Cell. Mol. Biol. 57 (1): 100-111
Published on February 12, 2011; DOI 10.1170/T908

IN VITRO INDUCTION OF THE UBIQUITOUS 60 AND 70KD HEAT SHOCK PROTEINS BY PESTICIDES MONOCROTOPHOS AND ENDOSULPHAN IN *Musca domestica*: POTENTIAL BIOMARKERS OF TOXICITY

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Abstract

This study has investigated the effect of two highly toxic pesticides, monocrotophos (organophosphate) and endosulphan (organochlorine), on the inducibility of two major heat shock proteins, the HSP60 and HSP70, essential for cell survival, in the house fly *Musca domestica*. The LC_{50} values of the two pesticides for larva and adult (monocrotophos: 0.05 ppm for larva and 0.025 ppm for adult; endosulphan: 15 ppm for larva and 2 ppm for adult) revealed monocrotophos to be potentially more toxic than endosulphan. The relative susceptibility (lethality) of adult to either of these pesticides appeared much higher than that of larva. The expression patterns of HSP60 and HSP70 were analysed in various larval and adult tissues, exposed to varying sub-acute doses of monocrotophos (0.00010 ppm - 0.00075 ppm for larva and 0.00010 ppm – 0.00050 ppm for adult) and endosulphan (0.5ppm – 2.0ppm for larva and 0.01ppm-0.10 ppm for adult). The immunoblots revealed significant correlation between the pattern of HSP's expression and the pesticides-induced tissue injury/ mortality, visualized by trypan blue dye exclusion test. Both the pesticides caused significant induction of these HSPs in a tissue and dose-dependent manner, suggesting their importance as molecular indicators (biomarker) in the assessment of cellular toxicity caused by endosulphan and monocrotophos.

Key words: Monocrotophos, Endosulphan, HSP60, HSP70, *Musca domestica*, Pesticide toxicity.

Article information
Received on December 17, 2010
Accepted on January 5, 2011

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Abbreviations: LC_{50}: Lethal Concentration 50; **ppm**: Parts per million; **HSPs**: Heat Shock Proteins; **kD**: Kilo Dalton; **PBS**: Phosphate Buffer Saline; **BSA**: Bovine Serum Albumin; **IgG HRP**: Immunoglobulin Horse Radish Peroxidase; **PBT**: PBS+BSA+Tween 20; **ECL**: Enhanced Chemiluminiscence ; **O. D.**: Optical Density.

INTRODUCTION

The heat shock proteins (HSPs) or the molecular chaperones are among the most indispensable cellular biomolecules, assisting in proper folding of nascent polypeptides, removal of denatured substrates, intracellular transport, prevention of stress induced protein aggregation and many other essential cellular functions under normal as well as stressed conditions (17, 47, 30, 25). Several studies have reported predominant induction of at least two major HSPs, HSP60 and HSP70, in response to exposure of cells to a variety of stressors, including heat, heavy metals, chemicals, pesticides, oxidative agents, etc. (4, 5, 6, 33, 34, 48, 25, 13, 15). Heat shock proteins are regarded as tier-II biomarkers, which respond to a wide variety of sub-lethal stressors. The specificity of HSPs to different stressors and their evaluation as biomarkers often varies in different

cases. Many of the characteristics of heat shock proteins, particularly of HSP60 and HSP70, are best suited to an ideal biomarker of exposure (37, 38, 56, 54, 25). This proposal has been further advocated in some of the recent studies also (31, 32, 48, 25, 13, 15).

Among several environmental contaminants, pesticides are the highly complex organic molecules causing varying levels of toxicity to both target and non-target organisms (44, 57). Monocrotophos and endosulphan are the two most common insecticides widely applied in the field for the management of insect pests. Both pesticides have been shown to act as potential neurotoxins and may cause severe health hazards to human health, if their applications are not strictly regulated (21).

Since heat shock response can act as a biosensor of stress induced tissue damage, it was thought to investigate the effects of monocrotophos and endosulphan on the expression of two most ubiquitous and abundant protein of molecular weight 60kD and 70kD in order to evaluate their suitability in the bio-monitoring of pesticide induced toxicity in the house fly *Musca domestica*. *Musca domestica* provides advantage over other animal models, mainly because of its closeness to the human habitation, economic and simple laboratory rearing conditions, and above all, being non-pathogenic under laboratory culture condition. Many investigators have used *M. domestica* as a useful insect model to understand the mode of action and development of resistance against insecticides/pesticides (41, 20, 25, 13). Thus, main objectives of this study were (i) to determine if, there exists a dose-response relationship between the pesticides and the expression of HSPs (HSP60 and HSP70) and, (ii) to evaluate the usefulness of HSPs as early physiological markers (biomarker/indicators) in the monitoring of pesticide induced cellular toxicity. The i*n vitro* exposures of various larval and adult tissues of *M. domestica* to endosulphan and monocrotophos revealed a significant correlation between the level of HSP60 and HSP70 induced and the tissue mortality, though in a tissue-specific manner.

MATERIALS AND METHODS

Fly culture

The laboratory culture of *Musca domestica* was maintained in the insectory for the last several years at 26 ± 2°C and 50% relative humidity. Larvae and adults were fed on rice condensed protein and sugar-milk meal, respectively.

Pesticide treatments

The commercial grade of endosulphan (35%) and technical grade of monocrotophos (100%) were obtained from Cheminova India Ltd. and Rallies Chemicals India Ltd, respectively. The LC_{50} values for both endosulphan and monocrotophos were determined by exposing second instar larvae (endosulphan: 05, 10 15, 20, 25 & 30 ppm, monocrotophos: 0.01, 0.05, 0.50, 1.00, & 2.00 ppm) or 2 days post-eclosed adults (endosulphan: 0.1, 0.5 1.00, 1.50, 2.00 & 2.5 ppm, monocrotophos: 0.001, 0.0025, 0.005, 0.0075, 0.010 ppm) to varying concentrations of the pesticides following the methods of Smith et al. (45). The pesticide dilutions were made in milk (5 ml volume), soaked in cotton plugs and placed in 50ml glass vials. Larvae or flies (N = 20) were transferred in the vials separately for feeding or exposure (contact) for 24 hrs. Triplicates of each treatment and parallel controls (on milk without pesticide) were also set. The vials were covered with muslin cloth to prevent escape of the larvae or flies and proper aeration. For *in vitro* treatment, pesticides from stock were diluted in Robb's medium (16). Adult and larval tissues were dissected out in Robb's medium and transferred to glass cavity blocks for pesticide treatment. The final range of pesticide concentrations selected for exposure of larvae or adults for determination of LC_{50} was based on the pilot experiments carried out on larval or adult survival or mortality (levels of induced toxicity) at various pesticide concentrations. Similarly, the concentrations of pesticides for *in vitro* treatment were selected using LC_{50} values as the base line concentrations. Tissue mortality was detected by trypan blue staining as described below.

Trypan blue staining

For *in vitro* exposure, larval and adult tissues were excised in Robb's medium (16) and exposed separately to various concentrations of the pesticides (larva: endosulphan: 0.5, 1.0 and 2.0 ppm, monocrotophos: 0.00010, 0.00050 and 0.00075 ppm. Adult: endosulphan: 0.01, 0.05 and 0.10 ppm, monocrotophos: 0.00010, 0.00025 and 0.00050 ppm), prepared in Robb's medium, for 1 hr each. Simultaneously, control tissues were incubated in medium only. Both treated and control tissues were stained with trypan blue dye for 30 min by constant shaking, followed by thorough washing in PBS buffer thrice for 10 min each in order to remove the excess of dye or non-specific dye binding (22). Trypan blue stained tissues were mounted in 50% glycerol for observation and photography under a Nikon photomicroscope (Japan).

Immunoblotting

Both larval and adult tissues were incubated in Robb's medium containing various doses of the two pesticides (larva-endosulphan: 1.0 and 2.0 ppm, monocrotophos: 0.00050 and 0.00075 ppm, Adult: endosulphan: 0.05 and 0.10 ppm, monocrotophos: 0.00025 and 0.00050 ppm) in glass cavity blocks. Control tissues were incubated in medium only. Treated tissues were washed once with the medium to remove traces of the pesticides. Both control and treated tissues were transferred to lysis buffer separately (23). The total protein in each sample was estimated by Bradford's method (9). Equal amount of proteins (50 µg) from pesticide treated and control tissues were electrophoresed on one-dimensional SDS-PAGE (12.5%

polyacrylamide) to separate the polypeptides. Proteins from gel matrix were transferred on nitrocellulose membrane using semi-dry electroblotting method (42, 43). The protein quantity and the efficiency of transfer in each lane were monitored by Ponceau-S (Sigma) staining in order to ensure equal quantity of proteins in all the lanes. The proteins were blocked in 5% skimmed milk powder for 2 hours to prevent non-specific antibody cross-reactivity. After thorough washing in PBT (1X PBS+ 0.3% BSA+0.1% Tween 20), the blots were incubated separately for 1 hr in Rabbit anti-HSP60 *Heliothis* polyclonal and mouse anti-HSP70 chicken monoclonal antibodies (Cat. No. SPA-805 and SPA-822, respectively; StressGen Biotechnologies, Canada). Primary antibody treatment was followed by a 30 min washing in PBT again and then incubation in secondary antibodies, anti-rabbit IgG-HRP conjugate for HSP60 and anti-mouse HRP conjugate for HSP70 for 1 hour. The fluorescence signals were detected using ECL-Plus Chemiluminiscence system (GE Healthcare).

Densitometric analysis

The relative intensities (density) of western blot signals (protein bands) for HSP60 and HSP70 in different tissues were measured as optical density (O. D. in arbitrary units) in an UVItech gel doc system (UVItech, UK) provided with UVIband software, in reference to a blank lane. The optical density values were used to draw line graphs for direct comparison of levels of control and pesticide induced HSP60 and HSP70 proteins in each tissue type exposed to endosulphan or monocrotophos.

Statistical analysis of data

The Student's t-test for significance of mortality at varying concentrations of the pesticides was carried out to determine LC_{50} values and was further ascertained by one-way analysis of variance (ANOVA), followed by Tukey's and Dunnet's post-hoc analysis using SPSS 13.0 and XLSTAT 2010 softwares. The p-values less than 0.05 were considered significant. Sperman rank correlation (r-value) for increased concentration of pesticide treatment for various tissues and HSPs induction or expression level was calculated by Analyse-it software.

RESULTS

Detection of LC_{50}

The levels of mortality of *M. domestica* larvae or adults exposed to various concentrations of endosulphan and monocrotophos are shown in Tables 1A, B, C and D. The values of LC_{50} for endosulphan at larval and adult stages were recorded as 15 ppm and 2 ppm, respectively. For monocrotophos, the observed LC_{50} values were much lower, being 0.05 ppm for larva and 0.025 ppm for adult. The significant values of student's t-test between groups are represented by (*) in each Table (Tables 1A-D), which was further ascertained by Statistical analysis that confirmed the significant death ($P< 0.05$) of larvae or adults with increase in the pesticide concentrations.

Effect of endosulphan and monocrotophos on tissue mortality and expression of HSPs (HSP60 and HSP70)

Larva:

Trypan blue staining

The characteristic property of trypan blue dye to identify dead or dying cells was used to assess the effects of pesticides on the larval and adult tissues. At 0.5 ppm endosulphan, the dye exclusion test for larval tissues (fat body, mid gut and hindgut) showed only partial cell death. Increase in the pesticide (endosulphan) concentration to 1ppm was observed to enhance the tissue mortality significantly more than at 0.5 ppm, specifically in midgut and fat body. Further increase in the mortality was observed at 2 ppm in all the tissues, showing intense cytoplasmic staining, particularly, in hind gut, covering most of the tissue area (Fig 1A). As observed for endosulphan, all the larval tissues tested (e.g. hindgut, midgut and fat body) showed increased mortality with increasing concentrations of monocrotophos. At 0.0001 ppm, small light blue patches of trypan blue stain appeared dispersed throughout. At higher concentrations (0.0005 ppm or 0.00075 ppm), however, the staining pattern appeared tissue-specific. For example, at 0.0005 ppm, hindgut appeared less affected than midgut and fat body. Similarly, while fat body was seen relatively mildly stained at 0.00075 ppm, hindgut was seen intensely stained throughout (Fig. 1B). Overall, the 0.00075ppm of monocrotophos appeared highly toxic to all the tissues examined.

Expression of HSP60 and HSP70

The results of immunoblot analysis of endosulphan and monocrotophos induced HSP60 and HSP70 proteins in larval tissues (e.g., mid gut, hind gut and fat body) of *M. domestica* are shown in Figs. 2A & B and 3A & B. The upper panels in each (Figs. 2AI & BI, 3AI & BI) show the cross-reacting bands (Western signals) for HSP60 and HSP70 proteins, respectively. The lower panels (Figs. 2AII & BII and 3AII & BII) represent the densitometric analysis (line graph), measuring the intensity (O. D. in arbitrary units) of the corresponding bands in the immunoblot.

Table 1. Endosulphan (E) and Monocrotophos (M) induced larval (A & C) and adult (B & D) mortality (three replicates of n=20 each) in *M. domestica*. Asterisks (*) in columns 5 in this, tables 2A, 2B, 2C and 2D represent highly significant t- test values (when $p<0.05$), when compared with each other. C = control.

A

Group no. 1	Conc. (ppm) 2	Mean mortality (± SE) 3	Value of t- test in reference to control 4	Inter-group t- test 5	
-	-	-	-	Group no.	t- test value
1	C	00.3 ± 0.4	-	-	-
2	E0.1	03.3 ± 0.6	04.1*	1 and 2	4.1*
3	E0.5	07.0	16.7*	2 and 3	6.1*
4	E1.0	10.6 ± 1.0	09.6*	3 and 4	3.6*
5	E1.5	12.0 ± 0.8	13.1*	4 and 5	1.1
6	E2.0	15.3 ± 1.1	13.6*	5 and 6	2.5
7	E2.5	20.0 ± 0.8	22.1*	6 and 7	3.6*
8	E3.0	27.0 ± 2.1	12.3*	7 and 8	3.1*

B

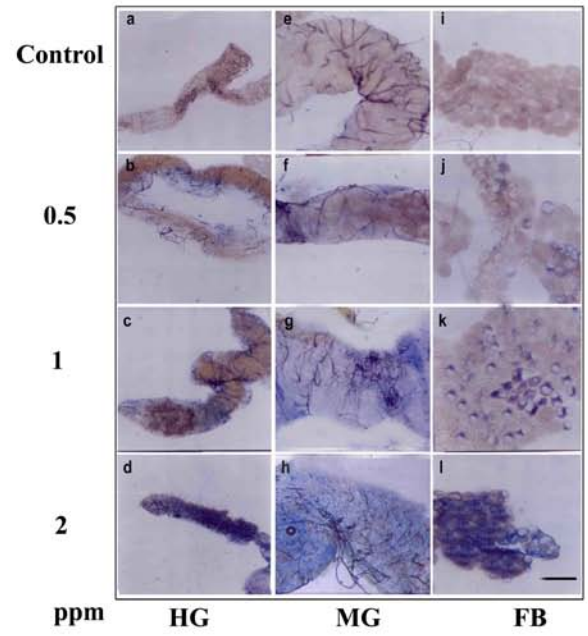
Group no. 1	Conc. (ppm) 2	Mean mortality (± SE) 3	Value of t- test in reference to control 4	Inter-group t-test 5	
-	-	-	-	Group number	t-test value
1	C	-	-		
2	E05	04.0 ± 0.8	05.00*	1 and 2	5.0*
3	E10	07.3 ± 1.4	05.20*	2 and 3	2.0
4	E15	10.0 ± 0.8	12.50*	3 and 4	2.7
5	E20	14.3 ± 1.1	13.00*	4 and 5	3.3*
6	E25	16.3 ±1.4	11.60*	5 and 6	1.1
7	E30	20.0±0.0	20.00*	6 and 7	2.6

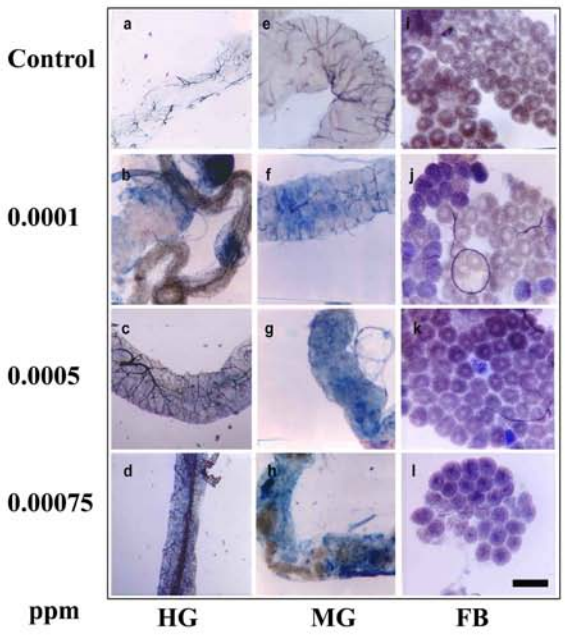
C

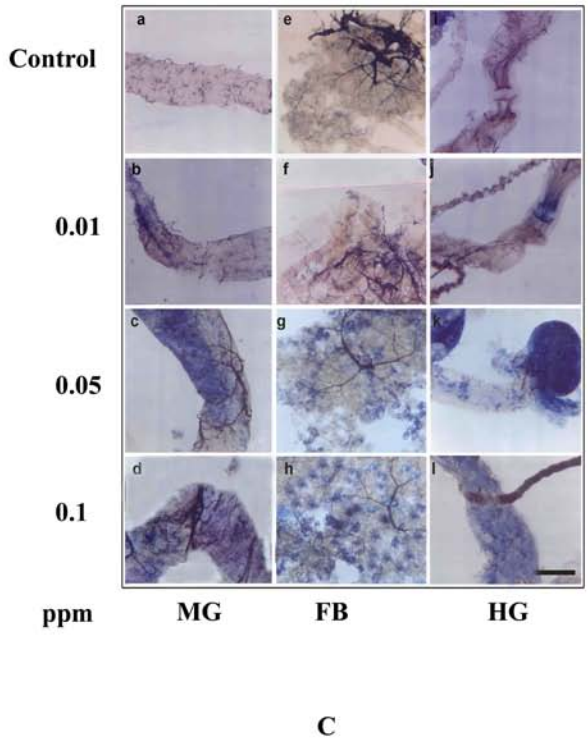
Group no. 1	Conc (ppm) 2	Mean mortality (±SE) 3	Value of t- test in reference to control 4	Inter-group t-test 5	
-	-	-	-	Group number	t- test value
1	C	00.3 ± 0.4	-	-	-
2	M0.01	07.0 ± 0.8	07.5*	1 and 2	7.5*
3	M0.05	12.3 ± 1.0	11.2*	2 and 3	4.4*
4	M0.50	15.0 ± 0.8	15.7*	3 and 4	2.25
5	M1.00	20.0	42.5*	4 and 5	6.25*
6	M2.00	20.0	42.5*	5 and 6	0.00

D

Group No 1	Conc. (ppm) 2	Mean mortality (± SE) 3	Value of t- test in reference to control 4	Value of inter-group t-test 5	
-	-	-	-	Group number	t- test value
1	C	-	-	-	-
2	M0.0010	06.3 ± 0.9	07.0*	1 and 2	7.0*
3	M0.0025	07.6 ± 1.0	07.6*	2 and 3	1.0
4	M0.0050	09.6 ± 1.2	08.0*	3 and 4	1.3
5	M0.0075	11.6 ± 1.3	08.9*	4 and 5	1.1
6	M0.0100	14.6 ± 1.4	10.4*	5 and 6	1.5







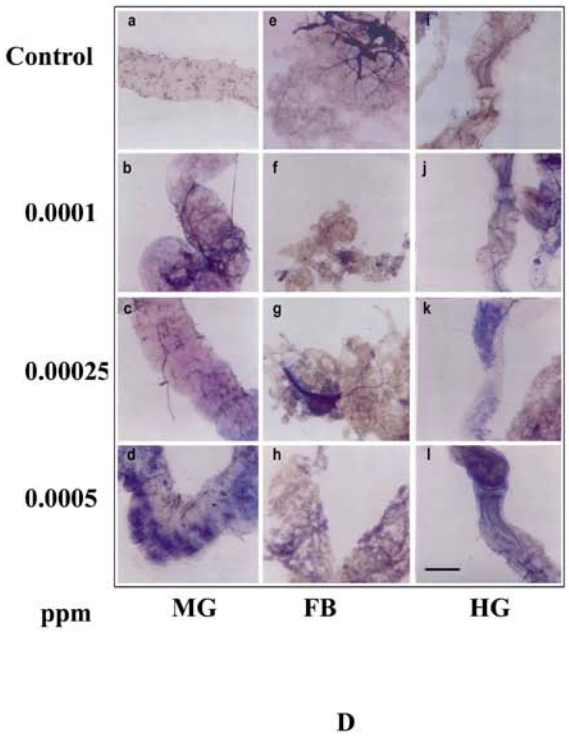


Figure 1. Trypan blue staining of larval tissue exposed to varying concentrations of endosulphan (A) and monocrotophos (B). The larval tissues are hindgut (HG, a-d), midgut (MG, e-h) and fat body (FB, i - l). Trypan blue staining of adult tissues exposed to varying concentrations of endosulphan (C) and monocrotophos (D). Adult tissues are midgut (MG, a-d), fat body (FB, e-h) and hind gut (HG, i-l). The concentrations of the pesticides (ppm) are given on the left side of each panel. The bars represent 150 µM scale.

Analysis of the expression of HSP60 (Figs. 2AI and 3AI) in larval tissues indicated its level to have increased significantly upon pesticide treatment as compared to that in control. At 2 ppm, the level of induction of HSP60 further increased only in midgut as compared to that at 1 ppm. However, no apparent increase in HSP60 level was noted in fat body and hindgut at this concentration. The analysis of the induced pattern of HSP70 expression in endosulphan treated tissues appeared comparable to that of HSP60. All the three tissues expressed increased level of HSP70 protein at both the pesticide doses (1 ppm and 2 ppm), as compared to their respective controls (Figs. 3A & B). Interestingly, 2 ppm endosulphan caused further increase in the HSP70 expression only in fat body tissue, but not in midgut, which showed enhanced expression of HSP60 at this concentration. In general, HSP60 (Figs. 2A & B) and HSP70 (Figs. 3A & B) both, showed tissue-specificity in their expression patterns, particularly at 2 ppm concentration of endosulphan.

The western blot and densitometric analysis of HSP60 and HSP70 expression in monocrotophos (0.0005 ppm and 0.00075 ppm) treated tissues (e.g., mid gut, hind gut and fat body) indicated a pattern comparable to that observed for endosulphan treated tissues (Figs. 2A and 3A). As compared to endosulphan, monocrotophos appeared as a weak inducer of HSP60, particularly in midgut and fat body tissues (Fig. 2B). At 0.00075ppm, however, hindgut showed a significant increase in HSP60 induction (Fig. 2B). In contrast, monocrotophos induced significantly high-level expression of HSP70 even at low pesticide concentration (0.0005 ppm) as compared to its level in control tissues (Fig. 3B). Except fat body, neither of the two larval tissues (mid gut and hind gut) showed any further increase in the level of HSP70 expression at 0.00075ppm (Fig. 3B). It was even noted drastically down regulated in mid gut tissue at higher pesticide concentration (0.00075 ppm; Fig. 3B). In all the above experiments distinct tissue-specific variations in the basal level (control) expression of both HSP60 and HSP70 were observed.

Adult:

Trypan blue staining of pesticide treated tissues of adult M. domestica

All the three tissues, (midgut, fat body and hindgut) appeared highly sensitive to increasing concentrations of endosulphan. The data presented in Figure 1C demonstrate marked mortality at 0.05 ppm as compared to that at 0.01 ppm. It was found increased further at higher concentration (0.1ppm), which is evident from the intensity and distribution of trypan blue staining (Fig. 1C). Among the adult tissues examined for monocrotophos toxicity, hindgut and midgut were found to be relatively more sensitive. The result of trypan blue staining of pesticide treated tissues is shown in Fig. 1D. At 0.0001 ppm, both mid gut and hind gut tissues showed mild staining (dispersed patches of blue stain), while fat body appeared relatively weakly stained. A further increase (2.5 to 5 times) in the pesticide concentration proved extremely lethal, particularly for midgut, which was stained more intensely than (dark blue staining throughout) hindgut or fat body (Fig. 1D).

Expression of HSP60 and HSP70 proteins in the tissues of adult M. domestica

In view of the higher mortality rates in adult caused by pesticides, different tissues (e.g., hindgut, fat body and midgut) were tested for their effect on the inducibility of HSP60 and HSP70 proteins. The tissues were treated with different concentrations of endosulphan (0.01 and 0.10 ppm) and monocrotophos (0.00025 ppm and 0.0005 ppm). As observed in larval tissues above, the western blots and densitometric (O.D.) analysis indicated a tissue-specific pattern of induction of both HSP60 and HSP70 in the three adult tissues examined (Figs. 2C & D, and 3C & D). The immunoblots for HSP60 in tissues treated with endosulphan showed distinct pattern of expression. As evident from figure 2C, fat body showed only a slight induction of HSP60 at 0.1 ppm, but there was no induction at 0.01 ppm when compared with the control. Hindgut expressed a very high level of HSP60 induction at 0.01 ppm endosulphan, which appeared drastically reduced at 0.1 ppm. In contrast, midgut showed a steady increase in the HSP60 expression with increase in the pesticide concentration when compared with the control tissues where HSP60 level was found much lower than that of hindgut and fat body.

A comparison between the three adult tissues, treated with endosulphan (0.01ppm-0.1ppm), indicated a tissue-specific pattern of induction of HSP70, as was noted for HSP60 (Fig. 3C). In this case also, fat body showed induction of HSP70 only at 0.1 ppm but not at

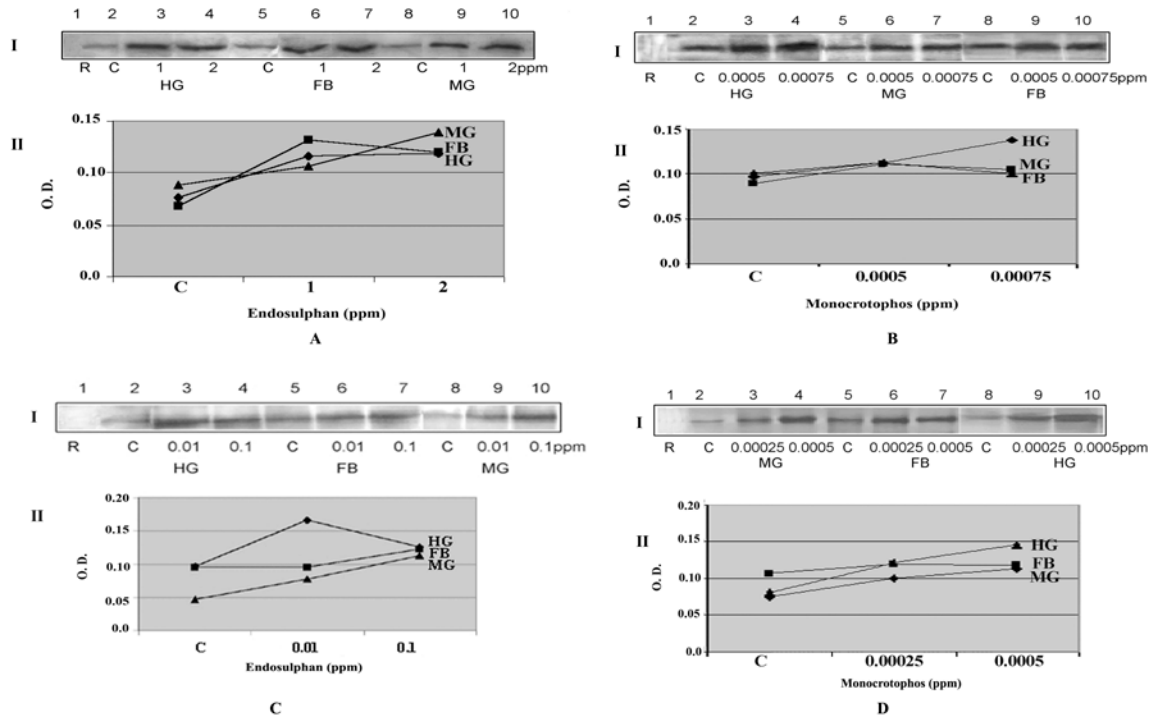


Figure 2. Western blots (I, upper panel in each) showing expression pattern of HSP60 in larval (A & B) and adult (C & D) tissues exposed to various concentration of endosulphan (A & C) and monocrotophos (B & D).The lower panel in each (II, A-D) show line graphs of densitometric measurements (O.D., Optical density/absorbance) of corresponding western signals (bands), to show the variations in the expression of HSP60 in larval and adult tissues of *M. domestica*. Abbreviations: MG = midgut, HG = Hind gut, FB = Fat body, R = Reference lane, C = Control.

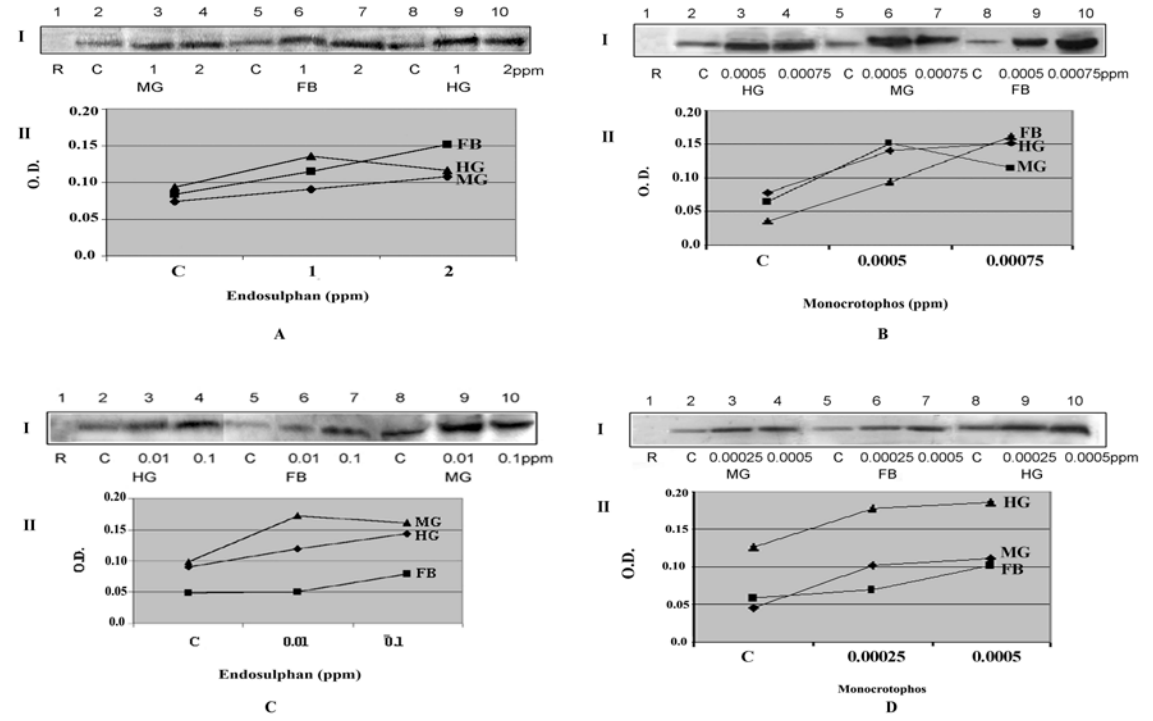


Figure 3. Western blots (I, upper panel in each) showing expression pattern of HSP70 in larval (A & B) and adult (C & D) tissues exposed to various concentration of endosulphan (A & C) and monocrotophos (B & D).The lower panel in each (II, A-D) show line graphs of densitometric measurements (O.D., Optical density/absorbance) of corresponding western signals (bands), to show the variations in the expression of HSP60 in larval and adult tissues of *M. domestica*. Abbreviations: MG = midgut, HG = Hind gut, FB = Fat body, R = Reference lane, C = Control..

lower pesticide concentration (0.01 ppm) (Fig. 3C). Likewise, instead of midgut, which showed a linear increase in the HSP60 expression, hindgut indicated a comparable trend in the expression of HSP70 (Fig. 3C). Further, the densitometric analysis for HSP70 expression indicated midgut to be most stress-responsive tissue, with maximal induction at 0.01ppm concentration. However, it appeared slightly down regulated at higher concentration (0.1ppm) of endosulphan, yet being higher than that in hindgut or fat body.

The western blot analysis of adult tissues treated with monocrotophos also indicated inducibility of HSP60 and HSP70 proteins when compared with the control untreated tissues. While there was a moderate induction of HSP60 in all the three tissues (hindgut, midgut and fat body) tested at both the pesticide concentrations (0.00025 and 0.0005 ppm), significantly high level of expression of HSP70 was observed in all the tissues treated with monocrotophos as compared to their respective controls (Figs. 2D and 3D). Among the three tissues, hindgut indicated maximal induction of HSP60 exhibiting an increasing trend with the increase in pesticide concentration (0.00025 ppm and 0.0005 ppm) (Fig. 2D). Immunoblots for HSP70 showed a very high level of expression of the protein in hindgut as compared to midgut or fat body (Fig. 3D). Midgut, showed relatively increased synthesis of HSP70 as compared to that in fat body at both pesticide concentrations (Fig. 3D). In general, the level of induction of both HSP60 and HSP70 by monocrotophos, appeared tissue-specific. A positive Spearman rank correlation (r value) was recorded between the pesticide (i.e., endosulphan & monocrotophos) concentrations for all the tissues of both the developmental stages (larva and adult) and the levels of HSP60 and 70 expression (r value is in the range of 0.5 to 1).

DISCUSSION

Our results show that endosulphan and monocrotophos both are potential toxicants for both larva and adult as indicated by LC_{50} values. As compared to endosulphan, monocrotophos proved extremely lethal and was able to inflict considerable cellular damage even at several folds lower concentration than that of endosulphan. Particularly, adults showed significant decrease in the survival rate when exposed to monocrotophos as compared to endosulphan. This was also reflected in the tissue-specific differential pattern of expression of HSP60 or HSP70 proteins in response to pesticide exposure of larval or adult tissues, which may likely be due to differences in their functional or metabolic conditions. In our earlier observation in *Musca domestica* on thermotolerance also, adults were found highly sensitive to heat stress as compared to larvae or pupae (52, 43). This observation is, however, not universal e. g., the adults of fleshfly *Sarcophaga crassipalpis* were found better adapted to increased environmental temperature (thermo-tolerance) compared to larvae (58). The reasons for such contrasting behaviors could more likely be due to the species-specific variations in the behavioural, morphological or physiological adaptabilities or adjustments to different environmental stressors.

Similarly, the differential susceptibilities of larval and adult stages to pesticide stress may also be associated, to a great extent, with their structural as well as physiological characteristics, which may facilitate or reduce the degree of exposure. As noted in this study, larva is more tolerant to pesticide toxicity, exhibited higher LC_{50} values than the adult with significantly low LC_{50}. It is possible that the relatively poor larval response to pesticide toxicity as compared to adult could also be due to the higher rate of metabolic breakdown of the pesticides by various hydrolyzing or detoxifying enzymes available in the gut of larva, followed by rapid excretion of these metabolites. In contrast, the fat body and Malpighian tubules both are poorly developed in adult that weaken the efficiency of detoxification and removal of toxic products, increasing their susceptibility to pesticide toxicity. Besides, both the pesticides are known to be potential neurotoxins, hence, can cause significant lethality to both larva as well as adult. Possibly, the states of cellular physiology of mitotically active larval and non-mitotic adult brain cells have significant relationship with their differential responses to pesticide toxicity.

Although above mechanism are expected to play significant role in the differential tolerance of larval and adult stage to pesticide toxicity, these are the adaptations of long-term exposure. In contrast, short-term exposures are often proved to be more lethal because of their rapid action before any stable physiological mechanism

comes into play. Under such circumstance, the heat shock response plays significant protective role for which it is evolved.

Our results showed that endosulphan and monocrotophos both induced significant level of HSP60 and HSP70 proteins in all the larval and adult tissues tested, generally, in a dose-dependent manner. The observed pattern of induced expressions of these HSPs revealed distinct tissue-specificity. For example, gut tissues (hind gut and midgut), which appeared more sensitive to pesticide toxicity than non- gut tissue (e.g., fat body), as indicated by trypan blue staining also, showed increased level of expression of HSP60 or HSP70 protein. The most likely reason for increased sensitivity of gut tissues to insecticide stressors lies in the fact that these are the first target tissues directly involved in the ingestion as well as metabolic breakdown of the insecticides. The other tissues floating in the haemolymph could be the secondary targets, exposed to the metabolites of the insecticides secreted in the haemolymph, e. g., fat body. In one of the earlier observations on heat stress response in *Drosophila* (22) also, gut tissues were found to be more sensitive to heat stress and appeared in a direct correlation between the tissue damage and the expression of HSP70 in the corresponding tissues. In mammalian cells (HeLa) also, a direct correlation between the intensity of the heat shock (temperature) and the quantity of HSPs expressed has been reported (1). However, when the temperature is raised further (43°C), the response attenuates and no further increase in the HSPs is observed (1). A similar study from our laboratory in house fly and blow fly (52, 53), demonstrated that increase in the incubation temperature from 37°C to 42°C/43°C, could induce enhanced level of HSPs (e. g., HSP70), which was significantly reduced when the heat shock temperature was raised further (43^{o}C). The current observation on the pesticides induced HSP60 or HSP70 expression with increasing concentration of pesticide in housefly, thus, appears correlated with the above studies. The HSP60 and HSP70 heat shock proteins are demonstrated to be major polypeptides involved in the maintenance of cellular homeostasis. While HSP70 is more general and ubiquitous in its functions, HSP60 is a sub-cellular or mitochondrial protein (28, 46). However, they contribute equally and significantly to several vital cellular functions including proper proteins folding, preventing misfolding during stress, removal of denatured protein substrates (17, 29, 11, 36) and growth and differentiation of germ cells (14). Under stressed conditions, which might perturb all or some of the above cellular processes, cells require these chaperones for protection and recovery from the trauma of stress. The likely reasons for the observed variations in the pesticide induced expression of HSP60 and HSP70 may be several. Tissue-specificity in the susceptibility to insecticides, varying rates of penetration of the two insecticides into larval and adult tissues, selective inhibition of these HSPs in a tissue- or dose-dependent manner, or even their differential degradation in different cell types, could be a few possibilities, which may potentially alter the pattern of heat shock gene expression in a dose tissue or developmental stage-specific manner (50). Further, in western blot, an antibody detects total antigen (HSPs) present in the cell, including the non-degraded pre-existing (constitutive) pool, irrespective of delayed inhibition or decreased expression due to insecticide toxicity. Though not experimentally tested, this might also contribute to the observed variations, to some extent. Thus, a careful assessment of expression of these proteins (HSP60 and HSP70), can provide more effective means to monitor the level of cellular toxicity inflicted by a stressor, be it an insecticide, metal or heat (40, 19).

Both monocrotophos and endosulphan are potential neurotoxins but exert strong toxic effect on other organs or tissue-systems as well, directly or indirectly, disrupting their normal functions (18). For example, phosphamidon, a primary metabolite of monocrotophos, competitively inhibits acetyl cholinesterase activity (27), resulting in termination of signal transduction in the cholinergic synapses in insects. Although there is no evidence available as yet to conclude, it is likely that some of the metabolites of these insecticides might be acting as the inducers of HSPs rather than insecticides themselves. In insects and rat, endosulphan is broken down to its sulphate, diol and some other derivatives, like ether, lactone and hydroxyether, which is interconvertible into diol and then to ether (3, 2, 26). Some of these metabolites or by products, like ether or alcohol, have also been reported to induce heat shock proteins (12, 55, 24, 8, 7, 35). Further, both the insecticides are known to induce oxidative stress and can cause DNA damage. It is expected that such conditions may themselves become some of the likely sources of

cellular stress leading to activation of heat shock genes (39, 51, 10).

The universality of the stress response and its non-specificity to tissue types, stressor or species under study, in some cases, may likely pose question on the suitability of HSPs as biomarkers (19, 49). However, careful evaluation with respect to dose (concentration), level of the response (HSPs) and cellular toxicity defined by cell death (e.g., trypan blue exclusion test) for a given stressor, tissue type or species, may overcome the above limitations to greater extent. Being simple, low cost and easy to perform, even in the field-based laboratories, HSPs can be valued as highly reliable, early and intrinsic biomolecular markers of exposure of several toxic agents, including pesticides, which also act as potent toxicants for even non-target organisms, such as human beings, cattle and wild lives. The peripheral blood may be used to estimate the expression of HSPs in case of suspected exposures compared with that of the unexposed healthy individual acting as controls.

Acknowledgement - We thankfully acknowledge the Department of Atomic energy (DAE, DST), Mumbai, Government of India for providing financial support through research grants to PKT.

Other articles in this theme issue include references (59-74).

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