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Oxidative Stress Induced by Different Pesticides in the Land Snails, *Helix aspersa*

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Abstract: The present study was designated to compare the ability of the two carbamate compounds methomyl and carbofuran, the organophosphorus compound chlorpyrifos and the bipyridylium compound paraquat to induce the oxidative stress and affect some biochemical targets in the terrestrial snail, *Helix aspersa*. LD_{50} values for these pesticides were determined 48 h following topical application. They were 240, 500, 900 and 920 μg/snail for methomyl, carbofuran, chlorpyrifos and paraquat, respectively. Some biomarkers of the oxidative stress such as Lipid Peroxidation (LP), Lactic Dehydrogenase (LDH) and Glutathione (GSH) as well as the inhibitory effects of these compounds against acetylcholinesterase (AChE) were carried out following topical application of ¼LD_{50} values. The results showed that carbofuran was the most potent to inhibit AChE in snails followed by methomyl, where the enzyme activities dropped to 9.86 and 28.82% of the control activity, respectively, 48 h following application. Non-significant increase in the levels of thiobarbituric acid-reactive species in the snail tissue homogenate intoxicated with methomyl, paraquat or carbofuran were found comparing to control value, while it was similar to control following chlorpyrifos treatment. On the other hand, the activities of LDH were increased following all tested pesticides. Also, the results showed that GSH level in the snail tissue homogenate was elevated following both methomyl and chlorpyrifos, while it decreased following either carbofuran or paraquat application. It could be concluded that methomyl was the most toxic pesticide followed by carbofuran against the land snail and their mode of action could be due to the induction of oxidative stress in addition to their anticholinesterase potencies. Chlorpyrifos or paraquat had slightly effects to alter the biomarkers of oxidative stress in the snail.

Key words: Snails, pesticides, oxidative stress, biomarkers

INTRODUCTION

The acute toxicity of organophosphorus esters and carbamates is essentially due to their anticholinesterase property, which is responsible for both insecticidal activity and mammalian toxicity[1-4]. Recently, organophosphorus pesticides have been reported to induce oxidative stress *in vivo* and *in vitro* in brain and hepatocytes of rats by inhibition of superoxide dismutase and glutathione peroxidase and enhancement of malondialdehyde production, lactate dehydrogenase leakage and DNA single strand breaks[5-8]. Chlorpyrifos was found to induce oxidative damage in female rats[8-11]. Carbamates have been also reported to induce oxidative damage in animal by inducing lipid peroxidation and glutathione depletion in rats[12]. The *in vivo* toxicity of benomyl was found to be associated with oxidative stress to cellular membranes[12]. On the other hand, bipyridylium compounds such as paraquat caused lung, liver and kidney injuries via enhancement the level of Lipid Peroxidation (LP) and Lactic Dehydrogenase (LD)[8-10,13-17]. Such pesticides mediate the transfer of electrons from NADPH to O_2, generating a flux of superoxide radicals (O_2^-). Oxidative damage or stress usually refers to the impairment of the function of cellular components (e.g. enzymes, nucleic acids, membranes and proteins) by Reactive Oxygen Species (ROS) such as superoxide radicals (O_2^-), hydroxyl free radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). A major form of cellular oxidation damages is lipid peroxidation, which is initiated by $^{\bullet}OH$ through the extraction of hydrogen atom from unsaturated fatty acids of membrane phospholipids[18]. The increased

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oxidative stress resulted in an increase in the activity of antioxidant enzymes such as superoxide dismutase and catalase. The enhancement of release of LDH is also indicative of cellular and membrane damage[8], while inactivation of superoxide dismutase and catalase are expected to enhance the generation of reactive oxygen species and consequently, pose an oxidative stress upon the system[19]. The increase in reduced glutathione content in erythrocytes may probably be an initial adaptive response to increase oxidative stress[20, 21]. There is no information available regarding the oxidative stress caused by paraquat, chlorpyrifos, methomyl or carbofuran in the land snails. Therefore, the present study was designated to study the ability of these pesticides to generate oxidative stress in the terrestrial snail, *Helix aspersa*.

MATERIALS AND METHODS

Chemicals: Pure samples of paraquat dichloride (98%), carbofuran (99%) and chlorpyrifos (99%) were supplied by Chem Service while pure methomyl (99%) was obtained from Dupont Co. All other chemicals used in this study were obtained either from Sigma or BDH Companies and they were of the highest grade available.

Tested animal: The land snail, *Helix aspersa* was collected from the farm of the Faculty of Agriculture, Alexandria University. Adults were left to acclimatize to the laboratory conditions for 30 days before starting the experiments and they were fed on lettuce leaves.

Toxicity study: Stock solutions of each compound were prepared in dimethyl sulfoxide (DMSO) except paraquat, which prepared in Distilled Water (DW). Different concentrations for each compound were prepared in the appropriate solvent and three replicates (10 animals for each) were kept in plastic jars covered with cloth netting and secured with a rubber band to prevent snails from escaping. Control snails were treated with either DMSO or DW. Twenty microliters of each concentration of the tested compounds were topically applied inside the shell upon the surface of the snail body using micropipet. Dead animals were recorded 48 h after treatment by the loss of response to a thin stainless steel needle according to the WHO procedure[22]. Data were subjected to probit analysis and effectiveness was expressed as LD_{50} values (μg/snail)[23].

Biochemical studies: For the biochemical studies, ¼ LD_{50} values of the tested compounds were topically applied as previously described. Snail tissues were dissected out 48 h following application. All tissues of each treatment were homogenized in 0.10 M phosphate buffer pH 7.4 (1:10 w/v) using a polytron homogenizer. The homogenates were centrifuged at 5000 rpm for 20 min at 4°C and the supernatants were taken to determine the activities of acetylcholinesterase (AChE) and lactic dehydrogenase (LDH), the levels of lipid peroxidation (LP) and glutathione (GSH) and protein contents.

Determination of acetylcholinesterase activity: Acetylcholinesterase activity (AChE) was determined by measuring the hydrolysis of acetythiocholine iodide (ASCh)[24]. An aliquot (0.02 mL) of 10% tissue homogenate in 0.1 mM phosphate buffer, pH 8.0 was added to a reaction mixture containing 0.075 M ASCh, 0.01 M of 5,5-dithiodinitrobenzoic acid (DTNB) in a final volume of 3.0 mL. The mixture was incubated at 37°C for 10 min and then the optical density was measured at 412 nm. AChE activity is expressed μmole of ASCh hydrolyzed/min/mg protein.

Lipid peroxidation assay: The determination of Lipid Peroxidation (LP) was based on the formation of thiobarbituric acid-reactive substances (TBARS)[25]. To 0.5 mL of the homogenate, 3 mL of 1% phosphoric acid and 1 mL of 0.6% thiobarbituric acid aqueous solution were added. The mixture was heated for 45 min in a boiling water bath and then cooled. Four milliliter of n-butanol was added to the above mixture and mixed vigorously. The butanol layer was separated by centrifugation and the absorbance was measured at 532 and 520 nm. Malondialdehyde (MDA) was employed as the standard and the molar absorptivity constant of 1.56×10^{-5} M cm^{-1} was used. LP is expressed as nmole MDA/mg protein.

Lactic dehydrogenase assay: The determination of lactic dehydrogenase (LDH) was based on the conversion of lactate to pyruvate or pyruvate to lactate[26]. The rate of NADH oxidation is proportional to LDH activity. Twenty microliter of tissue homogenate was added to 1 mL of 50 mM phosphate buffer pH 7.5 containing 0.6 mM sodium pyruvate, 0.9 g L^{-1} sodium azide and 0.18 mM NADH. The mixture was gently mixed and incubated at 30°C. The rate of NADH oxidation was measured at 340 nm. LDH activity is expressed as unit/mg protein.

Determination of reduced glutathione content: Reduced glutathione content (GSH) were measured according to the method of Anderson[27] and expressed as μmole/mg protein.

Determination of protein: The protein contents in snail was determined using bovine serum albumin as the standard[28].

Statistical analysis: Data were calculated as mean±SD and analyzed using analysis of variance technique (ANOVA) followed by Least Significant Difference (LSD). Probability of 0.05 or less was considered significant. All statistical analysis was done with Costat Program[29] on a personal computer.

RESULTS AND DISCUSSION

Pesticides toxicity against the terrestrial snail, *Helix aspersa*: The results indicated that methomyl was the most potent compound followed by carbofuran, chlorpyrifos and paraquat where their corresponding LD_{50} values were 240, 500, 900 and 920 μg/snail, respectively, 48 h following topical application (Table 1). Thus the carbamate compounds, methomyl and carbofuran were found to be more toxic to the land snail compared with either the organophosphorus compound, chlorpyrifos or the bipyridyium compound, paraquat. These results are in agreement with other the study of Crowell[30] who found that carbamate compounds were the most toxic compounds to the terrestrial snails comparing with pesticides from other groups[30].

Anticholinesterase potencies of the tested pesticides: The results showed that carbofuran was found to be the most inhibitory compound to AChE of snail followed by methomyl, where the percentages of activity of the enzyme were 9.86 and 28.85, respectively (Table 2). It is well known that the most significant biochemical effect of carbamates are the ability to reversibly inhibit AChE, which is responsible for molluscicidal activity. Some carbamates such as methomyl and thiodicarb were found to strongly inhibit AChE in snails[31-33]. In spite of the acute toxicity of organophosphorus compound is also essentially due to their anticholinesterase properties[2], chlorpyrifos had less effect on the activity AChE in *H. aspersa* and was similar to the paraquat effect.

Biomarkers for oxidative stress induced by the tested pesticides: The TBARS, the activity of LDH and the glutathione levels (GSH) in the tissue homogenate of the snail intoxicated with ¼ LD_{50} of tested pesticides were investigated. There were no observed differences between control values treated with either DMSO or DW.

Level of Lipid Peroxidation (LP): In spite of the major form of cellular oxidation damages is the lipid peroxidation[18], the enhancements of the levels of TBARS following methomyl, paraquat and carbofuran showed nonsignificant increment in lipid peroxidation comparing to control value (Table 3). On the other hand, the LP level following chlorpyrifos treatment was approximately close to control value. However, paraquat and chlorpyrifos were found significantly to enhance the level of LP in lung, kidney, liver, brain and serum of intoxicated rats[8,9].

Table 1: Toxicity of different pesticides against the terrestrial snail, *Helix aspersa* 48 h following topical application

Pesticide	LD_{50} (μg/snail)
Methomyl	240
Carbofuran	500
Chlorpyrifos	900
Paraquat	920

Table 2: Acetyl cholinesterase activity of the tissue homogenate of the land snail, *Helix aspersa* 48 h following pesticides application

Treatment	Dose (μg/snail)	Specific activity[1]	% of control
Control	0	12.27±0.63^{d}	100.00
Methomyl	60	3.54±0.33^{b}	28.85
Carbofuran	125	1.21±0.42^{a}	9.86
Chlorpyrifos	225	10.88±0.42^{c}	88.67
Paraquat	230	10.50±1.33^{c}	85.57
$LSD_{0.05}$		1.33	

[1]Specific activity is expressed as μmole/min/mg protein
Values are means±SD for (n=3)
Values followed by the same letter are not significantly different at $p \leq 0.05$

Table 3: Lipid peroxidation level in the land snail, *Helix aspersa* 48 h following pesticides application

		Lipid peroxidation level	
Compound	Dose (μg/snail)	nmole MDA/mg protein	% of control
Control	0	212.35±5.4^{a}	100.00
Methomyl	60	240.05±18.8^{a}	113.05
Carbofuran	125	226.08±17.5^{a}	106.47
Chlorpyrifos	225	211.15±4.5^{a}	99.44
Paraquat	230	233.00±16.7^{a}	109.73
$LSD_{0.05}$		51.59	

Values are means±SD (n=3)
Values followed by the same letter are not significantly different at $p \leq 0.05$

Table 4: Lactic dehydrogenase activity in the land snail, *Helix aspersa* 48 h following pesticides application

		Lactic dehydrogenase activity	
Compound	Dose (μg/snail)	Unit/mg protein	% of control
Control	0	23.58±5.9^{c}	100
Methomyl	60	141.48±37.9^{a}	600
Carbofuran	125	124.97±8.4^{a}	530
Chlorpyrifos	225	54.23±9.6^{b}	230
Paraquat	230	26.53±2.5^{c}	112.5
$LSD_{0.05}$		20.99	

Values are means±SD (n=3)
Values followed by the same letter are not significantly different at $p \leq 0.05$

Activity of lactic dehydrogenase (LDH): Table 4 indicated that methomyl, carbofuran and chlorpyrifos significantly increased the level of LDH, while paraquat insignificantly rose LDH level compared with the control treatment. The enhancement of release of LDH is indicative of cellular

Table 5: Glutathione content in the land snail, *Helix aspersa* 48 h following pesticides application.

Compound	Dose (µg/snail)	Glutathione content µmole/mg protein	% of control
Control	0	47.98±3.11[b]	100.00
Methomyl	60	83.12±3.86[d]	173.24
Carbofuran	125	38.40±5.18[a]	80.03
Chlorpyrifos	225	70.73±3.02[c]	147.42
Paraquat	230	42.55±3.11[ab]	88.68
$LSD_{0.05}$		5.65	

Values are means±SD (n=3)
Values followed by the same letter are not significantly different at $p \leq 0.05$

and/or membrane damage. In this concern, among treatment, methomyl had proven to be very strong compounds to enhance the release of LDH followed by carbofuran and then chlorpyrifos, where they increased activity by 6, 5.3 and 2.3 folds of control value, respectively. However, nonsignificant elevation in LDH activity following paraquat treatment was observed. Paraquat and chlorpyrifos significantly enhanced the level of LDH in lung, kidney, liver, brain and serum of intoxicated rats[8,9].

Glutathione content (GSH): The results indicated that methomyl and chlorpyrifos significantly increased the level of GSH in the snails compared with the control treatment (Table 5). However, carbofuran and paraquat significantly and nonsignificantly declined the GSH level compared with the control treatment. The enhancement of GSH level may be reflected the oxidative stress caused by the compound because GSH is antioxidant and its level should be rose to protect the animal from such effect.

Finally it could be concluded the alteration shown in some of the tested biomarkers from the normal values denote biochemical impairment and reflect the possibility of the effects of these pesticides to the land snails. In this respect, methomyl was the most toxic pesticide followed by carbofuran against the land snail and their mode of action could be due to the induction of oxidative stress in addition to their anticholinesterase potencies. Although chlorpyrifos and paraquat have proven to induce the oxidative stress in rats[8], the present results indicated that both compounds were slightly potencies to cause this effect in the snails. These may be attributed to the biological differences between rats and snails.

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