen cellularity as the number of PFC per spleen.

Lymphocyte Proliferation Test

Splenocyte single cell suspension was prepared by up-downing 4m1 RPMI-1640 in spleen and after omitting RBCs using 0.75% NH4C1 in Tris (0.02%, pH=7.2) buffer (adding 6 ml buffer to 2 ml cell suspension after 3 minutes centrifuging at 1000g for 2 min). Concentration was adjusted to 2×10^6 cell/ml in RPMI-1640 supplemented by 10% fetal calf serum, 2 mM L-Glutamine, 25 mM HEPES. One hundred microliters of diluted cell suspension were dispensed into 96-well flat

Table 1. Effect of different doses of propoxure (PPX) on functional tests of the immune system

Treatment group (n)	HA (Log_2 titre)	IgM-PFC		DTH' (%)		Lymphocyte Proliferation[2]
		PFC/10 cells	PFC/Spleen	24hrs	48hrs	
Control (Vehicle) (6)	5.83±0.31	1025±57	96297±7082	55.99± 4.20	20.28 ± 2.52	0.894±0.0069
PPX 10mg/kg (6)	5.2±0.31	913±28	86223±4224	34.77±4.33*	15.06±3.07	0.917±0.0260
PPX 2mg/kg (6)	6.7±0.21*	1195±35*	121925±7482*	49.94±2.80	18.89±3.98	0.908±0.0116
PPX 0.2mg/kg (5)	5.8±0.37	986±51	90668±3345	58.85±3.76	24.45±4.14	0.894±0.0089
Positive Contro13 (6)	3.8±0.31**a	382±78**a	18277±3345**a	11.86±7.09**b	5.261±4.90*b	0.641±0.0169* * b

1) Percent of increase in paw thickness (edema) 2) Optical density obtained by MTT assay
3) a: Dexamethasone 4mg/kg IP for 5 days, b: Cyclophosphamide 10 mg/kg 1P for 5 days *P <0.5, ** P<0.01

bottom culture plate. Mitogen phytohemmaglutinin-A (PHA) was added at 5 µg/ml final concentration to each well and the volume was adjusted to 0.2 m1. After incubating for 72 h at 37°C and 5% CO_2 humid atmosphere air, cell proliferation was determined by MTT assay method (8). Briefly, 10% of (3-(4, 5 diamethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium) (MTT) (5 mg/ml) was added to each well and plates were incubated at 37°C in CO_2 humid atmosphere for 4 h. The blue formazan precipitate was dissolved in acidic Isopropanol and its optical density was measured in 570 nm using Elisa Reader (Stat-FaXTM). Each dose was tested in triplicates.

Delayed Type Hypersensitivity (DTH) to SRBC

The mice were sensitized by injecting 10^8 SRBCs suspended in 50 µ1 FCA subcutaneously (s.c.) to their hind paws at the day 18 (or day 2 for positive control groups). After 10 days secondary 10^8 SRBC suspended in 50 µl FICA was injected s.c. to the same hind paw. The edema (as percentage of increase in thickness) at 24 and 48 h after injection were measured by micrometer and compared to the control paw (50 µl s.c. injection of saline) (6).

Spleen T-Cell subtyping

T-Cell subtyping was performed as previously described method (9). Briefly, splenocyte single cell suspension in RPMI-1640 (10^6 cell/ml) was prepared and after counting viable cells by trypan-blue dye exclusion method, spleen cellularity was obtained. The $CD4^+/CD8^-$ and $CD4^-/CD8^+$ T-Cell subtypes were measured using Epics® flow cytometer and rat anti-CD4 and CD8 monoclonal antibody conjugated with Flouresceine-isothio-cyanate (FITC). By multiplying differential ratios of each CD4 and CD8 subtypes to the total spleen cell contents, their total amounts in spleen were calculated.

Statistical analysis

Homogenous variance data were analyzed statistically by student t-test and one-way ANOVA with Dunnett's post test, using GraphPad Prism® 3.03 software. Data are presented as mean ±SE.

Results and Discussion

There is little information in the literature regarding the dose dependent effect of PPX on immune system. The latest comprehensive study on PPX immunotoxicity screening was conducted by Seth *et al* in a rat model (4). In our study, female inbred C57bl/6 mice were used, which are more susceptible than rats to PPX (LD50 i.p. <15 mg/kg) and also are an accepted model for immunotoxicological studies (10, 11). Our previous studies on this

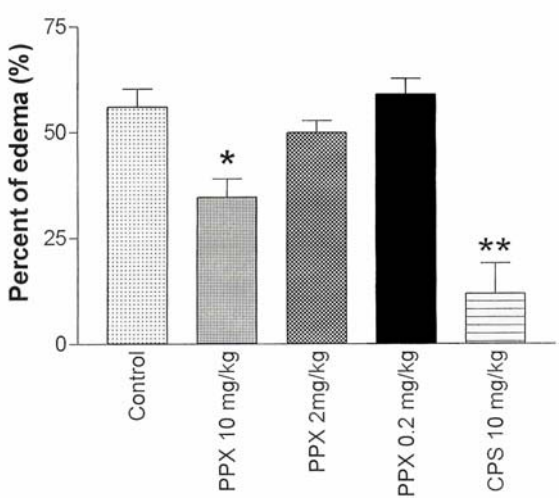

Figure 1. Effect of different doses of Propoxure (PPX) on Delayed Type Hypersensitivity response (percent of edema 24 h after secondary SRBC injection to the mice hind paws) compared to the positive (Cylophosphamide 10 mg/kg/day) and negative (saline 10 ml/kg) controls .
*P<0.05, **P<0.01

Table 2. Effects of different doses of Propoxure (PPX) on spleen T cell subtypes

Treatment group (n)	$CD4^+$T-Cell (%)	$CD8^+$T-Cell (%)	CD4/CD8 Ratio	Spleen $CD4^+$ Content (x 10^7)	Spleen $CD8^+$ Content (x 10^7)
Control (Vehicle) (6)	24.7±0.90	13.25±0.34	1.83±0.09	2.70±0.12	1.47±0.04
PPX 10mg/kg (6)	25.1±1.93	15.82±0.35*	1.58±0.09	2.23±0.22	1.43±0.06
PPX 2mg/kg (6)	24.7±0.63	13.6 ±0.69	1.83±0.06	2.72±0.09	1.50±0.07
PPX 0.2mg/kg (5)	23.2±0.59	13.6 ±0.38	1.72±0.07	2.58±0.13	1.50±0.03
Cyclophosphamide (6)	30.8±1.30 **	18.29±0.53**	1.68±0.06	2.02±0.16**	1.13±0.10**

*P<0.05, * * P<0.01 statistically different from the control group.

model showed that subchronic exposure to PPX at high dose produced histopathological changes in immune system (unpublished data). So as the tier I of PPX immunotoxicology tests (12), we examined the effect of i.p. administered PPX on mice immune functions. Allowed daily intake (ADI) for PPX is 0.02 mg/kg/days (1), so the dose of 0.2 mg/kg/day was chosen as the lowest treatment dose. PPX has no bioaccumulation and its cholinesterase inhibitory effect would be reversed in few hours after its administration (13). High dose of PPX (10 mg/kg/day) decreased DTH response at 24 h after secondary antigen injection (Figure l), but did not show statistically significant effect at 48 h after injection (Table 1). Furthermore, there was an increase in spleen $CD8^+/CD4^-$ T-Cell subtype; however, other immunological responses did not change at this dose (Table 2). PPX at medium dose (2 mg/kg/day) increased the number of Plaque Forming Cells (IgM-PFC) and serum anti-SRBC antibody titer (HA) (Figures 2 and 3). At low dose of PPX (0.2 mg/kg/day) no marked changes in any of these parameters were observed (Tables 1 and 2).

Immunosuppression is a common side effect in subchronic exposure to most of insecticides including carbamates (3, 14). We also observed an immunosuppressive effect induced by PPX on DTH response, which confirms previous reports about PPX immunotoxic effects (4). However, possibly due to the different toxicokinetic profiles in rat and mice, the minimum immunosuppressive doses in these two models are different. This immuno-suppressive effect, at least in some extent, may be initiated from an elevation in $CD8^+$ T-Cell percentage which could lead to the decline in cellular immune response (15). PPX at lower dose (2 mg/kg/day) showed an immuno-stimulatory effect in humoral responses. Results of this experiment showed that PPX at low dose may stimulate humoral system while at higher dose the inhibitory effect in this system is dominant. These results also indicated that PPX at 0.2 mg/kg had no observable adverse effect on murine immune system, which is also in concordance with the recommended no observable adverse effect level (NOAEL) of PPX (0.02 mg/kg/day) by WHO.

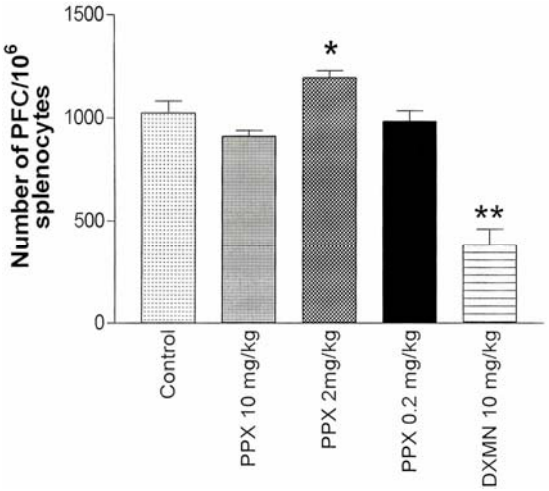

Figure 2. Effect of different doses of Propoxure (PPX) on the number of Plaque Formating Cells (PFC) per 10^6 splenocytes compared to 4mg/kg Dexamethasone (DXMN) as positive control and 10 mg/kg saline as negative control.
*P<0.05, **P<0.01

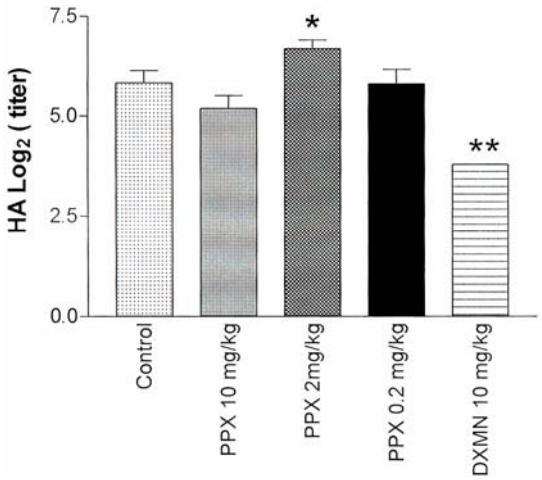

Figure 3. Effect of different doses of Propoxure (PPX) on serum Hemagglutination titer compared to 4 mg/kg Dexamethasone (DXMN) (as positive control) and 10 ml/kg saline (negative control).
*P<0.05, **P<0.01

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