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Organochlorine pesticide, endosulfan induced cellular and organismal response in *Drosophila melanogaster*

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ABSTRACT

The effect of endosulfan (0.02–2.0 μg mL^{-1}) to *Drosophila melanogaster* (Oregon R$^+$) at the cellular and organismal levels was examined. Third instar larvae of *D. melanogaster* and the strains transgenic for *hsp70*, *hsp83* and *hsp26* were exposed to endosulfan through food for 12–48 h to examine the heat shock proteins (hsps), reactive oxygen species (ROS) generation, anti-oxidant stress markers and xenobiotic metabolism enzymes. We observed a concentration- and time-dependent significant induction of only small hsps (*hsp23* > *hsp22*) in the exposed organism in concurrence with a significant induction of ROS generation, oxidative stress and xenobiotic metabolism markers. Sub-organismal response was to be propagated towards organismal response, i.e., delay in the emergence of flies and decreased locomotor behaviour. Organisms with diminished locomotion also exhibited significantly lowered acetylcholinesterase activity. A significant positive correlation observed among ROS generation and different cellular endpoints (small hsps, oxidative stress markers, cytochrome P450 activities) in the exposed organism indicate a modulatory role of ROS in endosulfan-mediated cellular toxicity. The study thus suggests that the adverse effects of endosulfan in exposed Drosophila are manifested both at cellular and organismal levels and recommends Drosophila as an alternative animal model for screening the risk caused by environmental chemicals.

1. Introduction

Pesticides have been in use worldwide for controlling pests to increase food production. Endosulfan, a widely used organochlorine pesticide, has been listed as one of the persistent organic pollutants (POPs) by Persistent Organic Pollutants Review Committee (POPRC) during the Stockholm Convention on POPs. The POPRC has recommended a ban on the use as well as production of endosulfan in several developing/developed countries [1,2]. However, its use for agricultural and other related purposes is still continuing in several countries including India [3]. Moreover, presence of endosulfan in different environmental compartments (soil, water, etc.) has been detected [3–5].

Endosulfan is highly lipophilic and exposure to it can lead to bioaccumulation and biomagnifications, resulting into adverse effects on target and non-target organisms. Summarizing the earlier studies, endosulfan has been shown to cause biological effects that include endocrine disruption and hepato-, neuro- and geno-toxicity along with increased oxidative stress and cell death [6–8]. Besides killing pests, it also affects the non-target organisms viz. fish, Daphnia, honeybees and other insects which are not pests and also higher mammals including humans [9–11]. In animals, the majority of the studies on endosulfan are based on its exposure through intra-peritoneal or dermal routes with limited information on dietary exposure [12,13].

Following exposure to endosulfan, an organism experiences xenobiotic stress. Stress elicits the primary protective response of an organism by the induction of a specific set of genes, known as heat shock genes (also stress genes), whose products are termed as heat shock proteins (hsps) (also stress proteins; sp). These genes are highly conserved across the taxa [14]. Hsps are one of the most studied proteins against chemical stressors in terrestrial and aquatic systems [15,16]. Among the heat shock genes, *hsp70* is one of the highly conserved genes which was first reported to be induced in Drosophila by different types of stressors including chemicals [17], a parasite-and a free living-protozoan [18], an isopoda [19] and a marine teleost [20]. Endosulfan induced *hsp70* expression in aquatic organisms like prawns and midges has been reported [21,22]. Conversely, non-induction of *hsp70* in different experimental model systems after endosulfan treatment has also been reported [23–27]. Therefore, *hsp70* expression projected as the first

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tier indicator of cellular stress against endosulfan exposure remains inconclusive from these studies. Besides *hsp70*, other members of the hsps family have been found to play important roles in exposure to xenobiotics vs. their effect [28]. Therefore, it is likely that in the event of refractoriness of *hsp70*, other members of hsps family (both small and large hsps) may provide the requisite defense to the organism exposed to xenobiotics.

Like hsps, all organisms are bestowed with anti-oxidant defense system against various stress stimuli that include both enzymatic and non-enzymatic components [29]. While information on either oxidative stress or hsps in endosulfan-exposed organism are available [22,30], however, studies pertaining to both the cellular defense systems operating together in the exposed organism are missing.

Exposure of an organism to xenobiotics results in the metabolism of the latter leading to the activation of xenobiotics metabolizing enzymes like cytochrome P-450 (CYP) and glutathione S-transferases (GSTs) [31]. Previous studies have reported activation of CYP1A1, CYP1A2, CYP2B1 and CYP2B2 in endosulfan-treated mammalian cells and in aquatic organisms [13,32]. On the contrary, information about activation of CYP enzymes in non-target insect species is limited.

Parallel to the sub-organismal effect of this pesticide on the exposed organism, organismal response against the same toxicant reflects an overall effect on population and therefore, such an effect may be causally linked to population dynamics wherein characteristics like survival, development, and behaviour of exposed individuals may be affected [33,34].

The present study, therefore, aims to examine the *in vivo* effect of endosulfan given to *Drosophila melanogaster* in the diet to understand the role of selected stress genes expression, xenobiotics metabolism and antioxidant defense systems. The study was further extended to examine the organismal response to this insecticide.

We used *D. melanogaster* because it is an invertebrate model organism that is closest to humans and has wide acceptability for genetic and developmental studies. During the last decade, this model has been extensively used for elucidating human diseases and for toxicological studies [35,36]. Drosophila has been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) [37] for promoting 3Rs (reduction, refinement and replacement) of laboratory animal use in toxicity studies and testing.

2. Material and methods

2.1. Fly strains

Wild type *D. melanogaster* (Oregon R^+) and transgenic strains for *hsp70* (*hsp70-lacZ*), Bg^9 [38], *hsp83* (*hsp83-lacZ*), *83Z-880* [39] and *hsp26* (*hsp26-lacZ*), *351-94A* [40] were used for the study. In transgenic strains, bacterial β-galactosidase is expressed as a response to stress. Flies and larvae of all the strains were reared at $24 \pm 1\,^{\circ}C$ on standard Drosophila diet as described previously [41]. Additional yeast suspension was provided for healthy growth of the organism.

2.2. Treatment schedule

Three concentrations (0.02, 0.2 and 2.0 $\mu g\,mL^{-1}$) of endosulfan (Analytical grade, 99.7% purity, Sigma Chemicals, St. Louise, MO, USA) were used in the study. Larvae/flies were grown on standard Drosophila diet either in the absence or presence of endosulfan dissolved in dimethylsulfoxide (DMSO) [final concentration of DMSO as 0.3%] [42] for 12–48 h. Based on the maximum residue level (MRL) in fruits (2.0 ppm), the various concentrations of endosulfan for the study were selected. Organisms fed on normal food or food mixed with 0.3% DMSO were used as control and vehicular control respectively.

2.3. Quantification of endosulfan in Drosophila larvae by gas liquid chromatography (GLC)

Quantification of endosulfan both in food and in the exposed organism was carried out using the method previously described [26]. In brief, control and endosulfan-exposed larvae (in three replicates) were homogenized in acetonitrile and extracted with *n*-hexane followed by cleaning and concentrating of the samples. The samples were applied on an Agilent GLC 7890A (Foster City, CA, USA) equipped with electron capture detector (ECD) to identify and quantify the residual level of endosulfan (isomers and metabolites) by applying recovery factors.

2.4. Emergence of flies

First instar Oregon R^+ larvae were transferred to normal food or food containing DMSO or food containing endosulfan (50 larvae/vial and 10 vials/group) and were allowed to grow till they emerge as flies. All the flies till the emergence of the last one were counted in each group. Emergence probability (number of flies emerging from the group/total number of larvae transferred to the group) of the flies in different groups was evaluated as described earlier [43,44].

2.5. Survivorship of adult flies

Effect of endosulfan on the life span of the adult flies was examined by feeding the newly emerged adult flies with food mixed with different concentrations of endosulfan from day one of their emergence. Flies were transferred to the fresh vials every alternate day and the number of dead flies was recorded till the death of the last fly [45].

2.6. Climbing assay

Climbing assay was performed as described previously [46] with some modifications. Twenty adult flies were placed in a vertical plastic tube (18 cm length × 2 cm diameter). Flies that crossed the 15 cm line within 30 s from the time they were tapped to the bottom of the vials were scored. The climbing scores represent the mean percentage of flies that crossed the 15 cm line among the total number of flies per experiment. The scores are the mean of the numbers of flies above 15 cm (n_{top}) and below 15 cm (n_{bot}), expressed as percentage of the total number of flies (n_{tot}). Results are presented as mean ± SD of the scores obtained from three independent experiments. For each experiment, a performance index (PI) was calculated which is defined as $1/2[(n_{tot} + n_{top} - n_{bot})/n_{tot}]$.

2.7. Jumping assay

For examining neuromuscular activity, jumping activity assay was performed. Threshold for the jumping response appears to be related to the speed of locomotor activity. Newly emerged flies, one at a time, were transferred to a vial marked 1–10 cm and the distance jumped by the fly was recorded from the bottom of the vial. The average number of jumps in five replicates was taken as the jumping activity. One hundred flies per group with five replicates for each group were used.

Table 1
Genes and their primer sequences used in RT-PCR amplification.

hsp22	Forward (F) 5′GGATGAACTGGACAAGGCTCTAAA3′
	Reverse (R) 5′ ATATGATTGGCGACTGCTTCTCC3′
hsp23	Forward (F) 5′GAGCCTTGCCGACGATTTG3′
	Reverse (R) 5′ GGCGCCCACCTGTTTCTC3′
hsp26	Forward (F) 5′CAAGCAGCTGAACAAGCTAACAATCTG3′
	Reverse (R) 5′GCATGATGTGACCATGGTCGTCCTGG3′
hsp60	Forward (F) 5′CCTCCGGCGGCATTGTCTTC3′
	Reverse (R) 5′ AGCGCATCGTAGCCGTAGTCACC3′
hsp70	Forward (F) 5′GAACGGGCCAAGCGCACACTCTC3′
	Reverse (R) 5′TCCTGGATCTTGCCGCTCTGGTCTC3′
hsp83	Forward (F) 5′CCCGTGGCTTCGAGGTGGTCT3′
	Reverse (R) 5′TCTGGGCATCGTCGGTAGTCATAGG3′
gapdh	Forward (F) 5′ AATTCCGATCTTCGACATGG 3′
	Reverse (R) 5′ GAAAAAGCGGCAGTCGTAAT 3′

2.8. Acetylcholinesterase (AchE) activity

AchE activity was estimated in fly brain tissues as described previously [47] with minor modifications [48]. Briefly, 10% homogenate of brains of flies from control and endosulfan-treated groups was prepared in 50 mmol L^{-1} Hepes buffer containing protease inhibitor, followed by centrifugation at 10,000 × *g* for 15 min. The assay mixture consisted of tissue homogenate, phosphate buffer, 5,5-dithiobis(2-nitrobenzoic) acid (DTNB) and acetylthiocholine iodide as the substrate. The degradation of acetylthiocholine iodide was measured at 412 nm and the results were expressed as μmol min^{-1} mg^{-1} protein.

2.9. hsp70, hsp83 and hsp26 promoter induction experiments

Third instar larvae of Bg^9, *83Z-880* and *351-94A* from control and treated groups were washed thoroughly with Poels' salt solution (PSS) [49] and then processed for *hsp70*, *hsp83* and *hsp26* expression studies as described below.

2.9.1. Soluble O-nitrophenyl-β-D-galactopyranoside (ONPG) assay in Bg^9, 83Z-880 and 351-94A larvae

ONPG assay was done essentially following the method of Stringham and Candido [50] with minor modifications [45]. Briefly, larvae of Bg^9, after washing, were placed in a microcentrifuge tube (20 larvae per tube, three replicates per group), permeabilized for 10 min with acetone, incubated for 12 ± 1 h at 37 °C in 600 μL of ONPG staining buffer and the reaction was stopped by adding 300 μL of 1 M Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm on a GBC-UV spectrophotometer (GBC Scientific Equipment, Melbourne, Australia).

2.9.2. RT-PCR analysis of hsp70, hsp83, hsp60, hsp26, hsp23 and hsp22 mRNA in third instar larval tissues of D. melanogaster (Oregon R+)

Total RNA from control and treated third instar larvae of Oregon R^+ was extracted using TRI reagent (Ambion, Austin, TX, USA). The mRNA was reverse transcribed into cDNA using oligo dT primer and Revert Aid™ H minus first strand cDNA synthesis kit (Fermentas, MD, USA) essentially following the manufacturers' instructions. Forward and reverse primers for *hsp83*, *hsp70*, *hsp60*, *hsp26*, *hsp23*, *hsp22* and *gapdh* (Table 1) were synthesized as previously described [51]. Following PCR, the amplicons were separated on an 1.5% agarose gel containing ethidium bromide at 5 V cm^{-1} and visualized under a VERSA DOC Imaging System Model 1000 (Bio-Rad, Hercules, CA, USA). The intensity of the bands was quantified by Quantity One software of Bio-Rad, CA, USA. Each experiment was carried out with three biological replicates prepared from independent pools.

2.10. Assay of oxidative stress markers

For evaluating oxidative damage, reactive oxygen species (ROS) generation, superoxide dismutase (SOD), catalase (CAT) and GST activities, protein carbonyl content and lipid peroxidation (LPO) product were assayed in third instar larvae of Oregon R^+. Except for ROS measurement for which single cell suspension was used, the rest of the above mentioned assays were carried out in 10% tissue homogenate.

2.10.1. Preparation of tissue homogenate

Larval midgut tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl as described previously [52]. After a centrifugation (10,000 × *g*) step, the supernatant was used for different assays and protein estimation.

2.10.2. Single cell preparation

Midgut tissues of 15 larvae from control and treated groups were incubated in collagenase (0.5 mg mL^{-1}) for 15 min at 24 ± 1 °C. The cells were passed through a nylon mesh (85 μm) and were washed with phosphate buffered saline (PBS) (three times) with gentle shaking to remove collagenase. Finally, the cells were processed for different end point measurements as described below.

2.10.3. Measurement of ROS

Intracellular ROS generation in midgut cells of control and treated larvae was measured by flow cytometry using a dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA; Sigma Chemicals, St. Louise, MO, USA) [53]. The data were analysed using cell quest software (Mac OS 8.6).

2.10.4. Superoxide dismutase (SOD) (superoxide:superoxide oxidoreductase EC 1.15.1.1)

SOD activity was assayed as described previously [54] with minor modifications [55]. Briefly, the reaction mixture had 10% tissue homogenate, sodium pyrophosphate buffer pH 8.3, distilled water, 186 μM phenazine methosulphate and 300.0 μM nitroblue tetrazolium. Reduced nicotinamide adenine dinucleotide and glacial acetic acid were added to start and terminate the reaction respectively. *n*-Butanol was added to the mixture to extract a coloured product. One unit of enzyme activity is defined as the enzyme concentration required for inhibiting chromogen production (optical density at 560 nm) by 50% in 1 min under assay condition and expressed as specific activity in units min^{-1} mg^{-1} larval protein.

2.10.5. Catalase (CAT) (H_2O_2:H_2O_2 oxidoreductase EC 1.11.1.6)

CAT activity was assayed by the method of Sinha [56]. Briefly, the assay mixture consisted of 10% tissue homogenate, 0.01 M phosphate buffer and distilled water. H_2O_2 (0.2 M) and dichromate/acetic acid reagent were added to start and terminate the reaction respectively. Absorbance of stable green colour of the chromic acetate formed was measured at 570 nm against a blank (distilled water). Enzyme activity was represented in terms of μmol H_2O_2 min^{-1} mg^{-1} larval protein.

2.10.6. Assay for lipid peroxidation (LPO)

Malonyl dialdehyde (MDA) content as a measure of LPO was assayed using tetraethoxypropane as an external standard [57] and lipid peroxide level was expressed in terms of nmol MDA formed h^{-1} mg^{-1} protein.

2.10.7. Determination of protein carbonyl (PC) content

We measured PC content in control and treated groups as described previously [58]. In brief, absorption of di-nitro phenyl hydrazine (DNPH) was determined at 370 nm using 2 M HCl as a

blank. The results were expressed as nanomol of DNPH incorporated mg^{-1} protein based on the molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

2.10.8. Protein estimation

Protein content in various samples was estimated by the method of Lowry et al. [59] using Folin reagent and BSA as the standard.

2.11. Preparation of microsomes

Microsomes from control and treated larvae were prepared as described previously [52]. In brief, 10% midgut tissue homogenate was centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant was centrifuged at 105,000 × *g* for 60 min to sediment microsomes followed by resuspension of pellets in microsomes dilution buffer [0.1 M potassium phosphate buffer, pH 7.25, 20% (v/v) glycerol, 0.25 mM PMSF, 0.01 M EDTA and 0.1 M DTT]. The microsomes were stored at −80 °C until further use.

2.11.1. Ethoxyresorufin-O-deethylase (EROD), 7-pentoxyresorufin-O-dealkylase (PROD) and methoxyresorufin-O-deethylase (MROD) activities

The activities of EROD, PROD and MROD in larval microsomes were determined following a previously described method [60], with minor modifications [52]. In brief, the reaction mixture had 0.1 M PBS pH 7.8, 1 mM methoxy, ethoxy or pentoxy resorufin and microsomal fractions. NADPH and methanol were added to start and terminate the reaction respectively. Levels of resorufin in the supernatant were measured at excitation wavelength of 550 nm and emission wavelength of 585 nm using a Perkin Elmer LS 55 luminescence spectrometer (California, USA). The catalytic activity of CYP1A1, CYP1A2 and CYP2B1was calculated in pmol resorufin min^{-1} mg^{-1} protein.

2.12. Glutathione S-transferase (GST, EC 2.5.1.18)

GST activity was assayed in control and treated groups as described previously [61] using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The formation of CDNB–GSH conjugate was measured at 340 nm for 3 min at every 30 s interval by monitoring the increase in absorbance and the enzyme activity was calculated as nmol CDNB reduced min^{-1} larval $protein^{-1}$ using molar extinction coefficient of $6.25 \times 10^3\ M^{-1}\ cm^{-1}$.

2.13. Evaluation of oxidative DNA damage by Comet assay

For this assay, single cells were prepared as already described above in Section 2.10.2. Viability of the cells was checked by trypan blue assay [62]. Base slide preparation was done according to the method of Tice et al. [63]. For alkaline Comet assay, slides were prepared in triplicate as described previously [64] and oxidized bases were detected essentially following the method of Collins et al. [65] with some modifications [66]. All the experiments were repeated three times. The cell suspension (80 μL) was mixed with 80 μL of 1.5% low melting point agarose (LMA; prepared in Ca^{2+} Mg^{2+} free PBS; final concentration 0.75%) (Sigma Chemicals, St. Louise, MO, USA). For each slide, 75 μL of the above mixture was immediately layered on a base slide. Coverslip was immediately placed over the second layer. The slide was then placed on a chilled plate for 10 min to allow complete polymerization of agarose. After removal of the coverslip, LMA (0.75%) was layered and covered with a coverslip and allowed to solidify on a chilled plate. Finally, the coverslip was removed and the slide was immersed for 2 h in freshly prepared chilled lysing solution (2.5 M NaCl, 100.0 mM EDTA, 10.0 mM Tris and 1.0% Triton X-100, pH 10). For enzyme treatment, the slide was removed from the lysis buffer and incubated with enzyme reaction buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, and 0.2 mg mL^{-1} BSA, adjusted to pH 8 with KOH) for 5 min. This was followed by the addition of 75 μL of either formamidopyrimidine DNA glycosylase (FPG) (catalyses excision of oxidized purines) (1:3000) or endonuclease III (Endo III) (catalyses excision on oxidized pyrimidines) (1:300) on the slide. The slide was covered with a coverslip and incubated at 37 °C for 30 min (FPG) or 45 min (Endo III). The enzyme control reaction was carried out with the enzyme reaction buffer only. After enzyme treatment, coverslip was removed and the slide was placed on a horizontal electrophoresis platform (Life Technologies, Gaithersburg, MD, USA), containing fresh, chilled electrophoresis buffer (1.0 mM Na_2EDTA and 300.0 mM NaOH, pH > 13) for 10 min for DNA unwinding. After unwinding, electrophoresis was conducted for 15 min at 0.7 V cm^{-1} (300 mA/25 V) at 4 °C using a power supply (Techno Source Pvt. Ltd., Mumbai, India). The slides were then washed three times with 0.4 M Tris buffer (pH 7.5) at 4 °C to neutralize excess alkali and stained with 20 μg mL^{-1} ethidium bromide in dark. After staining, the slides were dipped once in chilled distilled water for removing excess stain and subsequently, cover slips were placed on the slides. All the slides were examined on a Leica DMBL microscope with fluorescence attachment (Wetzlar, Germany). The images were transferred to a computer through a charge coupled device (CCD) camera and analysed using Komet 5.0 software (Kinetic Imaging, Liverpool, UK). One hundred and fifty cells from each group (50 cells/slide, 3 slides/experiment group, 3 experiments/group) were examined. Three different parameters are generally used as indicators of DNA damage, (i) tail DNA (%) (TD): shows the ratio of DNA present in tail to the total DNA content, measured by its pixels intensity and is expressed in percentage and (ii) tail length (μm) (TL): a measure of the distance from the nuclear core to the end of DNA migration and (iii) tail moment (TM) (arbitrary units): depicts percentage tail DNA multiplied with the distance between the

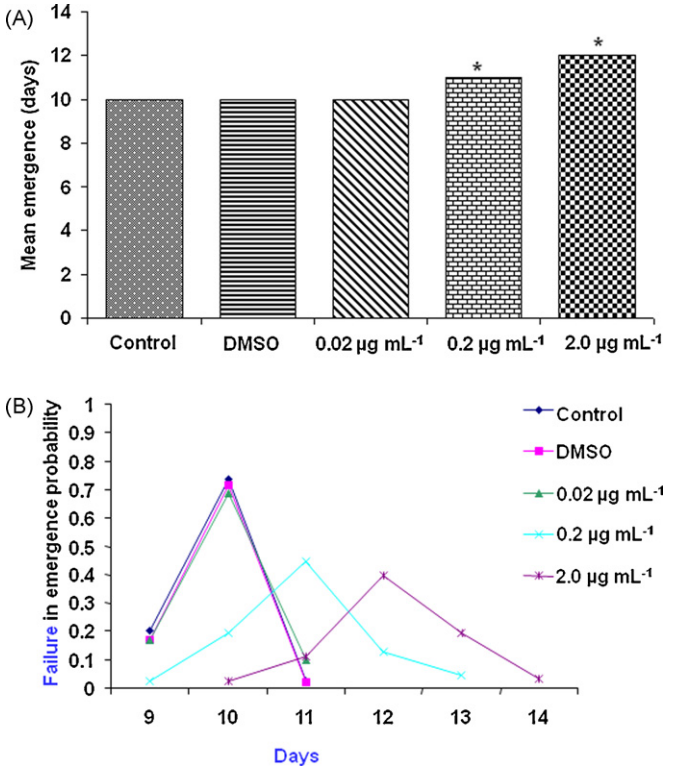

Fig. 1. Mean emergence of flies (days) of *D. melanogaster* (Oregon R^+) grown on control and food mixed with different concentrations of endosulfan (A) and failure in emergence probability (B); significance ascribed as $^*P < 0.05$.

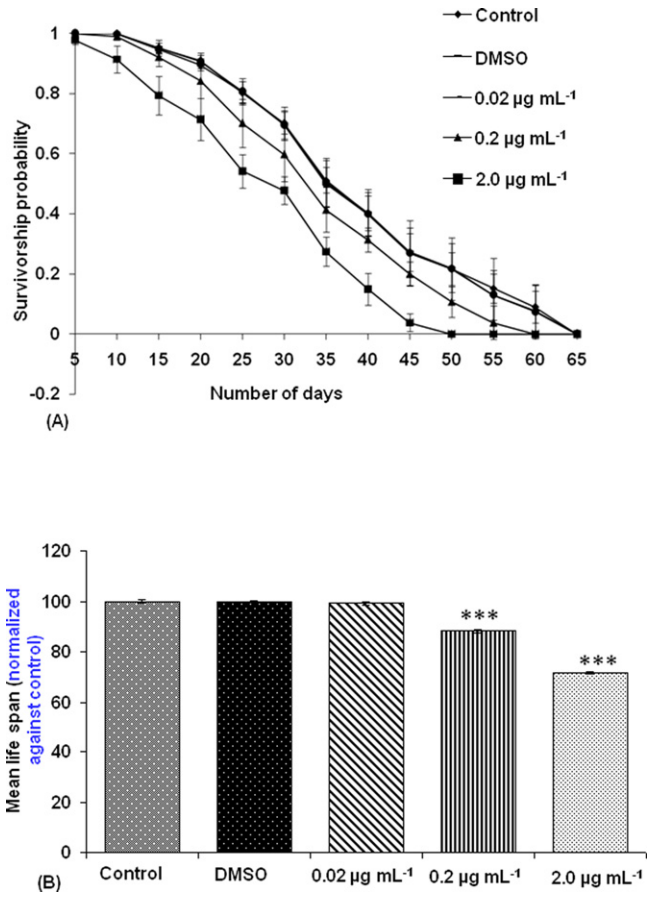

Fig. 2. Survivorship probability (A) and mean life span (B) in *D. melanogaster* (Oregon R^+) flies exposed to different concentrations of endosulfan; significance ascribed as $^{***}P < 0.001$.

centre of mass of the tail and the centre of mass of the head [67]. Since TM does not provide any additional information over TD and TL, we did not include TM in the results.

2.14. Statistical analysis

Statistical significance of the mean values of different parameters was monitored in control and endosulfan-treated groups using two-way ANOVA followed by Bonferroni's test for multiple comparisons after ascertaining the homogeneity of variance and normality of data. We considered each endpoint as dependent variable and concentration and exposure time as independent variables. $P < 0.05$ was considered statistically significant. Pearson's correlations were calculated and then linear regression analysis was carried out. Prism computer program (GraphPad version 4.0, San Diego, CA, USA) was used for statistical analysis. Kaplan–Meier analyses were used for survivorship analysis with stratified log rank tests using SPSS software version 13.0, (SPSS Inc. Chicago, USA).

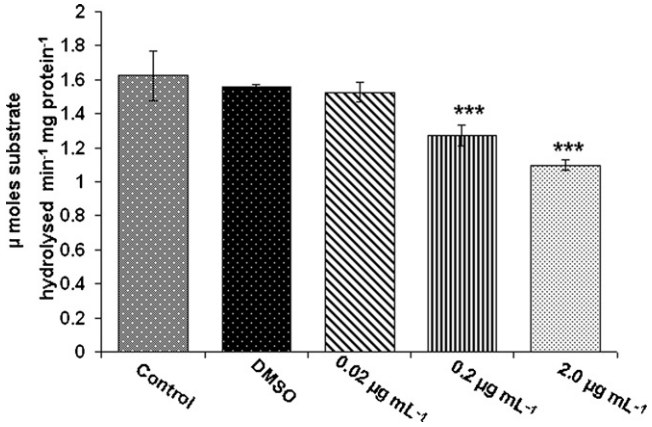

Fig. 3. Acetylcholinesterase activity in the fly brain of control and endosulfan-treated groups. Data represent mean ± SD ($n = 3$); significance ascribed as $^{***}P < 0.001$.

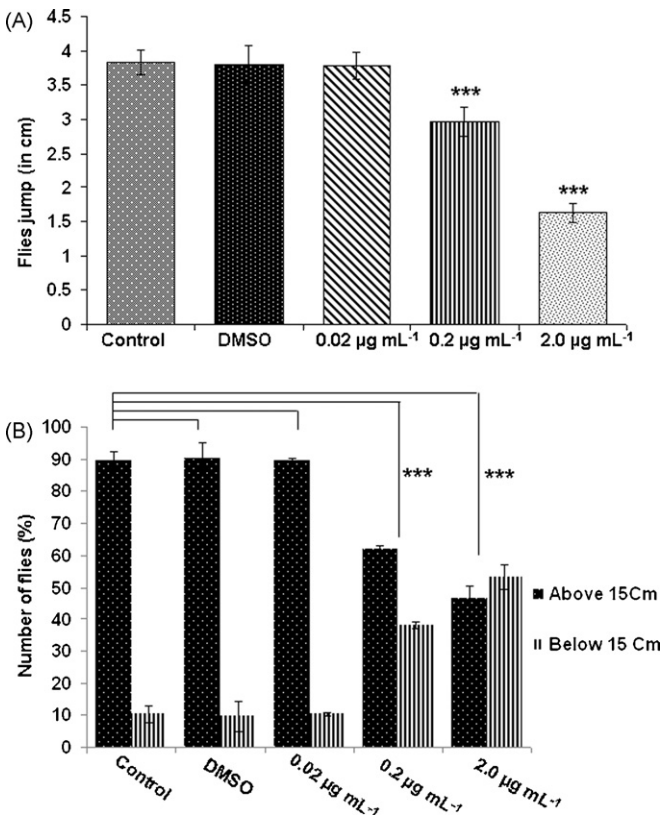

Fig. 4. Jumping (A) and climbing (B) activity of *D. melanogaster* (Oregon R^+) flies exposed to different concentrations of endosulfan; significance ascribed as $^{***}P < 0.001$.

3. Results

During the course of study, endosulfan-exposed larvae did not show any overt signs of toxicity, however, at the highest tested concentration, the larvae displayed sluggish movement. Since DMSO-treated organism did not elicit any significant alterations in any of the end points examined in comparison to control, only control was included for comparison.

3.1. Detection of endosulfan in Drosophila larvae

Analytical results established >96% homogeneity of endosulfan in food and showed the presence of its isomers [α-($1.19 \pm 0.6\ \mu g\ g^{-1}$) and β- ($0.75 \pm 0.31\ \mu g\ g^{-1}$) endosulfan] and metabolites [endosulfan-sulfate ($3.2 \pm 1.19\ \mu g\ g^{-1}$) and -ether ($0.43 \pm 0.03\ \mu g\ g^{-1}$)] respectively in the organism exposed for 48 h.

3.2. Endosulfan causes reduced and delayed emergence of Oregon R^+ flies

Fig. 1 shows the effect of endosulfan on development of the organism. Emergence pattern of the flies from control and $0.02\ \mu g\ mL^{-1}$ endosulfan-treated groups was not significantly

different. Conversely, emergence of the flies was significantly ($P < 0.05$) delayed at 0.2 μg mL^{-1} (one day delay) and 2.0 μg mL^{-1} (two day delay) concentrations (Fig. 1A). Concurrently, we also observed reduced emergence of flies at these two concentrations (on day 10, 76 and 96%-reduction in emergence for 0.2 and 2.0 μg mL^{-1} endosulfan respectively as compared to control) (Fig. 1B).

3.3. Endosulfan exposure compromises with the life span of the exposed organism

Flies, grown on 0.02 μg mL^{-1} of endosulfan, showed a non-significant change in their life span as compared to control. We observed a significant ($P < 0.001$) reduction in life span of the organism exposed to higher concentrations of endosulfan [13- and 29%-decline in life span of the organism after 0.2 and 2.0 μg mL^{-1} of endosulfan treatment respectively in comparison to control] (Fig. 2A and B).

3.4. Endosulfan inhibits AchE activity in the exposed organism

We observed a significant ($P < 0.001$) inhibition in AchE activity in the brain of flies emerging from 0.2 and 2.0 μg mL^{-1} endosulfan-contaminated food as compared to control (22 and 33% inhibition in 0.2 and 2.0 μg mL^{-1} endosulfan-treated groups respectively) (Fig. 3).

3.5. Endosulfan affects locomotor behaviour in the exposed organism

Concomitant with a non-significant change in the jumping activity of 0.02 μg mL^{-1} endosulfan-exposed flies, we observed a significantly ($P < 0.001$) decreased jumping behaviour of the flies in the higher two concentrations of endosulfan as compared to control (24- and 58%-reduction after 0.2 and 2.0 μg mL^{-1} endosulfan treatment respectively) (Fig. 4A). A similar trend in the climbing behaviour of endosulfan-treated organism was observed, wherein,

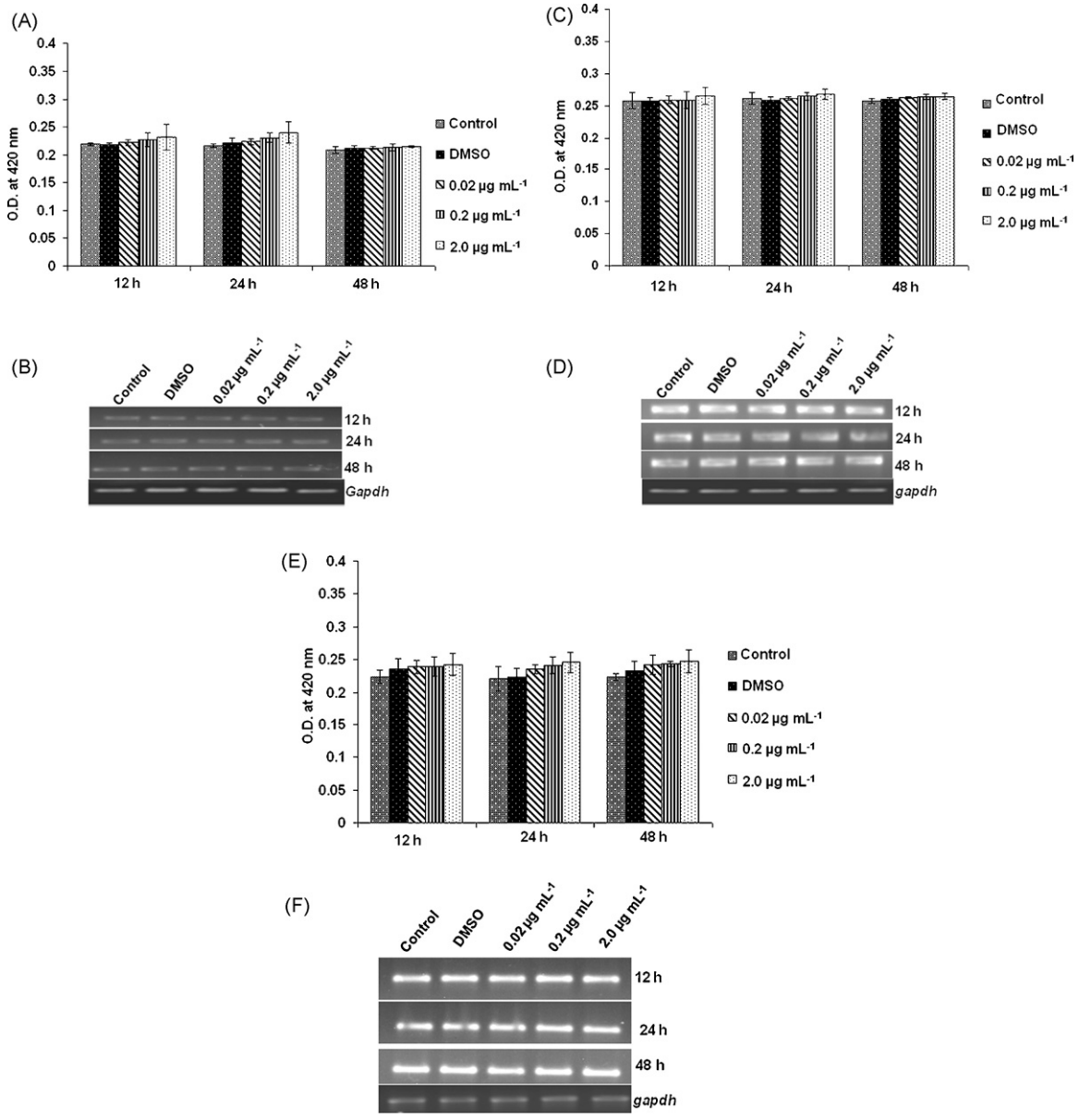

Fig. 5. Soluble ONPG assay showing β galactosidase activity in transgenic *D. melanogaster* (*hsp70-lacZ*) (A), (*hsp83-lacZ*) (C), (*hsp26-lacZ*) (E) and RT-PCR analysis of *hsp70* (B), *hsp83* (D) and *hsp26* (F) in *D. melanogaster* (Oregon R$^+$) in control and endosulfan-treated third instar larvae. Data represent mean ± SD ($n = 3$); significance ascribed as $^*P < 0.05$.

only 62 and 47% of the flies of 0.2 and 2.0 μg mL^{-1} endosulfan-treated groups crossed the 15 cm height within 30 s respectively (Fig. 4B).

3.6. Endosulfan evoked induction of hsp23 and hsp22 in the exposed organism

To examine endosulfan-induced expression of selected stress genes in the exposed Oregon R$^+$ larvae, we carried out RT-PCR assay for *hsp83*, *hsp70*, *hsp60*, *hsp26*, *hsp23* and *hsp22*. Simultaneously, we also assayed β-galactosidase activity for *hsp83*, *hsp70* and *hsp26* in transgenic strains (*83Z-880*, *Bg*^{9} and *351-94A*). We observed a non-significantly ($P > 0.05$) changed expression of *hsp83*, *hsp70*, *hsp60* and *hsp26* in larvae exposed to 0.02–2.0 μg mL^{-1} endosulfan for 12–48 h when compared to respective controls (Figs. 5A–F and 6A). Unlike the above, a concentration- and time-dependent increase in the expression of *hsp23* and *hsp22* was observed in the exposed organism (Fig. 6B and C) and after 48 h, higher expression of *hsp23* (5.2 fold) than *hsp22* (3.1 fold).

3.7. Elevated ROS generation in endosulfan exposed third instar D. melanogaster (Oregon R$^+$) larvae

Fig. 7 shows measurement of ROS generation in endosulfan-exposed larvae by flow cytometry. ROS generation in control and 0.02 μg mL^{-1} endosulfan-treated groups was comparable. In rest of the groups, a concentration- and time-dependent significant ($P < 0.001$) increase in ROS generation was observed with maximum ROS generation (5.5-fold) in 2.0 μg mL^{-1} endosulfan-treated larvae after 48 h.

3.8. Effect of endosulfan on anti-oxidant markers in exposed third instar D. melanogaster (Oregon R$^+$) larvae

Fig. 8A shows Cu–Zn SOD activity in control and endosulfan-exposed larvae. We observed a non-significant ($P > 0.05$) change in SOD activity in 0.02 μg mL^{-1} endosulfan-treated larvae and in rest of the treated groups, a concentration- and time-dependent increase in the enzyme activity with maximum activity (2.3 fold) at 2.0 μg mL^{-1} endosulfan concentration after 48 h. A similar trend was observed for CAT activity with maximum enzyme activity (2.8 fold at 2.0 μg mL^{-1} endosulfan after 48 h) (Fig. 8B). Induction of both MDA and PC contents in the exposed larvae showed a trend as observed for SOD and CAT activities with maximum MDA (1.8 fold) and PC (2.7 fold) content after 48 h (Fig. 8C and D).

3.9. EROD, PROD, MROD and GST activities in D. melanogaster (Oregon R$^+$) larvae exposed to endosulfan

Figs. 9 and 10 show the EROD, PROD, MROD and GST activities in control and endosulfan-exposed groups wherein, a concentration- and time-dependent increase in EROD, PROD and GST activities was observed. MROD activity was found to be significantly ($P < 0.001$) increased in 0.2 and 2.0 μg mL^{-1} endosulfan-treated organism after 24 and 48 h as compared to respective controls. Maximum activity of EROD, PROD, MROD and GST was 4.7-, 3.8-, 2.5- and 7.2-fold respectively in organism exposed to 2.0 μg mL^{-1} endosulfan for 48 h.

3.10. Increased oxidative DNA damage in endosulfan exposed D. melanogaster (Oregon R$^+$) larvae

Drosophila larvae exposed to endosulfan exhibited a varied increase in DNA migration in their gut cells as evident by a significant ($P < 0.05$–$P < 0.001$) increase in the measured Comet parameters [TD (%)] in presence of FPG/ENDOIII (Table 2). DNA

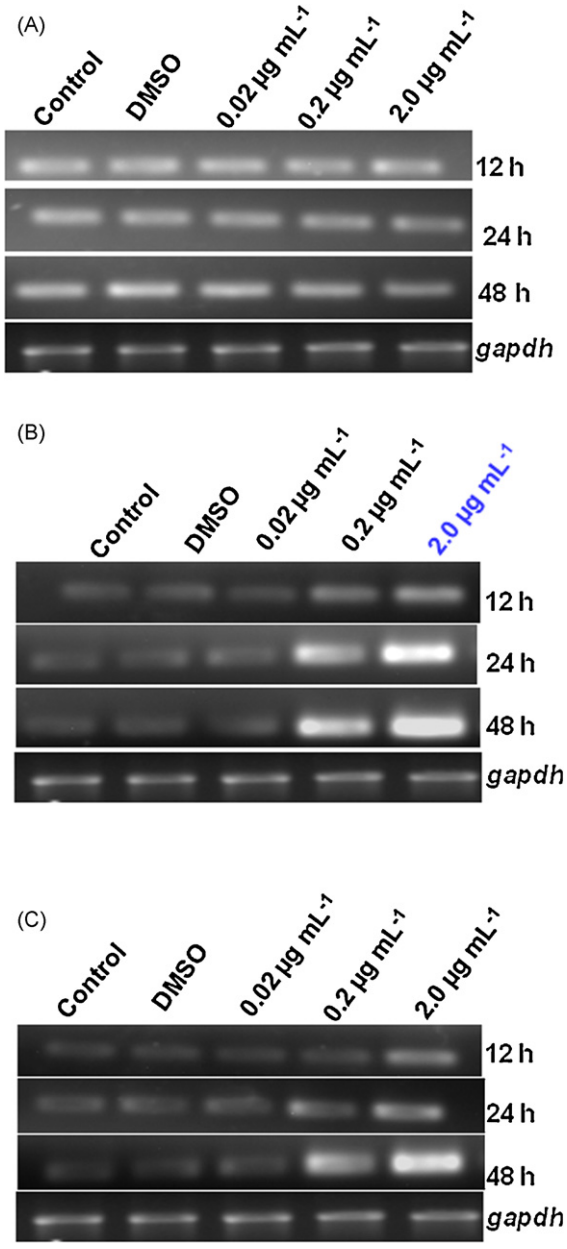

Fig. 6. RT-PCR analysis of *hsp60* (A), *hsp23* (B) and *hsp22* (C) expression in control and endosulfan-treated third instar larvae of *D. melanogaster* (Oregon R$^+$). Data represent mean ± SD ($n = 3$); significance ascribed as $^*P < 0.05$.

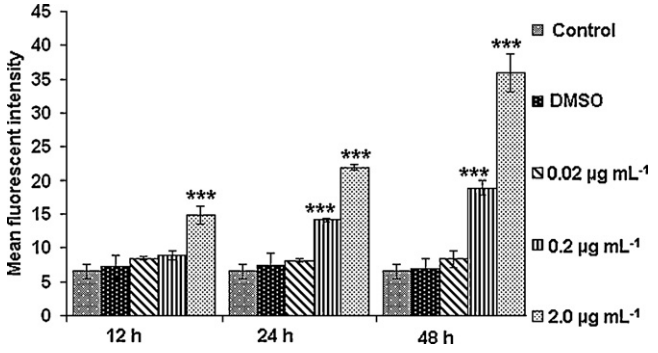

Fig. 7. Mean fluorescent intensity indicating ROS generation in control and endosulfan-exposed third instar larvae of *D. melanogaster* (Oregon R$^+$). Data represent mean ± SD ($n = 3$); significance ascribed as $^{***}P < 0.001$.

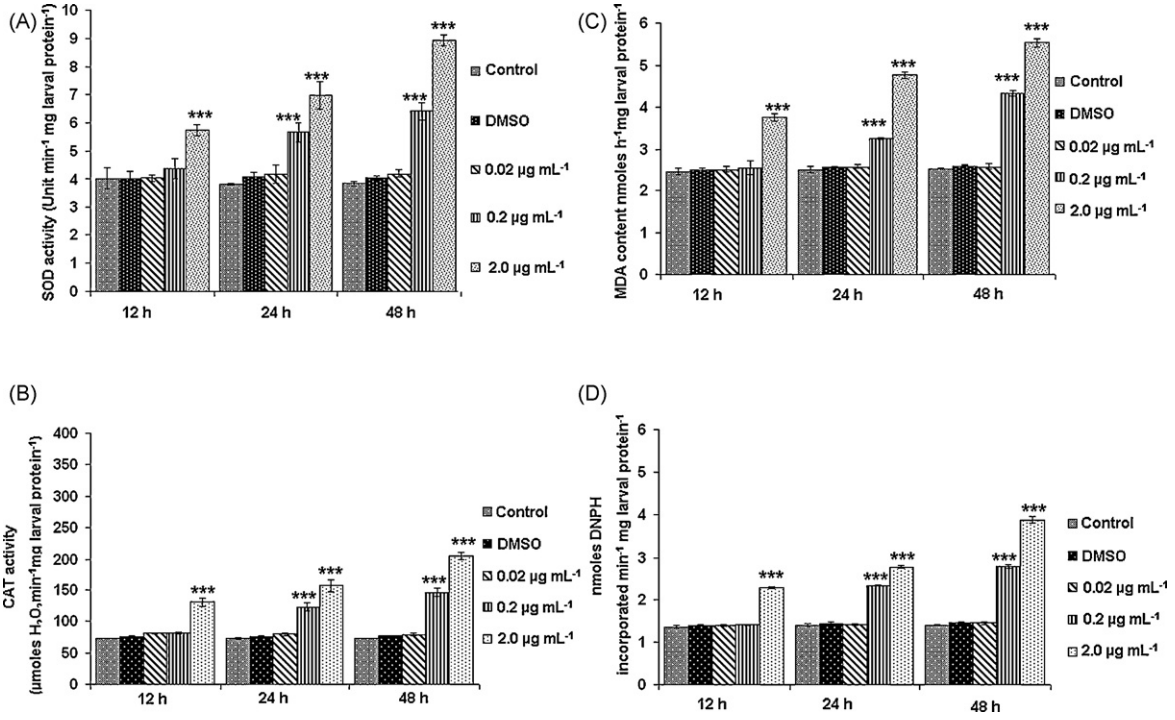

Fig. 8. Cu–Zn SOD (A) and CAT (B) activities and MDA (C) and PC content (D) in control and endosulfan-treated third instar larvae of *D. melanogaster* (Oregon R$^+$). Data represent mean ± SD ($n = 3$); significance ascribed as $^{***}P < 0.001$.

migration in the organism treated with 0.02 μg mL^{-1} endosulfan for 12–48 h and 0.2 μg mL^{-1} for 12 h was not significantly ($P > 0.05$) affected in the presence of FPG or ENDOIII as compared to that observed with buffer. Conversely, we observed a significant ($P < 0.05$–$P < 0.001$) change in Comet parameter in larvae exposed to 0.2 μg mL^{-1} and 2.0 μg mL^{-1} endosulfan for 12–48 h. At 2.0 μg mL^{-1} concentration, maximum change in Comet parameter [3.0 fold increase in TD (%)] was observed in the exposed larvae after FPG treatment, while ENDOIII treatment evoked only 1.6 fold increase in TD (%).

3.11. Correlation among different stress parameters

We drew a correlation among different endpoints studied in the exposed organism in the context of stress and oxidative stress along with other parameters relevant to endosulfan-induced toxicity (Tables 3 and 4). For example, we observed a significant positive correlation between *hsp23* and oxidative stress end points [vs. ROS generation ($r = 0.99$), vs. SOD ($r = 0.96$), vs. PC content ($r = 1.0$)] and between ROS generation and oxidative DNA damage ($r = 0.95$). Further, a strong positive correlation ($r = 1.0$) was drawn between AchE activity and locomotor behaviour at different concentrations of endosulfan.

4. Discussion

The present study demonstrates endosulfan-induced cellular and organismal toxicity in a non-target organism, *D. melanogaster*.

Presence of endosulfan in the organism has its relevance for the exposure-effect. We detected the isomers and metabolites of the pesticide in the exposed organism indicating that the pesticide is being metabolized in the organism.

The adverse effect of endosulfan on the development of the organism was evident by a delay in the emergence along with a significant decrease in the number of adult flies. This observation is in concurrence with the previous studies on the effect of different environmental chemicals or mixtures and industrial wastes, on different organisms wherein, delayed and reduced emergence of organisms was observed [44,51,68–70]. The present observation finds support from a previous study [26], in which the organism was fed endosulfan contaminated food (2.0 μg mL^{-1}). The adverse effects of the pesticide were in the form of deformity in hind leg (truncation and/or fusion of tarsal segments (tarsomeres) of the enclosed flies, indicating the teratogenic potential of the pesticide. One of the possibilities for such a developmental change may be due to environmental and genetic factors as reported earlier [43]. Concomitant with this observation, we also observed significantly higher negative impact of endosulfan on the life span of

Table 2
Effect of endosulfan on tail DNA (%) of DNA in exposed Drosophila larvae after incubation with or without the lesion-specific-endonucleases (FPG and ENDOIII).

Groups	Buffer			+FPG			+ENDOIII		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
Control	6.1 ± 0.5	6.3 ± 0.8	6.7 ± 0.4	6.8 ± 0.6	7.0 ± 0.7	6.9 ± 0.6	7.1 ± 0.9	6.8 ± 0.6	7.1 ± 0.6
DMSO	7.1 ± 0.4	7.4 ± 0.7	7.1 ± 0.5	7.0 ± 0.9	7.2 ± 0.7	7.1 ± 0.5	7.2 ± 0.5	7.1 ± 0.5	7.2 ± 0.4
0.02 μg mL^{-1}	7.1 ± 0.8	7.7 ± 0.9	7.9 ± 0.7	7.6 ± 0.7	7.6 ± 0.7	8.1 ± 1.3	7.7 ± 0.6	7.6 ± 0.6	7.3 ± 0.6
0.2 μg mL^{-1}	7.8 ± 1.2	8.7 ± 1.3	11.4 ± 1.5**	9.4 ± 1.6	13.2 ± 0.9$^{***\$\$\$}$	16.3 ± 0.4$^{***\$\$\$}$	9.6 ± 1.2	12.8 ± 1.3$^{**\$\$\$}$	15.3 ± 0.8$^{***\$\$\$}$
2 μg mL^{-1}	9.4 ± 1.1**	11.4 ± 1.2**	13.6 ± 1.6**	13.8 ± 1.7$^{**\$\$\$}$	16.6 ± 1.1$^{***\$\$\$}$	25.6 ± 1.4$^{***\$\$\$}$	12.3 ± 0.5$^{**\$\$}$	15.4 ± 1.1$^{***\$\$\$}$	18.6 ± 1.3$^{***\$\$\$}$

Data represent mean ± SD of three experiments (150 cells). Significance ascribed as $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control as well as $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ vs. buffer. FPG = formamidopyrimidine DNA-glycosylase; ENDOIII = endonuclease III.

Table 3
Correlation among different parameters measured in endosulfan exposed *Drosophila melanogaster* (Oregon R$^+$) larvae.

	Hsp22	Hsp23	SOD	CAT	LPO	PC	ROS	GST	EROD	FPG	ENDOIII
Hsp22	1.00										
Hsp23	0.96**	1.00									
SOD	1.00**	0.96**	1.00								
CAT	0.99**	0.98**	1.00**	1.00							
LPO	0.99**	0.90**	0.99**	0.97**	1.00						
PC	0.98**	1.00**	0.98**	0.99**	0.94**	1.00					
ROS	0.99**	0.99**	0.99**	1.00**	0.96**	1.00**	1.00				
GST	0.97**	1.00**	0.97**	0.99**	0.92**	1.00**	1.00**	1.00			
EROD	1.00**	0.97**	1.00**	1.00**	0.98**	0.98**	0.99**	0.98**	1.00		
FPG	1.00**	0.95**	1.00**	0.99**	0.99**	0.97**	0.98**	0.96**	1.00**	1.00	
ENDOIII	1.00**	0.95**	1.00**	0.99**	0.99**	0.97**	0.98**	0.96**	1.00**	1.00**	1.00

$n = 20$ (degree of freedom).
** Significance ascribed as $P < 0.001$ vs. concentration.

exposed Drosophila with an increasing concentration of the pesticide in food. The observation finds support from a previously conducted study on tadpoles wherein endosulfan and mancozeb greatly reduced their survival [71]. Earlier studies suggested that treatments that increase oxidative stress, reduce the life span of an organism possibly due to increased ROS generation [72,73]. However, we are unable to extrapolate uncritically the present findings to field conditions and this stands valid only to this organism under the present experimental laboratory conditions.

The behaviour of an organism reflects its normal physiological activity [74,75]. In this context, jumping and climbing activities

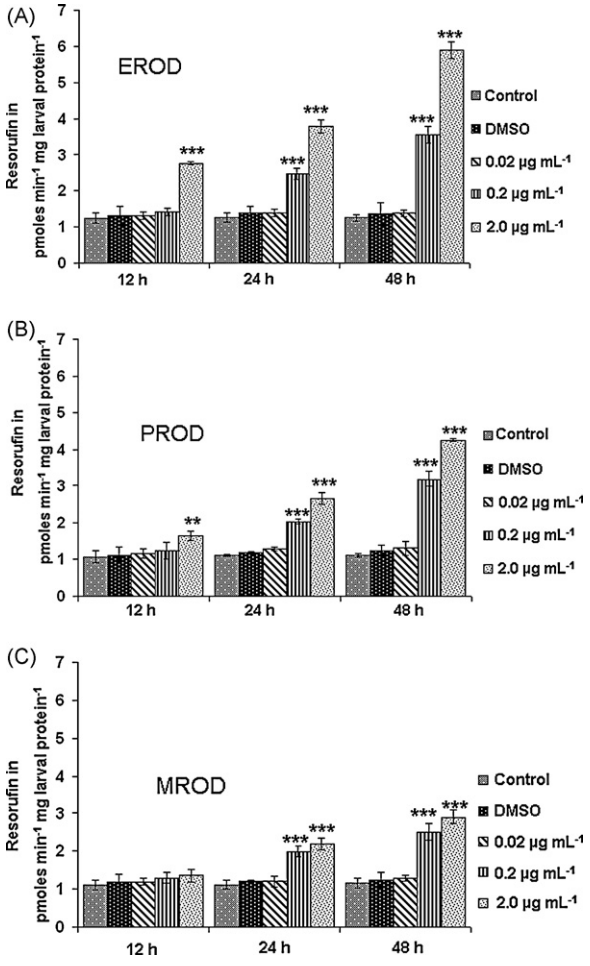

Fig. 9. EROD (A), PROD (B) and MROD (C) activities in microsomes isolated from endosulfan-treated third instar larvae of *D. melanogaster* (Oregon R$^+$). Data represent mean ± SD ($n = 3$); significance ascribed as $^{**}P < 0.01$, $^{***}P < 0.001$.

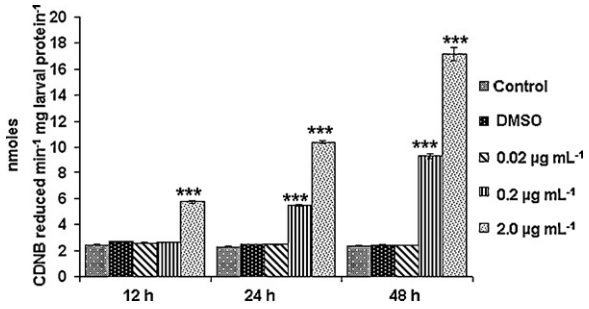

Fig. 10. GST activities in control and endosulfan-exposed third instar larvae of *D. melanogaster* (Oregon R$^+$). Data represent mean ± SD ($n = 3$); significance ascribed as $^{***}P < 0.001$.

reflect the organism's physiological status. Therefore, we argued that the extent of jumping and climbing activities in endosulfan-exposed Drosophila could be the effect of the chemical on the physiology of the organism. Significantly reduced jumping and climbing activities observed in the exposed organism followed by an inhibition of AchE activity supports the adverse effect of the pesticide on the organism. This was further strengthened by a strong positive correlation drawn between inhibition of AchE activity vs. locomotor activity (please see Table 4), thereby indicating interrupted coordination between nervous and muscular junctions following endosulfan exposure. Previously, inhibition of AchE activity was reported to be an indicator of poor locomotor activity [76].

Concurrent with the observed adverse organismal effects of endosulfan, a significant induction of small hsps (shsps) (*hsp22* and *hsp23*) and a non-significant induction of high molecular weight (hmw) hsps (*hsp83*, *hsp70* and *hsp60*) and also one of the shsps (*hsp26*) was observed in the exposed organism. Hsps are known to confer primary cellular defense through a number of specific adaptive stress response pathways that endeavour to mitigate damage and maintain or re-establish homeostasis against a variety of stressors [77]. Hsps are triggered through a mechanism of toxicity that involves generation of abnormal proteins and alteration of cellular function [78]. In the present study, induction of shsps could be due to the presence of the pesticide in cellular milieu. Mukhopadhyay et al. [79] proposed earlier that elevated levels of Hsp70 can

Table 4
Correlation between jumping and AchE activity in endosulfan exposed *D. melanogaster* (Oregon R$^+$).

	Jumping activity	AchE activity
Jumping activity	1	
AchE activity	0.96**	1

$n = 4$ (degree of freedom).
** Significance ascribed as $P < 0.001$ vs. concentration.

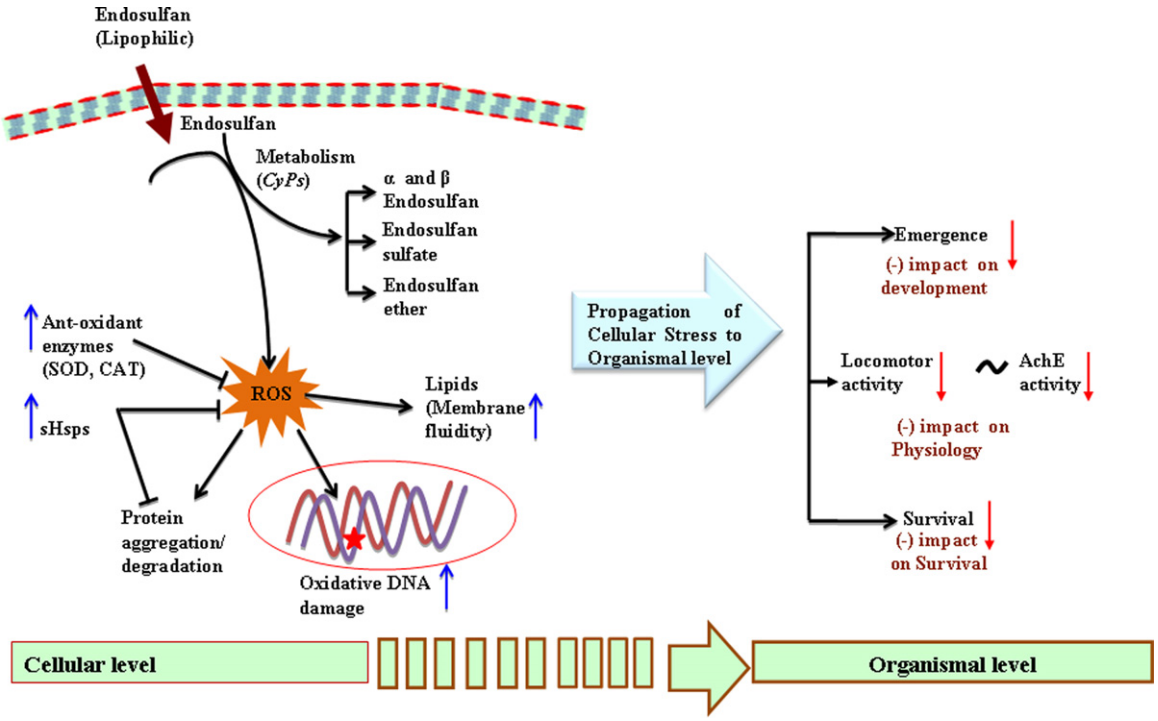

Fig. 11. Schematic representation of endosulfan-induced cellular and organismal response in *D. melanogaster*. Endosulfan modulated several cellular responses viz. upregulation of shsps and oxidative stress vis a vis ROS generation in the exposed organism and caused oxidatively damaged DNA in the exposed organism. Such cellular perturbation can be seen transmitted to organismal responses.

be used as the first tier bio-indicator of cellular toxicity induced by environmental chemicals. Contrary to the above, we observed non-induction of *hsp70* in endosulfan-exposed organism which finds support from a recent observation from this lab on another herbicide, diuron (Sharma and Kar Chowdhuri, 2011, unpublished data). To have a holistic picture of cellular toxicity caused by various pollutants due to differences in their potential to induce stress proteins, we advocated that multiple stress genes should be tested under various environmental conditions instead of one particular stress gene. [28]. sHsps (two members significantly up-regulated in this study) have been reported to perform a variety of functions in cells under stress, i.e., basic chaperoning activity [80], cytoskeleton protection [81] and modulation of the apoptotic process [82]. In *D. melanogaster*, induction of *hsp23* was reported in response to heat stress [77], and also in response to other stresses including heavy metal exposure, desiccation and anoxia in insects [83,84]. Similarly, upregulation of *hsp22* by exposure to a heavy metal [85], genotoxic agents [83], and teratogens in Drosophila embryonic cells [86] were also reported. In the present context, we believe that *hsp22* and *hsp23* in the absence of hmw–hsps, predominantly take up the role of protecting Drosophila against endosulfan-mediated cellular stress.

Elevated levels of sHsps have been shown to buffer with ROS generation for protecting cellular structures from stress [87,88]. Further, ROS have previously been reported to play an important role in the modulation of gene expression by activating transcription factors that, in turn, mediate induction of proteins involved in cellular response to environmental conditions [89,90]. Oxidative modification is also a consequence of ROS. A positive correlation among ROS, *hsp22* or *hsp23* expression and PC suggests that sHsps play a role in protecting the cell from the adverse effect of ROS. In agreement with the previous studies [76,91], we also observed increased SOD and CAT activities in the exposed organism which is due to increased ROS generation after endosulfan exposure. We drew a positive correlation between *hsp22* and *hsp23* expression and primary anti-oxidant defence enzyme activities (SOD and CAT). This tempted us to speculate that primary defence systems work together for cellular defence against the toxic effect of xenobiotics.

ROS generation can happen during metabolism of environmental chemicals. Significantly increased activities of EROD, PROD and MROD observed in the exposed organism indicate active metabolism of endosulfan in the organism. We observed increased activities of EROD and PROD (EROD > PROD) in the exposed organism and this is supported by an earlier observation made by Dehn et al. [32] in endosulfan-exposed HepG2 cells. Our observation of significantly increased GST activity in the exposed organism indicates phase II metabolism in conjugation with GSH. Earlier studies also showed endosulfan mediated GST activity in different model systems [35,92,93]. Since ROS are produced as a result of the metabolism of environmental chemicals through CYPs [94], a positive correlation drawn between ROS generation and EROD activity in this study (Table 3) suggests that increased endosulfan toxicity is probably mediated through the above mentioned mechanism.

Oxidative stress and DNA damage have been reported to be closely associated [95]. Increased ROS generation can induce oxidative damage to DNA, including strand breaks and base and nucleotide modifications [96]. Using modified version of alkaline Comet assay utilizing lesion-specific endonucleases (FPG and ENDOIII), we detected a significantly increased DNA damage in the exposed organism. FPG specifically recognizes the number of oxidized purine bases and other ring-opened purines [97], while ENDOIII recognizes oxidized pyrimidines [65,98]. Further, detection of more FPG sensitive sites than the ENDOIII sites in DNA of the exposed organism indicates that endosulfan oxidizes purines including 8-oxoGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde) as well as other ring-opened purines [99,100]. A significant positive correlation between ROS and DNA damage in the exposed organism supports that the pesticide induced ROS generation can play an important role in

damaging DNA in the exposed organism. Though *in vitro* and *in vivo* studies conducted earlier documented endosulfan mediated genotoxicity [6,101], to the best of our knowledge, this is the first report to demonstrate *in vivo* oxidatively damaged DNA induced by this pesticide using specific DNA repair enzymes.

5. Conclusion

The study suggests the negative impact of endosulfan on Drosophila, a non-target organism, at sub-organismal and organismal level with production of oxidatively damaged DNA as a consequence of the metabolism of the pesticide (Fig. 11). We recommend the use of *D. melanogaster* as an alternative animal model for *in vivo* assessment of toxicity induced by environmental chemicals which might have relevance to higher organisms.

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