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Hematological changes in *Theba pisana* induced by sublethal treatments of certain pesticides

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ABSTRACT

The sublethal effects of three pesticides (methomyl, chloropyrifos and metaldehyde) on the hemolymph of land snail *Theba pisana* including number of hemocytes, hemocytic lysosomes as well as hemolymph free amino acids using wheat bran laboratory formulated toxic baits were investigated. The results revealed that hemocyte counts were increased significantly with all tested pesticides, except chlorpyrifos which did not appear any effect on the hemocyte counts. Maximum increase was observed after 6 hours of exposure, with time increase, the number of hemocytes decreased but still higher than control. Neutral red retention time assay revealed an effect of sublethal concentrations of tested pesticides on hemocytic lysosomal membrane. Retention time was reduced when concentrations of all tested pesticides increased. Free amino acids in the hemolymph were affected by pesticide treatments. There was no patterned profile of responses, but free amino acids sometimes increased and sometimes decreased with treatments. Aspartic acid concentration elevated when *T. pisana* was treated by methomyl or chlorpyrifos. Also, histidine and arginin concentrations elevated due to chlorpyrifos treatment. In contrary, cystein concentration decreased as a result of methomyl and metaldehyde treatments.

Keywords: Land snail, pesticides, hemolymph, hemocytes, lysosomes, amino acids, sublethal effects.

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INTRODUCTION

Gastropods play an escalator role as agricultural pests, as they cause a damage in agriculture, horticulture and forestry. They attack any part of the plant and they have a wide range of host plants (El-Okda, 1979 and Abdallah *et al*., 1992). Furthermore, they are of importance in medical and veterinary practice, since they serve as intermediate hosts for certain parasitic worms of man and his domestic animals that cause serious endemic diseases as schistosomiasis and fashiola in several countries as well as Egypt (Godan, 1983 and Triebskorn *et al*., 1996).

As non-target organisms, gastropods are exposed to sublethal concentrations of insecticides, herbicides, fungicides, etc., during control of different pests that live in the same area.

The present study aimed to investigate some responses of *T. pisana* after exposure to sublethal concentrations of different pesticide types named chlorpyrifos, methomyl or metaldehyde at cellular level (hemocytes), subcellular level (lysosomes) and molecular level (amino acids).

MATERIALS AND METHODS

1. Snails and their treatments: Adult snails of *T. pisana* were collected from uncontaminated nurseries and allowed to acclimatize to laboratory conditions for 3 weeks. They were fed on lettuce *ad libitum*.

T. pisana was treated with chlorpyrifos, methomyl or metaldehyde. Preliminary series of experiments were carried out to determine the sublethal concentrations of each pesticide. The snails were fed on a diet as described by Beeby and Richmond (2001) with slight modification (Abo-Bakr, 2004). Control snails were fed on pesticide-free bait or diet.

2. Changes in number of hemocytes due to pesticides exposure: After 6, 24, 72 and 168 hours of pesticides exposure, the number of hemocytes in the snails' hemolymph was checked. Ten snails were selected randomly for each concentration and each time of exposure. The hemolymph of *T. pisana* was collected according to the method of Svendsen and Weeks (1995), that cited in Snyman *et al*. (2002).

3. Hemocyte counts: The number of hemocytes with pseudopodia was determined using a light microscope (400X). Hemocytes were simultaneously counted in the corresponding control snails. Results were expressed as means ± S. E. of cell number per mm^3 hemolymph.

4. Effect of pesticides exposure on hemocytic lysosomal membrane: Five snails were randomly selected to determine the effect of these pesticides on hemocytic lysosomal membraneaccording to Snyman *et al*. (2002) at the end of the exposure time (7 days).

5. Free amino acids analysis in the hemolymph: Free amino acids were extracted according to the method described by Hamilton (1962). Individual free amino acids were estimated by the method described by Spackman *et al*. (1958) using a Beckman 119 CL amino acid analyzer. The response of the amino acid analyzer was checked by analyzing a standard mixture of seventeen commonly occurring amino acids in protein, and the obtained recoveries were used to calculate the amounts of the amino acids in various samples.

RESULTS AND DISCUSSION

1. Effect of pesticides on hemocyte counts: Changes in hemocyte counts after different times of *T. pisana* exposure to different concentrations of methomyl, chorpyrifos or metaldehyde are shown in Tables 1, 2 and 3 and regression lines are presented in Figs. 1, 2 and 3, respectively. Data in Table 1 revealed the increases of hemocyte counts of methomyl-exposed *T. pisana* with all concentrations and at all times as compared to control. The maximum mean values of hemocyte counts were 1270 ± 92.7 (3.8-fold increase) and 1240 ± 160.2 (3.6-fold increase) cells/mm^3 with 200 μg/g after 24 and 72 hours of exposure time, respectively.

Regression analysis (Fig. 1-a) showed a positive relation between methomyl concentrations and the number of hemocytes at all exposure times, but the strongest relation was at 72 h of exposure, whereas the regression coefficient (r^2) was 0.79 and the slope was 3.99. Figure 1-b showed the negative relation between exposure time and hemocyte counts at different levels of methomyl concentrations. The best r^2 was 0.97 at 50 μg/g concentration and b was -2.14. No or limited effect of chlorpyrifos on the number of hemocytes was observed as shown in Table 2. Regression lines

revealed no relation occurred between chlorpyrifos and number of hemocytes (Fig. 2-a), for example, r^2 at 72 h was 0.02 and b was 0.0001. Also, the number of hemocyted was not affected by exposure time as illustrated in Fig. 2-b.

Table (1): Hemocyte counts (cell/mm3) of methomyl-treated *Theba pisana* after different times of exposure

Conc.	Exposure time (hrs)			
µg/g	6	24	72	168
0	335 ± 42.3	330 ± 60.8	340 ± 3.7	310 ± 29.7
10	345 ± 33.6	710 ± 110.7	450 ± 86.5	510 ± 97.3
20	910 ± 107.6	1040 ± 166.8	765 ± 114.7	515 ± 80.2
50	970 ± 88.6	930 ± 61.6	885 ± 87.1	620 ± 58.6
100	870 ± 102.9	1190 ± 119	1015 ± 92.4	785 ± 143.1
200	765 ± 35.6	1270 ± 92.7	1240 ± 160.2	760 ± 81.7

Values are means ± S.E.

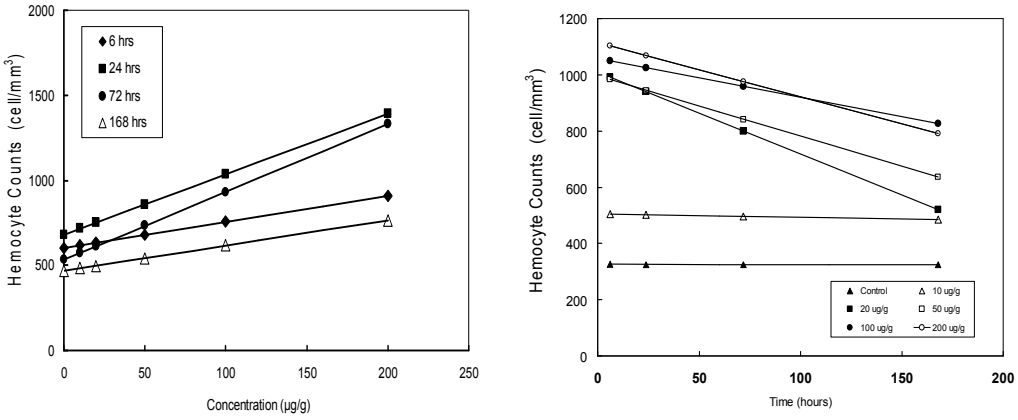

Fig. (1): Regression lines of hemocyte counts at (a) different methomyl concentrations after (b) different exposure times

Table (2): Hemocyte counts (cell/mm3) of chlorpyrifos-treated *Theba pisana* after different times of exposure

Conc.	Exposure time (hrs)			
µg/g	6	24	72	168
0	308 ± 48.8	325 ± 29.3	310 ± 39.6	340 ± 45.8
500	425 ± 73.8	390 ± 102.1	340 ± 64.04	265 ± 44.2
1000	390 ± 80.1	340 ± 65.2	380 ± 50.4	315 ± 55.3
2500	415 ± 65.8	335 ± 62.05	390 ± 134.4	335 ± 50.3
5000	445 ± 47.3	350 ± 53.8	415 ± 65.5	405 ± 69.4
10000	345 ± 61.8	335 ± 48.7	305 ± 46.1	335 ± 89.6

Values are means ± S.E.

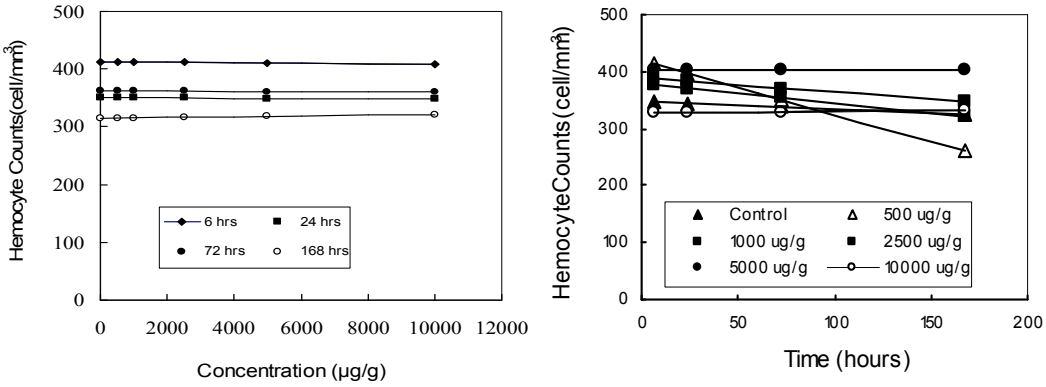

Fig. (2): Regression lines of hemocyte counts at (a) different chlorpyrifos concentrations after (b) different exposure times

Dramatic increases of the number of hemocytes were observed after 6 h exposure of snails to metaldehyde, with mean values reached 1540 ± 91.7 (4.2-fold increase) and 1475 ± 257 (4.04-fold increase) cells/mm^3 for 200 and 500 µg/g, respectively (Table 3). During time course, the number of hemocytes began to decrease, but still higher than that of control.

Regression analysis showed the positive relation between metaldehyde concentrations and hemocyte counts (Fig. 3-a). The strongest relation between these two variants appeared at 24 h exposure time (r^2 = 0.74 and b = 1.39). Figure 3-b showed a weak and moderate negative effect of time course on the number of hemocytes of metaldehyde-treated *T. pisana*. The highest r^2 was 0.6 at 50 µg/g metaldehyde concentration, while the lowest was 0.3 at 100 µg/g concentration.

Table (3): Hemocyte counts (cell/mm^3) of metaldehyde-treated *Theba pisana* after different times of exposure

Conc.	Exposure time (hrs)			
µg/g	6	24	72	168
0	365 ± 39.1	330 ± 50.4	370 ± 49.3	345 ± 55.5
20	770 ± 177.5	560 ± 69.9	530 ± 125.5	550 ± 52.3
50	950 ± 273.9	720 ± 115.2	545 ± 96.4	545 ± 42.6
100	1135 ± 74.7	710 ± 57.5	710 ± 139.1	720 ± 89.9
200	1540 ± 91.7	1055 ± 63.9	820 ± 29.3	844 ± 56.4
500	1475 ± 257	142 ± 112.5	750 ± 39.1	835 ± 82.1

Values are means ± S.E.

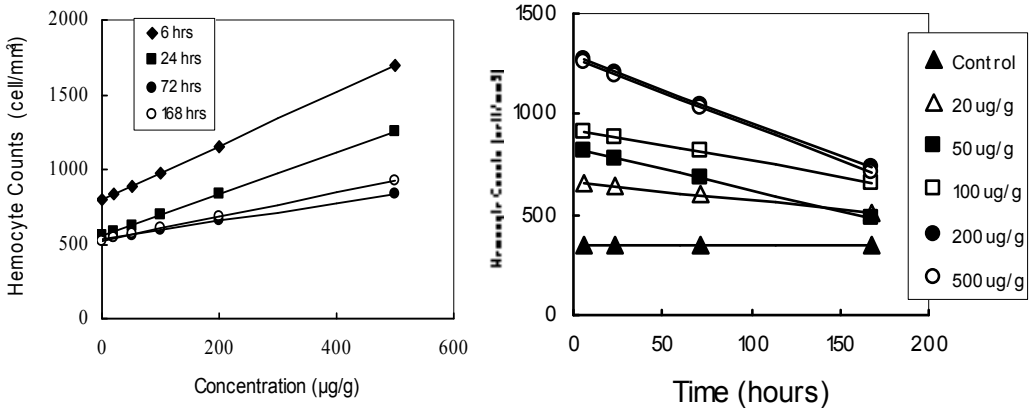

Fig. (3): Regression lines of hemocyte counts at (a) different metaldehyde concentrations after (b) different exposure times

In general, methomyl and metaldehyde were responsible for dramatic increases in the number of hemocytes at different exposure times, especially after 6 h of exposure. Decreases in cells density with time course were observed, especially at high concentration.

Pollutant-induced quantitative changes in hemocyte populations have been reported in a number of bivalve mollusks, and elevation of circulating cells is the most commonly observed response. Increases of hemocyte number were observed in oysters, clams and mussels exposed to various environmental organic contaminants (Runddell and Rains, 1975; Seiler and Morse, 1988 and Anderson, 1993). Invertebrates, like vertebrates, are protected against invading microorganisms by an internal defense system.

2. Effect of pesticides on hemocytic lysosomal membrane: Table 4 shows the neutral red retention (NRR) times (min) of *T. pisana* hemocytic lysosomes as affected by methomyl, chlorpyrifos and metaldehyde treatments. Hemocytic lysosomes of *T. pisana* exposed to different concentrations of methomyl exhibited significantly shorter NRR times compared to control. Remarkable reduction of NRR times was observed with all methomyl concentrations.

Table (4): Effect of pesticides on *Theba pisana* hemocytic lysosomal membrane.

Parameter	Pesticide						L.S.D$_{0.05}$
	Methomyl						
Concentration (µg/g)	Control	10	20	50	100	200	
NRR* time (min)	72.8^{a}±2.20	31.8^{b}±3.89	33.6^{b}±3.71	20.2^{c}±1.92	20.0^{c}±2.24	20.8^{c}±4.15	6.12
	Chlorpyrifos						
Concentration (µg/g)	Control	500	1000	2000	5000	10000	
NRR* time (min)	74.4^{a}±3.97	76.6^{a}±7.70	69.8^{a}±7.60	58.2^{b}±4.09	38.2^{c}±5.89	34.0^{c}±6.20	10.79
	Metaldehyde						
Concentration (µg/g)	Control	20	50	100	200	500	
NRR* time (min)	79.8^{a}±3.77	74.4^{a}±2.78	65.0^{b}±3.36	58.4^{bc}±4.31	50.8^{c}±3.83	38.4^{d}±2.13	8.61

*NRP: Neutral red retention time
Values are means ±S.E.
Values that share the same superscript(s) are not significantly different.

Both of chlorpyrifos and metaldehyde gradually reduced NRR times in concentration-dependent manner. The significant reduction of NRR times due to chlorpyrifos appeared for the third concentration (2000 μg/g) and higher ones, while in the case of methomyl treatment the significant time reduction occurred for the second concentration (50 μg/g) and higher ones compared to control as shown in Table 4.

Lysosomes are known to be linked to pathological changes in plants and animals and these changes have been shown to be associated with a variety of diseases induced by environmental pollutants. A remarkable feature of lysosomes is their ability to accumulate a diverse range of toxic metals and organic chemicals; however, this results in enhanced toxicity and cell injury through lysosomal damage (Lowe and Pipe, 1994).

In the present study, all tested pesticides shortened NRR time of hemocytic lysosomes, that means damage of somehow took place to lysosomal membrane. This result, especially for methomyl treatment, is supported by the finding of Beshr (2000), who used the transmission electron microscope in ultra structural study to investigate the effect of methomyl on cell organelles of the digestive gland cells of *T. pisana* snail. The microphotographs illustrated increased number and enlargement of the lysosomes compared to control.

Snyman *et al*. (2002) used this lysosomal assay in field application as biomarker of *Helix aspersa* snail exposure to copper oxychloride, and they found significant differences in NRR times between control and treated sites. Hence, they reported that NRR time assay has been shown to be an early warning of stress induced by the fungicide copper oxychloride. From the results of the present study, NRR time assay may be useful as a biomarker for different groups of pesticides either in terrestrial or aquatic environments.

3. Effect of pesticides on hemolymph free amino acids: Changes in individual, total non-polar, total uncharged polar, total (acidic or basic) charged polar and total free amino acids concentration in the hemolymph of pesticides-treated *T. pisana* are shown in Table 5.

Table (5): Changes in free amino acid concentrations (µg/µl) in the hemolymph of *Theba pisana* treated with different pesticides.

Group		Amino acids	Treatment			
			Control	Methomyl	Chlorpyrifos	Metaldehyde
Non Polar	Neutral	Proline	10.82	8.11	12.98	7.57
		Alanine	11.69	13.36	8.91	15.04
		Valine	5.12	1.36	4.10	2.05
		Methionine	5.02	2.23	4.46	4.46
		Isoleucine	1.93	2.32	3.87	1.55
		Leucine	5.39	4.10	5.39	5.39
		Phenylalanine	3.44	3.13	5.01	2.82
		Total	43.41	34.61	44.72	38.88
Uncharged Polar		Threonine	40.18	50.81	32.50	24.82
		Serine	11.02	14.50	21.75	9.86
		Glycine	23.71	35.12	20.96	13.06
		Cysteine	2.58	0.75	3.68	1.47
		Tyrosine	3.08	2.15	4.61	2.46
		Total	80.57	103.33	83.50	51.67
Charged Polar	Acidic	Aspartic acid	7.54	8.79	11.08	11.84
		Glutamic acid	9.91	8.32	11.10	10.70
		Total	17.45	17.11	22.18	22.54
	Basic	Histidine	26.01	25.25	37.50	20.57
		Lysine	12.84	12.84	14.71	12.02
		Arginine	2.30	1.92	4.61	2.96
		Total	41.15	40.01	56.82	35.28
Total amino acids			182.58	195.06	207.22	148.37

Seventeen free amino acids were identified and quantified in *T. pisana* hemolymph. Seven of these amino acids are non-polar, five are uncharged polar and five are charged polar (2 acidic and 3 basic). Little increase (6.8 %) was observed in total amino acids concentration in the hemolymph of methomyl-treated *T. pisana* compared to control. Slight increase (28 %) than control in total uncharged polar amino acids concentration was detected. This is attributed to the increase of threonine, serine and glycine concentrations, which exceeded the control by 26, 31 and 48 %, respectively. Remarkable reduction (71 %) in cystein concentration was measured. Methomyl treatment reduced the concentration of total non-polar amino acids by 20 % compared to control, valine and methionine were responsible for this reduction, where they were reduced by 74 and 56 %, respectively compared to control.

Chlorpyrifos treatment slightly induced the total concentration of amino acids by 13 % compared to control. The highest increase between amino acid groups was in basic amino acid, that reached 13.9 % compared to control. This increase is mainly due to the dramatic increase (2-fold increase) of arginine. Increasing of aspartic acid concentration (47 %) and glutamic acid (12 %) raised the total concentration of acidic amino acids by 27 % than control. In the group of uncharged polar amino acids, concentrations of 3 amino acids (serine, cystein and glycine) markedly increased by 97, 42 and 50%, respectively compared to control due to chlorpyrifos treatment. Although there were almost no differences in the total non-polar amino acid concentrations, isoleucine exhibited dramatic increase (2-fold increase) compared to control.

Metaldehyde is the only pesticide that reduced the concentration of total amino acids of *T. pisana* hemolymph, where the reduction percentage was 19 % compared to control. Uncharged polar amino acids group was the most affected one than others, the reduction reached 44 % than control. Concentrations of glycine, cystein and threonine reflected the total group concentration reduction, where their concentrations were 55, 57 and 60 %, respectively compared to control. Acidic and basic amino acid groups showed an opposite behaviour, while concentration of the former increased by 29 %, that of the latter decreased by 14% compared to control. The reduction of total basic amino acids concentration was due to histidine concentration decrease by 21 %, while increasing total acidic amino acids concentration was mainly due to increasing aspartic acid concentration by 57 % compared to control (Table 5).

All non-polar amino acid exhibited a reduction in their concentration except alanine due to metaldehyde exposure. Valine is the most affected amino acid in this group, its concentration was reduced by 60 % compared to control. Alanine was the only amino acid of this group that exhibited an increase (29 %) in opposite behaviour than the other members of this group.

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