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Chlorpyrifos induces apoptosis and DNA damage in Drosophila through generation of reactive oxygen species ☆

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ABSTRACT

The present study investigated the apoptosis and DNA damage inducing potential of chlorpyrifos (CP) in *Drosophila melanogaster*. Third instar larvae of Drosophila were treated with different concentrations of CP (0.015–15.0 μg/L) for 2–48 h. Reactive oxygen species (ROS) generation, oxidative stress markers, DNA damage and apoptotic cell death end points were measured in them. A significant increase in DNA damage was concomitant with apoptotic mode of cell death in 15.0 μg/L CP-treated organisms for 24 and 48 h. Depolarization in mitochondrial membrane potential and increased casapase-3 and caspase-9 activities in these organisms indicated both as potential targets of CP. A significant positive correlation was observed among ROS generation, apoptosis and DNA damage. The study suggests that (i) ROS may be involved in inducing apoptosis and DNA damage in the CP-exposed larvae of Drosophila and (ii) *D. melanogaster* may be used as an alternative in vivo animal model for xenobiotics hazard assessment.

1. Introduction

Aerobic organisms are exposed to ROS in a continuous and unavoidable manner. The ROS are generated as a part of the normal metabolism of a cell. Additionally, they can also be derived from exogenous sources following exposure of a cell/organism to environmental chemical insult including pesticides. Although ROS regulate various biological processes by regulating an array of signal transduction pathways when present in transient amounts, at high and/or sustained levels, they can cause severe damage to DNA, protein and lipids (Lau et al., 2008).

Various stressors present in the environment including pesticides are capable of reacting with DNA and causing DNA damage (Wilson et al., 2003). Given that stressors have the capability to generate ROS, one of the possible mechanisms for the induction of DNA damage may be through the generation of ROS. Excessive ROS production may lead to cellular dysfunction culminating in cell death. Apoptosis is one of the major forms of cell death, in which the cell designs and executes the program of its own demise. Recently, ROS produced during oxidative stress have been reported to initiate signaling cascades leading to apoptosis (Yuan et al., 2008). Although a mechanism for ROS-induced apoptosis has not been delineated, increasing amounts of research have indicated the involvement of mitochondria in this process (Ratha et al., 2007).

Chlorpyrifos (CP) is a broad spectrum organophosphorous pesticide that is widely used throughout the world in agriculture and non-agriculture applications. It has been reported to cause immunological abnormalities (Trasher et al., 2002) and to induce oxidative stress (Goel et al., 2005) and tissue damage (Jett and Navoa, 2000). The main toxicity of CP is neurotoxicity, which is caused by the inhibition of acetylcholinesterase (Yu et al., 2008). Nevertheless, the toxicity of CP may involve mechanisms other than the inhibition of cholinesterase. One such mechanism may be the inhibition of mitochondrial ATP production through the uncoupling of oxidative phosphorylation that could lead to the generation of ROS (Ishii et al., 2004).

Previous studies from this laboratory have shown that CP can induce toxicity in *D. melanogaster* (Gupta et al., 2007a; Nazir et al., 2001) wherein ROS have been considered to be a possible signaling molecule causing cellular toxicity. Therefore, the present study was conducted to examine the role of ROS in

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inducing DNA damage and apoptosis in the third instar larvae of *D. melanogaster* after CP exposure.

In recent years, one of the fundamental concerns for researchers has been toward reducing the number of higher laboratory animals for research and testing due to ethical issues with an emphasis on the use of alternative animal models. In the present study, Drosophila was used as a model for its well-defined genetics, molecular and developmental biology. During the last decade, the model has been used extensively for toxicological studies (Chowdhuri et al., 1999; Mukhopadhyay et al., 2003; Nazir et al., 2003; Siddique et al., 2005a), drug discovery and targeting pathway genes of human diseases (Chang et al., 2008; Ratnaparkhi et al., 2008). The model raises few ethical concerns and is exempted by the animal rights organizations (Benford et al., 2000).

2. Materials and methods

2.1. Fly strain and culture

The wild type fly and larvae of *D. melanogaster* (Oregon R^+) were reared at 22 ± 1 °C on a standard Drosophila diet containing agar-agar, maize powder, sugar, yeast, nepagin (methyl-p-hydroxy benzoate) and propionic acid. For healthy growth of the organism, additional yeast suspension was provided.

2.2. Treatment schedule

Technical grade CP (97.15%) obtained from DE-NOCIL Crop Protection Ltd., Mumbai, India was used during the study. Four different concentrations (0.015, 0.15, 1.5 and 15.0 μg/L) of the test compound corresponding to different fractions of the LC_{50} value of CP in third instar larvae of Drosophila for 48 h (75.0 μg/L) was used. The chemical was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 0.3% in food (Nazir et al., 2003) and fed to the larvae. Two sets of control were used: in set I control, larvae received standard Drosophila food, while in set II control, 0.3% DMSO mixed food was fed to the larvae. Larvae were allowed to feed on normal or test chemical contaminated food for different time intervals (2, 4, 6, 12, 24, and 48 h).

2.3. Analysis of purity of CP

The reference standard (Supelco, USA) and technical grade CP were separately weighed (10 mg each) and dissolved in n-hexane. Both were further diluted to a final concentration of 5 μg/mL and applied on GLC equipped with electron capture detector. Purity of the CP was estimated by comparing both the chromatograms.

2.4. Stability test of prepared stock solutions of test CP

In order to examine the stability, a stock of technical grade CP (2000 μg/mL) was prepared in DMSO and diluted to 15 μg/L. The stability of final diluted CP was carried out by analyzing in GLC equipped with electron capture detector at 0, 24 and 48 h against a standard reference solution of CP.

2.5. Estimation of CP in food mixed with 15 μg/L

The control and test sample (50 g each) in triplicate were extracted in acetonitrile and partitioned with 30 mL n-hexane three times. The concentrated extract (2–3 mL) was cleaned using Fluorisil column with eluting solvent (15% dichloromethane in n-hexane). The resultant cleaned extract (1–2 mL) was made up to 5 mL and analyzed on GLC. The recovery was found to be 89.5% with limit of detection (LOD, 3 μg/kg) and limit of quantification (LOQ, 10 μg/kg) (Soderberg, 2005).

2.6. Assay of oxidative stress markers

Oxidative stress inducing potential of CP was assayed by examining superoxide dismutase (SOD), catalase (CAT) activities, contents of malondialdehyde (MDA), protein carbonyl (PC), glutathione (GSH) and ROS generation in the exposed organisms. Enzymes were assayed using 10% homogenate of the midgut tissues of the larvae prepared in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl. ROS generation was quantified using single cell suspension prepared from midgut tissues of the larvae after incubation of the explanted tissues in collagenase (0.5 mg/mL) for 15 min at 24 ± 1 °C, after which cells were passed through a nylon mesh (60 μm). Final suspension of the cells was in 500 μL of 50 mM PBS (pH 7.4) (Gupta et al., 2007b).

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

Cytosolic Cu–Zn SOD was assayed following the modified version of a method (Gupta et al., 2005) described previously (Nishikimi et al., 1972). Briefly, the assay mixture consisted of sodium pyrophosphate buffer, phenazine methosulfate, nitroblue tetrazolium, reduced nicotinamide adenine dinucleotide and the homogenate. The enzyme activity was measured following the absorbance of formazan formed during the reaction at 560 nm. One unit of enzyme activity is defined as the enzyme concentration required for inhibiting chromogen production (optical density 560 nm) by 50% in 1 min under assay condition and expressed as specific activity in units/min/mg protein.

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

CAT activity was measured by following the ability of the enzyme to split H_2O_2 within 1 min of incubation time. The reaction was stopped by adding dichromate/acetic acid reagent (5% solution of $K_2Cr_2O_7$:glacial acetic acid, 1:3 by volume), and the remaining H_2O_2 was determined by measuring chromic acetate at 570 nm, which is formed by reduction of dichromate/acetic acid in the presence of H_2O_2 (Sinha, 1972).

2.9. Glutathione (GSH) content

GSH was quantified using Ellman's reagent (Ellman, 1959). The assay mixture consisted of 0.2 M phosphate buffer (pH 8.0), 0.01% 5,5′-Dithiobis-2-nitro benzoic acid (DTNB) and the larval homogenate. The reaction was monitored at 412 nm, and the amount of GSH was expressed in terms of nmoles/mg protein.

2.10. Assay for lipid peroxidation (LPO)

MDA content as a measure of LPO was assayed using tetraethoxypropane as an external standard (Ohkawa et al., 1979). Briefly, the assay mixture consisted of 10% sodium dodecyl sulfate (SDS), 0.8% thiobarbituric acid (TBA) and tissue homogenate. Lipid peroxide level was expressed in terms of nmoles MDA formed/h/mg protein.

2.11. Determination of protein carbonyl (PC) content

We followed the method of Levine et al. (1990) with minor modifications for the determination of PC content. Two equal aliquots of supernatant fraction were taken, one treated with an equal volume of 2,4-Dinitrophenyl hydrazine (10 mM dissolved in 2 M HCl) (test sample) and the other with 2 M HCl (blank). Each mixture was incubated for 1 h, followed by precipitation with 20% TCA and subsequently extracted with ethanol:ethylacetate mixture (1:1). The pellets were then dissolved in 1.0 mL 6 M guanidine hydrochloride. The spectrum of the DNPH treated sample versus the HCl blank was determined at 370 nm, and results were expressed in terms of nmoles DNPH incorporated/mg protein based on molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.12. Protein estimation

The estimation of protein in all of the required fractions was carried out using BSA as a standard (Lowry et al., 1951).

2.13. Measurement of ROS generation

Intracellular ROS was analyzed using a fluorescent dye 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen) (Srivastava et al., 2006). Cells from control and treated organisms were suspended in Schneider's Drosophila medium containing 10 μM DCFH-DA and incubated for 1 h at 24 ± 1 °C in dark. The cells were then harvested in 0.1 M PBS (pH 7.4) and analyzed immediately by FACScan (Becton Dickinson, USA). A total of 10,000 events were counted per sample and values were expressed in terms of mean fluorescence intensity.

2.14. Dye exclusion assay

Tissue damage, if any, after exposure of the organisms to CP was assayed by trypan blue dye exclusion assay (Krebs and Feder, 1997). Explanted larval tissues were immersed in trypan blue (0.02% in 50 mM PBS, pH 7.4) at 24 ± 1 °C and immediately scored for trypan blue positive tissues.

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2.15. Assays for detecting apoptosis

To investigate apoptotic potential of CP, single cells (described in assay for oxidative stress markers section) prepared from the explanted midgut tissues from both control and treated organisms were used. For caspase assay, 10% larval midgut homogenate (described in assay for oxidative stress markers) was used.

2.16. Assay for Rpr, Hid and Grim expression

Cells were fixed in PBS containing 0.25% paraformaldehyde for 1 h at 4 °C, permeabilized in PBST (0.1% Triton X-100, 0.5% BSA, 1 M PBS; pH 7.4) and incubated in primary antibody against Rpr, Hid and Grim (anti-goat polyclonal IgG, 1:50 in 1 M PBS, pH 7.4 containing 2% BSA; Santacrutz), respectively, for 1 h at 4 °C. The cells were then incubated in FITC conjugated secondary antibody (anti-goat IgG, 1:100 in 1 M PBS, pH 7.4 containing 2% BSA; Sigma) for 1 h at 4 °C. A total of 10,000 events were analyzed immediately for each sample by FACScan (Becton Dickinson, USA). The results were expressed in terms of percent of positive cells expressing the particular protein (Siddique et al., 2007).

2.17. Determination of mitochondrial membrane potential ($\Delta\Psi$)

Mitochondrial membrane potential was determined using a fluorescent probe; JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide) (Invitrogen) that detects mitochondrial depolarization by an increase in green fluorescence (FL1). Approximately 5×10^5 cells were incubated in Schneider's Drosophila medium containing 10 μM JC-1 for 30 min and $\Delta\Psi_m$ was determined in 10,000 cells by FACScan. The data were expressed as the percent of depolarized mitochondria.

2.18. Phosphatidylserine (PS) externalization assay

Cells were stained with Annexin V-FITC and propidium iodide (PI) following essentially the manufacturer's protocol (Oncogene). We counted 10,000 events by FACScan (Becton Dickinson, USA) for analysis. The FITC signal was detected by FL1 (FITC detector) at 518 nm and Pl was detected by FL2 (phycoerythrin fluorescence detector) at 620 nm.

2.19. Assay for cysteine-aspartate specific proteases [caspase-3 (DRICE) and caspase-9 (DRONC)] activities

The assay was performed following the manufacturer's protocol (Bio Vision). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptides DEVD-pNA and IETD-pNA, respectively. The assay mixture consisted of larval midgut homogenate, chilled cell lysis buffer, 2× reaction buffer containing 10 mM dithiothreitol (DTT) and 200 μM substrate. The reaction mixture was incubated at 37 °C for 1.5 h and absorbance of the colored product was measured at 405 nm on a Cintra 20 ultraviolet spectrophotometer (GBC Scientific Equipment, Australia).

2.20. Analysis of DNA damage by Comet assay

Single cell gel electrophoresis or Comet assay is one of the simple, sensitive and rapid methods for the detection and quantification of DNA damage (Singh et al., 1988). We essentially followed the method described by Mukhopadhyay et al. (2004). Slides were prepared in duplicate per group. The cell suspension (80 μL) was mixed with equal volume of 1.5% low melting point agarose (final concentration 0.75%). Seventy-five microliters of the mixture was quickly layered on an end frosted slide that was pre-coated with 1.0% normal melting point agarose. Slides were then immersed in freshly prepared, chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% Triton X-100, pH 10.0) for 2 h. After lysis, the slides were transferred to a horizontal electrophoresis platform (Life Technologies, Gaithersburg, MD) containing fresh, chilled electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13) for 10 min for DNA unwinding. After DNA unwinding process, electrophoresis was carried out in the same buffer for 15 min at 0.7 V/C m (300 mA/25 V) at 4 °C. The slides were then washed three times with neutralizing buffer (0.4 M Tris–HCl, pH 7.4) at 4 °C to neutralize excess alkali and stained with 20 μg/mL ethidium bromide in the dark. After staining, the slides were dipped in chilled distilled water to remove excess stain and subsequently the coverslips were placed over the slides. The slides were examined under a fluorescent microscope (Leica DMBL, Wetzlar, Germany). The images were transferred to a computer through a CCD camera and analyzed using Komet 5.0 software (Kinetic Imaging, Liverpool, United Kingdom). A total of 150 cells from each group (25 cells/slide from 2 slides/experimental group, 3 experiments/group) were examined. Three different parameters were used as indicators of DNA damage: tail moment (TM) (arbitrary units), tail DNA (%), and tail length (μm). These parameters have been described previously in detail (Olive et al., 1992).

2.21. Statistical methods

Different parameters were monitored in normal and exposed organisms. The analysis of variance was carried out to find the significant differences in means considering each end point as dependent variable and concentration (0.015, 0.15, 1.5 and 15.0 μg/L) and duration of exposure (2, 4, 6, 12, 24 and 48 h) as independent variables. Prior to the analysis, the normality assumption of the data and homogeneity of variance among the different groups were tested and data were found to hold the above two assumptions. To make a comparison between the two groups post-hoc analysis was carried out considering linear contrasts. Pearson's correlations were calculated and then linear regression analysis was carried out. The level of statistical significance was set as $p<0.05$. Systat 9.0 software (Binary Semantics Ltd., New Jersey, USA; www.binarysemantics.com) was used for analysis of variance.

3. Results

During the course of the study, no overt sign of toxicity was observed in test chemical treated organisms. Since DMSO itself was unable to induce any significant change in the end points examined as compared to control, only control was included for the comparison. The data presented are for a single significant concentration of CP (15.0 μg/L) to show time-course. Additionally, a single significant time point was also chosen to show the dose-response curve of significant values at that time.

3.1. Purity and actual concentration of CP

The purity of test CP was found to be 96.15% and stability of its prepared solution was found to be insignificantly different at different time points (0, 24 and 48 h). Actual concentration of CP in the prepared food was found to be 14.81 ± 0.47 μg/L which was insignificantly different ($p>0.5$) from its nominal concentration. Actual concentration of CP at <1.5 μg/L could not be analyzed due to higher LOQ of the method.

3.2. CP evoked an induction of Cu–Zn SOD and CAT activities and GSH content in the exposed D. melanogaster larvae

The enzyme (Cu–Zn SOD and CAT) activities and GSH content remained unaltered in the larvae fed 0.015–1.5 μg/L CP mixed food throughout the exposure time (2–48 h). At its highest concentration (15.0 μg/L), CP evoked a significant ($p<0.05$) 1.7- and 2.3-fold increase in SOD activity in the exposed organisms after 24 and 48 h, respectively (Fig. 1A). A respective 1.7- and 2.2-fold increase in CAT activity was observed in 15.0 μg/L CP-exposed organisms after 24 and 48 h (Fig. 1B). A prominent ($p<0.05$) reduction in the GSH content was observed at the highest concentration (15.0 μg/L) of CP (a respective 0.3- and 0.4-fold reduction after 24 and 48 h as compared to control) (Fig. 1C).

3.3. CP exposure induces lipid peroxidation and protein oxidation in the D. melanogaster larvae

An insignificant increase ($p>0.05$) in MDA and PC contents was observed in 0.015 and 0.15 μg/L CP-treated larvae after 48 h in comparison to control. At 1.5 μg/L CP, a significant ($p<0.05$) 1.8-fold increase in MDA content (Fig. 2A) and 1.6-fold increase in PC content (Fig. 2C) was observed in the exposed larvae after 48 h as compared to control. Larvae exposed to 15.0 μg/L CP exhibited a significant ($p<0.05$) 1.8- to 2.9-fold increase in MDA content after 12–48 h exposure, respectively, in comparison to control (Fig. 2B). At this concentration, a respective 1.6- to 2.6-fold

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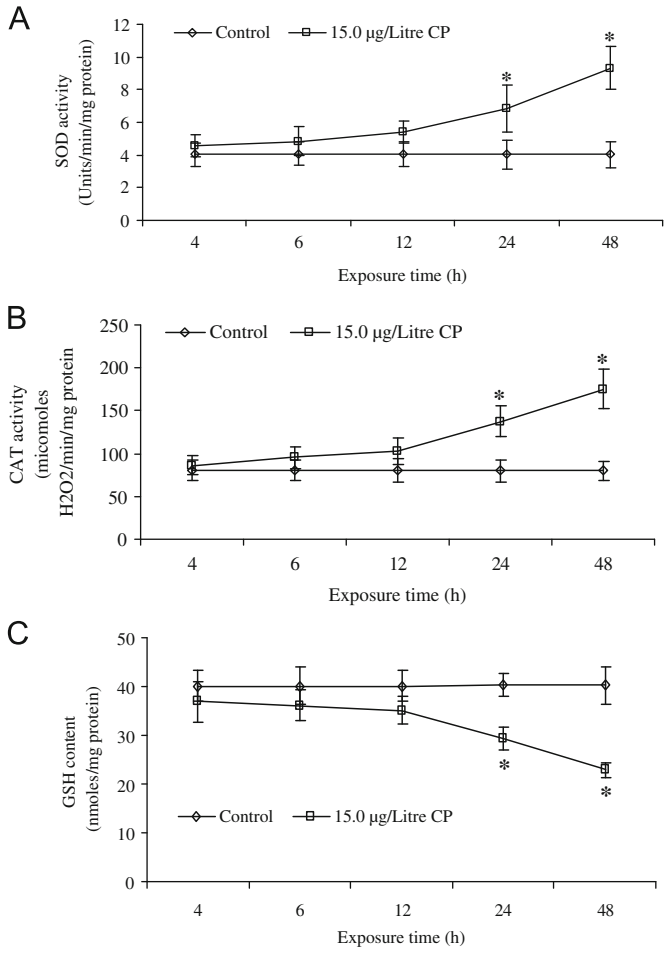

Fig. 1. Cu–Zn SOD (A), CAT (B) activities and GSH content (C) in normal and in 15.0 µg/L CP-treated organisms for 4–48 h. Data represent mean ± SD of five identical experiments made in five replicates. Significance is ascribed as $^{*}p < 0.05$. CP, chlorpyrifos.

increase in PC content was observed in the larvae exposed for 12–48 h (Fig. 2D).

3.4. Effect of CP on ROS generation in D. melanogaster larvae

CP at its 0.015–0.15 µg/L concentrations did not evoke any significant induction ($p > 0.05$) in ROS generation in the exposed organism after 48 h as compared to control. Organisms exposed to 1.5 µg/L CP for 24 and 48 h exhibited a significant ($p < 0.05$) 2.2- and 3-fold increase in ROS generation, respectively. At the highest concentration of CP (15.0 µg/L), a time-dependent significant increase ($p < 0.05$) in the ROS generation was observed in the exposed organisms in comparison to control (Fig. 3A–C(a), (b)).

3.5. Increased trypan blue staining in tissues of D. melanogaster larvae exposed to CP

An exposure of 15.0 µg/L CP to the larvae for 48 h resulted in a blue staining in their tissues (86% of exposed larvae showed a moderate blue staining in midgut, salivary gland, gastric caeca and brain ganglia) (Fig. 4A, B). Larvae exposed to 0.015–1.5 µg/L CP for 48 h exhibited little or no staining in their tissues (figure not shown).

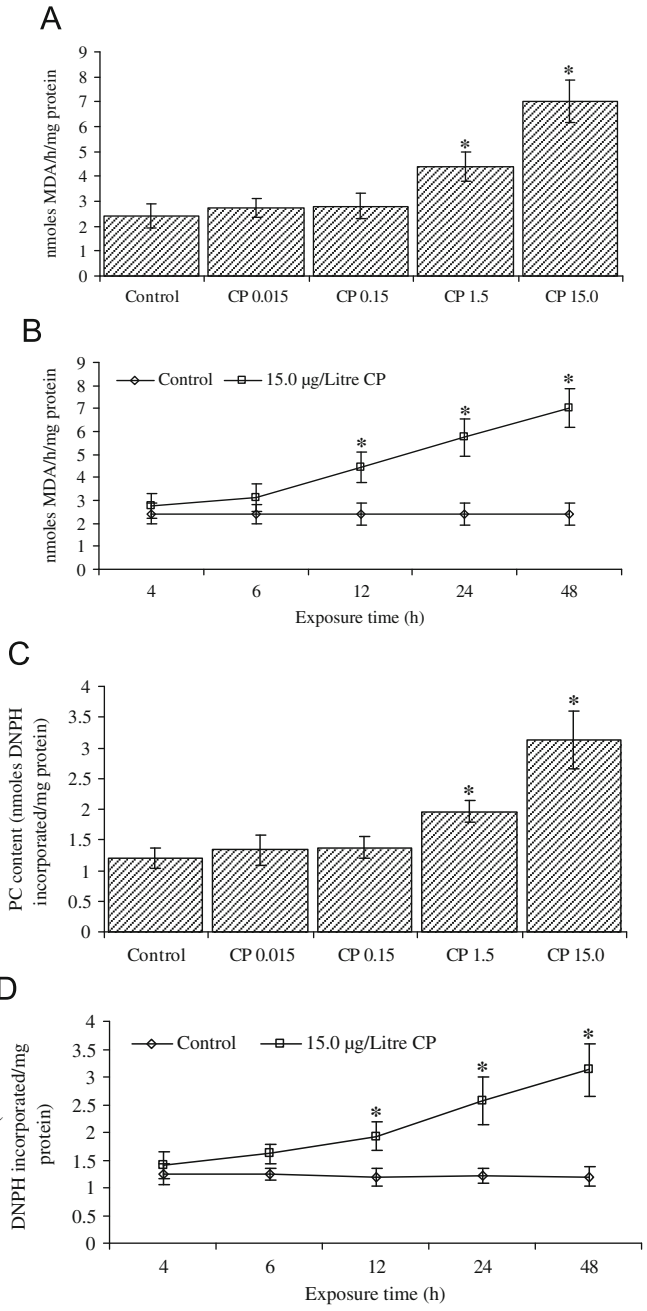

Fig. 2. MDA (A, B) and PC (C, D) contents in normal and in 0.015–15.0 µg/L CP-treated third instar larvae of Drosophila after 48 h exposure (A, C) and in normal and 15.0 µg/L CP-treated organisms for 4–48 h (B, D). Data represent mean ± SD of five identical experiments made in five replicates. Significance is ascribed as $^{*}p < 0.05$. CP, chlorpyrifos.

3.6. Induction of apoptosis in CP-exposed Drosophila larvae

D. melanogaster larvae exposed to 15.0 µg/L CP showing trypan blue staining prompted us to examine apoptotic potential of the test chemical. Since CP at its 0.015–1.5 µg/L concentrations did not evoke significant changes in apoptosis in the exposed larvae, data for 15.0 µg/L CP-exposed organisms were shown.

3.7. Increased expression of rpr, hid and grim indicate apoptosis in CP-exposed Drosophila larvae

A significant increase in Rpr and Grim expression was observed in the larvae exposed to 15.0 µg/L CP for 24 and 48 h,

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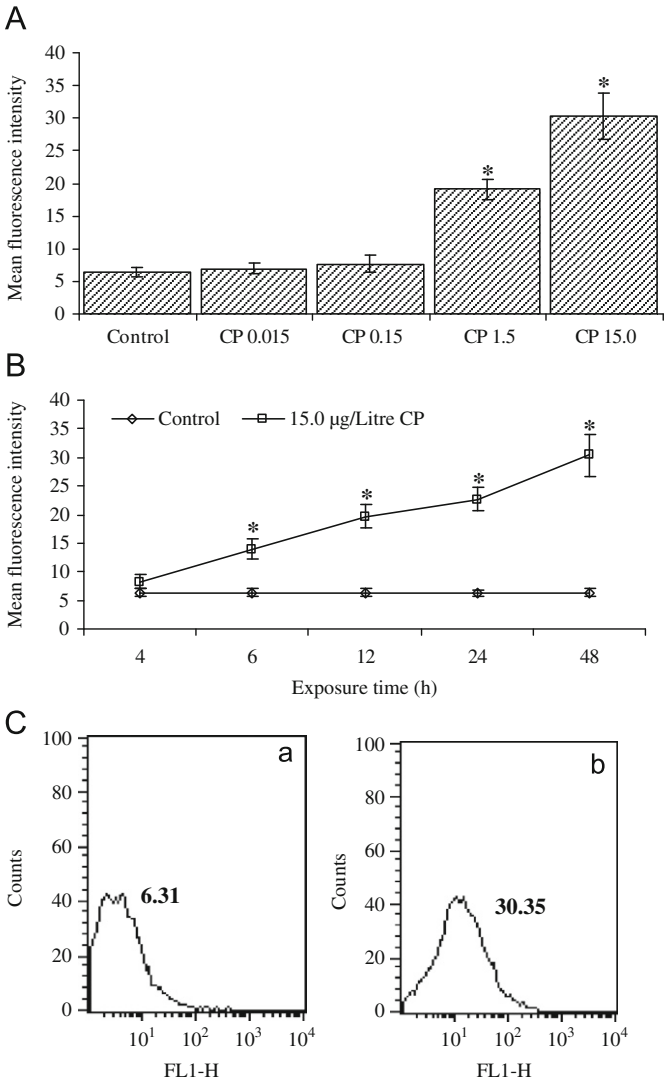

Fig. 3. ROS generation in normal and in 0.015–15.0 µg/L CP-treated third instar larvae of Drosophila after 48 h exposure (A) and in normal and 15.0 µg/L CP-treated organisms for 4–48 h (B). Data represent mean ± SD of five identical experiments made in five replicates and significance is ascribed as $^{*}p < 0.05$. CP, chlorpyrifos. (C) Depicts the flow cytometric representation in control (a) and in 15.0 µg/L CP-exposed organisms after 48 h (b), respectively. Values across the peak in (C) (a, b) represent mean fluorescence intensity.

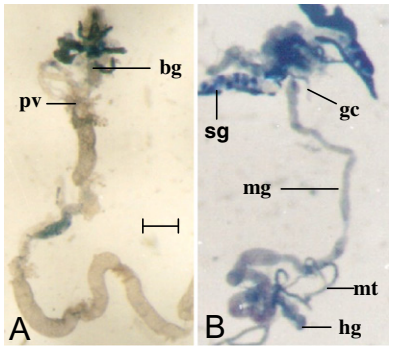

Fig. 4. Trypan blue staining in the tissues of third instar larvae of Drosophila in control (A) and in 15.0 µg/L chlorpyrifos treated organisms after 48 h (B). bg=Brain ganglia, sg=salivary gland, pv=proventriculus, gc=gastric caeca, mg=midgut, hg=hind gut, mt=Malpighian tubules. Bar represents 100 µm.

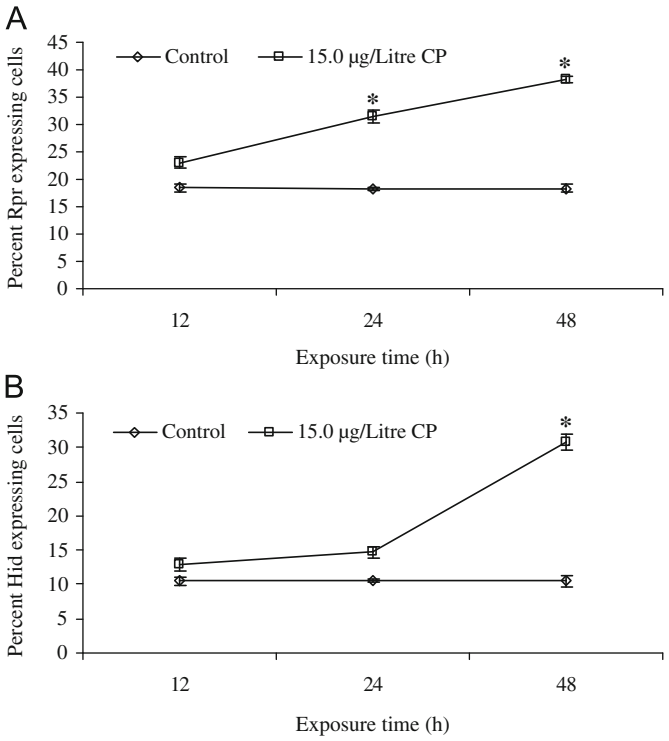

Fig. 5. Expression of Rpr (A) and Hid (B) in normal and in 15.0 µg/L CP-exposed organisms for 12–48 h. Data represent mean ± SD of three identical experiments made in triplicates and significance is ascribed as $^{*}p < 0.05$. CP, chlorpyrifos.

respectively (Fig. 5A; data for grim expression not shown). Hid expression was significantly increased in CP-treated larvae only after 48 h (Fig. 5B).

3.8. Increased depolarization of mitochondrial membrane potential in CP-exposed Drosophila larvae

CP exposure to *D. melanogaster* larvae for 48 h at 15.0 µg/L evoked a significant dissipation in $\Delta\Psi$ (5.7-fold) in comparison to control as evidenced by a shift from red fluorescence to a higher intensity of green fluorescence (Fig. 6(A), (B)).

3.9. Effect of CP on caspase-3 and caspase-9 activities

Initiator and executioner caspases are key components of apoptotic machinery that are activated during apoptosis. Fig. 7A, B shows the relative intensities of the cleaved chromophore (*p*-nitroanilide) obtained after specific cleavage by caspases (caspase-3 and caspase-9) in larvae exposed to 15.0 µg/L CP for 12–48 h. A significant ($p < 0.05$) 1.9-fold increase in the caspase-3 activity was observed in the 15.0 µg/L CP-treated organisms after 48 h as compared to control (Fig. 7A). A similar trend was observed in caspase-9 activity of the treated third instar larvae (Fig. 7B).

3.10. Enhanced externalization of phosphatidylserine (PS) in Drosophila larvae after CP exposure

Under defined salt and calcium concentrations, annexin V is predisposed to bind PS that is externalized onto the cell surface in the very early stages of apoptosis. Fig. 8 shows the percent distribution of annexin V positive early apoptotic cells in the midgut tissues of normal and treated larvae. A significant increase

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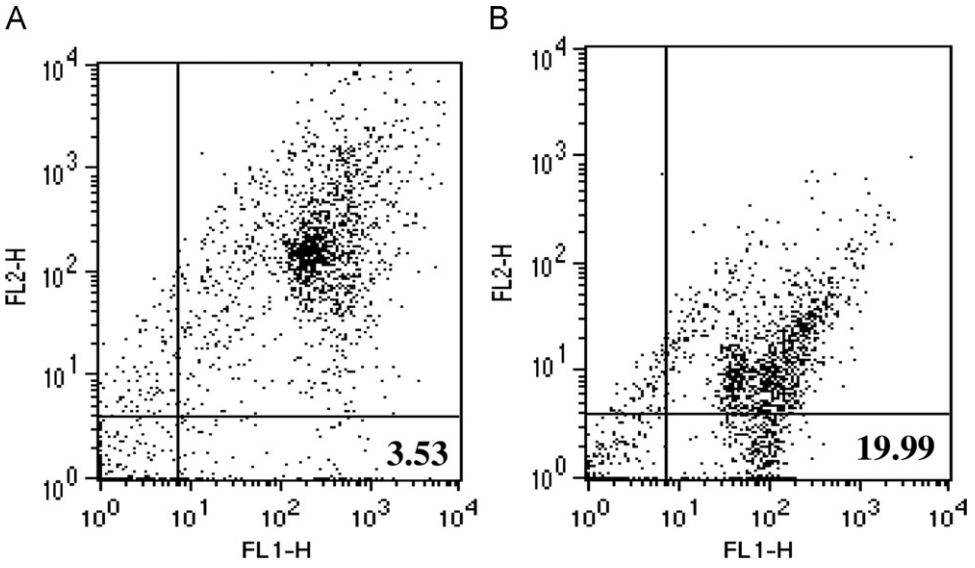

Fig. 6. Flow cytometric representation showing changes in MMP in midgut tissues of control (A) and in 15.0 μg/L CP-treated organisms after 48 h exposure (B), respectively. CP, chlorpyrifos.

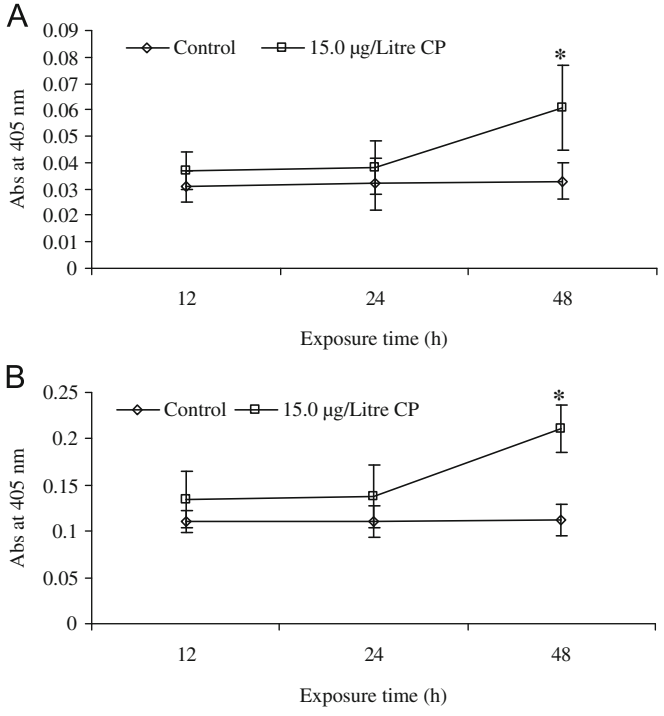

Fig. 7. Caspase-3 (A) and caspase-9 (B) activities in normal and in 15.0 μg/L CP-exposed organisms for 12–48 h. Data represent mean ± SD of five identical experiments made in five replicates and significance is ascribed as $^{*}p < 0.05$. CP, chlorpyrifos.

(6.2-fold) in annexin V positive cells was observed in the 15.0 μg/L CP-treated larvae after 48 h in comparison to control.

3.11. CP exposure resulted in DNA damage in Drosophila larvae

To examine whether CP has an ability to induce DNA damage, three different Comet parameters viz., tail moment (TM), tail DNA (%) and tail length (μm) were analyzed. Larvae exposed to 15.0 μg/L CP for 24 and 48 h exhibited significant increase in Comet parameters in comparison to control (Table 1). Larvae exposed to 0.015–1.5 μg/L CP exhibited an insignificant ($p > 0.05$) increase in Comet parameters as compared to control.

3.12. Effect of ROS generation on apoptosis and DNA damage in CP-treated third instar larvae of Drosophila

To examine the effect of ROS generation on apoptosis and extent of DNA damage in CP-treated larvae, a comparison was made among ROS generation, apoptotic end points and tail moment (Comet parameter) in the organisms exposed to 15.0 μg/L CP for 2–48 h. ROS generation was found to precede the changes in apoptotic end points and extent of DNA damage (Table 2A). A significant positive correlation ($p < 0.05$) was observed between ROS generation and (i) caspase activities ($r = 0.65$ and 0.63 for caspase-3 and caspase-9 activities, respectively), (ii) $\Delta\Psi$ ($r = 0.82$), (iii) PS externalization ($r = 0.80$), (iv) tail moment ($r = 0.86$), (v) MDA content ($r = 0.92$), and (vi) PC content ($r = 0.87$). A significant negative correlation was observed between ROS generation and GSH content ($r = -0.76$) (Table 2B).

4. Discussion

In the present study, exposure of third instar larvae of *D. melanogaster* to CP was found to induce oxidative stress, apoptosis and DNA damage in the exposed organisms.

Chlorpyrifos has been in extensive use for various purposes and therefore, environmental presence and subsequently effect on biota is of concern. In a field study, 0.12 μg/L CP was detected in Royal lake, Washington (Gruber and Munn, 1998). A compilation of >60 studies in a review by Schulz (2004) showed a maximum of 3.8 μg/L CP in surface water and 1.3–720 μg/kg in sediments. Curwin et al. (2007) reported the presence of CP (0.24–1.96 μg/kg) in farm and non-farm children of Iowa. Therefore, in this study, we have used a range of concentrations of CP that have environmental relevance and also fall within the fraction of LC_{50} value of CP in the third instar larvae of Drosophila after 48 h. While a number of studies reported the organismal effect of CP in aquatic and soil invertebrates (Moore et al., 1998; Lydy et al., 1999; Jantunen et al., 2008), the study of suborganismal effect of this insecticide to model laboratory organism can throw insight of its damaging effect to the non-target organisms.

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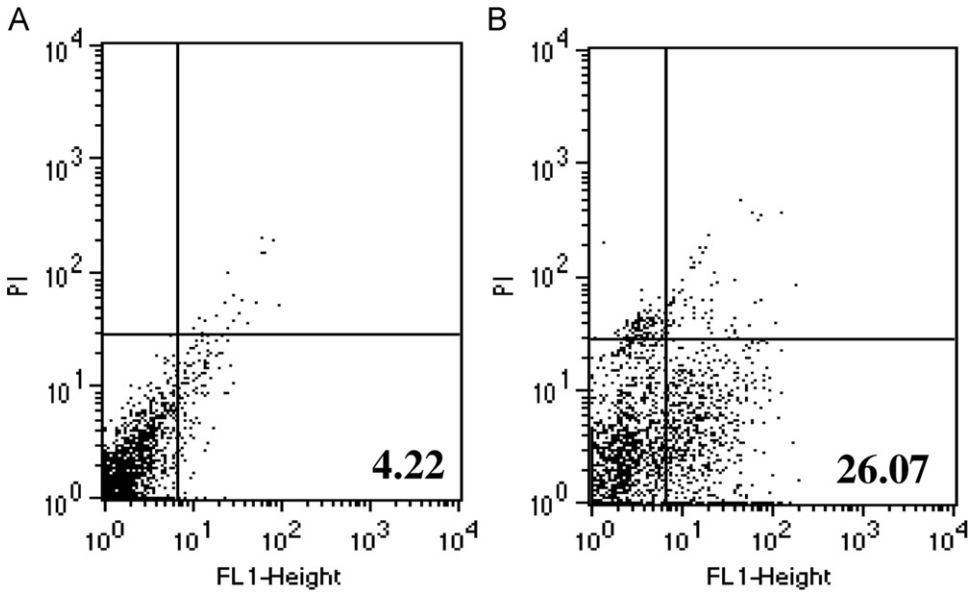

Fig. 8. Flow cytometric representation showing changes in annexin V positive cells in midgut tissues of control (A) and in 15.0 μg/L CP-treated organisms after 48 h exposure (B), respectively. CP, chlorpyrifos.

Table 1
DNA damage in midgut cells of third instar larvae of *D. melanogaster* exposed to chlorpyrifos for 24 and 48 h.

Group	Tail length (μm)		Tail moment (arbitrary units)		Tail DNA (%)	
	24 h	48 h	24 h	48 h	24 h	48 h
Control	8.60 ± 0.93	9.54 ± 1.33	0.83 ± 0.06	0.91 ± 0.04	9.35 ± 2.37	9.09 ± 1.79
1.0 mM EMS	23.09 ± 0.65*	26.87 ± 2.33*	2.01 ± 0.15*	2.48 ± 0.78*	14.78 ± 0.87*	21.91 ± 2.32*
1.5 μg/L CP	9.63 ± 0.70	9.87 ± 1.00	1.06 ± 0.13	1.17 ± 0.49	9.65 ± 1.12	1.09 ± 0.39
15.0 μg/L CP	19.26 ± 1.88*	28.21 ± 2.99*	1.89 ± 0.51*	2.99 ± 0.15*	18.94 ± 1.72*	27.14 ± 2.12*

Values given are mean ± SE of three experiments per group ($n=3$). CP, chlorpyrifos; EMS, ethylmethanesulfonate; EMS was used as positive control.

Table 2A
Summary of oxidative stress markers, apoptotic markers and DNA damage in chlorpyrifos-exposed third instar larvae of *D. melanogaster*.

Group and time	Oxidative stress markers				Apoptotic markers		DNA damage
	ROS	SOD, CAT	GSH	LPO, PC	*rpr, grim*	*hid*, Casp-3, Casp-9, MMP and PS	
1.5 μg/L CP							
24 h	↑	–	–	–	–	–	–
48 h	↑	–	–	↑	–	–	–
15.0 μg/L CP							
6 h	↑	–	–	–	–	–	
12 h	↑	–	–	↑	–	–	
24 h	↑	↑	↓	↑	↑	–	↑
48 h	↑	↑	↓	↑	↑	↑	↑

↑ = Significant induction, ↓ = significant depletion and – = insignificant compared to control. CP = chlorpyrifos. Significance is ascribed as $p < 0.05$. Rest of the concentrations and time intervals are not presented due to an insignificant change compared to control.

The oxidative stress inducing potential was evident from the ability of CP to increase ROS generation, activities of anti-oxidant defense enzymes, contents of MDA (major aldehyde product of LPO) and protein carbonyl, and depletion in GSH content in the exposed organisms. Lipid peroxidation is a free radical driven reaction which causes tissue membrane damage by reaction of oxygen with polyunsaturated fatty acids (PUFAs) (Chattopadhyay et al., 2006). A significant positive correlation between ROS generation and the contents of MDA and PC indicates the involvement of ROS in inducing LPO and protein modification and possibly membrane damage in the exposed organisms. SOD and CAT are the important anti-oxidant defense enzymes that are meant to scavenge superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively. An increase in the activities of these enzymes in the exposed organisms may be an attempt by them to counterbalance the damage induced by these species. A significant negative correlation between ROS generation and GSH content in the exposed organism indicates the rapid requirement of later under conditions of oxidative stress. A previous study on thallium interacting with glutathione-dependent anti-oxidant

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Table 2B
Correlation among ROS generation, apoptotic markers, oxidative stress markers and DNA damage in the third instar larvae of *D. melanogaster* exposed to 15.0 μg/L CP for 2–48 h.

Regression equation	Correlation (r)
$Y_{casp\text{-}3}=0.001X_R+0.022$	0.65
$Y_{casp\text{-}9}=0.003X_R+0.091$	0.63
$Y_{\Delta\Psi}=0.526X_R-0.617$	0.82
$Y_{PS}=0.716X_R-2.391$	0.80
$Y_{TM}=0.084X_R-0.012$	0.86
$Y_{MDA}=0.193X_R+0.991$	0.92
$Y_{PC}=0.075X_R+0.703$	0.87
$Y_{GSH}=-0.539X_R+42.269$	−0.76

(+) Value of 'r' represents positive correlation while (−) value represents negative correlation. R=ROS, casp-3=caspase-3, casp-9=caspase-9, $\Delta\Psi$=mitochondrial membrane potential, PS=phosphatidylserine externalization, TM=tail moment, MDA=malondialdehyde, PC=protein carbonyl, GSH=glutathione.

defense system is in accordance with the present observation (Villaverde et al., 2004).

CP-induced DNA damage was observed in Drosophila larval midgut tissues, as evidenced by a significant increase in the Comet parameters, viz, tail length (μm), TM (arbitrary units) and tail DNA (%) in the exposed organisms. Earlier ROS was shown to cause chemical modifications and alterations in DNA and nucleoproteins, including modified bases and sugars and even strand breaks (Jaruga et al., 1994; Linn, 1998). In this context, a positive correlation was drawn between ROS generation and tail moment in the exposed organisms in the present study. Therefore, one of the possibilities for the induction of DNA damage in the exposed organisms may be due to the generation of ROS. While CP alone can generate ROS, alternatively, chlorpyrifos oxon (CPO) generated through a P-450 mediated desulfuration reaction may also act as another source of ROS that could lead to DNA damage in the exposed organism.

Along with DNA damage, we observed an apoptotic mode of cell death in the exposed organism. To our knowledge, this is the first report on CP-induced apoptosis in Drosophila larvae in vivo. However, a couple of in vitro studies showed an apoptotic mode of cell death in CP-exposed human monocyte cell line U937 (Nakadai et al., 2006) and in human placental choriocarcinoma cells (Saulsbury et al., 2008). To examine the mechanism of CP-induced apoptosis, we focused on mitochondria and found that apoptosis was associated with the loss of mitochondrial membrane potential. Thus, the mitochondrial membrane integrity and functions and caspases may be potential targets of CP action. ROS generated through oxidative stress caused by CP preceded the changes in the mitochondrial membrane potential. The possibility that ROS may affect mitochondrial membrane integrity and function cannot be ruled out in the present study.

Among the various possibilities for the causation of apoptosis, ROS arising during oxidative injury to cell has been implicated as signaling molecules for inducing apoptosis (Fleury et al., 2002; Loh et al., 2006). In this context, a significant positive correlation between ROS generation and apoptotic end points measured in this study supports a key role of ROS during apoptosis in Drosophila. GSH has been reported to act as free radical scavenger and also to replenish intracellular stores of endogenous antioxidants, or as thiol-reducing agents (Valko et al., 2007). Thus our observations suggest a possibility of CP-induced oxidation of thiol group of GSH, which may allow the accumulation of ROS, redox modulation, oxidative DNA damage and cell apoptosis. While our study was in progress, Saulsbury et al. (2008) reported CP-induced apoptotic cell death in human placental cells showing activation of p38 Mitogen Activated Protein Kinase (p38 MAPK) as integral to the protection of cells against CP-induced injury. Conversely, our study showed ROS as a key inducer in CP-induced apoptosis and DNA damage. Recently, Chen et al. (2009) showed p38 MAPK activation by ROS. Therefore, it is tempting to speculate that extensive ROS generation by CP may abrogate the protective role of p38 MAPK thereby causing DNA damage and cell death.

We used Drosophila as a surrogate animal model in this study. During the last decade, the use of model organisms, especially lower eukaryotes like *Caenorhabditis elegans* and Drosophila, generated much interest after the unraveling of the genome sequences of these organisms. This is more so since the majority of Drosophila genes have functional homolog in humans. In this context, a pertinent question is whether flies are equally as sensitive to toxicants as higher mammals. Previous studies have shown that flies and mammals have similar a dose-response relationship (Hirsch et al., 2003; Siddique et al., 2005b). Thus, laboratory based experimental evidence using Drosophila is useful in generating information that could be of value for their efficient extrapolation to higher mammals.

5. Conclusion

In conclusion, the present study provides evidence of CP-induced negative impact (viz. DNA damage and apoptosis) on Drosophila larvae which may be attributed due to generation of ROS. The study further demonstrates that mitochondria and caspases are potential targets of CP in the exposed organism. However, further studies to understand the mechanism involved in ROS-induced changes in the mitochondrial membrane potential and activation of caspases following CP exposure to Drosophila larvae is warranted.

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References

Benford, D.J., Hanley, A.B., Bottrill, K., Oehschlager, S., Ball, M., Branca, F., Categnaro, J.J., Descotes, J., Hemminiki, K., Lindsay, D., Schilter, B., 2000. Biomarkers as predictive tools in toxicity testing. ATLA 28, 119–131.

Chang, H.C., Dimlich, D.N., Yokokura, T., Mukherjee, A., Kankel, M.W., Sen, A., Sridhar, V., Fulga, T.A., Hart, A.C., Vactor, D.V., Artavanis-Tsakonas, S., 2008. Artavanis–Tsakonas, modeling spinal muscular atrophy in Drosophila. PLoS ONE 3, e3209.

Chattopadhyay, I., Bandhopadhyay, U., Biswas, K., Maity, P., Banerjee, R.K., 2006. Indomethacin inactivates gastric peroxidase to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen. Free Radic. Biol. Med. 40, 1397–1408.

Chen, K.C., Kao, P.H., Lin, S.R., Chang, L.S., 2009. Upregulation of Fas and FasL in Taiwan cobra phospholipase A2-treated human neuroblastoma SK–N–SH cells through ROS- and Ca^{2+}-mediated p38 MAPK activation. J. Cell Biochem. 106, 93–102.

Chowdhuri, D.K., Saxena, D.K., Vishwanathan, P.N., 1999. Effect of hexachlorocyclohexane (HCH), its isomers, and metabolites on Hsp70 expression in transgenic *Drosophila melanogaster*. Pestic. Biochem. Physiol. 63, 15–25.

Curwin, B.D., Hein, M.J., Sanderson, W.T., Striley, C., Heederik, D., Kromhout, H., Reynolds, S., Alavanja, M.C., 2007. Pesticide dose estimates for children of Iowa farmers and non-farmers. Environ. Res. 105, 307–315.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Fleury, C., Mignotte, B., Vayssiere, J.L., 2002. Mitochondrial reactive oxygen species in cell death signaling. Biochimie 84, 131–141.

Goel, A., Dani, V., Dhawan, D.K., 2005. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. Chem. Biol. Interact. 156, 131–140.

Gruber, S.J., Munn, M.D., 1998. Organophosphate and carbamates insecticides in agricultural waters and cholinesterase (ChE) inhibition in common carp (*Cyprinus carpio*). Arch. Environ. Contam. Toxicol. 35, 391–396.

Gupta, S.C., Siddique, H.R., Mathur, N., Mishra, R.K., Mitra, K., Saxena, D.K., Chowdhuri, D.K., 2007a. Adverse effect of organophosphate compounds dichlorvos and chlorpyrifos in the reproductive tissues of transgenic *Drosophila melanogaster*: 70 kDa heat shock protein as a marker of cellular damage. Toxicology 238, 1–14.

Gupta, S.C., Siddique, H.R., Mathur, N., Vishwakarma, A.L., Mishra, R.K., Saxena, D.K., Chowdhuri, D.K., 2007b. Induction of *hsp70*, alterations in oxidative stress markers and apoptosis against dichlorvos exposure in transgenic *Drosophila melanogaster*: modulation by reactive oxygen species. Biochim. Biophys. Acta 1770, 1382–1394.

Gupta, S.C., Siddique, H.R., Saxena, D.K., Chowdhuri, D.K., 2005. Hazardous effect of organophosphate compound, dichlorvos in transgenic *Drosophila melanogaster* (*hsp70-lacZ*): induction of *hsp70*, antioxidant enzymes and inhibition of acetylcholinesterase. Biochim. Biophys. Acta 1725, 81–92.

Hirsch, H.V., Mercer, J., Sambaziotis, H., Huber, M., Stark, D.T., Torno-Morley, T., Hollocher, K., Ghiradella, H., Ruden, D.M., 2003. Behavioral effects of chronic exposure to low level lead in *Drosophila melanogaster*. Neurotoxicology 24, 435–442.

Ishii, N., Senoo-Matsuda, N., Miyake, K., Yasuda, K., Ishii, T., Hartman, P.S., Furukawa, S., 2004. Coenzyme Q10 can prolong *C. elegans* lifespan by lowering oxidative stress. Mech. Ageing Dev. 125, 41–46.

Jantunen, A.P.K., Tuikka, A., Akkanen, J., Kukkonen, J.V.K., 2008. Bioaccumulation of atrazin and chlorpyrifos to *Lumbriculus variegates* from lake sediments. Ecotoxicol. Environ. Saf. 71, 860–868.

Jaruga, P., Zastawny, T.H., Skokowski, J., Dizdaroglu, M., Olinski, R., 1994. Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer. FEBS Lett. 341, 59–64.

Jett, D.A., Navoa, R.V., 2000. In vitro and in vivo effects of chlorpyrifos on glutathione peroxidase and catalase in developing rat brain. Neurotoxicology 21, 141–145.

Krebs, R.A., Feder, M.E., 1997. Tissue specific variation in *hsp70* expression and thermal damage in *Drosophila melanogaster* larvae. J. Exp. Biol. 200, 2007–2015.

Lau, A.T., Wang, Y., Chiu, J.F., 2008. Reactive oxygen species: current knowledge and applications in cancer research and therapeutic. J. Cell Biochem. 104, 657–667.

Levine, R.L., Garland, D., Oliver, C.N., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.

Linn, S., 1998. DNA damage by iron and hydrogen peroxide in vitro and in vivo. Drug Metab. Rev. 30, 313–326.

Loh, K.P., Huang, S.H., De Silva, R., Tan, B.K., Zhu, Y.Z., 2006. Oxidative stress: apoptosis in neuronal injury. Curr. Alzheimer Res. 3, 327–337.

Lowry, O.H., Rosenbrough, N.J., Farr, A.A., Randall, R.J., 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193, 265–275.

Lydy, M.J., Belden, J.B., Ternes, M.A., 1999. Effects of temperature on the toxicity of M-parathion, chlorpyrifos and pentachlorobenzene on *Chironomus tentans*. Arch. Environ. Contam. Toxicol. 37, 542–547.

Moore, M.T., Huggett, D.B., Gillespie, W.B., Rodgers Jr., J.H., Cooper, C.M., 1998. Comparative toxicity of chlordane, chlorpyrifos and aldicarb to four aquatic testing organisms. Arch. Environ. Contam. Toxicol. 34, 152–157.

Mukhopadhyay, I., Saxena, D.K., Chowdhuri, D.K., 2003. Hazardous effects of effluent from the chrome plating industry: 70 kDa heat shock protein expression as a marker of cellular damage in transgenic *Drosophila melanogaster* (*hsp70–lacZ*). Environ. Health Perspect. 111, 1926–1932.

Mukhopadhyay, I., Chowdhuri, D.K., Bajpayee, M., Dhawan, A., 2004. Evaluation of in vivo genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline Comet assay. Mutagenesis 19, 85–90.

Nakadai, A., Li, Q., Kawada, T., 2006. Chlorpyrifos induces apoptosis in human monocyte cell line U937. Toxicology 224, 202–209.

Nazir, A., Mukhopadhyay, I., Saxena, D.K., Chowdhuri, D.K., 2001. Chlorpyrifos induced *hsp70* expression and effects on reproductive performance in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9. Arch. Environ. Contam. Toxicol. 41, 443–449.

Nazir, A., Mukhopadhyay, I., Saxena, D.K., Chowdhuri, D.K., 2003. Evaluation of the No-observed adverse effect level of solvent dimethyl sulphoxide in *Drosophila melanogaster*. Toxicol. Mech. Methods 13, 147–152.

Nishikimi, M., Rao, N.A., Yagi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. Biochem. Biophys. Res. Commun. 46, 849–854.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.

Olive, P.L., Wlodek, D., Durand, R.E., Banath, J.P., 1992. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. Exp. Cell Res. 198, 259–267.

Ratha, J., Majumdar, K.N., Dhara, K., Singh, S.K., Saha, K.D., Bhadra, R., 2007. Attenuated Leishmanial sphingolipid induces apoptosis in A375 human melanoma cell via both caspase-dependent and -independent pathways. Mol. Cell Biochem. 304, 143–154.

Ratnaparkhi, A., Lawless, G.M., Schweizer, F.E., Golshani, P., Jackson, G.R., 2008. A Drosophila model of ALS: human ALS-associated mutation in VAP33A suggests a dominant negative mechanism. PLoS ONE 3, e2334.

Saulsbury, M.D., Heyliger, S.O., Wang, K., Round, D., 2008. Characterization of chlorpyrifos-induced apoptosis in placental cells. Toxicology 244, 98–110.

Schulz, R., 2004. Field studies on exposure, effects and risk mitigation of aquatic nonpoint source. Insecticide pollution: a review. J. Environ. Qual. 33, 419–448.

Siddique, H.R., Gupta, S.C., Dhawan, A., Murthy, R.C., Saxena, D.K., Chowdhuri, D.K., 2005a. Genotoxicity of industrial solid waste leachates in *Drosophila melanogaster*. Environ. Mol. Mutagen 46, 189–197.

Siddique, H.R., Chowdhuri, D.K., Saxena, D.K., Dhawan, A., 2005b. Validation of *Drosophila melanogaster* as an in vivo model for genotoxicity assessment using modified alkaline Comet assay. Mutagenesis 20, 285–290.

Siddique, H.R., Gupta, S.C., Mitra, K., Murthy, R.C., Saxena, D.K., Chowdhuri, D.K., 2007. Induction of biochemical stress markers and apoptosis in transgenic *Drosophila melanogaster* against complex chemical mixtures: role of reactive oxygen species. Chem. Biol. Interact. 169, 171–188.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Sinha, A.K., 1972. Colorimetric assay of catalase. Anal. Biochem. 47, 389–394.

Soderberg, D., 2005. Pesticide and industrial chemical residues. In: Horwitz, W., Latimer, G.W. (Eds.), Official Methods of Analysis of AOAC International 18th Ed. AOAC International, MD, USA (Chapter 10).

Srivastava, A., Tiwari, M., Sinha, R.A., Kumar, A., Balapure, A.K., Bajpai, V.K., Sharma, R., Mitra, K., Tandon, A., Godbole, M.M., 2006. Molecular iodine induces caspase-independent apoptosis in human breast carcinoma cells involving the mitochondria-mediated pathway. J. Biol. Chem. 281, 19762–19771.

Trasher, J.D., Heuser, G., Broughton, A., 2002. Immunological abnormalities in human chronically exposed to chlorpyrifos. Arch. Environ. Health 57, 181–187.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44–84.

Villaverde, M.S., Hanzel, C.E., Verstraeten, S.V., 2004. In vitro interactions of thallium with components of the glutathione-dependent antioxidant defense system. Free Radic. Res. 38, 977–984.

Wilson, D.M., Sofinowski, T.M., McNeill, D.R., 2003. Repair mechanisms for oxidative DNA damage. Front. Biosci: A Journal and Virtual Library 8, d963–d981.

Yu, F., Wang, Z., Ju, B., Wang, Y., Wang, J., Bai, D., 2008. Apoptotic effect of organophosphorous insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E. Exp. Toxicol. Pathol. 59, 415–423.

Yuan, B., Ohyama, K., Bessho, T., Uchide, N., Toyoda, H., 2008. Imbalance between ROS production and elimination results in apoptosis induction in primary smooth chorion trophoblast cells prepared from human fetal membrane tissues. Life Sci. 82, 623–630.

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