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# Comparative toxic potential of market formulation of two organophosphate pesticides in transgenic *Drosophila melanogaster* (*hsp70-lacZ*)

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## Abstract

This study investigated the working hypothesis that two widely used organophosphate pesticides; Nuvan and Dimecron, exert toxic effects in *Drosophila*. Transgenic *D. melanogaster (hsp70-lacZ)* was used as a model for assaying stress gene expression and AchE activity as an endpoint for toxicity and also to evaluate whether stress gene expression is sufficient to protect against toxic insult of the chemicals and to prevent tissue damage. The study was extended to investigate the effect of the pesticides on the life cycle and reproduction of the organism. The study showed that Nuvan affected emergence of the exposed flies more drastically than Dimecron and the effect was lethal at the highest tested concentration (0.075 ppm). While Nuvan at 0.0075 and 0.015 ppm concentrations affected reproduction of the flies significantly, the effect of Dimecron was significant only at 0.015 and 0.075 ppm. Nuvan-exposed third-instar larvae exhibited a 1.2-fold to 1.5-fold greater *hsp70* expression compared to Dimecron at concentrations ranging from 0.0075 to 0.075 ppm following 12 and 18 h exposure. While maximum expression of *hsp70* was observed in Nuvan-exposed third-instar larval tissues following 18 h exposure at 0.075 ppm, Dimecron at the same dietary concentration induced a maximum expression of *hsp70* following 24 h exposure. Further, concomitant with a significant induction of *hsp70*, significant inhibition of AchE was observed following chemical exposure and temperature shock. Concurrent with a significant decline in *hsp70* expression in Nuvan-exposed larvae after 48 h at 0.075 ppm, tissue damage was evident. Dimecron-exposed larvae exhibited a plateau in *hsp70* induction even after 48 h exposure and moderate tissue damage was observed in these larvae. The present study suggests that Nuvan is more cytotoxic than Dimecron in transgenic *Drosophila melanogaster*.

*Abbreviations:* AchE, acetylchloinesterase; DAB, diaminobenzidine tetrahydrochloride; ECVAM, European Center for the Validation of Alternative Methods; HSP, heat shock protein; HRP, horseradish peroxidase; MRL, maximum residue limit; NOAEL, no observed adverse effect level; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; ppm, parts per million; OP, organophosphate pesticide; PSS, Poels' salt solution

## Introduction

Pesticides are used for crop protection in India and elsewhere. Although their use in agricultural practice is essential for protection of crops against damage induced by pests, exposure to them produces adverse effects in exposed populations, including humans (John et al., 2001).

Among the pesticides, organophosphate pesticides (OPs), which are esters, amides, or simple derivatives of phosphoric or thiophosphoric acids, are synthetic chemicals designed to be toxic, somewhat volatile, and stable enough to remain in their toxic form for relatively short periods of time (Poovala et al., 1998); these are often used indiscriminately. OPs are a group of chemicals that act primarily by inhibiting the enzyme acetylcholinesterase (AchE) at cholinergic junctions of the nervous system (Gallo and Lawryk, 1991); as a result, acetylcholine levels accumulate in the synapse, followed by enhanced stimulation of cholinergic receptors on postsynaptic cells and subsequent alteration of receptor-mediated signaling pathways, e.g., alteration of intracellular cAMP levels. These cellular changes may lead to functional changes at the tissue/organism level (Pope et al., 2005). In addition, OPs are reported to be associated with various other esterases including neurotoxic (or neuropathy target) esterase (NTE), butyrylcholinesterase, carboxylesterase, and A-esterases. Inhibition and subsequent "aging" of NTE is correlated with initiation of organophosphorus-induced delayed neurotoxicity (OPIDN) (Johnson, 1970; Pope et al., 2005). Among the organophosphate group of pesticides, Nuvan (dichlorvos 76% EC) and Dimecron (phosphamidon 86% EC) are used widely in developing countries such as India for agricultural purposes.

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate), a contact and stomach poison for control of insects, is reported to be carcinogenic (National Toxicology Program, 1989), hepatotoxic (Yamano and Morita, 1992), genotoxic (Cakir and Sarikaya, 2005) and neurotoxic (Isoda et al., 2005). It is reported to be toxic to some nontarget organisms such as *Daphnia magna* and *Scenedesmus subupicatus* (Tomlin, 1994).

Phosphamidon, another widely used organophosphate (Jokanovic et al., 1995) with neurotoxic properties, has been reported to cause histopathological changes in rat epididymis (Akbarsha and Sivasamy, 1998). It has been reported to be mutagenic in both animal and plant systems (Panda and Sharma, 1979), and causes significant decrease in hemoglobin percentage, red blood cell number and $O_2$-carrying capacity of blood (Anand-Kumar et al., 2001). Acute and chronic exposure to phosphamidon has been reported to create disturbances in normal functioning at the cellular level in the tissues of prawns (Reddy and Rao, 1990).

Cells from all organisms respond at the cellular level to adverse changes in their environment, such as temperature change or exposure to xenobiotics, UV, oxidizing agents, heavy metals, mutagens, carcinogens and gene expression inhibitors, by a protective mechanism called stress response/heat shock response (Nover, 1991). This response, originally observed in *Drosophila melanogaster* (Ritossa, 1962), has been recognized to represent a universally conserved (from bacteria to human) cellular defense program. The response is mediated by the increased expression of genes encoding a group of proteins referred to as the heat shock proteins (HSPs) or stress proteins (Schlesinger, 1986; Feder and Hofmann, 1999; Lakhotia and Prasanth, 2002). Stress proteins are classified into families according to their apparent molecular weights and respective inducers (Rokutan et al., 1998). The HSPs are thought to assist the cells during adverse conditions by transiently reprogramming cellular metabolic activity, which protects cells from further oxidative and heat damage (Mathew and Morimoto, 1998). Among the various families of stress genes, those of one family, *hsp70*, and their products, HSP70 proteins, are not only highly conserved and the largest of all the stress protein families but also one of the most extensively studied families (Leigh-Brown and Isha-Horowicz, 1981; Hightower, 1991; Morimoto, 1993; Tavaria et al., 1996; Yokoyama et al., 2000). Under adverse environmental conditions, the synthesis of Hsp70 increases and it takes on new but related roles to protect the cells from proteotoxicity (Hightower, 1991).

In recent years, HSPs have shown potential for use as a first-tier tool to screen for adverse biological effects induced by environmental chemicals (Rossner et al., 2003). Because HSPs are part of the machinery for defense, repair, and detoxification of cells, they become direct and specific markers of exposure and effect (Bierkens, 2000). Previous studies have shown that organophosphate pesticides can stimulate stress response in the exposed organisms (Bagchi et al., 1996; Nazir et al., 2001). Recently, our laboratory, as well as others, has shown that *hsp70* can be used as a candidate gene for predicting cytotoxicity of environmental chemicals (Lewis et al., 1999; Mukhopadhyay et al., 2003; Nazir et al., 2003c).

*Drosophila* was chosen as an experimental model for the study because of its well known genetics and developmental biology and because its genome can easily be manipulated by P-element-mediated germline transformation and the expression of a given gene can be studied by reporter gene assay. Moreover, use of *Drosophila* falls within the recommendations of the European Center for the Validation of Alternative Methods (ECVAM) (Festing et al., 1998), whose goal is to promote the scientific and regulatory acceptance of alternative methods that are of importance in the field of bioscience and that reduce, refine, or replace the use of laboratory animals (Benford et al., 2000).

The present study was carried out to examine the comparative toxic potential of Nuvan and Dimecron by examining *hsp70* expression, both qualitatively (*in situ*) and quantitatively, and AchE activity in *Drosophila* transgenic for the *hsp70* gene. Tissue damage, if any, either due to compromise in stress gene expression or due to pesticide exposure *per se* was also examined. The study was extended to investigate whether these pesticides have any effect on the life cycle of *Drosophila* by examining the emergence pattern, hatchability, fecundity, fertility and reproductive performance of the exposed organisms.

## Materials and methods

### Drosophila *strain*

A transgenic *Drosophila melanogaster* line that expresses bacterial β-galactosidase under the control of wild-type *hsp70* promoter (*hsp70-lacZ*) $Bg^9$ as a response to stress (Lis et al., 1983) was used in the study. In this strain of *Drosophila*, the transformation vector is inserted within a P-element. The line contains wild-type *hsp70* sequence up to the *lacZ* fusion point. The flies and larvae of stock $Bg^9$ were cultured on standard *Drosophila* food containing agar, corn meal, brown sugar, and yeast at 24 ± 1°C. Additional suspension of yeast was provided in Petri dishes for healthy growth.

### *Treatment schedule*

The two organophosphate pesticides, Nuvan (dichlorvos 76% EC) and Dimecron (phosphamidon 86% EC), used in the present study were of market formulation, manufactured by Hikal Limited, Bharuch, Gujarat and marketed by Syngenta India Ltd, Mumbai. Four different concentrations of pesticides (0.0015, 0.0075, 0.015 and 0.075 ppm), corresponding to 1.5%, 7.5%, 15% and 75% of the MRL value, respectively, of dichlorvos in fruits (0.1 ppm) (Toxics Link, 2000) were used in the study. Nuvan was soluble in dimethyl sulfoxide (DMSO) and Dimecron in water. The final concentration of DMSO as solvent in food was 0.3% as reported previously (Nazir et al., 2003a). Where DMSO was used, two sets of controls (sets I and II) were used. In set I, larvae received normal food; in set II, food mixed with DMSO was fed to the larvae.

### *Hatchability of eggs*

A method described earlier (Marchal-Segault et al., 1985) was followed to examine the effects' if any, of Nuvan and Dimecron on early development

by examining hatchability of eggs. Briefly, flies were allowed to lay eggs on normal food for 0.5 h, followed by transfer of the eggs to normal food, 0.3% DMSO food and food with different concentrations of Nuvan and Dimecron. After 24 h, the number of nonhatched eggs was recorded, which gives a hatchability index of control and treated flies.

*Emergence of flies*

First-instar larvae hatching from the eggs following synchronous egg laying for 30 min were transferred to control food, DMSO control food and, food contaminated with different concentrations of pesticides and were allowed to grow on these throughout their development. Fifty larvae were transferred in each vial and 10 vials were taken in each group. The number of emerging flies was recorded daily until all the flies emerged. From the data generated, the emergence of the flies in different groups was evaluated according to the method of Gayathri and Krishnamurthy (1981).

*Fecundity, fertility, and reproductive performance of flies*

For the calculation of fecundity, fertility, and reproductive performance of flies, first-instar larvae were transferred to control and contaminated food. Virgin male and female flies emerging from both the control and treated food were separated and pair-mated in vials having normal *Drosophila* food. Ten pairs of flies were included in each group. Flies were transferred to fresh vials everyday for the next 10 days, and the number of eggs laid during this period was scored. From these data, total fecundity (number of eggs) and mean egg production by a female for 10 days were calculated. For reproductive performance, the total number of flies produced by each pair for 10 days was counted and the mean number of flies per pair for a period of 10 days was calculated (Gayathri and Krishnamurthy, 1981).

*Stress gene assay*

To examine the cytotoxic effects of these two pesticides on the organism, third-instar larvae were allowed to feed on normal food, DMSO food, or food contaminated with different concentrations of pesticides for different times (2, 4, 6, 12, 18, 24 and 48 h) at 24 ± 1°C. At the termination of the treatment, the larvae were removed from the food, washed thoroughly with Poels' salt solution (PSS) (Lakhotia and Mukherjee, 1980), and subsequently used for *hsp70* expression studies by quantitative (spectrophotometric) and qualitative (histochemical staining) stress gene assays as indicated below.

*Soluble O-Nitrophenyl-β-D galactopyranoside (ONPG) assay (quantitative)*

A modified version (Nazir et al., 2001) of the method described earlier (Stringham and Candido, 1994) was followed for quantitative assay. Briefly, after washing, the larvae were placed in a microcentrifuge tube (20 larvae per tube, 5 replicates per group), permeabilized for 10 min with acetone, and incubated overnight at 37°C in 600 µl of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 µl of 1 mol/L $Na_2CO_3$. The extent of reaction was quantified by measuring the absorbance at 420 nm on Cintra 20 GBCUV spectrophotometer.

In-situ *histochemical β-galactosidase activity (qualitative)*

At the termination of the treatment, the larvae were washed with PSS to remove food material adhering to the larvae, and internal tissues were explanted in PSS. After brief fixing in 2.5% glutaraldehyde and post-washing in 50 mmol/L phosphate-buffered saline (PBS) (pH 8.0), staining was performed as described earlier (O'Kane and Gehring, 1987; Kar Chowdhuri et al., 1999). For each group, 45–50 larvae were taken.

*Immunohistochemical staining*

A modified version of the method described earlier (Krebs and Feder, 1997a) was followed. Briefly, the internal tissues of control, DMSO control and treated larvae were explanted and dissected in PSS and then fixed in 2.5% glutaraldehyde and permeabilized in PBS with Triton X-100 (PBST). They were then incubated with 7Fb rat monoclonal antibody, which detects only the inducible form of Hsp70 in *Drosophila melanogaster* (Velaquez and Lindquist, 1984), challenged with HRP-conjugated rabbit anti-rat secondary antibody (Bangalore Genei, India), and stained with the chromogenic substrate diaminobenzidine (DAB) as described earlier (Sambrook et al., 1989).

*Dye exclusion assay*

Tissue damage, if any, occurring in the exposed organism due to exposure to the pesticides was examined by trypan blue exclusion assay as described previously (Krebs and Feder, 1997b). Control, DMSO control and treated larvae were dissected out in PSS to explant the internal tissues. The internal tissues were briefly washed in PBS and stained with trypan blue for 30 min at $24 \pm 1^{\circ}$C. The larvae were washed three times in PBS and immediately scored for trypan blue-positive tissues. For each treatment group, 15–20 larvae were scored.

*Measurement of acetylcholinestearase (AchE) activity*

A modified version of the method described earlier (Ellman et al., 1961) was followed for the measurement of AchE activity. Briefly, 10% homogenate of brain ganglia of both control and treated larvae was made in 50 mmol/L Hepes buffer containing protease inhibitor, followed by centrifugation at 11 800*g* for 15 min. Supernatant was then removed and used for enzyme assay. The assay mixture comprised tissue homogenate, phosphate buffer, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) and acetylthiocholine iodide as substrate. The absorbance was measured at 412 nm for 5 min at intervals of 30 s and the enzyme activity was expressed in terms of μmoles of substrate hydrolyzed per minute per gram of tissue.

*Temperature shock treatment*

For the positive control group, healthy third-instar larvae were placed on a Petri-dish lined with moist filter paper and were given a temperature shock at $37 \pm 1^{\circ}$C for 1 h as described previously (Lakhotia and Singh, 1989).

During the course of the study, larvae were observed for any signs of overt toxicity.

*Statistical analysis*

Statistical analysis was carried out by Student's *t*-test after ascertaining homogeneity of variance. Significance was calculated at $p < 0.05$.

## Results

During the course of study, larvae did not show any overt signs of toxicity, as evident from their active crawling and boring habit. However, first-instar larvae transferred to the highest concentration of Nuvan-contaminated food exhibited 100% mortality during third-instar stage. In contrast, third-instar larvae exposed to the same dietary concentration of Nuvan showed 50% mortality; in the rest, sluggish movement was observed.

*Effect on hatchability of eggs*

Nonhatched eggs were counted to examine the effect of Nuvan and Dimecron on early development of the organism. No significant effect on hatchability pattern of the eggs was observed in any of the treated groups (data not shown).

*Effect of Nuvan and Dimecron on emergence pattern of the flies*

The emergence pattern of adult flies was observed (Figure 1A,B) to examine the effect of environmental chemicals on development of the flies. In control, DMSO control, 0.0015 ppm Nuvan and Dimecron, and 0.0075 and 0.015 ppm Dimecron treated groups, respectively, 87%, 85%, 83%, 82%, 80%, and 76% of flies emerged on day 10. At 0.0075 ppm Nuvan a delay of 1 day was observed in the 30% exposed flies, and in the 0.015 ppm Nuvan exposed group a delay of 2 days in emergence of flies was observed. At the highest concentration of Nuvan (0.075 ppm), no flies emerged, owing to larval death, while Dimecron at the same concentration affected the emergence of the flies by a day.

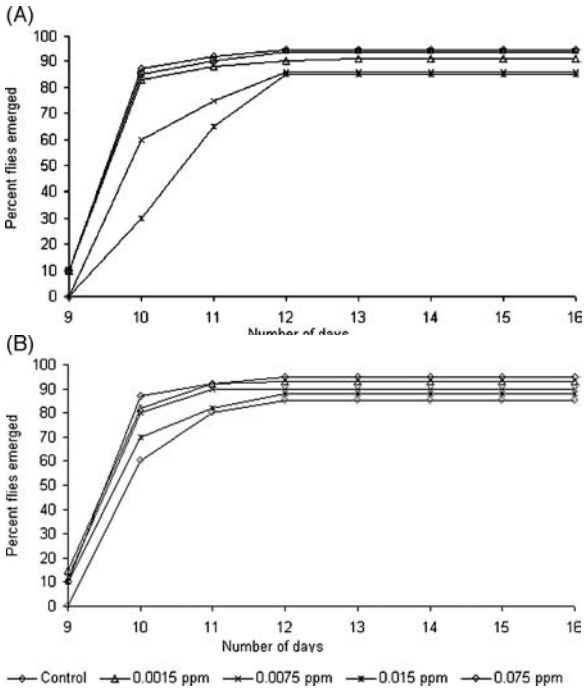


*Figure 1.* Emergence pattern of transgenic *Drosophila melanogaster (hsp70-lacZ)* in control and DMSO control groups and at different concentrations of Nuvan (A) and Dimecron (B).

*Effect on reproduction*

Table 1 shows the effect of Nuvan and Dimecron on reproduction. While Nuvan at 0.0075 ppm dietary concentration induced a significant decrease ($p < 0.05$) in the reproductive performance and mean daily egg laying of the exposed organisms, Dimecron at this concentration failed to evoke any significant effect. At 0.015 ppm concentration of both the pesticides, reproductive performance of the organisms was significantly affected. In addition, Nuvan significantly ($p < 0.05$) affected mean daily egg laying of the exposed flies. At 0.075 ppm Dimecron, mean daily egg laying and reproductive performance were significantly affected ($p < 0.05$).

*Quantitative detection of* hsp70 *expression in larval tissues of Bg*[9]

Figure 2A,B shows *hsp70* expression as a measure of toxicity by quantitative β-galactosidase activity in control and treated larvae. At the lowest concentration of both the pesticides (0.0015 ppm), no significant change in *hsp70* expression was observed in the exposed larvae compared to control and DMSO control. Larvae exposed to 0.0075 ppm Nuvan exhibited a significant ($p < 0.05$) 27% increase in β-galactosidase activity after 18 h and 37% increased activity after 48 h compared to control. However, larvae exposed to 0.0075 ppm Dimecron exhibited a significant ($p < 0.05$) 27% increase in β-galactosidase activity after 48 h. A further increase in dietary concentration of Nuvan (0.015 ppm) resulted in a significant ($p < 0.05$) increase in β-galactosidase activity (42%) after 12 h and a peak in the activity after 18 h (72% increase) compared to control, with an insignificant decline ($p > 0.05$) in β-galactosidase activity (14% regression) after 48 h compared to that after 18 h. Dimecron at this concentration induced a significant ($p < 0.05$) 32% increase in β-galactosidase activity in the exposed larvae after 24 h followed by a plateau in the activity after

*Table 1.* Effect of Nuvan and Dimecron on reproduction in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg[9]

| Group | Total fecundity (eggs) | Daily egg laying per female (Mean ± SEM) | Fertility (%) | Reproductive performance (± SEM) |
|---|---|---|---|---|
| Control | 2160 | 21.6 ± 0.57 | 95 | 204.9 ± 4.97 |
| DMSO control | 2080 | 20.8 ± 0.58 | 94 | 195.5 ± 5.54 |
| Nuvan | | | | |
| 0.0015 ppm | 2035 | 20.35 ± 0.73 | 94 | 190.9 ± 9.97 |
| 0.0075 ppm | 1859 | 18.59 ± 0.72*[a] | 87 | 160.8 ± 7.89*[a] |
| 0.015 ppm | 1809 | 18.09 ± 0.41*[a] | 76 | 137.9 ± 4.13*[a] |
| 0.075 ppm | [c] | [c] | [c] | [c] |
| Dimecron | | | | |
| 0.0015 ppm | 2138 | 21.38 ± 0.24 | 94 | 199.8 ± 7.41 |
| 0.0075 ppm | 2090 | 20.90 ± 0.66 | 92 | 191.2 ± 6.66 |
| 0.015 ppm | 2076 | 20.76 ± 0.27 | 92 | 190.1 ± 2.94*[b] |
| 0.075 ppm | 1888 | 18.88 ± 0.24*[b] | 81 | 153.7 ± 3.11*[b] |

Statistically significant at $^{*}p < 0.05$.
[a]Versus DMSO control.
[b]Versus control.
[c]No flies emerged owing to larval death.

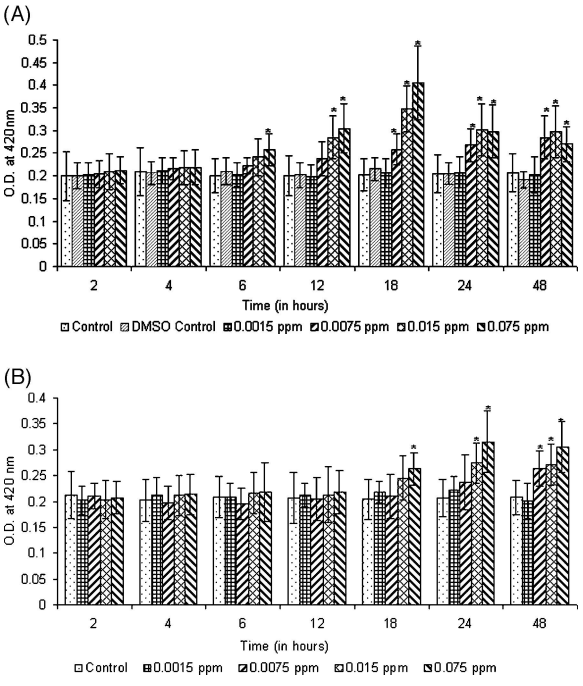


*Figure 2.* Soluble ONPG assay showing β-galactosidase activity in the third-instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) in control and DMSO control groups and at different concentrations of Nuvan (A) and Dimecron (B) treatment for different times (2, 4, 6, 12, 18, 24 and 48 h). Values are mean ± SD of five identical experiments ($n = 5$) made in five replicates. $^{*}p < 0.05$.

48 h. At 0.075 ppm Nuvan, a similar trend was observed to that at the previous concentration except that a significant decline ($p < 0.05$) (33% regression) in β-galactosidase activity in the exposed larvae was observed after 48 h compared to that at 18 h. Interestingly, larvae exposed to Dimecron at this concentration evoked a significant increase ($p < 0.05$) in the β-galactosidase activity following 18–48 h exposure (29% increased activity after 18 h, a maximum 52% increased activity after 24 h, and a plateau in the activity after 48 h). A comparison of β-galactosidase activity in Nuvan and Dimecron exposed larvae at the higher concentrations (0.0075 to 0.075 ppm) for 12–18 h revealed a 1.2-fold to 1.5-fold more β-galactosidase activity in the former. However, such a trend was not evident with further lengthening of the exposure period.

### In-situ *histochemical staining pattern in the larvae exposed to different concentrations of pesticides for different time intervals*

Figure 3A–I shows the β-galactosidase staining pattern in different tissues of control, DMSO control, and treated larvae. Larvae in control, DMSO

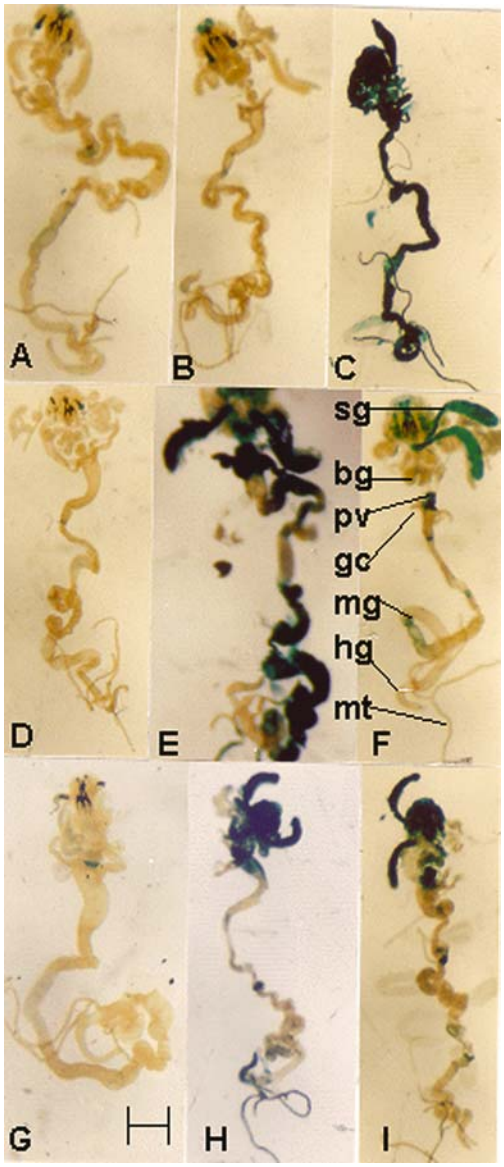


*Figure 3*. β-Galactosidase staining pattern in the tissues of third-instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*): (A) control; (B) DMSO control; (C) temperature-shocked; (D) 0.075 ppm Nuvan for 2 h; (E) 0.075 ppm Nuvan for 18 h; (F) 0.075 ppm Nuvan for 48 h; (G) 0.075 ppm Dimecron for 2 h; (H) 0.075 ppm Dimecron for 24 h; (I) 0.075 ppm Dimecron for 48 h. bg, brain ganglia; sg, salivary gland; pv, proventriculus; gc, gastric caeca; mg, mid gut; hg, hind gut; mt, malpighian tubules. Note the maximum β-galactosidase staining in the tissues of larvae exposed to 0.075 ppm Nuvan after 18 h followed by an appreciable decline in staining pattern after 48 h. Bar 200 μm.

control, and 0.0015 ppm Nuvan and Dimecron groups did not show β-galactosidase activity in their tissues except in 2%, 3%, 6%, and 4% of larvae, respectively, where a pale blue staining was observed in the proventriculus (Figure 3A,B). Temperature-shocked larvae (positive control) showed a maximum β-galactosidase activity (dark blue staining) in all the larval tissues except in malpighian tubules (Figure 3C). Larvae exposed to 0.0075 ppm Nuvan showed a time-dependent increase in the staining pattern in their tissues; 85% of the exposed larvae showed a dark blue staining in proventriculus, moderate blue staining in salivary gland, gastric caeca, midgut and in brain ganglia, and pale blue staining in hindgut, while in the other 15% no staining was observed after 48 h (not shown). On the other hand, 70% of 0.0075 ppm Dimecron-exposed larvae exhibited a moderate blue staining in proventriculus, salivary gland and gastric caeca, while in the other 30% pale blue staining was observed in brain ganglia, midgut and hindgut after 48 h (not shown). While larvae exposed to 0.015 ppm Nuvan showed an increase in staining intensity in the above-mentioned tissues after 18 h (84% of the exposed larvae showed dark blue staining in proventriculus and in gastric caeca, and moderate blue staining in midgut, salivary gland, and brain ganglia, while the other 16% showed pale blue staining in hindgut) (not shown), Dimecron exposure at this concentration resulted in a similar activity to that at the preceding concentration (0.0075 ppm) after 48 h (not shown). At 0.075 ppm Nuvan, exposed larvae showed maximum staining after 18 h (88% of the exposed larvae showed dark blue staining in proventriculus, salivary gland, brain ganglia, gastric caeca and midgut, and in the other 12% moderate blue staining in hindgut of the exposed larvae was observed) and a decline in the staining pattern after 48 h of exposure (Figure 3E,F). Dimecron at this concentration evoked dark blue staining in proventriculus, and moderate blue staining in midgut, salivary gland, gastric caeca, and brain ganglia in

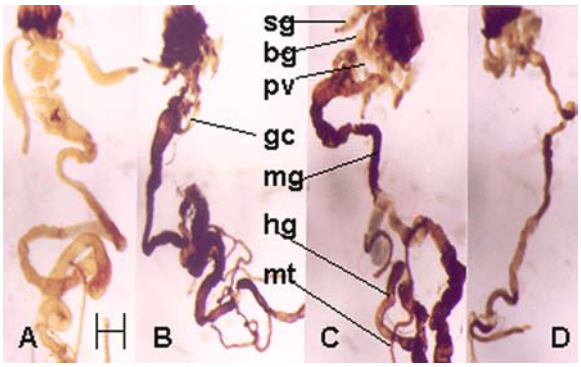


*Figure 4*. *In-situ* immunohistochemistry using anti-Hsp70 antibody in the midgut tissues of third-instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*): (A) control, (B) temperature-shocked; (C) 0.075 ppm Nuvan for 18 h; (D) 0.075 ppm Dimecron for 24 h. Bar 200 μm.

86% exposed larvae, while in the other 14% pale blue staining in hindgut after 24 h and 48 h was observed (Figure 3H,I).

In-situ *immunohistochemistry*

To examine whether the reporter gene activity in the larvae exposed to different concentrations of the test chemicals mimics the resident Hsp70 activity, *in-situ* immunohistochemical staining was performed. The patterns of Hsp70 expression in the tissues of larvae treated with Nuvan and Dimecon were comparable to those observed with the reporter gene assay (Figure 4A,D).

*Vital dye staining for tissue damage*

Trypan blue, a vital dye excluded by living cells, gives a reliable measure of tissue damage. Figure 5A–C shows trypan blue staining in third-instar larval tissues of transgenic *Drosophila* in control, DMSO control, and pesticide-treated groups. Larvae exposed to the highest concentration (0.075 ppm) of Nuvan and Dimecron for 48 h exhibited dark blue staining in the tissues with the former and moderate blue staining with the latter (Figure 5B,C). No appreciable staining was observed in the rest.

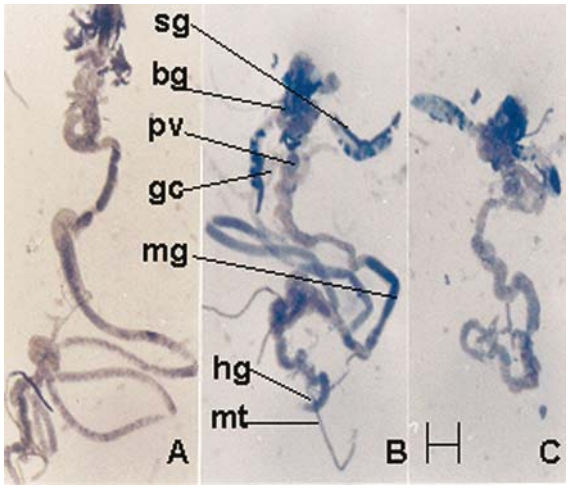


*Figure 5*. Trypan blue staining in the tissues of third-instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*): (A) control; (B) 0.075 ppm Nuvan for 48 h; (C) 0.075 ppm Dimecron for 48 h. bg, brain ganglia; sg, salivary gland; pv, proventriculus; gc, gastric caeca; mg, mid gut; hg, hind gut; mt, malpighian tubules. Bar 200 μm.

*Effect of Nuvan and Dimecron on AchE activity*

Figure 6 shows AchE activity in the brain ganglia of control and exposed larvae. In control, DMSO control, and 0.0015 ppm Nuvan and Dimecron treated groups, AchE activity was comparable throughout the exposure period (2–48 h). Larvae exposed to 0.0075 ppm Nuvan showed a significant inhibition ($p < 0.05$) in AchE activity following 18 h to 48 h exposure (18% and 24% inhibition in the activity following 18 h and 48 h treatment, respectively). However, exposure of the larvae to Dimecron at this concentration resulted in a significant inhibition ($p < 0.05$) in AchE activity only after 48 h exposure. At 0.015 ppm Nuvan, exposed larvae exhibited a significant inhibition ($p < 0.05$) in AchE activity following 12–48 h treatment. Larvae exposed to Dimecron at the same concentration showed a significant inhibition ($p < 0.05$) in AchE activity following 24 h exposure. At the highest concentration of Nuvan (0.075 ppm), exposed larvae showed a significant inhibition ($p < 0.05$) in the enzyme activity after

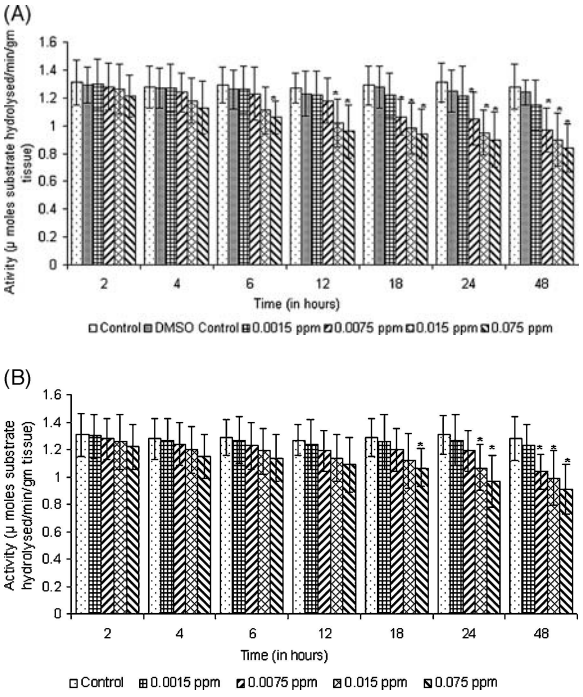


*Figure 6.* AchE activity in the brain ganglia of control and DMSO control groups and at different concentrations of Nuvan (A) and Dimecron (B) treatment for different times (2, 4, 6, 12, 18, 24 and 48 h). Values are mean ± SD of five identical experiments ($n = 5$) made in five replicates. $^{*}p < 0.05$.

6 h and maximum inhibition after 48 h (34%), while exposure to Dimecron resulted in a significant inhibition ($p < 0.05$) of the enzyme activity after 18 h exposure. Temperature-shocked larvae also exhibited a significant inhibition of AchE activity ($p < 0.05$) as compared to control (data not shown).

### *Comparison between AchE activity and* hsp70 *expression in the brain ganglia of temperature-shocked, Nuvan and Dimecron treated larvae*

Table 2 summarizes the results, showing that a significant induction of *hsp70* in the treated and temperature-shocked larvae was accompanied by a significant inhibition of AchE activity.

*Table 2.* Summary of *hsp70* expression and AchE activity in the brain ganglia of third-instar larvae of transgenic *D. melanogaster* following treatment with different concentrations of Nuvan and Dimecron and temperature shock

| Treatment group | Exposure time | *hsp70* expression | AchE activity |
|---|---|---|---|
| Nuvan | | | |
| 0.0015 ppm | 2–48 h | – | – |
| 0.0075 ppm | 2–12 h | – | – |
| | 18–48 h | ↑ | ↓ |
| 0.015 ppm | 2–6 h | – | – |
| | 12–48 h | ↑ | ↓ |
| 0.075 ppm | 2–4 h | – | – |
| | 6–48 h | ↑ | ↓ |
| Dimecron | | | |
| 0.0015 ppm | 2–48 h | – | – |
| 0.0075 ppm | 2–24 h | – | – |
| | 48 h | ↑ | ↓ |
| 0.015 ppm | 2–18 h | – | – |
| | 24–48 h | ↑ | ↓ |
| 0.075 ppm | 2–12 h | – | – |
| | 18–48 h | ↑ | ↓ |

↑, significant induction; ↓, significant inhibition; –, insignificant as compared to the control.

## Discussion

In the present study, toxic effects of Nuvan and Dimecron were studied by *hsp70* expression, AchE activity in terms of tissue damage, and overall biological effects. Nuvan was found to affect the emergence of flies more drastically than Dimecron and also caused larval mortality at its highest concentration. This indicates that the test chemicals caused lengthening of the developmental period or caused mortality. Such a lengthening of the developmental period is an indication of somatic effects caused by the test chemicals (Luning, 1966). Gayathri and Krishnamurthy (1981) using Agallol 3, a mercuric fungicide, and Nazir et al. (2001), using chlorpyrifos, an organophosphate pesticide, observed similar effects. The exact mechanism by which OPs exert their ultimate lethality is subject to debate (Bird et al., 2003) although the major pathway is believed to be through AchE inhibition (Canadas et al., 2005). Thus, it is likely that the lethal effects

of the chemicals on organisms are due to its robust inhibitory effect on AchE activity.

Environmental factors have great influence on the lifespan and fecundity of *Drosophila* (Lints, 1971). Apart from factors such as genotype and age, fecundity is affected by the conditions to which the larvae are exposed (Gruvez et al., 1971). A significant effect ($p < 0.05$) on reproductive performance of flies as observed in the present study, was more pronounced in Nuvan-exposed organisms than in those exposed to Dimecron. This may be due to the adverse effect of the chemicals either during maturation of sperm or ova or on the overall reproductive physiology of the organisms, including reduced oviposition as reported in grain weevils by Chadwick (1962) using pyrethroids.

Organophosphate pesticides have been reported to produce neurobehavioral damage during development (Pope, 1999; Slotkin, 2004), but developing organisms have been found to recover more quickly from cholinesterase inhibition than do exposed adults (Song et al., 1997). No significant effect in the early development of the organism, as evidenced by no delay in hatchability of eggs following exposure to Nuvan and Dimecron in the present study, is intriguing. However, it is likely that the subtle effect at the initial developmental stage may elicit a more pronounced effect at a later stage, as evidenced by larval mortality at the highest concentration of Nuvan.

The measure of toxic effects evoked by a test chemical can be achieved by analyzing various endpoints, including pathological lesions, enzymatic imbalances, anatomical disorders, and the like. Such studies usually allow the potency of the toxicant to be known only at a much later stage. Toxicologists have exploited HSPs for protective roles in living organisms by using them in the prediction of toxic effects of environmental chemicals. Being responsive to even minor insaults, HSPs are reported to be efficient biosensors (Ait-Aissa et al., 2003). Previously, Werner and Nagel (1997), Lewis et al. (1999), Mukhopadhyay et al. (2003), and Nazir et al. (2003c) have reported the toxicity of test chemicals in different organisms by employing *hsp70* expression as an endpoint. An insignificant induction of *hsp70* was observed in larvae exposed to the lowest concentration (0.0015 ppm) of both the pesticides. Thus, this concentration may be suggested as the No Observed Adverse Effect Level (NOAEL) of the test chemicals.

It has been reported that induction of *hsp70* by certain environmental chemicals is generally correlated with early cytotoxic events and is a secondary consequence of damage that affects cellular integrity. The lipophilic nature of the compounds allows them to pass easily through the plasma membrane to alter cellular functions before interacting with cellular proteins. This in turn may trigger denaturing of proteins to stimulate stress protein induction (Steinmetz and Rensing, 1997; Ait-Aissa et al., 2000). The possibility that both Nuvan and Dimecron, being lipophilic compounds, could evoke *hsp70* induction in the exposed organism in a similar way cannot be ruled out.

A significant decline in *hsp70* expression was observed in larvae exposed to 0.075 ppm Nuvan after 48 h. Whether such a decline is due to instability of the reporter gene product after 48 h or due to reduction in the number of cells available for Hsp70 assay was also addressed. Our results with immunohistochemistry showing *hsp70* expression levels comparable to those observed in the reporter gene assay negate the first possibility. However, strong trypan blue staining in the larval tissues observed under such conditions concurrently with a decline in the *hsp70* expression in larvae exposed to Nuvan could well be due to a reduction in the number of viable cells. Similarly, moderate trypan blue staining in larvae exposed to the highest concentration of Dimecron indicated tissue damage. The uniform trypan blue staining observed in the head region of the larvae in all the groups may be due to tissue damage during dissection.

It is important to remember that the effects of test chemicals on the expression of

stress proteins are tissue-specific (Stringham and Candido, 1994). Both test chemicals at their higher dietary concentrations induced mild to strong *hsp70* expression in brain ganglia of the exposed larvae, with a more severe effect caused by Nuvan. Organophosphate insecticides are known as potent neurotoxins in insects (Baillie and Wright, 1985). A significant inhibition in AchE enzyme activity in the brain ganglia of larvae that were temperature-shocked and treated with higher concentrations of the chemicals was concurrent with significant induction of *hsp70*. In this context, Yang et al. (2002) have shown a similar effect in primary cultured skeletal muscle cells following exposure to dimethoate. Like brain ganglia, salivary glands and midgut tissues of the exposed larvae were also found to express *hsp70* at the higher concentrations. In this context, although a direct mechanistic link between the chemical exposure and *hsp70* induction in these tissues could not be established, it is likely that the inhibition of AchE enzyme activity, a key component of nerve function, may trigger damage to many cellular components, including protein, and thereby may stimulate induction of *hsp70.* Interestingly, malpighian tubules were found to be refractory to *hsp70* expression following chemical exposure. This may possibly be explained by the time of assaying the β-galactosidase activity in the cells of the malpighian tubules, as reported earlier from this laboratory (Nazir et al., 2003b). Recent studies have shown that vulnerable target organs for toxicants can be identified by stress gene expression (Hightower, 1991) and regulation of stress gene expression is achieved in a stress-specific manner. It is therefore likely that larval tissues exhibiting *hsp70* expression are vulnerable to the toxicant.

During the past decade, issues of animal use and care in toxicology research and testing have become fundamental concerns for scientists not only for ethical reasons but also because of the difficulty in interpreting data owing to interspecies variation and exorbitant cost (Benford et al., 2000). This has led researchers to encourage the use of alternative organisms in toxicology. The tiny fruit fly *Drosophila*, a well-established animal model for geneticists and developmental and molecular biologists, has been shown in recent years to be successfully employed as an alternative animal model for toxicological research (Kar Chowdhuri et al., 1999; Mukhopadhyay et al., 2002a,b, 2003; Nazir et al., 2001, 2003a,b,c).

In conclusion, it is suggested that Nuvan is more toxic than Dimecron in transgenic *D. melanogaster* as evidenced by *hsp70* expression, inhibition of AchE, and other developmental and biological effects. The study further supports the use of stress genes (Ryan and Hightower, 1996; Mukhopadhyay et al., 2003; Nazir et al., 2003c) and AchE (Linonetto et al., 2003) as biomarkers in environmental monitoring.

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