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Effect of Sublethal Concentration of Malathion Insecticide on Innate Immune System, Immune Function and Hemocytes of Adult *Drosophila melanogaster*

***[1]BINDHANI, B; [1]MAITY, S; [1]CHAKRABARTI, I; [2]SAHA, SK**

**[1]Department of Zoology, Dinabandhu Andrews College (affiliated to University of Calcutta), Garia, Kolkata-700084, West Bengal, India*
[2]Department of Zoology, West Bengal State University, Barasat, Kolkata- 7000126, West Bengal, India

**Corresponding Author Email: bindhanibanani@gmail.com*
Email of other authors:sulagna.maity@gmail.com, ipsitchakrabarti2011@gmail.com, samir0804@gmail.com

ABSTRACT: Invertebrate organisms mostly have innate immune system. Hemocytes are the immune cells of the innate immunity in invertebrates. Our study was designed to observe the effect of malathion insecticide on the innate immune system, immune function and hemocytes of *Drosophila melanogaster*. A hemocytometer was used for total count of hemocytes which showed a reduced percentage of live cells (from 91.63% to 44.57%) due to the treatment. For differential count, the hemolymph was spread as a smear on a clean grease free slide. It was observed that the plasmatocyte count considerably decreased under the effect of malathion (from 78% to 44.66%). Lamellocytes also showed a decline in their number (from 18.33% to 12.61%). But, a trivial increase (from 3.67% to 3.98%) in crystal cell count was recorded. Our result suggests that malathion has mostly negative effect on hemocytes. This study could help in understanding of insect defense mechanism against such hazardous chemicals. Additionally, the function of human macrophages is analogous to the function of plasmatocytes. Hence, this work could possibly aid us in studying the response of human immune cells against organophosphates like malathion.

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Hemocyte is a type of cell found within the hemolymph of invertebrates (Pham and Schneider, 2008). In *Drosophila* larvae, hemocytes are able to adhere to the body wall at the site of infection, though they move freely throughout the hemolymph (Pham and Schneider, 2008). Hemocytes in *Drosophila* sp. are of three types – Plasmatocytes, Crystal cells, and Lamellocytes (Evans et al., 2003; Lanot et al., 2001; Wertheim et al., 2005). Plasmatocytes comprise about 90-95% of circulating hemocytes and can adhere strongly *in vitro*. They are largely involved in phagocytosis (Meister and Lageux, 2003). Gold and Bruckner (2015) already demonstrated the homology between plasmatocyte and mammalian phagocytes which was later strengthened by Diwanji and Bergmann (2020). In *Drosophila* adult flies, plasmatocyte is higher in number in the hemolymph than other hemocyte cells (Meister and Lageux, 2003). Crystal cells are non-adhesive cells that build around 5% of the hemocytes in circulation (Strand, 2008) and responsible for humoral melanization, a process of melanin synthesis (Meister and Lageux, 2003). Lamellocytes are large, flat cells which generally differentiate from prohemocytes after being attacked by parasitoid wasp (Rizki and Rizki, 1992) and at the time of metamorphosis (Lanot et al., 2001). They take part in encapsulation of parasitoids (Meister and Lageux, 2003; Strand, 2008). Pesticides have become common in agricultural field to destroy or control harmful pests that damage crops (US EPA, 2018). Insecticides are the type of pesticides which target the insects. They mainly include organochlorines, organophosphates, carbamates and pyrethroids. Organophosphate pesticides block the action of enzyme acetylcholinesterase through irreversible covalent inhibition (Peter *et al.*, 2014). Application of

**Corresponding Author Email: bindhanibanani@gmail.com*

organophosphate emerged when organochlorine got banned in 1970s (Chavoshani et al., 2020). Malathion is an organophosphate pesticide used broadly in agricultural purpose as well as pest control programs. Like other organophosphates, this insecticide also kills the insect by inhibiting the release of enzyme acetylcholinesterase at synaptic junction (Cabello et al., 2001). Malathion toxicity probably results in permanent nerve damage as well as weakness and fatigability (Rosenthal and Cameron, 1991). It has also been reported to have a probable neurodegeneration effect on *Drosophila melanogaster* (Mehdi and Qamar, 2011). Apart from affecting the nervous system, insecticides could also influence other systems of *Drosophila*. In recent years, researchers are working on effect of organophosphates on insects (Kalita et al., 2016; Perveen and Ahmad, 2017). *Drosophila*, commonly known as fruit fly, has been widely used as a model species for research work in genetics, developmental biology, biochemistry and biomedical sciences. The objective of our study was therefore to evaluate the effect of malathion insecticide on the innate immune system and hemocytes of *Drosophila*.

MATERIALS AND METHOD

Preparation of Drosophila culture medium: In India, most laboratories use Agar-maize powder-brown sugar culture medium containing water (2900 ml), agar agar (25 g), maize powder (250 g), Brown sugar (250 g), dried yeast (75 g), nipagin dissolved in ethanol (2 g) and propionic acid (2 ml) (Poddar et al., 2015). We prepared the same culture medium for our experiment. The larvae were incubated at 19^0 C of temperature till they develop into imago or adult flies. Adult flies of *Drosophila* were chosen as experimental organisms to assess the toxicity of malathion.

Malathion treatment: 0.76 µg of malathion was mixed with 10 ml of *Drosophila* culture medium in a clean vial. The vial contained 30 adult flies. The effect of the treatment was observed after 24 hours. Out of 30, 10 adult flies were dead and 20 adult flies were alive. But, they showed very less movement or activity.

Collection of hemocyte from adult flies: The 20 adult flies, taken for control, were anesthetized with diethyl ether for 1 minute and hemolymph was collected as shown by Laura Palanker Musselman (www.youtube.com. 2013). The samples were centrifuged at 3000 rpm for 5 minutes at normal room temperature. Same procedure was applied to 20 adult experimental flies, but not anesthetized.

Total count of hemocytes: 10 µl of the resulting supernatant was collected and was stained with equal volume of 0.4 % Trypan Blue. After 5-10 minutes, 10 µl of the sample was transferred to hemocytometer (Fein-Optik Blankenburg), one chamber filled with controlled cells and other chamber with treated cells. It was examined at 40X magnification under a compound binocular microscope (from ZEISS India).

Differential count of hemocytes: The resulting supernatant was collected and diluted with 60 µl of phosphate buffered solution (PBS). A smear was drawn on a clean, grease free slide. Then, the hemolymph smear was air-dried and fixed with methanol for 10-15 minutes followed by staining with methylene blue for 15 minutes. Stained slide was washed with distilled water, blot-dried and observed at 40X magnification.

Statistical analysis: The mean and the standard error of the mean (SEM) for control and treatment category were calculated for four times. Student's t test and one way analysis of variance (ANOVA) were carried out to test the level of significance for changes in treatment category and control category.

RESULT AND DISCUSSION

Total Count: The live cells were recognized as small, transparent, circular cells (Plate 1), whereas the dead cells were visible as dark round dots (Plate 2). The mean percentage of the viable or live hemocytes is 91.63 % in normal untreated condition. This mean value remarkably decreased to 44.57% when the *Drosophila* flies were subjected to LC_{50} dose of malathion (Table 1). There is also an important change in the number of dead hemocytes. The mean percentage of dead hemocytes is 8.37% in normal condition which increased to 55.43% when the flies were treated with malathion (Table 2). The changes are statistically significant ($p<0.05$).

Differential Count: Plasmatocytes appeared as large cells with prominent nuclei and round or irregular cell membrane (Plate 3). Lamellocytes were visible as flat or elongated cells with clear nuclei (Plate 4). Crystal cells looked like darkly stained balls (Plate 5). Here, we observed the deflection in the number or mean percentage of the respective cells after the adult flies were exposed to malathion through the culture medium (Table 3). Plasmatocyte showed a drastic reduction in the count. Though lamellocyte count was fewer than the count of plasmatocyte in the hemolymph, it also decreased after the treatment. But, surprisingly, crystal cell showed very trivial fluctuation. There is a very little increase in its number.

Table 1: LC_{50} value determination, 20 Drosophila/vial / 10 ml food

Hours	Concentration (µl)	log_{10} (conc.)	% dead	Probit Value	LC_{50}	Final LC_{50} Value
24 hours LC_{50} value determination	2	0	15	3.96	LC_{50}= 1.28	LC_{50}=1.2µl
	1.7	0.113943352	25	4.33		
	1.5	0.176091259	85	6.04		
	1.3	0.230448921	85	6.04		
	1.1	0.301029996	100	7.33		
48 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.25	
	1.7	0.230448921	85	6.04		
	1.5	0.176091259	75	5.67		
	1.3	0.113943352	50	5		
	1.1	0.041392685	35	4.61		
72 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.2	
	1.7	0.230448921	90	6.28		
	1.5	0.176091259	85	6.04		
	1.3	0.113943352	60	5.25		
	1.1	0.041392685	40	4.75		
96 hours LC_{50} value determination	2	0.301029996	99	7.33	LC_{50}= 1.1	
	1.7	0.230448921	90	6.28		
	1.5	0.176091259	85	6.04		
	1.3	0.113943352	70	5.52		
	1.1	0.041392685	55	5.13		

Table 2: Mean percentage of live hemocytes and dead hemocytes in Control and Treated Condition

	Normal Condition (mean ± SEM)	Treated with Malathion (mean ± SEM)
Live cells	91.63±1.66 %	44.57±5.11 %
Dead cells	8.37±1.65 %	55.43±2.82 %

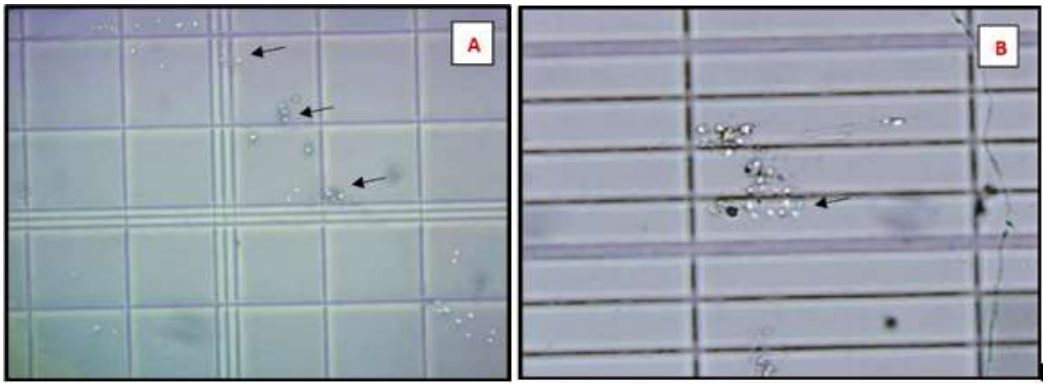

Plate 1. Plate A and B shows live hemocytes as white crystal dots in control adult flies (Magnification Under 40X)

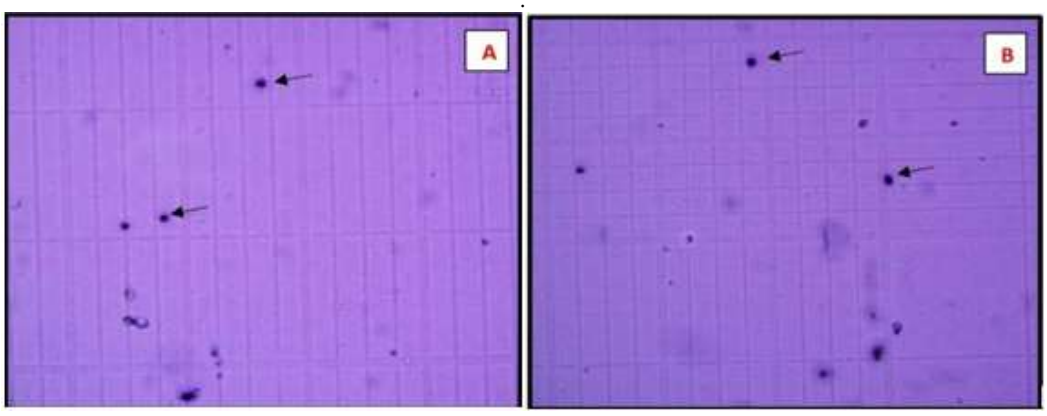

Plate 2. Plate A and B shows dead hemocytes as black dots in treated adult flies (Magnification Under 40X)

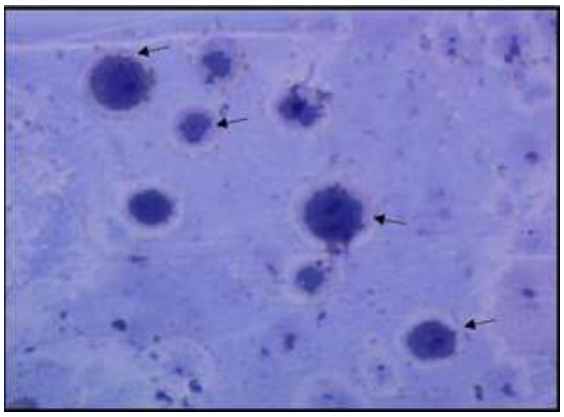

Plate 3. Plasmatocytes with irregular cell margin and prominent nuclei (Magnification Under 40X). They are more abundant in hemolymph than other hemocytes.

Plate 4. Plate 4 shows lamellocytes as elongated cells with nuclei (Magnification under 40X)

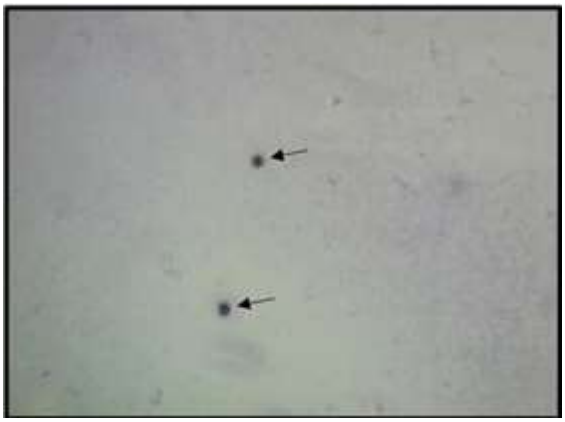

Plate 5. Plate 5 shows Crystal cells as dark stained mass (Magnification under 40X)

Table 3: Comparison among the mean percentage of Plasmatocyte, Lamellocyte and Crystal cell in Control and Treated Condition

	Normal Condition (mean ± SEM)	Treated with Malathion (mean ± SEM)
Plasmatocyte	78±1.73 %	44.66 ±3.75 %
Lamellocyte	18.33±1.85 %	12.61±1.15%
Crystal cell	3.67±0.33 %	3.98±0.3%

The changes in plasmatocyte and lamellocyte count were found to be statistically significant ($p<0.05$). But, the variation in crystal cell count was not significant ($p>0.05$). Our present experiment with adult *Drosophila* showed that the population of live hemocyte cells became very low due to presence of an organophosphate (Malathion) whereas the dead hemocyte count was high. This signifies that malathion negatively affects the viability of the hemocytes. An experiment by Oostingh et al., (2009) with organophosphates like chlorpyrifos and diazinon showed that chlorpyrifos caused a marked decline in cell viability, though diazinon only reduced cytokine induction. Our experiment revealed that malathion decreased the cell viability by 51.37% in a 24 hour treatment. In a study with human blood sample, the number of leukocytes also suffered a decline by 40% within 42 hours of high malathion treatment (Sharma et al., 2016). Because malathion has been reported to stimulate apoptotic cell death (Tripathi et al., 2007; Venkatesan et al., 2017), hemocyte cells could possibly be attacked by malathion leading to the reduction in the count. This could be the reason for the drastic decrease in live hemocytes in our study because dead hemocytes were found to be higher in number after exposure to malathion. We even found a critical decrease in the count of plasmatocyte by 42.74% and that of lamellocyte by 31.2% (Table 3). In a study by Rajak et al., (2015), reduction in the plasmatocyte and lamellocyte count was observed because of the effect of acephate. The decline in plasmatocyte and lamellocyte count in our study could be due to the induction of apoptosis by malathion. We observed a slight rise in the count of crystal cell in the treatment category, though it was not statistically significant ($p>0.05$). Our result for crystal cell count could find a support from the results of Sharma et al., (2016) where it has been shown that malathion had no impact on WBC if less quantity was used. Previously, crystal cells showed an increased count as a result of gradual rise in the treatment concentration of acephate (Rajak et al., 2015). Crystal cells have been known to stimulate melanization which is a crucial tool against physical and chemical stress (Hamilton and Gomez, 2002). Therefore, a slightly high crystal cell count due to exposure to an organophosphate may suggest that these cells, perhaps, divide and proliferate to produce ample melanin. Also, the quantity of malathion or any organophosphate could be an important factor in causing variations to the hemocyte count.

Conclusion: In conclusion, our findings may point out the possible effect of malathion on immune cells of human and other mammals because insect hemocytes share some similarities with the phagocytes found in mammalian innate immune system. Humans and other

vertebrates also consume fruits and vegetables that are being contaminated with these organophosphate insecticides. Vertebrate immune system could probably show some defense mechanism against those hazardous insecticides. Therefore, our experiment could be helpful in future for studying the interaction between organophosphate insecticides and human immune cells.

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