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INVOLVEMENT OF PARAOXONASE (PON) IN OXIDATIVE STRESS INDUCED BY CHLORPYRIFOS IN ALBINO RATS

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ABSTRACT

Organophosphorus insecticides (OPs) exert their toxic effect through inhibition of acetylcholinesterase (AchE). In addition, accumulated evidences indicated that oxidative stress involved in OPs neurotoxicity and cytotoxicity. However, the mechanism underlay OPs induces oxidative stress still so far unclear. Recent reports were focused on the role of paraoxonase (PON), the HDL-associated A-esterase, in the degradation of the oxon form of the organophosphate. This enzyme, which known to play a critical role in OPs metabolism has a vital role in preventing both LDL and HDL from Peroxidation. Based on the previous observation we propose that the interaction of OPs with PON is the key step in initiating lipid Peroxidation and oxidative stress. To test this suggestion, chlorpyrifos (CPF) was orally gavaged at dose levels of 3, 6, and 12 mg/Kg/day to adult male albino rats for 28 consecutive days. Animals were weighed weekly and blood samples were withdrawn after 7, 14, 21 and 28 days from the start of treatment. At the end of the experiment, animals were sacrificed and organs were weighed. The brain and the liver were frozen and kept till the biochemical analysis. Malondialdehyde (MDA), reduced glutathione (GSH), triglyceride (TG), total cholesterol, HDL-cholesterol and total protein level were determined. Also, PON, carboxyl esterase (CE), acetylcholinesterase (AchE), and glutathione-S-transferase (GST) activity were determined. In addition, PON and CE isozymes were separated on Native-PAGE electrophoresis. Results indicated that CPF produced constant and dose dependent inhibition in both plasma ChE and liver ChE whereas brain AchE was weakly affected. A pattern of adaptation was observed in plasma CE. Brain CE was the most affected, whereas, plasma PON did not affected by (CPF) treatment. Brain PON seemed to be highly susceptible and the effect was dose dependent. The same pattern was observed with AE. The native PAGE confirmed the biochemical determinations. Treatment also increased plasma MDA. GSH levels in blood, liver and brain were increased whereas, GST activity increased only in plasma. The cholesterol and triglyceride levels increased, whereas, HDL level didn't affect by treatment. The analysis of correlation also showed significant correlation between PON and MDA in brain and liver. These results support the suggestion that OPs interfere with the antioxidant system. In addition, it was clear that animals were developed an adaptation mechanism as a result of repetition chlorpyrifos treatment and more work should be offer to clear the role of PON in oxidative stress induced by OPs.

Keywords: Organophosphorus insecticides, chlorpyrifos, oxidative stress, Paraoxonase, carboxyl esterase, acetyl cholinesterase, glutathione-s-transferase, reduced glutathione, Malondialdehyde, lipid profile male rats.

INTRODUCTION

The primary target of organophosphorus insecticides (OPs) is the critical enzyme acetylcholinesterase (AchE) (Carr, *et al.,* 2002), and the mechanism of their acute neurotoxicity recently became well established (Dickson, *et al.,* 2003). Organophosphorus insecticides inhibit AchE by phosphorylating the serine hydroxyl group at the catalytic triad site (Abou-Donia, 2003), leading to the

accumulation of acetylcholine at both the mascarinic and nicotinic receptors in both central and peripheral nervous system. Excess acetylcholine initially causes excitation and then paralysis of cholinergic systems (Casida and Quistad, 2004).

Most OPs are found as phosphorothioate, the form that is weak inhibitor of AchE and require metabolic activation by cytochrome P-450 to exert the active form "oxon" which is responsible for the cholinergic toxicity (Amitia, 1998; Tang *et al.,* 2001). In addition to AchE, OPs may interact with other esterases such as Arylesterase (AE), Paraoxonase (PON) (Eckerson, *et al.,* 1983) and Carboxylesterase (CE) (Chanda, *et, al.,* 1997) the reaction that leads directly or indirectly to the reduction of the OPs cholinergic toxicity.

Carboxylesterase acts as "molecular scavenger" by binding to the active form of OPs "oxon" thereby reducing number of molecules available for inhibiting AchE (Yang and Dettbarn, 1998). In contrast, paraoxonase (PON) is HDL associated enzyme that hydrolyzes the oxon form of the OPs to its corresponding acid and alcohol (Mackness et al., 2001) PON also involved in prevention of LDL form peroxidation (Aviram *et al.,* 1998). Other enzymes and macromolecule involved in OPs detoxification are Glutathione-s-transferase (GST) and the reduced glutathione (GSH) (Abel, *et al.,* 2004; Van Beerendonk, *et al.,* 1995).

Although the cholinergic neurotoxicity is the major mechanism of OPs, It is also suggested that mechanisms other than acetylcholinesterase inhibition may be involved in the progression of type I and type II paralysis in acute organophosphate poisoned patients (Venkatesh, *et al.,* 2006). Also accumulated evidences strongly indicated that OPs toxicity is accompanied with reactive oxygen species (ROS) production and oxidative stress. Cankayali *et al.,* (2005), concluded that Organophosphate compounds might cause oxidative stress by interfering with antioxidant defense mechanisms. Also Milatovic *et al.,* (2006) observed an excessive formation of F2-isoprostanes and F4-neuroprostanes, (the *in vivo* biomarkers of lipid peroxidation) and generation of reactive oxygen species (ROS), and of citrulline, (a marker of NO/NOS) and reactive nitrogen species (RNS) generation when animals were treated with OPs.

In addition, OPs were found to increase blood Malondialdehyde (MDA) and decrease the reduced glutathione (GSH) level in mice (Yurumez, *et al.,* 2007), and also in both liver and brain of rats (Sharma *et al.,* 2005). In addition, treatment with OPs decrease erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) activity and increase the activities of glutathione-s-transferase (GST) and glutathione reductase (GR) in rat (Singh, *et al.,* 2006). As these evidences declare that OPs induce oxidative stress however, the mechanism underlies OPs induce oxidative stress unclear so far.

Due to the deep involvement of PON in OPs detoxification/intoxication and its role in prevention LDL peroxidation and limitation oxidative stress, the present work was aimed to investigate the suggestion that the overload on PON as a result of OP exposure could lead to evoke LDL peroxidation and consequently initiate oxidative stress.

MATERIAL AND METHODS

Animals and treatment:

Twenty adult albino rats with average body weight of 150 g were withdrawn from the breading colony of the Mammalian Toxicology Dept., Central Agricultural Pesticides Lab., and randomly divided into 4 groups (control and 3 treated groups) and housed in plastic cages with grill stainless steal covers. Animals were fed on a well balanced chow (Animals Food Manufactory of the ministry of Agricultural, Embaba, Giza, Egypt) and tap water *ad libitum*. The cages were kept in air conditioned room at a temperature of 22 C° and a relative humidity of 55% (55 - 70 %) and normal light / dark cycle.

Chlorpyrifos formulation (Pestban 48 % EC) was given by oral intubation daily (6 times/week) for 28 days at doses of 12, 6, and 3 mg Chlorpyrifos ai/kg body weight to the three treated groups whereas, the control group received water.

At each time-point i.e after 7, 14, 21 and 28 days animals of all groups were anesthetized and blood sample was withdrawn from retro-orbital plexus according to the method of Schalm (1986) into a heparinized tube. Part of the sample was used in reduced glutathione determination and the rest was centrifuged at 3600 r.p.m for 15 min. at 4^{o}C in refrigerated centrifuge. The resulting plasma was saved and kept frozen at – 20^{o}C till the biochemical assay.

At the end of the last period rats of each group were decapitated by cervical dislocation. Organs were quickly removed, weighed and both brain and liver were kept frozen at – 20^{o}C till the preparation for the enzymes assay.

Tissues preparation:

Brain and liver were homogenized in cold 35 mM Tris-HCl buffer pH 7.4 (1: 10 w/v) using Potter Teflon homogenizer. Part of the resulting homogenate was used in estimation of reduced glutathione level whereas, the rest were centrifuged at 10000 r.p.m for 15 min. and 4^{o}C in refrigerated centrifuge, the resulting supernatant saved and kept at – 20^{o}C till the enzyme assay.

Determination of AchE activity:

Acetylcholinesterase activity was assayed spectrophotometrically in 1 ml reaction mixture containing 38 mM tris-HCl pH 8.5, 1 mM dithio-bis- 2 nitro benzoic acid, 1 mM actylthiocholine iodide (Actch) and tissue supernatant at 412 nm according to the method of Ellman *et al.,* (1961) with the modification proposed by Bisso *et al.,* (1991).

Determination of Paraoxonase activity:

Paraoxonase activity was measured spectrophotometrically using p-nitrophenyl acetate as a substrate according to the method described by Gan *et al.,* (1991) with a slight modification. The reaction mixture (1 ml) contains 10 mM tris-HCl buffer 1 mM $CaCl_2$ 1 mM p-nitrophenyl acetate and sample. Change in absorbance at 412 nm was measured over 3 min and one unit of the enzyme activity was expressed as 1 uMole p-nitrophenol released per minute at room temperature.

Determination of arylesterase (AE) activity:

The AE activity was determined as the procedure described by Watson et al. (1995) using phenyl acetate as substrate. The samples pre-diluted in 900 ul of 10 mM Tris (pH 8.0) with 1.0 mM $CaCl_2$ and transferred to round-bottom snap cap polypropylene tubes. To the tubes, 100 ul of 10 mM phenyl acetate was added (final concentration 1.0 mM), thoroughly mixed, and immediately transferred to quartz spectrophotometer cuvettes. Absorbance at 270 nm was taken every 15 s for 120 s or until a linear rate of change in optical density was measured by spectrophotometer. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzyme unit is expressed as M phenyl acetate hydrolyzed/min/ml serum.

Determination of carboxylesterase activity:

Carboxylesterase activity was measured spectrophotometrically using p-nitrophenyl acetate as a substrate according to the method of Clement and Ehardt (1990) in 1 ml reaction mixture contains 100 mM tris-HCl buffer 2 mM EDTA 1 mM p-nitrophenyl acetate and sample. Change in absorbance at 412 nm was measured over 3 min and one unit of the enzyme activity was expressed as 1 uMole p-nitrophenol released per minute at room temperature.

Determination of GST activity:

The activity of GST was determined using 2,4-dichloronitrobenzene (DCNB) as a substrate and GSH as a cosubstrate in a reaction mixture containing 0.1m potassium phosphate buffer pH 6.5. The activity was measured spectrophotometrically at 340 nm by following the increase in optical density as a result of the conjugation of CDNB with GSH by glutathione-s-transferase according to the method of Habig *et al.* (1974).

Determination of GR activity:-

The activity of GR was determined using the method of Racker (1955) as modified by Coldberg and Spooner (1983). The enzyme activity was measured spectrophotometrically by following the decrease in absorbance of NADPH at 340 nm in a sodium phosphate buffer (50 mM, pH 7.6) containing 1 mM EDTA, 0.17 mM NADPH, and 3.3 mM oxidized glutathione.

Determination of lipid peroxidation:-

Lipid peroxidation was estimated through the determination of malondialdehyde (MDA) level. This was measured according to Yoshioka *et al.* (1979).

Determination of GSH level:

Level of GSH was determined according to Beutler *et al.* (1963). The method was based on the determination of the yellow color resulting from the reaction of 5,5 dithio-bis(2-nitrobenzoic acid) with the SH group of glutathione.

Determination of triglyceride, cholesterol and HDL-cholesterol content in plasma:

Plasma cholesterol was estimated according to the method of Allain *et al.,* (1974) and triglyceride according to the method of Dryer (1970) using the commercial kits of Stanbio, USA., by following the instruction of the manufacture.

Determination of total protein content:

The quantitative assay of protein contant in tissue fraction was done based on the method of Lowry *et al.,* (1951).

Native PAGE elelctrophoresis

native-PAGE were performed using either 7.5 acrylamide resolving gels and 4% acrylamide stacking gels (Laemmli, 1970). Samples were diluted in 0.062 M Tris-HCl buffer (pH 6.8) containing 10% glycerol and 0.001% bromphenol blue. A 0.05 M Tris buffer (pH 8.3) with 0.384 M glycine was used as electrode buffer. Electrophoresis was conducted at constant voltage (200 V) for 1 hr. Developed gels were incubated in 200 ml of buffer containing 1.0 mM $CaCl_2$ and incubated in a shaking water bath (37 °C, 5 min). Gels were stained for A-esterase activity (Gomori, 1953) by addition of α- naphthyl acetate in ethanol and Triton X-100 (final concentrations of 5.0 mM, 0.25 and 0.005%, respectively) in 0.05 M Tris-HCl (pH 7.4) containing 0.05% Fast Blue RR. Substrate development was performed at room temperature until bands appeared. Gel was fotographed using digetal camera.

Statistical analysis

One-Way ANOVA test (using Equal Variances Assumed LSD with significant level of 0.05) were used for statistical analysis of data. The analysis was undertaken using SPSS for windows, release 8.0.0 (22 Dec 1997) software.

RESULTS

Effect of chlorpyrifos (CPF) on body weight:

Treatment with chlorpyrifos (12, 6, and 3 mg/kg body weight) daily for 28 consecutive days (Fig. 1) did not produce any significant changes in the body weight at any dose level compared with control

Effect of chlorpyrifos on acetylcholinesterase (AChE):

Plasma ChE affected by chlorpyrifos treatment as it may expected (Table 1 and Fig. 2). But the valuable observation in this regard was that the inhibition level of ChE seemed to be constant during the course of experiment with inhibition level between (50.9 – 63.3%), (37.9 – 50.4%) and (32.4 – 42.9%) for the doses 12, 6, 3 mg/kg, respectively. In other words, it could be said that, the inhibition of plasma ChE was dose dependent but not time dependent and it seemed that chlorpyrifos did not have accumulation effect upon plasma ChE.

Also liver ChE was affected by chlorpyrifos treatment recording inhibition of 35.8, 30.4 and 11.7 % at dose level of 12, 6, and 3 mg/kg, respectively after 28 days treatment. Whereas, brain AchE was the less affected parameter recording 17.8% and decrease in AchE activity only with the high dose (12 mg/kg) after 28 days treatment.

Effect of chlorpyrifos on carboxyl esterase (CE) activity:

Treatment with Chlorpyrifos (CPF) produced a constant inhibition in plasma CE (40.5, 36.7, 39.5 and 22.5 % in the high dose level group, 31.8, 28.8, and 32.5 % in the medium dose level group as well as 28.7, 17.7 and 38.2 % in the low dose level group during the course of treatment). liver CE was the most affected parameter, recording 59.9, 53.1 and 38.0 % inhibition in the high, medium and low dose level respectively, after 28 days treatment. On the other hand, brain CE slightly affected by CPF treatment recording 11.4 % decrease in CE with the medium dose after 28 days treatment (Table 2 and Fig 3).

Effect of chlorpyrifos on Paraoxonase (PON) activity:

Plasma PON did not significantly affected by CPF treatment except in the case of the high dose which produced 19.1 % inhibition after 21 days treatment, whereas liver PON significantly inhibited by 48.8 % and 42.5 % in the high and medium dose groups respectively, (Table 3 and Fig 4). On the other hand, brain PON seemed to be unaffected by CPF treatment.

Effect of chlorpyrifos on Aryl esterase (AE) activity:

Unlike PON treatment with CPF increased plasma AE activity (Table 4 and Fig 5) by 19.6 and 21.2 % in the medium and low dose level groups respectively. On the other, hand the high dose produced inhibition of 13.0% after 21 days treatment. Also, liver AE slightly inhibited (12.6 % decrease compared with control group) only with the medium dose. Whereas, the brain AE inhibited by 32.2 and 22.7 % as a result of treated with the high and medium doses for 28 days.

Effect of chlorpyrifos on glutathione-s-transferase (GST) activity:

The high and medium dose of CPF produced significant increase in plasma GST activity (56.4 and 20.0 %, respectively after 28 days treatment). Also, the low dose increased plasma GST activity by 28.9 and 19.6 but after treatment for 21 and 28 days, respectively. On the other hand, the medium dose inhibited the liver GST activity by 18.1 after 28 days treatment. Brain GST activity remained unchanged (Table 5 and Fig 6).

Effect of chlorpyrifos on glutathione (GSH) level:

Treatment with the high dose of CPF produced increase in blood GSH level of 46.4, 40.6 and 71.3 % after 7, 14 21 days and increase of 29.1, 26.0, 27.7 and 33.4 % after 7, 14, 21 and 28 days, treatment with the medium dose, respectively. Whereas, the low dose increased GSH level by 32.8, 19.9 and 29.6 after 7, 14, and 21 days treatment, respectively. Also, liver GSH level increased by 58.8, 44.8, and 68.8 % as a result of treatment with the high, medium and low doses, for 28 days respectively (Table 6 and Fig 7). On the other hand, brain GSH level slightly affected by CPF treatment recorded only 18.6 % decrease after treatment with the low dose for 28 days.

Effect of chlorpyrifos on Malondialdehyde (MDA) level:

The high dose of CPF produced increase in plasma MDA level by 54.3 and 65.6 after 7 and 14 days treatment, respectively. But the MDA level returned to the normal level afterward. The same pattern of effect was observed with the other two dose levels (MDA increased by 49.2 and 70.7 after 7 and 14 days treatment with the medium dose and by 51.7 and 35.8% after 7 and 14 days treatment with the low dose). On the other hand, no significant changes were observed in MDA level in brain and liver after treatment with CPF (Table 7 and Fig 8).

Effect of chlorpyrifos on plasma lipid profile (PLP):

CPF at the high dose level significantly increased the plasma total cholesterol by 24.1 and 22.8 % after 14 and 28 days treatment. Also the medium and low doses increased the plasma cholesterol level during the course of treatment but not significantly. In contrast, the triglyceride significantly decreased in

the high and medium dose level groups where, the low dose significantly increased the triglyceride level after 14 and 28 days treatment (Table 8 and Fig. 9 and 10). The High density lipoprotein (HDL) was increased during the course of treatment with the high dose of CPF till the end of the day 21st of treatment. Afterward, a slight but significant decrease was occurred (Table 9 and Fig 11), whereas, no changes in HDL were observed in the groups that were treated with the medium or the low dose of CPF.

Effect of chlorpyrifos on plasma total protein (PTP):

The plasma total protein remained unchanged during the course of the experiment and till the end of treatment for all the treated groups

Analysis of correlation:

The analysis of the correlation between plasma ChE and the other parameters revealed that plasma ChE was significantly correlated with plasma and liver carboxyl esterase (CE) with Pearson correlation of 0915, 0.802 and 0.753 for plasma and 0.834 for liver. Whereas brain CE did not showed any correlation against plasma ChE. Also, there was significant –ve correlation between plasma ChE and blood GSH (-0.719, -0.602 and –0.726 for the periods 7, 14, 21 days, respectively). Plasma malondialdehide (MDA) was significantly linked to plasma ChE but only at the period 7 and 14 days (-0.454 and -0.691, respectively). Whereas, cholesterol was significantly correlated with ChE at the periods 7 and 28 days (-0.475 and -0.532, respectively). At the period 28 days, significant +ve correlation was observed between plasma ChE and plasma triglyceride (TG), brain AChE, liver AChE, liver paraoxonase (PON), liver glutathione-s-transferase (GST), liver GSH, and liver arylesterase (AE) (0.531, 0.825, 0.660, 0.702, 0.453, and 0.480, respectively). Whereas, significant –ve correlation was observed between plasma ChE and plasma GST and brain GSH (-0.575 and -0.581, respectively).

In contrast, brain AChE was significantly correlated with plasma GST, plasma cholesterol (-0.456 and -0.492, respectively) whereas, +ve correlation was observed between brain AChE and plasma TG, brain PON, brain CE, brain AE, liver ChE, liver PON, liver CE, liver GST and liver AE (0.705, 0.452, 0.488, 0.605, 0.582, 0.763, 0.745, 0.540 and 0.628, respectively).

Significant negative correlation was observed between liver ChE and plasma GST (-0.721), and significant +ve correlation was observed between liver ChE and plasma TG (0.470), liver CE (0.571), liver GST (0.667) and liver AE (0.555).

Native PAGE electrophoresis:

The native PAGE electrophoresis has separated the plasma PON to two distinct bands in both the control and the treated groups. No significant change was observed in the density of the separated bands except a reduction in the density of the bands No. two of the high dose group and increase in the density of the same bands in the low dose treated group, in comparison with control after 7 days treatment (figs. 12-15).

Separation of liver homogenate by the native PAGE electrophoresis resulted in the appearance of 7 clear bands expressed PON activity in the control group. In contrast, at least one band was deleted in the high dose treated group in addition to decreases in density of almost all the bands in the other groups (fig. 20). Brain PON was separated into two bands in both the control and the treated groups with uniform in bands density except in the low dose treated group in which the bands density was greater (fig. 22).

Plasma native PAGE electrophoresis separated the CE to 3 bands in both the control and the treated groups but the bands density of the treated groups was less than that observed in the control group tell the period 21st day (figs. 16-18) by the end of the experiment the separated bands of both the control and treated groups seemed the same (fig. 19).

Liver homogenate gave 6 separate bands with CE activity in both the control and the treated groups however, the bands density of the treated groups was less than that observed in the control (fig.21). In brain the native PAGE separation of the homogenate resulted in the appearance of 2 bands in both the control and treated groups with bonds density equal in both (fig. 23).

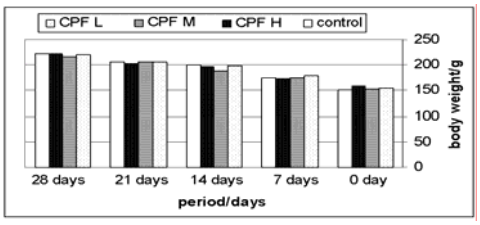


Fig. 1: weekly body weight of male rats treated with chlorpyrifos (CPF) (12, 6 and 3 mg/kg/day) for 28 consecutive days.

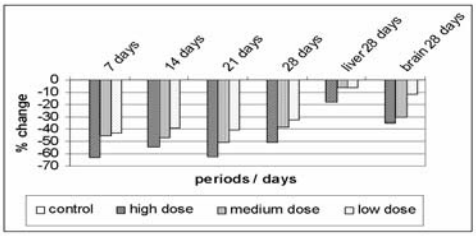


Fig. 2: Percentage of the change in plasma and liver ChE and brain AChE Activity.

Table 1: plasma and liver ChE, and brain acetyl cholinesterase (AchE) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	Brain 28 days
	7 days	**14 days**	**21 days**	**28 days**		
Control	366.243 ± 8.825	321.235 ±3.908	334.561 ±20.199	318.382 ±12.708	2177.64 67.24	1556.44 ± 30.26
CPF H (12 mg/kg)	134.314 ±11.416^{a,c,d}	147.184 ±10.476^{a,d}	127.433 ±10.669^{a,d}	156.233 ±9.915^{a,c,d}	1789.70 ± 70.12^{a,c,d}	1006.00 ± 45.62^{a,d}
CPF M (6 mg/kg)	200.834 ±14.021^{a,b}	171.245 ±19.151^{a}	165.899 ±15.187^{a}	197.576 ±8.929^{a,b}	2038.92 ± 59.22^{b}	1083.21 ± 26.39^{a,d}
CPF L (3 mg/kg)	208.990 ±11.447^{a,b}	195.889 ±14.088^{a,b}	197.776 ±24.284^{a,b}	215.101 ±7.375^{a,b}	2044.02 ± 66.16^{b}	1373.18 ± 34.28^{a,b,c}

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = ChE activity expressed as uM substrate hydrolyzed/min/ml plasma, $^{\Psi}$ = AChE activity expressed as uM substrate hydrolyzed/min/g tissue
Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 2: plasma, liver and brain carboxyl esterase (CE) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	$^{\Psi}$Brain 28 days
	7 days	**14 days**	**21 days**	**28 days**		
Control	633.024 ± 19.875	652.216 ±19.708	625.572 ±14.368	586.094 ±14.368	86997.9 7968.9	2847.14 ± 42.620
CPF H (12 mg/kg)	376.087 ±13.291^{a,d}	412.686 ±19.111^{a,d}	378.348 ±24.050^{a}	455.624 ±59.145^{a,c,d}	35691.1 ± 7199.0^{a}	2607.560 ± 44.210
CPF M (6 mg/kg)	431.454 ±27.150^{a}	464.300 ±30.200^{a}	422.090 ±13.263^{a}	645.894 ±55.187^{b}	40751.1 ± 6718.0^{a}	2522.300 ± 234.640^{a,b}
CPF L (3 mg/kg)	450.848 ±18.634^{a,b}	536.629 ±12.321^{a,b}	386.499 ±32.305^{a}	626.940 ±19.931^{b}	53858.8 ± 8071.0^{a}	2917.260 ± 105.470

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = CE activity expressed as uM substrate hydrolyzed/min/ml plasma, $^{\Psi}$ = CE activity expressed as uM substrate hydrolyzed/min/g tissue
Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L.

Table 3: plasma, liver and brain paraoxonase (PON) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	$^{\Psi}$Brain 28 days
	7 days	**14 days**	**21 days**	**28 days**		
Control	1712.05 ± 82.450	1713.87 ± 108.030	1707.24 ± 68.890	1631.38 ± 68.833	64291.48 2017.44	9763.28 ± 225.58
CPF H (12 mg/kg)	1549.85 ± 69.410^{c,d}	1818.85 ± 75.474	1380.09 ± 117.635^{a}	1366.43 ± 90.495^{c}	32883.60 ± 4984.50^{a,d}	9345.64 ± 158.35
CPF M (6 mg/kg)	1805.70 ± 39.251^{b}	1883.36 ± 25.395	1620.36 ± 87.461	1772.34 ± 121.683^{b}	36916.70 ± 6278.59^{a,d}	8817.60 ± 466.55^{a,d,}
CPF L (3 mg/kg)	1450.848 ± 61.885^{,b}	1916.90 ± 62.476	1616.91 ± 77.609	1573.71 ± 96.435	53785.60 ±7562.62^{b,c}	9834.10 ± 197.38^{c}

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = PON activity expressed as uM substrate hydrolyzed/min/ml plasma, $^{\Psi}$ = PON activity expressed as uM substrate hydrolyzed/min/g tissue
Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

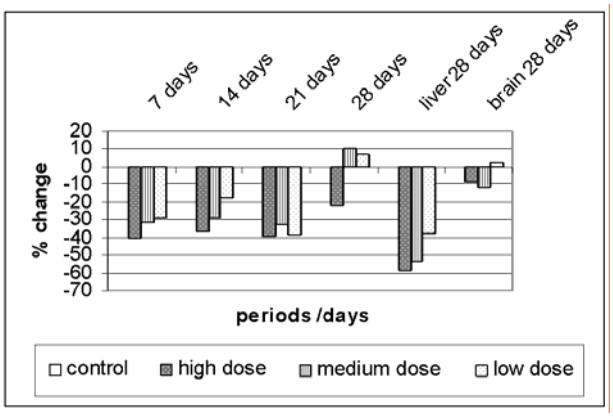


Fig.3: Percentage of the change in EC activity in plasma, liver and brain of the treated animals

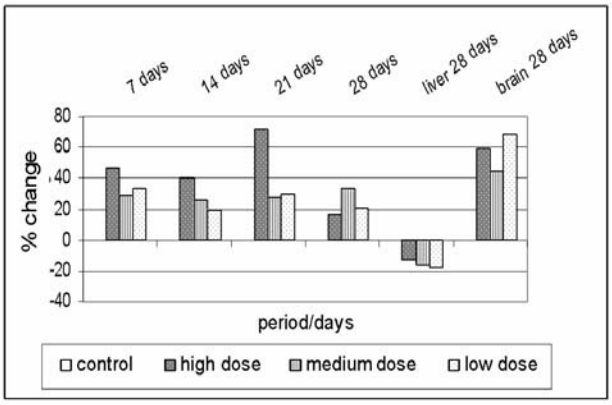


Fig.7: Percentage of the change in GSH activity of plasma, liver and brain of the treated animals

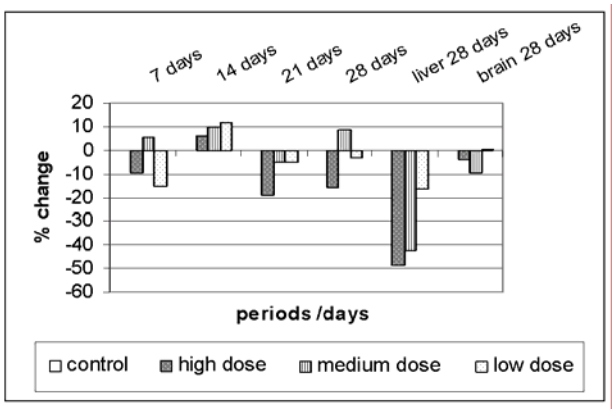


Fig.4: Percentage of the change in PON activity of plasma, liver and brain of the treated animals

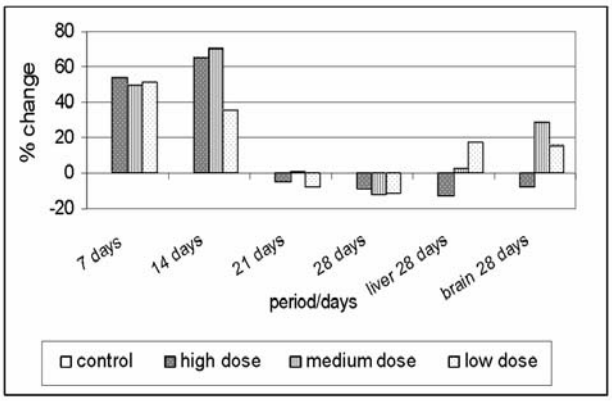


Fig.8: Percentage of the change in MAD livel in plasma, liver and brain of the treated animals

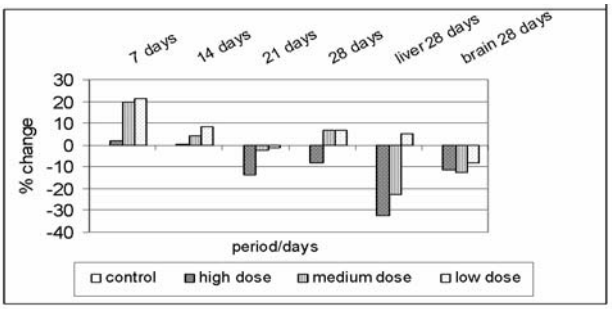


Fig.5: Percentage of the change in AE activity in plasma, liver and brain of the treated animals

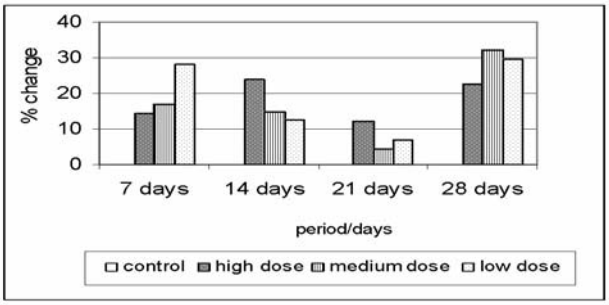


Fig.9: Percentage of the change in plasma cholesterol in the treated animals

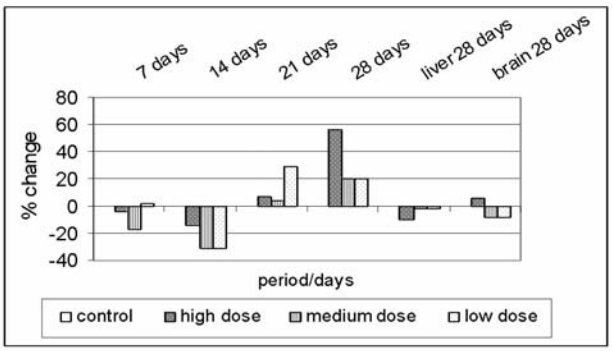


Fig.6: Percentage of the change in AE activity in plasma, liver and brain of the treated animals

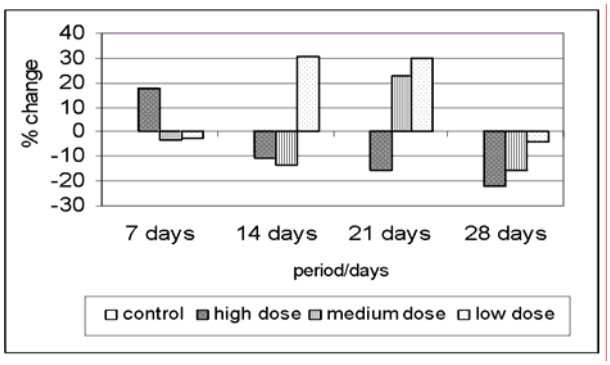


Fig.10: Percentage of the change in plasma triglyceride in the treated animals

Table 4: plasma, liver and brain Aryl esterase (AE) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	$^{\Psi}$Brain 28 days
	7 days	14 days	21 days	28 days		
Control	83.137 ± 2.935	85.085 ± 2.715	81.703 ± 2.699	84.344 ± 4.395	179.320 11.410	6.930 ± 0.312
CPF H (12 mg/kg)	84.678 ± 3.816	85.525 ± 3.484	71.075 ± 4.829[a,d]	77.611 ± 6.579	121.150 ± 9.480[a,d]	6.117 ± 0.137
CPF M (6 mg/kg)	99.505 ± 4.528[a]	88.784 ± 1.327	79.687 ± 1.811	90.281 ± 4.874	138.470 ± 10.900[a,d]	6.055 ± 0.245[a,]
CPF L (3 mg/kg)	100.802 ± 3.011[a]	92.200 ± 2.739	80.666 ± 2.130[b]	90.135 ± 3.024	188.860 ± 16.610[b,c]	6.361 ± 0.354

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = AE activity expressed as uM substrate hydrolyzed/min/ml plasma, $^{\Psi}$ = AE activity expressed as uM substrate hydrolyzed/min/g tissue

Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 5: plasma, liver and brain glutathione-s-transferase (GST) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	$^{\Psi}$Brain 28 days
	7 days	14 days	21 days	28 days		
Control	356.110 ± 41.827	365.832 ± 14.215	343.596 ± 15.531	321.407 ± 34.338	21324.87 353.27	1505.86 ± 67.821
CPF H (12 mg/kg)	341.388 ± 41.486	313.266 ± 41.645	368.116 ± 27.557	502.986 ± 56.804[a]	19289.58 ± 758.81[a]	1599.36 ± 51.757c
CPF M (6 mg/kg)	297.221 ± 64.340[a]	251.663 ± 13.475[a]	358.332 ± 40.225	386.110 ± 37.047	20826.39 ± 405.135	1233.12 ± 111.394[a,b,]
CPF L (3 mg/kg)	364.999 ± 45.204	251.166 ± 39.795[a]	442.945 ± 32.565[a]	384.722 ± 26.039	20895.83 ± 548.47	1386.106 ± 27.915

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = GST activity expressed as uM substrate hydrolyzed/min/ml plasma, $^{\Psi}$ = GST activity expressed as uM substrate hydrolyzed/min/g tissue

Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 6: plasma, liver and brain reduced glutathione (GSH) activity level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	$^{\Psi}$Brain 28 days
	7 days	14 days	21 days	28 days		
Control	24.437 ± 0.758	26.108 ± 0.819	29.608 ± 1.258	23.561 ± 1.155	212.762 ± 11.151	4.690 ± 0.7407
CPF H (12 mg/kg)	35.784 ±1.878[a]	36.721 ± 1.161[a,d]	50.723 ± 3.416[a,c,d]	27.405 ± 1.641	184.622 ± 9.507	7.448 ± 0.877[a]
CPF M (6 mg/kg)	31.560 ±1.429[a]	32.914 ± 0.944[a]	37.814 ± 1.065[a,b]	31.435 ± 2.916[a]	179.294 ± 12.678	6.793 ± 0.6166[a]
CPF L (3 mg/kg)	32.469 ±3.127[a]	31.315 ± 2.300[a,b]	38.378 ± 1.605[a,b]	28.445 ± 2.446	172.996 ± 11.119[a]	7.918 ± 0.7817[a]

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = GSH content expressed as mg GSH/dl whole blood $^{\Psi}$ = GSH content expressed as mg GSH/g tissue

Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 7: plasma, liver and brain Malondialdehyde (MDA) level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$Plasma				$^{\Psi}$Liver 28 days	Brain 28 days
	7 days	**14 days**	**21 days**	**28 days**		
Control	11.3278 ± 0.9837	15.0917 ± 2.3077	13.8468 ± 0.3805	12.2124 ± 0.4805	67.289 7.260	122.210 ± 21.040
CPF H (12 mg/kg)	17.4820 ±3.2442	25.0060 ± 1.6169[a]	13.0728 ± 0.3203	11.1919 ± 0.9316	58.600 ± 6.721	113.010 ± 14.500
CPF M (6 mg/kg)	16.9016 ± 1.6197	25.7650 ± 2.0351[a]	13.9738 ± 0.1986	10.6793 ± 0.8494	69.230 ± 5.776	157.300 ± 41.150
CPF L (3 mg/kg)	17.1908 ± 1.1935	20.4992 ± 2.3820	12.3968 ± 0.3584	10.7946 ± 1.2679	78.840 ± 8.642	141.270 ± 25.470

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = MDA level expressed as nM MDA/ml plasma,
$^{\Psi}$ = MDA level expressed as nM MDA/g tissue
Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 8: plasma Cholesterol and triglyceride level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$ Cholesterol				$^{\Psi}$ Triglyceride			
	7 days	**14 days**	**21 days**	**28 days**	**7 days**	**14 days**	**21 days**	**28 days**
Control	65.710 ± 0.839	63.096 ± 2.300	68.096 ± 2.300	59.296 ± 2.638	112.412 ± 6.84	105.847 ± 8..961	108.687 ± 10.558	119.746 ± 6.483
CPF H (12 mg/kg)	75.110 ± 5.165	78.216 ± 6.457[a]	76.404 ± 5.823	72.774 ± 1.862[a]	92.439 ± 7.078	94.533 ± 6.010[a,d]	91.998 ± 13.009	93.443 ± 6.478[a]
CPF M (6 mg/kg)	76.888 ± 2.792	72.614 ± 4.657	71.188 ± 1.755	78.499 ± 3.307	108.290 ± 8.226	91.464 ± 4.187[a,d]	133.117 ± 7.147[b,d]	100.673 ± 2.260[a]
CPF L (3 mg/kg)	84.369 ± 4.779	71.057 ± 4.533	72.739 ± 3.526	76.874 ± 4.563	109.387 ± 8.228	138.102 ± 4.461[a,b,c]	141.452 ± 10.441[a,b,c]	115.240 ± 3.662

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = Cholesterol level expressed as mg/dl plasma,
$^{\Psi}$ = triglyceride level expressed as mg/dl plasma
Significant at P < 0.05 ANOVA test (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L

Table 9: plasma High density lipoprotein (HDL) and total protein level of male rats treated with chlorpyrifos for 28 consecutive days.

	$^{\Phi}$ HDL				$^{\Psi}$ Total protein			
	7 days	**14 days**	**21 days**	**28 days**	**7 days**	**14 days**	**21 days**	**28 days**
Control	36.960 ± 2.234	39.416 ± 3.556	36.936 ± 2.561	39.835 ± 1.792	6.154 ± 0.233	6.774 ± 0.387	6.024 ± 0.399	5.792 ± 0.197
CPF H (12 mg/kg)	45.920 ± 2.095	45.472 ± 5.814[a]	39.704 ± 1.412	35.718 ± 3.884[a]	6.169 ± 0.223	6.813 ± 0.111[a,d]	5.734 ± 0.394	6.090 ± 0.411
CPF M (6 mg/kg)	39.480 ± 5.264	34.720 ± 1.957	37.016 ± 2.468	40.787 ± 4.037	5.903 ± 0.194	6.860 ± 0.219	5.323 ± 0.408	5.906 ± 0.301
CPF L (3 mg/kg)	48.160 ± 4.961	38.884 ± 2.555	37.632 ± 2.228	38.438 ± 4.628	6.349 ± 0.178	6.526 ± 6.526	5.156 ± 0.222	6.208 ± 0.298

Each value represents the mean ± SE of 5 animals, $^{\Phi}$ = HDL content expressed as mg/dl plasma, $^{\Psi}$ = Protein content expressed as g/L plasma **Significant at P < 0.05 ANOVA test** (a) Significant compared to control, (b) Significant compared to CFP H (c) Significant compared to CPF M (d) Significant compared to CPF L.

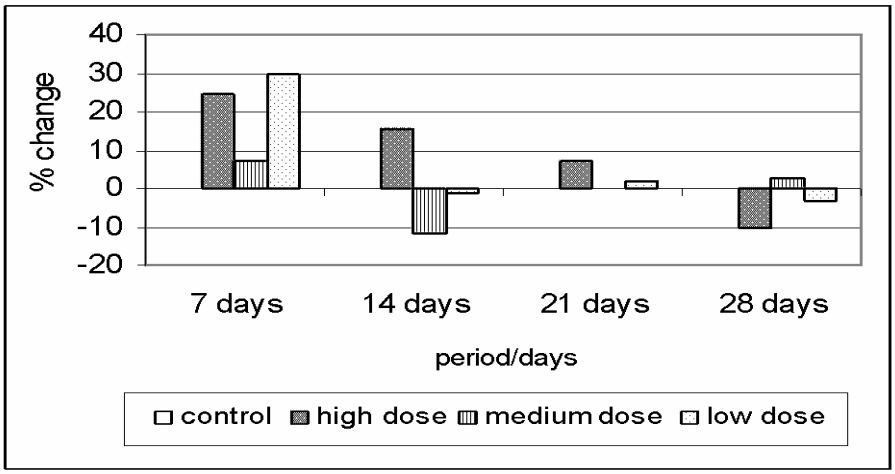


Fig. 11: Percentage of change in plasma HDL of the treated animals

Fig. 12: Native PAGE electrophorasis of plasma PON after 7 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose the arrows indicated to the separated isozymes.

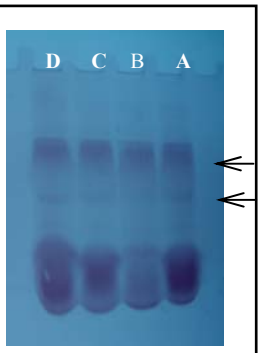


Fig. 13: Native PAGE electrophorasis of plasma PON after 14 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

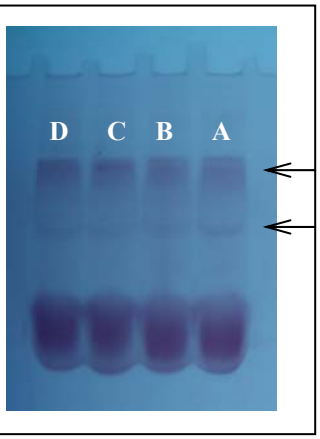


Fig. 14: Native PAGE electrophorasis of plasma PON after 21 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the mediam dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

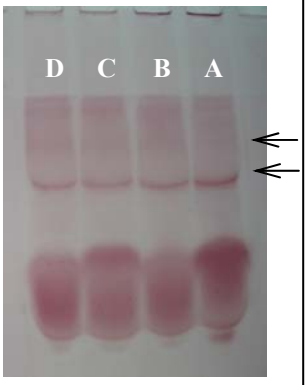


Fig. 15: Native PAGE electrophorasis of plasma PON after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the mediam dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

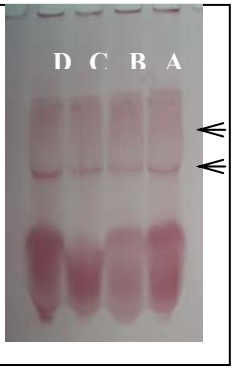


Fig.16: Native PAGE electrophorasis of plasma CE after 7 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose
The arrows indicated to the separated isozymes.

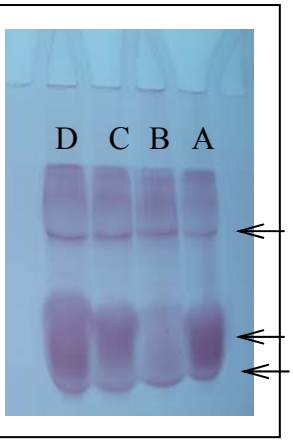


Fig. 17: Native PAGE electrophorasis of plasma CE after 14 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

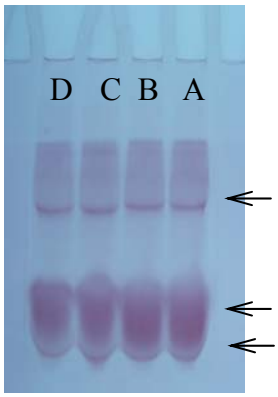


Fig. 18: Native PAGE electrophorasis of plasma CE after 21 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

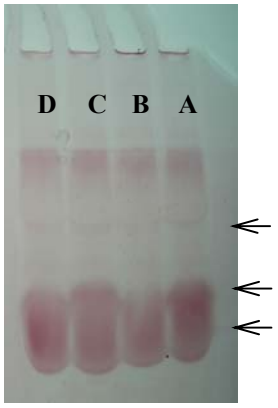


Fig. 19: Native PAGE electrophorasis of plasma CE after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

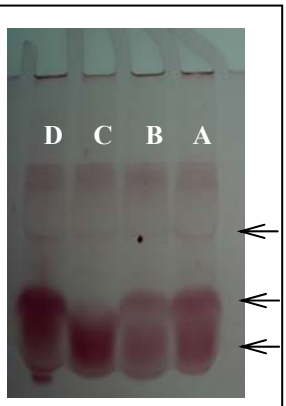


Fig. 20: Native PAGE lectrophorasis of liver PON after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

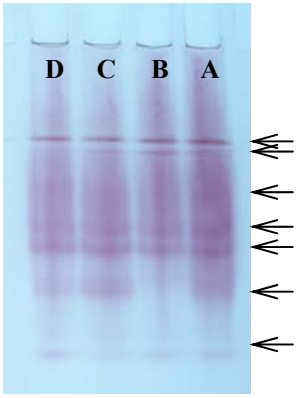


Fig. 21: Native PAGE ectrophorasis of liver CE after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

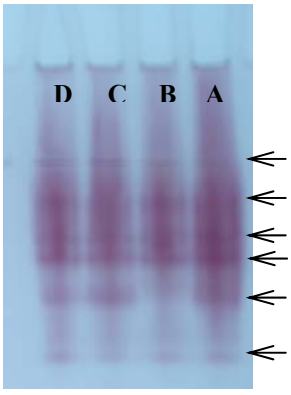
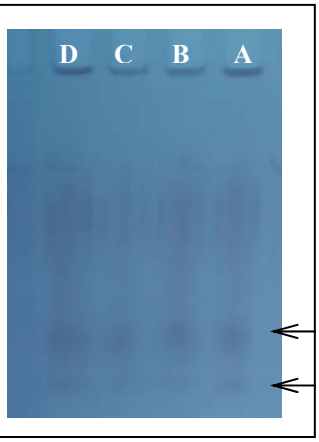


Fig. 22: Native PAGE electrophorasis of brain PON after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes

Fig. 23: Native PAGE electrophorasis of brain CE after 28 days treatment with chlorpyrifos (CPF) lane (A) is the control, lane (B) the high dose, lane (C) the medium dose and lane (D) the low dose.
The arrows indicated to the separated isozymes



DISCUSSION

It has been established that OPs induce their neurotoxicity via the inhibition of AchE (Chanda *et al.*, 1997; Abou-donia, 2003), however, there are differences in the relative neurotoxicity between the individual compounds (Atterberry, *et al.*, 1997). In this regard, at least two major factors are influenced and responsible for these differences. The first factor is the affinity strength between AchE and the oxon form of OPs and the other factor is the capacity of PON to catalysis this oxon (Li, *et al.*, 1993; Pond, *et al.*, 1998).

In addition to these major factors, there are many other factors that influence the neurotoxicity of OPs. Among of them is the cytochrome P450 mono-oxygenase which is responsible for converting OPs to its corresponding oxons (Poet, *et al.*, 2003). Also, the GST/GSH system represents the final step in eliminating the OPs metabolites from the body, this occurs by conjugating the metabolites with GSH and then secretion with urine (Ahmad and Forgash, 1976; Abel, *et al.*, 2004).

PON and GST/GSH system are also involved in the elimination of the free radicals and prevent the lipid peroxidation so they protect the cells from oxidative stress (Hayes and Pulford, 1995; Aviram *et*

al., 2000). The bifunction of these enzymes could be the reason that the neurotoxicity of OPs is accompanied by oxidative stress and the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Therefore, the present study was aimed to evaluate the hypothesis that the overload on the GST/GSH system and PON as a result of OPs intoxication is the reason for the accumulation of free radicals and increase lipid peroxidation which leads to the oxidative stress.

At least, two major classes of acetylcholinesterase could be defined based on their difference in their structure, origin, substrate specificity and susceptibility to inhibitors. The first is the true acetylcholinesterase (AchE) which is recently called the read-through acetylcholinesterase (Soreq and Seidman, 2001). AchE is abundant in brain, muscle and erythrocytes membrane and is inhibited by OPs (Silmon and Sussman, 2005). In contrast, Butyrylcholinesterase (BchE) or pseudocholinesterase (also called cholinesterase) is synthesized in liver but also found in lung, kidney and in an abundant amount in plasma. The physiological function of BchE is still unclear however, recent evidences suggested that this enzyme plays an important role in metabolizing wide spectrum of ester-containing drug such as cocaine, aspirin and amitripyline (Cokugras, 2003).

In addition, it is suggested that chE is involved in OPs detoxification since it works as scavenger for OPs and prevent the active metabolites (oxons) from reach to the target enzyme AchE (Chamber and Oppenheimer, 2004). As inhibition of AchE and ChE is the common characteristic of exposure to OPs, the assay of plasma ChE are widely used for confirming and assessing exposure. (Kamanyire and Karalliedde, 2004). In the present study, chlorpyrifos (CPF) produced steady, dose-dependent inhibition in both plasma ChE (which seemed the more affected) and liver ChE whereas, brain AchE affected only by the high dose. CPF was known to inhibit plasma ChE of OPs applicators (Geer, *et al.,* 2004) and both brain AchE and plasma ChE of developing (Campbell, *et al.,* 1997) and adult (Nostrandt, et al, 1997; Karanth, *et al.,* 2004) rats.

The steady inhibition of plasma ChE which observed during the course of the treatment indicated that there is no accumulation effect of CPF. Also, the dose dependent inhibition of plasma ChE clearly indicated that the capacity of CYT-P450 to convert CPF to CPF-oxon is more than the capacity of the detoxification system (CE, PON and GST/GSH) to eliminate the CPF-oxon at all dose levels. Our results also support the suggestion that ChE is a part of the detoxification system (Cokugras, 2003; Chamber and Oppenheimer, 2004) since the inhibition of both plasma and liver ChE as a result of treatment with the medium and low dose did not accompanied with inhibition of AchE in the brain. In other words, both the medium and the low doses are ineffective upon AchE. The result of the CPF's effect on carboxyl esterase activity also supports the previous suggestion.

In the present study, treatment with CPF produced inhibition in both plasma and liver CE and in this respect liver seemed to be the most affected organ but brain was the less. The native PAGE electrophoresis of the CE showed the same results. These results are consistent with the previous reports (Chanda, *et al.,* 1997; Chambers and Chambers, 1990; Karanth and Pope, 2000; Mileson, *et al.,* 1998). The most amazing observation was that plasma CE tended to become more resistant and showed less influence at the latest periods. Moreover, no inhibitory effect was observed at the end of the experiment with both the medium and low doses. Carboxylesterases are a large family of broad-specificity esterases that are widely distributed in various tissues and appear to serve a broad range of physiological functions. (Wallace, *et al.,* 1999). Both chE and carboxylesterases were inhibited by OPs, and are called "B" esterases (Aldridge, 1953). One of the physiological functions of B-esterase is the detoxification of OPs by irreversible binding of the compounds at the carboxylesterase active site (Jakonovic, *et al.,* 1996). The adaptation and/or tolerate behavior seen in long term exposure to OPs that observed in the present study and other (Bushinell, *et al.,* 1993) could be attributed in part to the adaptation and induction of CE that observed at the end of the experiment.

Concerning the other esterases (PON and AE), first it is worthy to mentioned that there is a confliction in define these two enzymes. Some reports refer to arylesterase as paraoxonase and vice versa (Karanth and Pope, 2000; Berkowitz, *et al.,* 2004). This confliction could be attributing to the fact that the two enzymes are sharing in many feature and characteristics. The evidences indicated that AE and PON are two separate enzymes that are different in thier response to specific inhibitors, ion strength, optimum pH and substrates affinity (Gan, *et al.,* 1991). Both PON and AE are calcium dependent HDL associated enzymes (Eckerson, *et al.,* 1983; Goue´dard, *et al.,* 2004). Aryleaterase hydrolyzes the exogenous substrate phenyl acetate and did not affect by NaCl, whereas, PON inhibited by phenyl acetate and is affected by NaCl and more specific for hydrolyzing the paraoxons of organophosphate (Gan, *et al.,* 1991). In the present study, we used both phenyl acetate and p-nitrophenyl acetate to differentiate the two enzymes' activity. The result of the present study indicated that the liver contains an abundant amount of PON and AE followed by plasma, whereas brain is the lowest. This result is

consistent with the previous finding (Primo-Parma *et al.,* 1996; Deakin, *et al.,* 2001). Also, it seemed that the effect of CPF on both PON and AE was varied according to the target tissues, treatment duration, and the dose level. In general, liver PON and AE were the highly affected parameters and the inhibition was dose dependent. Brain PON and AE were less affected and surprisingly, only the medium dose was the effective. The activity of plasma's PON and AE were inhibited after 21 days. The more interesting observation was that AE but not PON was increased at the first period.

Because both PON and AE are HDL associated enzymes, we evaluated the plasma lipid profile during the course of treatment. It seemed that both HDL and triglyceride contents were increased whereas, cholesterol was decreased. The involvement of HDL in the antioxidant properties of PON and AE was reported previously (Aviram, *et al.,* 1997). Evidences indicated that HDL is essential for expressing the antioxidant properties of the two enzymes, since the pure enzymes did not show any antioxidant properties (John, *et al.,* 2004). Therefore, the decrease in PON activity which observed in the present study may be attributed to the disturbance that occurred in the lipid profile. Also we suggest that the effect of CPF on lipid profile is independent.

In the present study we used both the GSH and MDA levels for the evaluation the oxidative stress potential of CPF where, OPs were found to produce oxidative stress in workers who formulate OPs (Shadnia, *et al.,* 2005), in the experimental animals (Altuntas and Delibas 2002; Sharma, *et al.,* 2005) and in vitro in erythrocytes (Altuntas, *et al.,* 2004).

Results extracted from the present study indicated that treatment with CPF initiated the oxidative stress process and stimulate the antioxidant system. As evidence, the MDA level increased during the first 14 days of treatment and the GSH level increased in both the plasma and the brain during the course of treatment. Similar result was mentioned by Verma and Srivastava, (2001) who found an increase in MDA and alteration in GSH/GSSG ratio in rat brain after treatment with chlorpyrifos. On the other hand, the GST activity decreased at the first 14 days but afterward the GST activity increased. This observation together with that of GSH indicate that CPF treatment stimulated the antioxidant system and that an adaptation mechanism was developed.

CONCLUSION:

The present study was aimed to investigate the involvement of PON in oxidative stress produced by CPF. Results indicated that the medium and low doses used in the present study were well tolerated by the animals whereas the high dose was affected regarding the brain AchE. In this respect it could be concluded that both plasma and liver ChE are well indicators for the OPs exposure whereas only brain AchE is the best as indicator for the neurological potential of OPs.

Also, it seemed that CPF produced disturbance in the lipid profile especially in HDL and triglyceride levels. We suggest that this disturbance is independent and it may affect the PON activity. Although, CPF was able to initiate the oxidative stress process and stimulate the antioxidant system, the treated animals have developed an adaptation mechanism by which the animals become more tolerance to the CPF effect and this adaptation was in progress with time. Result clearly indicted that CE was a significant factor in the developed adaptation mechanism together with both GSH and GST. As PON and AE are significant in OPs detoxification, more effort should be done to explain the correlation between these enzymes and the other detoxification and antioxidant enzymes system. The present study is just a little step on this road.

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