

Activities of brain antioxidant enzymes, lipid and protein peroxidation

Research Article

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Abstract: Organophosphate pesticides are known to induce oxidative stress and cause oxidative tissue damage, as has been reported in studies concerning acute and chronic intoxication with these compounds.

Our objective was to investigate the activities of brain antioxidant enzymes and malonyldialdehyde, as well as the level of carbonyl groups, in rats sub-chronically intoxicated with chlorpyrifos at doses of 0.2, 2 and 5 mg per kg of body weight per day.

It was found that chlorpyrifos induces change in brain antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, but to a different degree in comparison to proper control values; however, the elevated antioxidant enzymes activities failed to check lipid and protein peroxidation in the brains of rats. Thus, in sub-chronic intoxication with chlorpyrifos, as evidenced by increased level of malonyldialdehyde and carbonyl groups, oxidative stress is induced.

Measurements of protein carbonyl groups appeared to give more consistent responses in the rats' brains when compared to the malonyldialdehyde level after sub-chronic chlorpyrifos treatment.

Keywords: *Chlorpyrifos • Antioxidant enzymes • Malonyldialdehyde • Carbonyl groups*

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1. Introduction

The organophosphate insecticide (OP), chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothionate), is widely used in agricultural and non-agricultural applications [1]. Households use chlorpyrifos to control cockroaches, fleas, and termites. Residual amounts of organophosphate pesticides have been detected in the soil, bodies of water, vegetables, grains and other food products [2]. As compared to other organophosphate pesticides, chlorpyrifos is a fairly stable and persistent type of OP [2].

OPs produce toxicity in mammals mainly by acetylcholinesterase (AChE) inhibition and consequently, by accumulation of the acetylcholine (ACh) in synaptic junctions. It leads to overstimulation of postsynaptic cells and cholinergic manifestation [3,4].

OPs are also known to induce oxidative stress and oxidative tissue damage. This has been reported in studies concerning acute and chronic intoxication with

these compounds in experimental animals, as well as in agricultural workers engaged in spraying OPs over cultivated areas [5-7]. These reports indicate that a change in the antioxidant defence mechanism leads to neurological disturbance and neuronal cell death. Thus, oxidative stress has been implicated in pesticide-induced neurotoxicity [8]. In OP toxicity reactive oxygen species (ROS) generation results from high energy consumption coupled with inhibition of oxidative phosphorylation, which has been described by Milatovic et al. [9]. High energy consumption leads to decreased ability of cells to maintain energy levels. For this reason, excessive amounts of ROS may be generated in different organs [9]. Thus, a disturbance in the cell redox system is the mechanism implicated in the generation of reactive oxygen species in OP exposure [6,8].

Our objective was to investigate the activities of brain antioxidant enzymes and malonyldialdehyde and the level of carbonyl groups in rats sub-chronically intoxicated with various low doses of chlorpyrifos.

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2. Materials and Methods

2.1. Animals

Adult male Wistar rats (weight 200–230 g) were obtained from the certified Laboratory Animal House, Brwinow, Poland. They were caged in groups of 9, with free access to food (standard pellet diet) and water and were maintained on a 12-hour light/dark cycle.

2.2. Treatment and tissue collection

Once a day the animals in the control groups received 0.1 ml/100 g of olive oil intragastrically with the use of a stomach tube; the experimental groups received an oil solution of chlorpyrifos, i.e., O,O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothionate, at doses of 0.2, 2 and 5 mg per kilogram of body weight. These doses were far below the lethal dose 50% (LD₅₀) for chlorpyrifos and caused no signs of cholinergic toxicity. The chlorpyrifos was administered for 14 or 28 days. The LD₅₀ for chlorpyrifos was established at 95 mg per kilogram of body weight (mg/kg b.w.) [1, 10]. Twenty-four hours after the last insecticide administration, the rats were killed under anaesthesia with vetbutal, and their brains were removed and washed in cold saline.

Chlorpyrifos (certified analytical standard, purity value, min. 99.8% (m/m) was obtained from Institute of Organic Industrial Chemistry, Warsaw, Poland. Chlorpyrifos was diluted with olive oil to the required concentration before treatment.

The experimental procedure was approved by the Local Ethics Committee at the Medical University in Białystok.

2.3. Biochemical estimation

The brains were homogenized in a potassium phosphate buffer (50 mM, pH 7.4) [11]. The homogenate was divided into two portions. The first portion was centrifuged at 700xg for 20 minutes to determine the levels of malonyldialdehyde (MDA) and carbonyl groups. The second portion was centrifuged at 8,500 xg for 30 minutes to determine the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the supernatant.

Brain MDA concentration was measured with a BIOXYTECH MDA-586™ Assay kit. This method is designed to assay free MDA and is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with malondialdehyde at the temperature of 45°C.

The concentration of MDA was expressed as micromoles of MDA per milligram of protein.

The oxidative damage of protein was assessed spectrophotometrically by determination of carbonyl groups base on carbonyl group reaction with

dinitrophenylhydrazine (DNPH), as described by Levine [12]; 800 µl of 10 mM DNPH in 2.5 M HCl, or only HCl for blanks, were added to 200 µl of homogenate supernatant and incubated for 1 hour in the dark at room temperature. Aliquots were precipitated by adding 1 ml of 20% trichloroacetic acid (TCA) for 5 minutes on ice and centrifuged at 10,000×g for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet resuspended in 1 ml of 10% TCA for 5 minutes on ice, and centrifuged at 10,000×g for 10 minutes. After proteins were precipitated with TCA, the pellets were washed three times with 1 ml of 1:1 ethanol: ethyl acetate with 15-minute standing periods to remove excess DNPH. The pellets were redissolved in 500 µl of guanidine hydrochloride solution. Samples were left for 30 minutes at room temperature, and the absorbance was read at 370 nm.

The carbonyl content in nmol/mg protein was calculated with a molar extinction coefficient of 22,000M⁻¹ cm⁻¹ after subtraction of the blank absorbance.

The level of carbonyl groups was expressed as nmol carbonyl groups per milligram of protein.

SOD, as well as GPx activity was analysed, according to the kit's instructions, with the BIOXYTECH SOD-525™ Assay kit and BIOXYTECH GPx-340™ Assay kit. The SOD-525™ method is based on the SOD mediated increase in the rate of autoxidation of 5,6,6a, 11b-tetrahydrobenzo[c]fluorene (R1) in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Change in absorbance was measured after the addition of R1. The SOD activity was determined from the ratio of the autoxidation rates in the presence and in the absence of SOD. The GPx-340™ assay is an indirect measure of the activity of GPx. To assay the activity of this enzyme, brain homogenate was added to a solution containing glutathione, glutathione reductase and NADPH. The enzyme reaction was initiated by adding the substrate, tert-butyl hydroperoxide, and absorbance was recorded. The rate of decreases in absorbance was directly proportional to the GPx activity in the sample.

Brain CAT activity was determined according to a method described by Aebi [13], which is based on the decomposition of H₂O₂ by catalase. The reaction mixture was composed of 50 mM phosphate buffer of pH 7.0, 10 mM H₂O₂ and brain homogenate. The reduction rate of H₂O₂ was followed at 240 nm for 30 seconds at room temperature.

The enzymatic activities (SOD, CAT, GPx) were expressed as units of the enzymatic activity per milligram of protein.

The protein levels were determined after dilution of proper supernatant by the Lowry method [14].

BIOXYTECH SOD-525™ Assay kit, BIOXYTECH GPx-340™ Assay kit, and BIOXYTECH MDA-586™ were produced by OXIS International, Inc., Portland, Oregon, USA.

2.4. Statistical analysis

The data are expressed as mean \pm standard derivation (SD) of nine observations. Differences among experimental groups were determined by one-way ANOVA. Comparison between means was carried out with a Tukey-Kramer multiple comparison tests. In all experiments, P values lower than 0.05 were considered to be statistically significant. Spearman correlations between the study parameters were calculated.

3. Results

After 14 days of chlorpyrifos treatment at doses 0.2, 2 and 5 mg/kg b.w., the brain MDA level in the experimental group increased in comparison to the control group. The

Table 1 Rats brain malonyldialdehyde and carbonyl groups levels in sub-chronic intoxication with chlorpyrifos.

Days	Dose (mg/kg/day)	MDA (μ mol/mg protein)	Carbonyl groups (nmol/mg protein)
14	0	1.8 \pm 0.2	5.26 \pm 1.05
	0.2	2.36 \pm 0.32 ^a	5.37 \pm 0.94
	2	2.39 \pm 0.18 ^a	10.95 \pm 1.70 ^{ab}
	5	2.70 \pm 0.27 ^a	9.96 \pm 2.14 ^{ab}
28	0	1.76 \pm 0.37	5.21 \pm 0.85
	0.2	1.75 \pm 0.30	7.38 \pm 1.15 ^a
	2	1.92 \pm 0.36	9.32 \pm 0.49 ^{ab}
	5	2.52 \pm 0.3 ^a	11.89 \pm 1.82 ^{abc}

Values are expressed as means \pm SD (n=9)

p<0.05 in relation to:

a – control groups

b – group intoxicated with chlorpyrifos at dose 0.2 mg/kg b.w./day

c – group intoxicated with chlorpyrifos at dose 2 mg/kg b.w./day

same result was observed after 28 days of treatment at a dose 5 mg/kg b.w. per day (Table 1). There were no significant differences between groups of rats exposed to different doses of chlorpyrifos.

When compared to the control group (except for the lowest dose group) the treatment with chlorpyrifos showed a significantly increased level of carbonyl groups in the brain after 14 days and a significant increase in a dose-dependent manner after 28 days of treatment (Table 1). There were no significant differences after 14 days of chlorpyrifos treatment in rats at doses of 2 and 5 mg/kg.

The levels of MDA and carbonyl groups were positively correlated (r=0.42, p<0.0001).

Subchronic intoxication with chlorpyrifos resulted in an increase of SOD activity in the rats' brains after 14 days of insecticide administration, as well as after 28 days of treatment (2 and 5 mg/kg b.w.). These changes were statistically significant compared with the control groups (Table 2). In comparison to the control group, brain SOD activity increased more than twice after 14 days of insecticide administration at doses of 0.2 and 2 mg/kg b.w. per day and more than four times after insecticide administration at a dose 5 mg/kg b.w. per day. There were no significant differences in SOD activity in the brains of rats exposed to insecticide at doses of 0.2 and 2 mg/kg b.w. per day. The enhancement of SOD activity was more pronounced after chlorpyrifos administration at a dose 5 mg/kg b.w. per day, reaching 421% of control value (statistically significant), than in smaller doses.

After 28 days of chlorpyrifos treatment, SOD activity in the brain decreased (statistically significant) in comparison to the shorter period of chlorpyrifos administration. A significant increase in brain SOD activity in comparison to the control group was observed when animals were treated with chlorpyrifos at doses of 2 and 5 mg/kg b.w. per day.

Table 2 Rats brain SOD, CAT and GPx activities in sub-chronic intoxication with chlorpyrifos

Days	Dose (mg/kg/day)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
14	0	6.68 \pm 0.29	13.26 \pm 1.77	62.4 \pm 5.5
	0.2	13.88 \pm 1.09 ^a	23.06 \pm 3.48 ^a	74.7 \pm 3.7 ^a
	2	12.65 \pm 1.06 ^a	39.26 \pm 5.72 ^{ab}	106 \pm .8
	5	28.15 \pm 1.06 ^{abc}	42.71 \pm 5.61	129.2 \pm .9
28	0	6.59 \pm 0.34	13.42 \pm 1.85	59.8 \pm 7.3
	0.2	8.51 \pm 1.62 ^d	24.83 \pm 3.58	68.2 \pm .4
	2	9.51 \pm 0.92 ^{ad}	30.69 \pm 4.29 ^{ab}	66.7 \pm 8.2 ^d
	5	11.84 \pm 1.62 ^{ad}	37.08 \pm .50	79.9 \pm .1

Values are expressed as means \pm SD (n=9)

p<0.05 in relation to:

a – control groups

b – group intoxicated with chlorpyrifos at dose 0.2 mg/kg b.w./day

c – group intoxicated with chlorpyrifos at dose 2 mg/kg b.w./day

d – 14 days exposition

The activity of another antioxidant enzyme, catalase, increased after 14 and 28 days of chlorpyrifos administration in comparison to the brains of the untreated rats. Administration of insecticide at doses of 2 and 5 mg/kg b.w. per day resulted in statistically significant, higher increases of CAT activity in comparison to the smallest dose (Table 1). There were no significant differences in CAT activity between the groups of rats exposed to chlorpyrifos for 14 and 28 days.

The Spearman correlation coefficient between SOD and CAT activities in brain after sub-chronic chlorpyrifos poisoning was $r=0.75$, $p<0.0001$.

GPx activity increase was found in the brains after 14 days of chlorpyrifos administration (Table 2). Chlorpyrifos treatment enhanced activity of this enzyme in a dose-dependent manner. Although, after 28 days of insecticide administration, GPx activity returned to the control value for the groups of rats treated with chlorpyrifos at doses of 0.2 and 2 mg/kg b.w. per day. It was still significantly higher (by 17%) when compared to the control group of rats treated with chlorpyrifos at a dose of 5 mg/kg b.w. per day.

A positive correlation was found between SOD and GPx activity ($r=0.744$, $p<0.0001$).

4. Discussion

Chlorpyrifos, as other organophosphate insecticides, poses a risk to people involved in its production and its use in agriculture or households, as well as to the population exposed to these compounds by consumption of contaminated food products or water [6,15]. Metabolic bioactivation is necessary for chlorpyrifos to exert cholinesterase inhibition, and this process occurs primarily in the liver by cytochrome P450 enzymes (CYP). The CYP2B6 enzyme metabolizes chlorpyrifos to chlorpyrifos-oxon by replacing the sulfur group with oxygen [16]. Chlorpyrifos is moderately toxic to rats. The oral LD_{50} for rats ranges from 95 to 270 mg per kilogram [10]. In the present study, the rats received chlorpyrifos at doses of 0.2, 2 or 5 mg/kg b.w. per day for 14 and 28 days.

OPs cause severe environmental contamination and potential health hazards including acute and sub-chronic cases of human accidental poisoning [6,15].

With acute exposure, the main mechanism of toxicity of OP is irreversibly binding to the enzyme AChE and inhibiting its activity. This results in the accumulation and prolonged effect of acetylcholine and consequently, acute muscarinic and nicotinic effects follow. In chronic and sub-chronic exposures, in addition to cholinesterase inhibition, induction of oxidative stress

has been reported as the main mechanism of toxicity [6,8,9,17].

In the present study, the increased SOD, CAT and GPx activities in the brain indicated an elevated antioxidant status. A parallel increase in levels of antioxidative enzymes, with an increase in the carbonyl groups and MDA level, shows that a higher number of free radicals was generated. The elevated activity of the antioxidant enzymes failed to check lipid and protein peroxidation. Similar observations have also been reported in the liver of rats (elevated levels of SOD, GPx and MDA but depressed level of CAT) after 8 weeks of chlorpyrifos treatment at a dose of 13.5 mg per kilogram daily [18].

The role of ROS has been well established in many chronic disorders [6,19]. Thus, the investigation of the role of OPs in the induction of oxidative stress has an important health implication.

The oxidative stress in the OP's toxicity may be due to their "redox-cycling" activity or their generating reactive oxygen species by making changes in normal antioxidant homeostasis resulting in antioxidant depletion [7,20,21]. OPs create anoxic conditions in the brain tissue and may result in an impairment of respiratory chain reactions. Thus, disturbance in the cell redox system is a mechanism implicated in the generation of reactive oxygen species in the brain after? OP exposure [6,9].

Free radicals can interact with various tissue components, for example, lipids or proteins, that result in dysfunction of tissues or organs [22]. The antioxidant enzymes SOD, GPx and CAT limit the effects of reactive oxygen species on tissues and are active in defense against oxidative cell injury [19]. These enzymes work together to eliminate reactive oxygen species.

In the present study, an increase in the superoxide dismutase activity in the brains of chlorpyrifos treated rats was observed as compared to the control groups. There were no significant differences in the brain SOD activity between the groups of rats after administration of chlorpyrifos at doses of 0.2 and 2 mg/kg b.w. per day. However, after insecticide administration at a dose of 5 mg/kg b.w. per day, the activity of this enzyme was significantly higher in comparison to groups with a lower intoxication level. The increase in the superoxide dismutase activity in the brains of chlorpyrifos exposed rats appears to be due to the intensified generation of superoxide anions, which are dismutated by SOD, the enzyme that scavenges superoxide anion radical and, as reported by other authors, contributes to overproduction of a more reactive particle than superoxide anion – hydrogen peroxide [23]. In this study, we have observed statistically significant differences in SOD activity

between doses of 2 and 5 mg per kilogram of chlorpyrifos only. This observation could be due to the higher rate of superoxide anion generation at a dose of 5 mg per kilogram and the higher stimulation of SOD activity to manage this increase. We have also observed that the increased SOD was only partially effective in combating the oxidative damage, because the levels of MDA and carbonyl groups were significantly enhanced. On the other hand, after 28 days of chlorpyrifos administration, the brain SOD activity decreased as compared to the earlier period of intoxication. Yet, for doses 2 and 5 mg/kg b.w. per day, it was still statistically significantly higher in comparison to the control group. The rate of decrease in activity of this enzyme after insecticide administration at a dose of 5 mg/kg b.w. per day was more pronounced (60%) in comparison to lower doses (25%). The decrease may have resulted from increased generation of H_2O_2 . Some studies have indicated that an increased hydrogen peroxide level could inhibit SOD activity, and this phenomenon is indicative of the rate of formation of free radicals [24,25]. Thus, we can assume that the rate of H_2O_2 generation was higher after chlorpyrifos administration at a dose of 5 mg/kg b.w. per day in comparison to lower doses of intoxication.

Catalase is the main enzyme responsible for removal of hydrogen peroxide from normal tissues. By breaking down hydrogen peroxide, this enzyme does not cause generation of any other reactive oxygen species [26,27]. The other enzyme that removes hydrogen peroxide, GPx, converts not only hydrogen peroxide but also lipid peroxides to water and alcohol. On the basis of its role in removing lipid peroxides, GPx seems to play a more important role than CAT in managing the oxidative stress. This antioxidant enzyme controls the rate of lipid peroxidation and participates in cell membrane stabilization [26]. The antioxidant enzymes work together to remove reactive oxygen species, and for this reason the small imbalance between them may have an effect on the level of oxidative lipids and protein damage [28].

Such an imbalance between antioxidant enzymes was observed in the current study; the rate of increase in activity of brain GPx in chlorpyrifos-treated animals was lower when compared to the increase in SOD activity. The activity of SOD did not change predominantly in a dose-dependent manner. Also, another study reported that a dose-dependence in malathion-induced oxidative stress in different tissues could not be predictably modified [29].

The results of our study suggest that subchronic intoxication with chlorpyrifos leads to an imbalance between antioxidative enzymes. It was found that SOD activity in the brains of chlorpyrifos-treated rats

increases at a higher rate (29% higher for dose 0.02 mg/kg b.w./day and 70% and 107% for doses 2 and 5 mg/kg b.w./day respectively) than activity of GPx. The small rise, especially in GPx activity, would possibly result in the enhancement of brain oxidative stress as it was demonstrated in this study and in previous ones [5].

The previous studies have demonstrated that a diminished level of reduced glutathione resulted in a small rise of GPx activity in the brains of rats subchronically intoxicated with chlorfenvinphos at a dose 0.3 mg/kg b.w. [5]. When the formation of oxidants exceed the ability of antioxidant system to remove ROS, oxidative stress occurs [22,28]. In fact, in this study, increased levels of both MDA and carbonyl groups in the brains of chlorpyrifos-treated rats were observed. Lipid peroxidation is thought to be one of the mechanisms involved in the toxicity of organophosphates [30]. In the brains of chlorpyrifos-treated rats, the MDA level increased after 14 days of insecticide treatment in all groups. There was no significant difference between the groups of rats receiving different doses of chlorpyrifos. In this study, normalization of MDA level after 28 days of chlorpyrifos treatment at doses of 0.2 and 2 mg/kg b.w./day was noticed.

Malonyldialdehyde is a lipid peroxidation product. By-products of lipid and free amino acid oxidation as well as ROS can lead to protein oxidation [22,31,32]. In the present study, the low correlation between the levels of MDA, a product of lipid peroxidation, and carbonyl groups was observed. The increased level of MDA and carbonyl groups in the rats' brains after chlorpyrifos treatment suggests enhanced production of reactive oxygen species, including hydroxyl radicals, which act on a component of membranes, unsaturated fatty acids of phospholipids [22,28]. Moreover, OPs have a lipophilic nature, which facilitates their interaction with the cell membrane and leads to perturbations of the phospholipids bilayer structure [33].

Protein carbonyl groups are the most common products of protein oxidation in biological samples [22,28,34]. Many of the assays involve derivitization of the carbonyl groups with dinitrophenylhydrazine, which leads to the formation of a stable dinitrophenyl hydrazone product. The use of levels of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison to the measurement of other protein oxidation products due to the relative early formation and relative stability of carbonylated proteins [35].

In this study, we used this method to assay the level of total carbonyl groups in brain of chlorpyrifos-intoxicated rats. Modification of enzymes during oxidative stress can have a severe effect on the cellular metabolism. The severity of the impact depends on the

percentage of proteins that are modified by ROS and the chronicity of this modification [28,36].

In the present study, it was found that enhancement of the carbonyl groups in brains of chlorpyrifos-treated rats depended on a dose, except for the lowest dose we used. Induction of protein oxidation at a dose of 0.2 mg/kg b.w. per day was observed only after 28 days of the chlorpyrifos treatment.

Based on proper control values, the percentage of change in the brain's content of carbonyl groups was higher in comparison to the percentage of change in the MDA levels, which suggests that protein oxidation is affected more by chlorpyrifos exposure than by lipid peroxidation. Carbonyl groups can be induced by almost all types of ROS and lipid peroxidation products, for example, MDA [22]. The results of our work suggest that in the brains of chlorpyrifos-treated rats the enhancement of ROS generation was evidenced by increased contents of carbonyl groups rather than by MDA level.

Protein oxidation appeared to be more important, because of the unique biological functions of proteins and unique functional consequences resulting from their modification [28]. The protein was affected at a higher degree by chlorpyrifos intoxication than lipids, as reported in our study.

Oxidized proteins can be removed from the cell or tissue by proteolysis. In this process, proteolytic enzymes can distinguish between oxidized and native

forms of a protein, but a defect in proteasome activity may be responsible for the accumulation of damaged proteins [28]. In some cases, oxidative protein changes will go unnoticed by any repair system. This may occur when one amino acid is converted to another, leading to conformational change, which is unnoticed by proteolytic or other repair systems [28].

Thus, the increased level of protein carbonyl groups seems to be a better biomarker of oxidative stress than the lipid peroxidation index after sub-chronic chlorpyrifos treatment.

As the oxidative stress is an important pathomechanism in other neurodegenerative diseases [19], results and effects reported in our study have important implications.

The findings suggest that brain oxidative stress induced in sub-chronic intoxication with chlorpyrifos is due to an imbalance between the antioxidant enzymes. In sub-chronic exposures to very small doses of chlorpyrifos (0.2 and 2 mg/kg b.w./day), the organism is capable of defending itself; after 28 days of treatment, the lipid peroxidation index returned to the control value, but after 28 days of chlorpyrifos treatment at a dose 5 mg/kg b.w. (increased level of MDA as well as carbonyl groups), we observed that the ability to manage the oxidative stress failed.

Measurements of protein carbonyl groups seem to give more consistent responses in the brains of rats than the MDA level after sub-chronic chlorpyrifos treatment.

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