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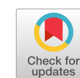
Lead modulated Heme synthesis inducing oxidative stress mediated Genotoxicity in *Drosophila melanogaster*



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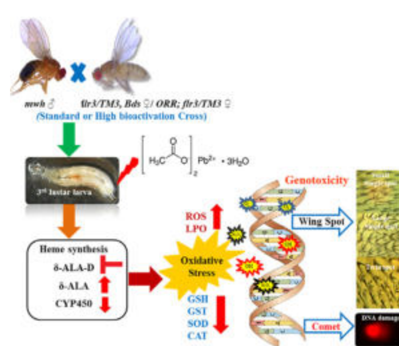
HIGHLIGHTS

- Lead (Pb) modulates the δ-ALA-D, δ-ALA and CYP450 levels in heme synthesis pathway.
- Accumulated δ-ALA induces ROS and LPO and depletes the antioxidants activities.
- Wing spot test confirms that Pb is mutagenic and weakly recombinogenic.
- Comet assay reveals genotoxicity of Pb.
- First report on Pb modulated heme synthesis pathway induced oxidative stress mediated genotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The mechanism of lead (Pb) modulated heme synthesis pathway induced oxidative stress mediated genotoxicity using standard (ST) and high bioactivation (HB) crosses of *Drosophila melanogaster* was addressed in the present study. Third instar larvae derived from the ST or HB crosses were reared in sub lethal concentrations of lead acetate (PbAc) treated food media and showed that Pb was readily taken up and accumulated in the said crosses. Pb modulated heme synthesis was evident by significant reductions of δ-aminolevulinic acid dehydratase (δ-ALA-D) and cytochrome P450 (CYP450) and increased accumulation of δ-aminolevulinic acid (δ-ALA). The results have also demonstrated that Pb induced oxidative stress by overproducing reactive oxygen species (ROS) and lipid peroxidation (LPO) and depletion of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-s-transferase (GST). Wing somatic mutation and recombination test (SMART) using ST and HB crosses revealed that Pb is mutagenic and weakly recombinogenic. By employing larval hemocytes, there was an increase in percent of tail DNA in alkaline comet compared to that of neutral comet revealing the DNA single strand breaks were the products of Pb modulated heme synthesis pathway induced oxidative free radicals. Based on these findings, it can be concluded that Pb modulated heme synthesis pathway induces oxidative stress that mediates the genotoxicity in *D. melanogaster*.

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1. Introduction

Lead (Pb) is one of the most useful heavy metals which causes global contamination of air, water and soil and also proved to be highly toxic (Cullen and McAlister, 2017). Pb causes a variety of deleterious effects

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in human systems such as the neurological, hematological, gastrointestinal, reproductive, immunological etc. (Mitra et al., 2017). Pb toxicity has been studied by employing heme synthesis pathway which is the best-known mechanism in organisms (Flora et al., 2012). Further it is proved that Pb modulation of heme synthesis by inactivating δ-aminolevulinic acid dehydratase (δ-ALA-D) enzyme, inturn reducing the production of hemeproteins - Cytochrome P450 (CYP450) of phase I of the xenobiotic metabolism (Carocci et al., 2016). Nevertheless, the Pb modulated enzymes and intermediates in the heme synthesis pathway also produce the reactive oxygen species (ROS) and induce the oxidative stress (Bechara, 1996). Carocci et al. (2016) suggested that increased free radical generation is one of the primary mechanisms of Pb-induced toxicity.

International Agency for Research on Cancer (IARC, 2006) listed Pb compounds as possible carcinogenic chemicals and weak mutagen. Epidemiological studies show that there is some evidence associating Pb exposure with increased risk of cancer, hematological and neurological disorders in humans (Ahamed et al., 2008; Ahamed et al., 2011; Alvarez-Ortega et al., 2017). Many studies have revealed positive and negative results of genotoxicity of Pb (Garcia-Leston et al., 2010). One of the possible mechanisms of Pb genotoxicity might be related to increased oxidative stress (Sanders et al., 2015). Studies revealed that oxidative DNA damage and oxidative stress in Pb-exposed workers (Singh et al., 2013; Dobrakowski et al., 2017) suggesting a potential genotoxic risk. However, Pb induced oxidative stress especially in heme synthesis and their subsequent deleterious effects, particularly induced genotoxicity are not well studied in any organisms including humans.

To understand this, *Drosophila melanogaster* is the best *in vivo* model organism (Hirsch et al., 2012). This is easy to maintain in laboratory conditions with short generation time, allowing a fast genotoxic evaluation and it has a metabolic activity analogous to that of the liver in mammals (Mishra et al., 2017). Further, in *Drosophila* and mammals, the heme synthesis pathway is highly conserved (Golombieski et al., 2008). Hemocytes in the hemolymph of *Drosophila* have the same role as lymphocytes in the blood of mammals (Irving et al., 2005). Furthermore, the availability of the *mwh*/*mwh*, *flare3* and *Oregon-flare3* transgenic fly strains are useful for studying the genotoxicity by the high bioactivation (HB) or standard (ST) crossing experiments (Vazquez-Gomez et al., 2010).

Thus, the aim of this study is to explore the Pb modulated heme synthesis enzymes and intermediates induced oxidative stress that mediates the genotoxicity in *D. melanogaster*. In the present study: (a) Pb modulated heme synthesis was analyzed by measuring the level of δ-ALA-D, δ-aminolevulinic acid (δ-ALA) and CYP450. (b) Pb modulated heme synthesis enzymes and intermediates induced oxidative stress was analyzed by measuring the oxidative stress markers: ROS and lipid peroxidation (LPO); and level of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-S-transferase (GST) antioxidants. In order to evaluate the involvement of oxidative stress in the genotoxicity of PbAc, wing somatic mutation and recombination test (SMART) and Comet assay were employed.

2. Materials and methods

2.1. Chemicals

Lead acetate (PbAc)-Pb $(CH_3COO)_2 \cdot 3H_2O$ (CAS-6080-56-4; 99.5% purity) and Nitric acid (HNO_3) (CAS-7697-37-2; 65% purity) was procured from Merck Millipore (Germany). Quercetin (CAS- 6151-25-3; 99% purity), Glutathione Reduced (GSH) (CAS-70-18-8; 99% purity), O-phthalaldehyde (OPT) (CAS- 643-79-8; 99% purity), Phenylthiourea (PTU) (CAS-103-85-5; 97% purity), Ethyl acetoacetate (CAS-141-97-9; 99% purity), Chloroform (CAS-67-66-3; 99% purity), Sodium chloride (NaCl) (CAS-7647-14-5; 99.5% purity), Potassium chloride (KCl) (CAS-7447-40-7; 99.5% purity), Sodium carbonate ($NaHCO_3$) (CAS-497-19-8; 99.9% purity), HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid)- (CAS-7365-45-9; 99.5% purity), Calcium chloride ($CaCl_2$) (CAS-10035-04-8; 99.5% purity), Glucose (CAS-50-99-77; 98% purity) were purchased from Sisco Research Laboratories, Mumbai, India. Ethyl methanesulphonate (EMS) (CAS-62-50-0; 100% purity), 5-5′-Dithiobis 2-nitrobenzoic acid (DTNB) (CAS-69-78-3; 98% purity), 1-chloro-2, 4-dinitrobenzene (CDNB) (CAS-97-00-7; 99% purity), Hydrogen peroxide (H_2O_2) (CAS-7722-84-1; 98% purity), 1,1,3,3-tetramethoxy propane (CAS-102-52-3; 99% purity), 5-aminolevulinic acid hydrochloride (CAS-5451-09-2; 97% purity) and 7- Ethoxyresorufin (CAS-5725-91-7; 98% purity) were purchased from Sigma Chemicals Co. St Louis, USA. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) (CAS - 4091-99-0; 98% purity) was purchased from Thermo Fisher Scientific, USA. N,N,N,N tetramethyl ethylene diamine (TEMED) (CAS-110-18-9; 99% purity), Sodium lauryl sulphate (SDS) (CAS-151-21-3; 85% purity), Ethylenediaminetetra acetic acid (EDTA) (CAS-6381-92-6; 99% purity), Thiobarbituric acid (TBA) (CAS-504-17-16; 98% purity), Trichloro acetic acid (TCA) (CAS-76-03-9; 98% purity), Copper sulphate ($CuSO_4$) (CAS-7758-99-8; 99.5% purity), Ehrlich reagent (CAS-100-10-7; 99% purity), Acetic acid (CAS-64-19-7; 99.5% purity), n-butanol (CAS-71-36-3; 99.8% purity), Ethidium bromide (CAS-1239-45-8; 95% purity), Triton X-100 (CAS-9002-93-1; 98% purity), Dimethyl formamide (CAS-68-12-2; 99% purity), normal melting point agarose (NMPA) and low melting point agarose (LMPA) (CAS-9012-36-6; 98% purity) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai.

2.2. Drosophila strains, culture and crossing experiments

The following *Drosophila melanogaster* strains and markers were used for the crosses: (i) *mwh*/*mwh* - the multiple wing hairs (*mwh*, 3–0.3) a recessive homozygous viable mutation, which is kept in homozygous condition. It produces multiple trichomes per cell instead of the normally unique trichome in the wing cells. (ii) *flr*^{3}/*In(3LR)TM3*, *ri p*^{p}. *sep l(3)89Aa bx*^{34e} *e Bd*^{s} (*flr*^{3}, 3–38.8) is a recessive mutation that affects the shape of wing hairs, producing malformed wing hairs that have a shape of flare. (iii) *ORR*/*ORR*, *flr*^{3}/*In(3LR)TM3*, *ri p*^{p}. *sep l(3)89Aa bx*^{34e} *e Bd*^{s} - Frolich and Wurgler (1989) constructed a strain (ORR) that has chromosomes 1 and 2 from a Dichloro-Diphenyl-Trichloroethane (DDT)-resistant Oregon line (*OR-R*), which constitutively overexpresses *Cyp* genes with increased constitutive levels of cytochrome P450 (CYP450) dependent xenobiotic metabolism which facilitates the detection of promutagens. More detailed information on these genetic markers and phenotypic descriptions of the strains used in this study are given in Lindsley and Zimm (1992). All the strains were the generous gift of Prof. Upendra Nongthomba, Molecular Reproduction and Developmental Genetics, Indian Institute of Science, Bangalore, India. The isogenic line of flies were maintained and cultured in bottles (50 mL) at the *Drosophila* laboratory, Department of Animal Science, Central University of Kerala, Kasaragod, India, under the standard conditions of 25 ± 1 °C and 60–70% relative humidity, 12 h/12 h light and dark cycle and on a standard wheat cream agar media with yeast granules as the protein source (Anupama et al., 2017).

Two different crosses were performed to produce the experimental larval populations to study the PbAc induced biochemical changes and genetic toxicity (wing spot and Comet assay). The standard (ST) and high bioactivation (HB) crosses were made using virgin females of the *flare3* and *Oregon-flare3* strains respectively, mated to *mwh*/*mwh* males. Generated eggs (offspring) from two different crosses were collected separately on agar- grape juice medium (containing 3% agar-agar, 1.2% sucrose, 2% ethanol, 1% acetic acid, and 27.2% grape juice without any preservative) for 2 h and transferred to standard food bottles. These were maintained at standard conditions. Three days later, the third instar larvae (72 ± 4 h) were washed out of the bottles with tap water (25 °C) through a fine nylon mesh and used as experimental larval populations for all assays.

2.3. Preparation of chemical food media

PbAc or EMS was added in desired concentration (weight/volume) to freshly prepared 50 mL of standard *Drosophila* food, thoroughly mixed, poured into experimental bottles and allowed to solidify. For each experiment, without PbAc food media as the negative control; food media with EMS (0.1 mM) a well-known mutagen, as a positive control (for wing spot and comet assay) and different concentrations of the PbAc treated food were prepared uniformly in the same batch.

2.4. Toxicity analysis and selection of PbAc concentrations for assays

LC_{50} toxicity assay in *Drosophila* larvae for PbAc was conducted for ST or HB crosses; i.e., the concentration at which 50% of larvae failed to reach the adulthood. To determine 50% lethal concentration of PbAc, 100 third instar (72 ± 4 h) larvae derived from the ST or HB crosses were transferred to the standard food medium containing different concentrations of PbAc ranging from 0.01–5.0 mM. In the control group, only standard *Drosophila* food was used (devoid of PbAc). Assay was conducted in triplicates and was repeated thrice. The larvae were maintained in the respective treated and untreated experimental food media where they fed for the remainder of their larval life (~48 h), pupated and emerged as adult flies. The emerged flies were counted every day from the day of eclosion to last day of emergence in each group. From this, the percent of viable flies emerged was noted. The data of each PbAc treated and control groups were used to determine the LC_{50} by Probit method (Finney, 1971). The calculated LC_{50} for PbAc: 1.42 mM for ST and 1.75 mM for HB cross offspring. Hence, less than half of the LC_{50} concentrations of PbAc, i.e. 0.2, 0.4, 0.6 and 0.8 mM were selected for analyzing the lead (Pb) induced biochemical and genetic (wing spot and Comet assay) effects in a concentration dependent fashion.

2.5. Metal content assay

Pb loads were measured in *Drosophila* larvae (3rd instar; 96 ± 4 h) derived from ST or HB crosses reared in control or in leaded medium (0.2, 0.4, 0.6 and 0.8 mM PbAc) in triplicate and experiments were repeated twice. 50 Larvae were washed three times with phosphate buffered saline (PBS 1×; pH 7.4) and were digested with a mixture of 65% HNO_3 and 30% H_2O_2 (1:1). The digested material was dried overnight at 95 °C. The dried residue was dissolved and diluted with 2% HNO_3 for estimating Pb loads (ng/individual) using atomic absorption spectrometer (AAS) (Perkin Elmer, USA) in the Department of Chemistry, Central University of Kerala, India.

2.6. Biochemical assays

All the biochemical assays were performed using third instar larvae (96 ± 4 h) of *Drosophila* derived from ST or HB crosses reared in control or in leaded medium (0.2, 0.4, 0.6 and 0.8 mM PbAc) in triplicate and the experiments were repeated thrice.

2.6.1. δ-aminolevulinate-dehydratase (δ-ALA-D)

The δ-ALA-D level was assayed using a modified method of Sassa (1982), by measuring the rate of porphobilinogen formation. 80 third instar larvae (96 ± 4 h) were anaesthetized with ice and homogenized in 5 mM Tris-HCl buffer, pH 8.5. The homogenate was centrifuged at 1878*g* for 10 min at 4 °C to yield a supernatant that was used for the enzyme assay. The reaction mixture made up of 10 μL of 0.5 M Tris-glycine (pH 8.5), 7 μL distilled water, 25 μL sample, and 8 μL of 31.25 mM 5-aminolevulinic acid hydrochloride was incubated at 37 °C for 3 h. Subsequently, 100 μL of 10% TCA containing 20 mM $CuSO_4$ was added to each reaction mixture, and centrifuged at 2935*g* for 10 min. After this, 100 μL from each of the supernatants obtained was added to 100 μL of Ehrlich reagent and incubated for 30 min at room temperature. The absorbance was measured at 555 nm in a plate reader and the data were expressed as percentage of the respective control.

2.6.2. δ-aminolevulinic acid (δ-ALA)

δ-ALA was determined by the modified method of Wada et al. (1969). 80 third instar larvae (96 ± 4 h) were homogenized in sodium phosphate buffer (0.1 M pH 8.0). 500 μL larval homogenate and 500 μL 20% acetic acid were mixed to which 3 mL n-butanol was added and the mixture was vigorously shaken. After phase separation, 250 μL of bottom layer was pipetted out and transferred to 2 glass tubes. 750 μL of a solution of 1 volume ethyl acetoacetate and 20 volumes 0.5 M sodium phosphate buffer pH 6.8 were added to one tube and 750 μL sodium phosphate buffer was added to other tube which was prepared as the blank. All tubes were kept in a boiling water bath for 10 min. After cooling to room temperature, 1 mL of Ehrlich reagent was added to each tube and then the contents were mixed well. After 10 min at room temperature, 2 mL chloroform was added to all tubes and shaken vigorously. Absorbance of the chloroform layer was read at 555 nm. The δ-ALA concentration was calculated from a standard calibration curve and values were expressed as μg/mL larval homogenate.

2.6.3. Cytochrome P450 (CYP450)

CYP450 was estimated following the method of Klotz et al. (1984) with some modifications. 80 third instar larvae (96 ± 4 h) were homogenized in 1 mL sodium pyrophosphate buffer (0.1 M pH 7.4). 1 mL larval homogenate, in sodium pyrophosphate buffer (pH 7.4) was centrifuged at 14000*g* for 20 min at 4 °C. Clear supernatant was used as an enzyme preparation for determination of CYP activity. Reaction mixture contained 70 μL larval homogenate, 50 μL Tris-HCl buffer (0.1 M, pH 7.8), 50 μL 0.1 M NaCl and 70 μL 2 μM 7-ethoxyresorufin. Reaction was initiated by adding 10 μL of 0.5 mM NADPH. Optical density was recorded at 572 nm after a reaction period of 2 min in a microplate reader (extinction coefficient 73 mM/cm). The values were expressed as μg resorufin produced/min/mg protein.

2.6.4. Sample preparation for oxidative stress assays

Samples were prepared by homogenizing 30 third instar larvae (96 ± 4 h) in 200 μL of ice cold sodium phosphate buffer (0.1 M; pH 7.4) containing protease inhibitor cocktail, followed by centrifugation at 2500*g* for 10 min at 4 °C. The filtered supernatant was collected for biochemical analysis.

2.6.5. Reactive oxygen species (ROS)

The level of ROS generation was measured by the method of Driver et al. (2000). An aliquot of homogenized larval sample (50 μL) was dispensed into tubes containing Locke's buffer solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 2 mM $CaCl_2$ and 10 mM glucose pH 7.4) to which 10 μL of DCFH-DA (5 μM) was added and incubated for 30 min at room temperature. The fluorescence was measured with excitation and emission wave lengths at 480 and 530 nm. The values were expressed as picomole of dichloro fluorescein formed/min/mg protein.

2.6.6. Lipid peroxidation (LPO)

The extent of LPO was measured by the method of Ohakawa et al. (1979) by measuring the thiobarbituric acid reactive substances (TBARS) in the larval homogenate. The reaction mixture contained 500 μL larval homogenate, 1.5 mL acetic acid (pH 3.5, 20% *v*/v), 1.5 mL of TBA (0.8% w/v) and 200 μL SDS (8% w/v). The mixture was heated in a boiling water bath for 45 min and adducts formed were extracted into 3 mL of 1-butanol and the color intensity was measured at 532 nm and quantified as malondialdehyde (MDA) equivalents using 1,1,3,3-tetramethoxy propane as standard. The values were expressed as μmole MDA/mg protein.

2.6.7. Superoxide dismutase (SOD)

SOD activity was determined following the method of Kostyuk and Potapovich (1989) by monitoring the inhibition of quercetin auto oxidation. To 1 mL reaction mixture containing 3–5 μg protein containing larval homogenate; 0.016 M sodium phosphate buffer (pH 7.8), 8 mM TEMED and 0.08 mM EDTA were added. Adding 0.15% quercetin dissolved in dimethyl formamide started the reaction. The rate of quercetin auto oxidation was monitored for 3 min at 406 nm. Following the addition of larval homogenate, the decrease in absorbance was monitored. The amount of protein that inhibits quercetin oxidation by 50% was defined as one unit. The values were expressed as unit/mg protein.

2.6.8. Catalase (CAT)

The CAT activity (Aebi, 1984) was determined as a measure of H_2O_2 (final concentration 8.8 mM) decomposition by the enzyme. It was monitored by the addition of an aliquot (20 μL) of the larval homogenate. The decrease in H_2O_2 was monitored for 3 min at 240 nm and resulted values were expressed as μM of H_2O_2 decomposed/min/mg protein.

2.6.9. Glutathione (GSH)

The total GSH content was measured following the method of Mokrasch and Tschke (1984). Thirty third instar larvae (96 ± 4 h) of *Drosophila* derived from ST or HB crosses were homogenized in ice-cold 10% TCA and 10 mM EDTA (1:1) and centrifuged at 5000*g* (15 min at 4 °C). 200 μL of the supernatant was added to the 3 mL reaction mixture containing Tris-buffer (0.2 M; pH 8.0) and 50 μL of DTNB. After 10 min of incubation at room temperature, the absorbance was read at 412 nm and values were expressed as μg GSH/mg protein.

2.6.10. Glutathione-S-transferase (GST)

GST activity was measured according to the procedure of Habig and Jakoby (1981) using CDNB as substrate. The assay reaction mixture was made up of 250 μL of a solution containing (10 μL of 0.25 M potassium phosphate buffer, pH 7.0, with 2.5 mM EDTA, 10 μL of distilled water, and 200 μL of 0.1 M GSH at 25 °C), 20 μL of sample (1:5 dilution), and 10 μL of 25 mM CDNB. The increase in the optical density was recorded for 3 min at 340 nm and the activity was expressed as μmol GS-DNB formed/min/mg protein using the molar extinction co-efficient ($\varepsilon =$ 9.6 mM/cm).

2.6.11. Protein estimation

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

2.7. Genotoxicity tests

2.7.1. Wing spot test

2.7.1.1. Treatment procedure, phenotypic characterization and preparation of microscopic slide. To perform wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster*, the third instar larvae (72 ± 4 h) derived from the ST or HB crosses were reared in negative, positive control (0.1 mM EMS) or sublethal concentrations of 0.2, 0.4, 0.6 and 0.8 mM PbAc treated food media. Advantage of selecting the third instar larvae (72 ± 4 h) for the wing spot test is that large number of cells in the wing discs are exposed simultaneously to the chemical (PbAc or EMS) that would develop large, recognizable wing spot helpful for easy interpretation of data. The five-bottles/experimental group (100 larvae/bottle; total 500 larvae/group) were maintained under standard conditions. Larvae were maintained in the respective treated and control food media where they fed for the remainder of their larval life (~48 h), pupated and emerged as adult flies. After completion of metamorphosis, all surviving flies were scored irrespective of sex and classified according to the presence or absence of the Bd^S phenotype. Adult flies produced from the experimental progeny of ST or HB crosses consisted of marker-heterozygous (MH) flies (*mwh* +/+ *flr3*) with phenotypically wild-type wings and balancer- heterozygous (BH) flies (*mwh* +/+ *TM3* Bd^S) with phenotypically serrate wings. These flies were stored in a 70% ethanol solution.

2.7.1.2. Wing spot analysis. Mounted wings (40 flies, i.e. 80 wings/experimental group) were observed under an optic microscope at 40× magnification for the occurrence of mutant spots. Mutant spots were classified into three types during wing microscopic analysis: (i) small single spots, consisting of 1 or 2 *mwh* or *flr3* cells; (ii) large single spots, consisting of three or more cells; and (iii) twin spots consisting of adjacent *mwh* and *flr3* cells (Graf et al., 1984). The number of spots and spot type on the wings were recorded in negative, positive controls and PbAc treated groups of ST or HB crosses of MH and BH genotype flies.

2.7.2. Detection of Pb induced DNA damage in hemocytes by comet assay

To perform comet assay, third instar (72 ± 4 h) larvae from the ST or HB crosses were reared in negative or positive control (0.1 mM EMS) or sublethal concentrations of 0.2, 0.4, 0.6 and 0.8 mM PbAc treated food media. The experiment was conducted in triplicate for each treatment, five-bottles/experimental group (100 larvae/bottle; total 500 larvae/group) were maintained. The larvae were maintained in the respective treated and control food media for ~24 h. The treated and control food fed larvae (96 ± 4 h) were used for the collection of hemolymph.

2.7.2.1. Collection of hemocytes. Hemocytes from larval hemolymph were collected according to the method of Marcos and Carmona (2013). Briefly, larvae (96 ± 4 h) were removed from food media, washed in distilled water, sterilized in 5% bleach and dried. The cuticle from each ($n = 50$) larva was disrupted with two fine forceps under a stereomicroscope. The hemolymph was directly collected into a drop of cold PBS solution containing 0.07% PTU in 1.5 mL micro centrifuge tube. Pooled hemolymph was centrifuged at 300g for 10 min at 4 °C, the supernatant was discarded, and the pellet containing hemocytes was resuspended in 20 μL of cold PBS.

2.7.2.2. Comet assay procedure. Comet assay was performed following the method of Dhawan et al. (2009) with some modifications. Neutral and alkaline Comet assays were carried out simultaneously in two different experimental sets. Comet assay slide preparation was performed by mixing 20 μL of hemocyte cell suspension in 75 μL of 0.75% LMPA. 20 μL of the mixture was layered on top of the slides that were pre-coated with 1% NMPA. The cell suspension was uniformly smeared with cover slips, and the slides were stored at 4 °C for 5 min until agarose solidification. After agarose solidification coverslips were removed and the slides were immersed for 2 h in freshly prepared, chilled lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, and 1% Triton X-100, pH 10) at 4 °C in dark chamber and lysis was performed. After lysis, the slides were subjected to neutral and alkaline gel electrophoresis: ***Neutral comet assay***- for the measurement of DNA double strand breaks, the slides after lysis were kept in electrophoresis buffer (300 mM CH_3COONa), 100 mM Tris-HCl (pH 8.5) for 1 h and then transferred to horizontal electrophoresis unit containing fresh buffer. Electrophoresis was carried out at a current of 60 mA for 1 h at 4 °C. ***Alkaline comet assay-*** for the measurement of DNA single strand breaks and alkali labile base damage, the slides after lysis were placed in chilled electrophoresis buffer (0.03 M NaOH, 1 mM Na_2EDTA, pH > 13) for 10 min for DNA unwinding. Subsequently, electrophoresis was conducted in chilled electrophoresis buffer for 15 min at 0.7 V/cm (300 mA/25 V) at 4 °C. The slides were gently washed three times with 0.4 M Tris buffer (pH 7.5) to neutralize excess alkali. ***Staining-*** Before staining, both the alkaline and neutral slides were dehydrated with ethanol for 10 min each and air-dried overnight. Slides were then stained with ethidium bromide (20 μg/mL: 25 μL per slide) for 10 min in dark. After staining, the slides were dipped once in chilled distilled water to remove excess stain and coverslips were placed over the slides.

Then the slides were examined using a fluorescence microscope (Leica, Germany) at 40× magnification. The images were transferred to a computer through a charge coupled device (CCD) camera and analyzed using CASP 1.2.3 software (CASPlab). One hundred twenty cells from each group (20 cells per slide with two slides per experimental group in triplicates) were examined. The tail intensity (% of DNA in tail) was used as parameter of Pb induced genotoxicity.

2.8. Data evaluation and statistical analyses

2.8.1. Biochemical assays

All the analyses were made in respective replicates and values were represented as mean ± SEM. Significant difference between the control and PbAc treated groups was obtained by one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test using SPSS (version 16.0) (SPSS Inc., Chicago, IL, USA).

2.8.2. Wing spot test

The data were pooled after verifying that the two independent experiments were in agreement with acceptable reproducibility. The wing spot data were evaluated with SMART PC-Version 2.1. For the frequencies of spots per wing, a multiple decision procedure is used to decide whether a result is positive, weakly positive, inconclusive or negative (Frei and Wurgler, 1995). The wing spot data of control (negative and positive) and treated groups were compared by conditional binomial test (Kastenbaum and Bowman, 1970) with significance levels set at $\alpha = \beta = 0.05$. Baseline data on the frequencies of the *mwh* clone at each PbAc concentrations used, control *mwh* clone frequency and numbers of wings analyzed in the MH and BH genotypes was used for the calculation of mean *mwh* clone size class and frequency of clone formation per 10^5 cells per cell cycle and percentage recombination (Frei et al., 1992). Regression analysis was performed using the combination of the mean *mwh* clone size class, frequency of clone formation per 10^5 cells per cell cycle and percentage recombination values from the different concentrations of PbAc treatment to establish the standardized clone induction frequency per mM PbAc in the *Drosophila* food media (Frei and Wurgler, 1995).

2.8.3. Comet assay

The % tail DNA data was obtained from 120 independent comet images per experimental group. The distribution of data is shown as box and whisker plot, the box represents the 1st quartile (25th percent) and the 3rd quartile (75th percent); the line in the box represents the median. Medians indicated those that are different from control. Non-parametric Kruskal Wallis test was applied to analyze the data for significance between the control and different treatment groups; and also analysis of variance (ANOVA) followed by Tukey HSD post hoc test was performed.

3. Results

3.1. Pb accumulation in larva

Fig. 1 gives the level of Pb load present in the third instar larvae of *D. melanogaster* in control and PbAc treated batches. Except in the lower concentration of Pb treatment, all others have significantly increased Pb level compared to control. For example at 0.8 mM PbAc treatment in ST cross, the Pb load is 30.37% more than HB cross (Fig. 1; $p < 0.001$).

3.2. Influence of Pb on enzymes and intermediates of heme synthesis

A significant reduction in the level of δ-ALA-D is noticed in third instar larvae derived from ST or HB crosses reared in PbAc treated food media compared to controls (Fig. 2a; $p < 0.001$). The percentage reduction of δ-ALA-D is more in ST cross larvae compared to the HB. Increased accumulation of δ-ALA was recorded in third instar larvae derived from ST or HB crosses reared in PbAc treated food medium compared to controls. In contrast to HB cross, ST have more accumulation of δ-ALA (Fig. 2b; $p < 0.05$). Similar to δ-ALA-D, CYP450 also had significant reduction compared to control. Here also ST is having more diminution compared to the HB cross (Fig. 2c; $p < 0.0001$).

3.3. Pb induced oxidative stress

The elevated ROS generation and increased level of LPO was noticed in the PbAc treated ST or HB cross third instar larvae than in the control (Fig. 3a and b). Significantly increased generation of ROS is 18.68%, 9.01%, 13.33%, 15.80% and 12.46% ($p < 0.05$) in control, 0.2, 0.4, 0.6 and 0.8 mM PbAc treated food media respectively in ST cross compared to HB cross. Similar results were observed in LPO assays.

3.4. Pb induced depletion of antioxidants

A significant reduction of SOD and CAT enzyme activities were recorded in the third instar larvae of ST or HB cross reared in different concentrations of PbAc treated food media compared to controls (Fig. 4a

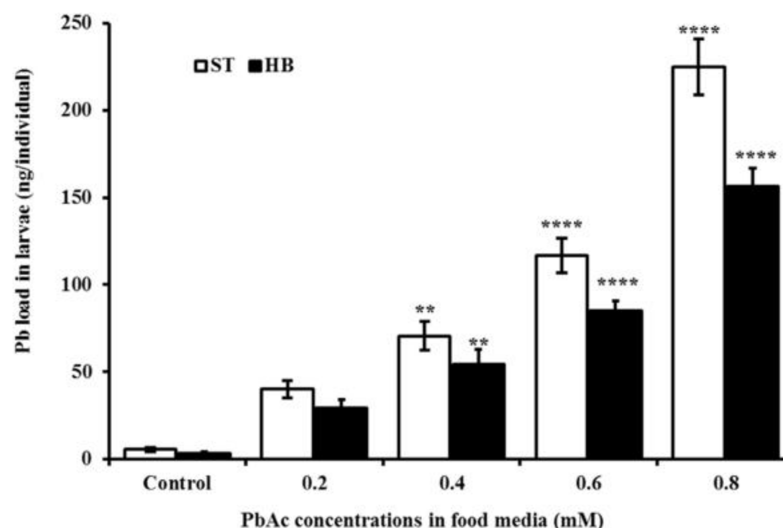

Fig. 1. Lead loads (mean ± SE) estimated in larvae of *Drosophila* derived from ST or HB crosses were reared in control or in leaded medium. Bars without the asterisk (*) are not significant compared to respective control at $p > 0.05$; and significant at $^{**}p < 0.01$; $^{****}p < 0.0001$ level.

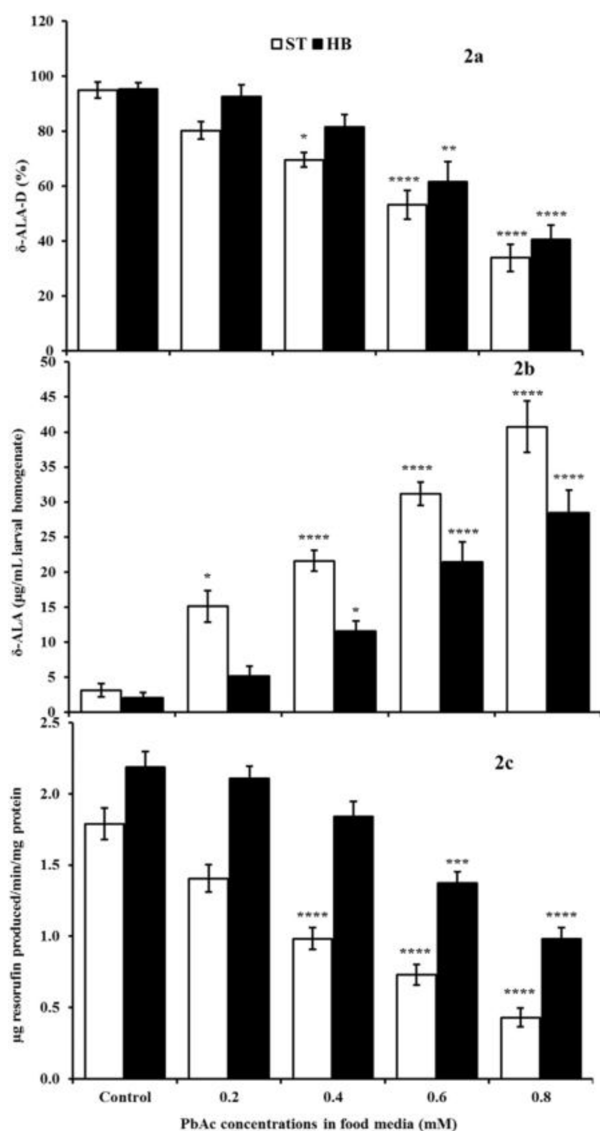

Fig. 2. ∂-aminolevulinate-dehydratase (δ-ALA-D) (a), δ-aminolevulinic acid (δ-ALA) (b) and cytochrome P450 (CYP450) (c) in third instar larvae (96 ± 4 h) derived from ST or HB crosses were reared in the control or PbAc treated groups. Values are presented in mean ± SE; bars without the asterisk (*) are not significant compared to control at $p > 0.05$; and significant at *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ level.

and b) ($p < 0.001$). Greater depletion of SOD and CAT enzyme activities were recorded in ST cross larvae compared to HB cross larvae ($p < 0.0001$). Similarly, the reduction of intracellular antioxidant GSH content and GST enzyme activity were noted in ST or HB cross larvae reared in higher concentrations except 0.2 mM PbAc treatment (Fig. 4c and d). The increased level of GST activity compared to control is recorded in ST (4.16%) and HB (3.53%) crosses larvae reared in 0.2 mM whereas the significant reduction of GST was recorded in ST or HB cross larvae reared in 0.8 mM PbAc treated food media (Fig. 4d; $p < 0.0001$).

3.5. Pb induced wing mutant spots

PbAc treated groups and 0.1 mM EMS treatment (positive control) have significant increase in the mutant spots per wing compared to negative control (without PbAc) in *D. melanogaster*. The results obtained for positive control showed a statistically significant increase in the small single spot, large single spot, twin spot and total spot per wing frequencies in the MH genotype flies of ST and HB crosses. The negative control frequencies of total spots per wing was 0.49 and 0.33 in ST cross and 0.35 and 0.24 in HB cross for the MH and BH genotype flies respectively. In the ST cross of MH genotype flies, all the PbAc treated concentrations produced positive results for small spots. Large spots showed their positive response in 0.6 and 0.8 mM PbAc treated groups. The weakly positive small single spots and inconclusive large single spots per wing frequencies were recorded in the ST cross of BH genotype flies (Table 1). HB cross of MH genotype flies, yielded a positive small spots per wing frequency at higher concentrations of 0.6 and 0.8 mM PbAc treated group and positive large single spots per wing frequency were observed at high concentration of 0.8 mM PbAc treated group. The PbAc treatment induced inconclusive twin spots per wing frequencies in ST and HB crosses of MH genotype flies (Table 2). The PbAc treatment resulted in inconclusive and negative total spots per wing frequencies in MH and BH genotype flies in ST and HB crosses. The higher frequency of clone induction formation per 10^5 cells was observed in the PbAc concentrations treated groups analyzed in flies of MH wings than in the BH wings of ST and HB crosses (Tables 1 and 2). The more frequencies of standardized *mwh* clone per 10^5 cells and less percentage of recombination were obtained in ST cross than HB cross per mM PbAc (Table 3).

3.6. Comet assay

Third instar larvae of ST or HB crosses reared in 0.1 mM EMS (positive control) showed a significant increase ($p < 0.0001$) in percent tail DNA in their hemocytes compared to negative controls (without PbAc). Dose dependent increase in percent tail DNA was observed in alkaline and neutral comets when different doses of PbAc were used. However, a significant increase in percent tail DNA was observed in hemocytes of ST or HB crosses larvae at the higher concentrations of PbAc tested groups compared to those of controls in alkaline and neutral comet except 0.2 mM. A significant increase in percent tail DNA is recorded in alkaline comet compared with neutral comet in all the concentrations of PbAc tested. Pb is inducing more percent tail DNA in hemocytes of ST cross larvae compared to HB cross larvae (Figs. 5 and 6).

4. Discussion

4.1. Pb accumulation in larva of D. melanogaster

It is important to confirm the uptake of Pb compound by the tested organism used for genotoxicity tests, especially in consideration of the inconsistent genotoxicity results reported in the literature (Garcia-Leston et al., 2010). In the present study, when Pb loads were measured in ST or HB crosses in third instar larvae it is readily accumulated in the larvae (Fig. 1), indicating greater absorption of Pb in treated groups. These results are on par with previous study in that *D. melanogaster* accumulated Pb in dose dependent manner throughout their life, third-instar larvae being the higher accumulation (Peterson et al., 2017). At this juncture it is pertinent to mention, that increased Pb accumulation is capable of interacting with the cell and cellular macromolecules including proteins, lipids and nucleic acids particularly at sulfhydryl groups and the phosphate backbone causing deleterious effects in an organism (Flora et al., 2012). The reason for the higher level of Pb in ST compared to HB is the constitutively enhanced CYP450 enzyme expression in HB (Fig. 2c and Saner et al., 1996).

4.2. Influence of Pb on enzymes and intermediates of heme synthesis

The δ-ALA-D can serve as a valuable biomarker of oxidative stress and also a biochemical indicator of Pb exposure (Gurer-Orhan et al., 2004). It is also well known that Pb interferes with the activity of δ-ALA-D modulating the heme synthesis (Akram et al., 2018). In the present study, the decrease of δ-ALA-D indicates the influence on heme synthesis and in turn increased the δ-ALA in cells of *Drosophila* in a dose dependent manner. Decreased δ-ALA-D activity in *Drosophila* may be due to the oxidation of sulfhydryl group present in this enzyme as has

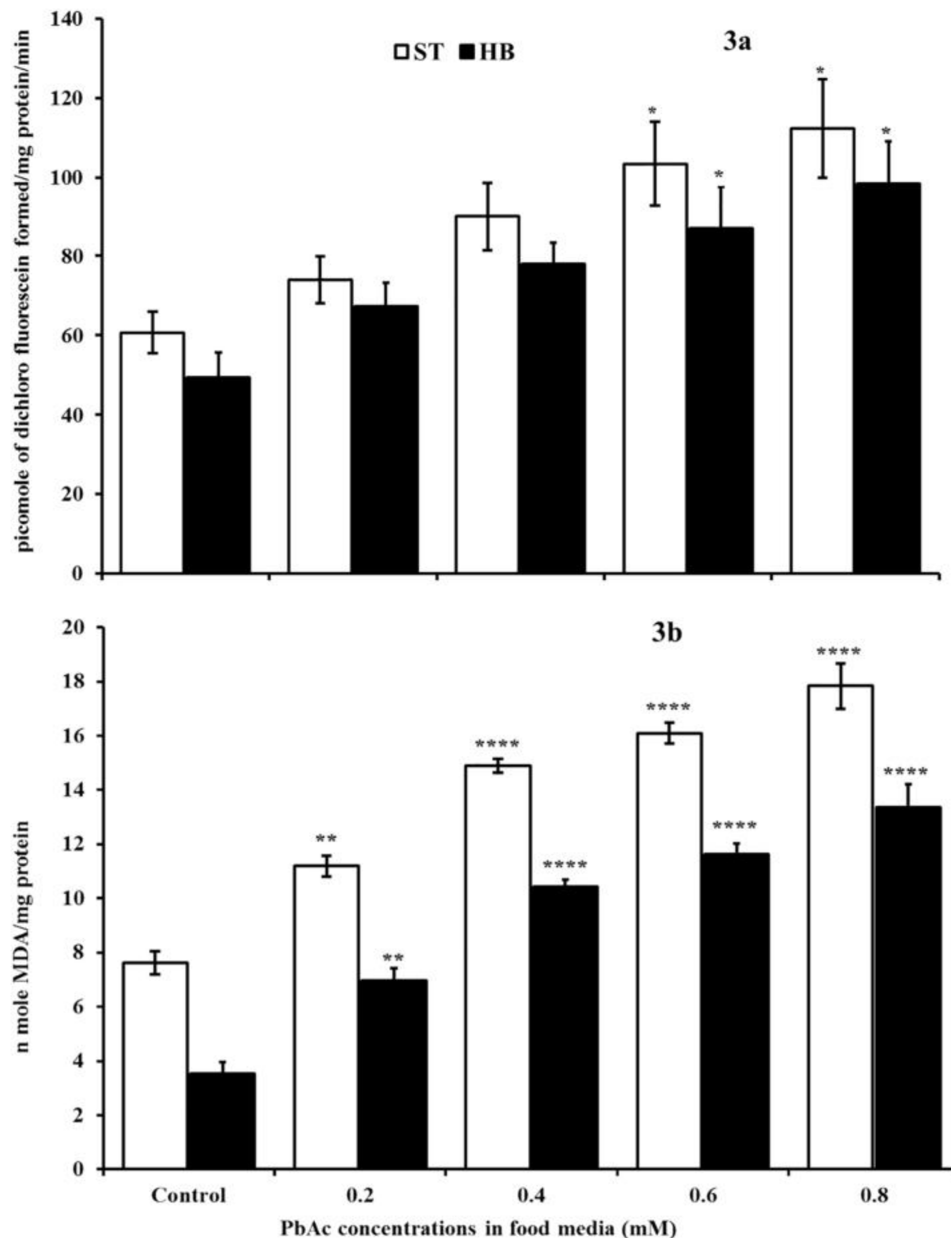

Fig. 3. Reactive oxygen species (ROS) generation (a) and Lipid peroxidation (LPO) level (b) in third instar larvae (96 ± 4 h) derived from ST or HB crosses were reared in the control or PbAc treated groups. Values are presented in mean ± SE; bars without the asterisk (*) are not significant compared to respective control at $p > 0.05$; and significant at *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ level.

been demonstrated by Golombieski et al. (2008). Several epidemiological studies showed higher levels of blood Pb (>10 μg/dL) in Pb exposed urban youngsters and battery plant workers, elevated level of LPO and lower δ-ALA-D activity and antioxidants, indicating that part of the pro-oxidant effect of Pb can be mediated by δ-ALA-D inhibition (Ahamed et al., 2011; Ahamed et al., 2008). The δ-ALA is a pro-oxidant compound and its accumulation during Pb exposure is known to stimulate ROS production (Bechara, 1996). Further it may rapidly undergoes enolization, auto-oxidation and generates reactive oxygen species (ROS) as superoxide ion ($O_2^{\cdot-}$), hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2) and also interacts with oxyhemoglobin (Laafi et al., 2014).

The δ-ALA-D is one of the enzymes of heme biosynthesis which converts ALA to porphobilinogen, the first precursor of pyrrole. The decreased activity of δ-ALA-D prevents δ-ALA from being converted to porphobilinogen, inhibiting incorporation of iron into the protoporphyrin ring, resulting in impaired heme biosynthesis (de Lucca et al., 2016). It has been evidenced that there was a significant reduction in the activity of δ-ALA-D and CYP450 (Fig. 2a and c) and increased accumulation of δ-ALA (Fig. 2b) in third instar larvae derived from ST or HB crosses reared in PbAc treated food medium compared with controls. Interference with heme production and subsequent reduction of the CYP450 pool is one of the key reasons of xenobiotic metabolism related disorders (Meyer et al., 2005). The generation of oxidative stress in Pb exposure strongly suggests that δ-ALA-D inhibition contributes to Pb toxicity by either disrupting the heme biosynthesis pathway and/or by increasing the concentration of the potential pro-oxidant δ-ALA (Rocha et al., 2012).

4.3. Pb induced oxidative stress

In this study, Pb caused overproduction of ROS in the ST or HB crosses of third instar larvae reared in PbAc treated food media (Fig. 3a). This intracellular build-up of ROS concentrations can induce oxidative damage in cellular macromolecules such as nucleic acids, proteins, lipids, and carbohydrates (Klaunig et al., 2010). Enhanced production of ROS leads to

membrane lipid bilayer disruption, elicited by the oxidation of polyunsaturated fatty acids known as LPO and Pb per se is also known to stimulate ferrous ion initiated membrane LPO, which leads to the formation of many toxic metabolites, including malondialdehyde (MDA), a widely used biomarker of oxidative stress (Adonaylo and Oteiza, 1999). The PbAc notably increased LPO levels in the larvae of ST or HB crosses suggesting an increased cellular oxidative stress as a result of increased ROS and depletion of antioxidant scavenger system (Figs. 3 and 4). The present study confirms that increased free radical production and reduced antioxidative enzymes like SOD, CAT, GSH and GST, which probably make the organism more susceptible to oxidative damage.

Antioxidant enzyme SOD requires Cu^{2+} and Zn^{2+} ions for its activity. Cu^{2+} ion plays a functional role in the reaction by undergoing oxidation and Zn^{2+} ions stabilize the enzyme (Ercal et al., 2001). High affinity of Pb to sulfhydryl (-SH) groups or metal cofactors in this enzyme, as Pb competes and replaces both the metal in their binding sites (Flora et al., 2012), might have decreased the level of SOD in the current study. On the other hand, CAT is a heme-containing enzyme, the depletion of the heme proteins might be proportional decrease in the CAT level (Flora et al., 2008). Thus, in the present study the inhibitory effects of Pb on SOD and CAT enzymes and increased free radical production would probably result in impaired antioxidant defenses in the cells and render cells more vulnerable to oxidative attacks.

The reduction of GSH which is indicator of oxidative stress in ST or HB crosses larvae reared in PbAc treated food media in the present study (Fig. 4c) could result from direct interaction of Pb with sulfhydryl group of GSH or impairment of GSH synthesis (Flora et al., 2012). In the current study, Pb induced overproduction of ROS and LPO in the larvae (Fig. 3a and b) caused a decrease in GSH content by rapid oxidation of GSH to disuphide GSSG (Ercal et al., 2001).

GSTs are actively involved in the Phase II detoxification metabolism of electrophilic molecules generated from the Phase I detoxification system by conjugation with GSH (Yologlu and Ozmen, 2015). Increased level of GST activity compared to control is recorded in ST or HB crosses larvae reared in 0.2 mM PbAc treated group (Fig. 4d) implying an adaptive response to the survival needs of larvae. Increase in generation of electrophilic reactive species from the Phase I detoxification system due to PbAc and impaired activation of Phase II detoxification system in the experimental condition, in turn lead to the significant reduction of GST in the higher concentrations of PbAc treated groups (Fig. 4d). Hence, the impaired capacity of larvae to completely detoxify Pb and its reactive species is leading to accumulation of free radicals.

4.4. Pb induced wing mutant spots

SMART is a versatile in vivo assay simultaneously detecting the potential of chemical to induce the mutagenic and recombinogenic activity in *Drosophila*. The negative control frequencies of total spots per wing obtained in the MH and BH genotypes of ST or HB cross larvae (Tables 1 and 2), is in accordance with the earlier works (Wurgler et al., 1985). The data of EMS (Tables 1 and 2) which was used as positive control, demonstrated that the strong mutagenic and recombinagenic activity in *D. melanogaster* somatic cells using the same concentration. This is on par with previous observations (Graf et al., 1984; Sarıkaya et al., 2016).

There was a statistically significant positive result for small and large single spots of MH genotype flies in the ST and HB crosses at the PbAc concentrations tested, compared to the spot frequencies observed in the negative control (Tables 1 and 2). These spots arise from various types of mutational events or somatic recombination (between the *mwh* and *flr* loci). The single spots detected in wings of MH genotype flies reflect only somatic mutations (point mutation, deletion, nondisjunction) (Frei and Wurgler, 1997). Twin spots indicating somatic recombination are less frequent (statistically inconclusive; Tables 1 and 2) than large and small single spots, because only somatic recombination events take place between the *flr3* locus and the centromere. Recombination events that take place between the marker loci are recovered as *mwh* single spots. The *flr3* single spots are infrequent because they probably arise from relatively rare events like point mutations at the locus (Frei et al., 1992). Since positive single spots were obtained in the MH genotypes of ST or HB cross, the BH genotype flies were analyzed for

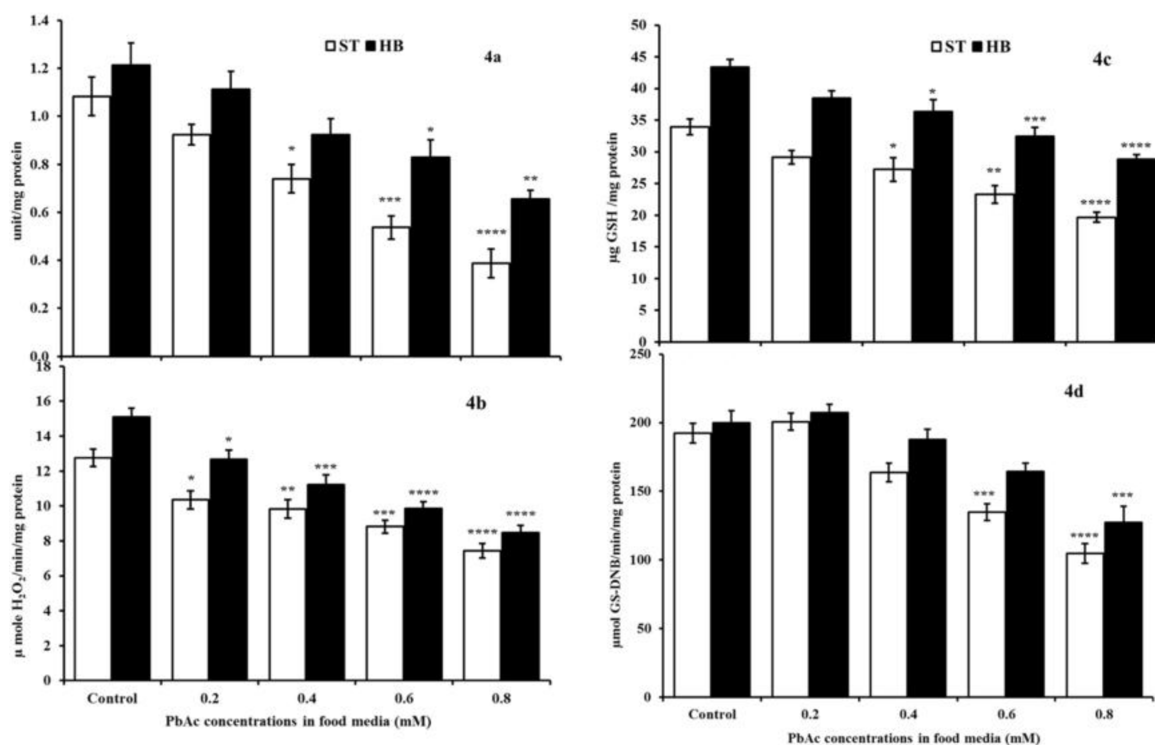

Fig. 4. Superoxide dismutase (SOD) (a), Catalase (CAT) (b), Glutathione (GSH) content (c) and Glutathione-S-transferase (GST) activity (d) in third instar larvae (96 ± 4 h) derived from ST or HB crosses were reared in the control or PbAc treated groups. Values are presented in mean ± SE; bars without the asterisk (*) are not significant compared to control at $p > 0.05$; and significant at *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ level.

Table 1
Frequency of wing mutant spots observed in the marker trans-heterozygous descendants (MH; *mwh/flr3*) and balancer heterozygotes (BH; *mwh/TM3*) of *D. melanogaster*, derived from the Standard Cross (ST) treated with different PbAc concentrations or positive control (EMS) or negative control.

Genotype	Treatment compound	Con. (mM)	Number of wings (N)	Spots per wing (no. of spots) statistical diagnosis[a]				Total *mwh* clones[c] (n)	Mean *mwh* clone size class[b] (î)	Frequency of clone formation per 10^5 cells[d]	
				Small single[b] spots (1–2 cells) (m = 2)	Large single[b] spots (>2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)			Without clone size correction $f_t = (n/NC) \times 10^5$	With clone size correction $f'_t = (2^{(\hat{i}-2)}) \times f_t$
MH; mwh/flr3	NC-without PbAc	0.0	80	0.44(35)	0.05(4)	0.00(0)	0.49(39)	0.49(39)	1.10	1.99	3.72
	PC: EMS	0.1	80	1.81(145)+	0.78(62)+	0.56(45)+	3.15(252)+	2.84(227)	2.93	11.62	22.17
	PbAc	0.2	80	0.68(54)+	0.06(5)−	0.01(1)i	0.75(60)i	0.74(59)	1.75	3.02	3.59
		0.4	80	0.78(62)+	0.06(5)−	0.01(1)i	0.85(68)i	0.75(60)	2.61	3.07	4.72
		0.6	80	0.90(72)+	0.29(23)+	0.05(4)i	1.24(99)i	1.03(82)	2.18	4.20	4.77
		0.8	80	1.04(83)+	0.38(30)+	0.10(8)i	1.51(121)i	1.39(111)	2.36	5.68	7.30
BH; mwh/TM3	NC-without PbAc	0.0	80	0.30(24)	0.03(2)	[e]	0.33(26)	0.31(25)	1.00	1.28	2.56
	PC: EMS	0.1	80	1.50(120)+	0.56(45)+		2.06(165)+	2.00 (160)	2.08	8.19	8.71
	PbAc	0.2	80	0.31(25)w	0.03(2)i		0.34(27)−	0.33 (26)	3.00	1.33	2.66
		0.4	80	0.31(25)w	0.05(4)i		0.36(29)−	0.35(28)	3.33	1.43	3.61
		0.6	80	0.34(27)w	0.08(6)i		0.41(33)i	0.39(31)	2.83	1.58	2.82
		0.8	80	0.35(28)w	0.11(9)i		0.46(37)i	0.44(35)	3.20	1.79	3.20

NC-without PbAc: Negative control; PC: Positive control; EMS: Ethyl methanesulfonate.
[a] Statistical diagnoses following Frei and Wurgler (1988): +, positive; −, negative; i: inconclusive; w: weak positive; m: multiplication factor; significance levels $\alpha = \beta = 0.05$.
[b] Including rare *flr3* single spots.
[c] Considering *mwh* clones from *mwh* single and twin spots.
[d] Frequency of clone formation per 10^5 cells = $(n/NC) \times 10^5$; n: the number of *mwh* clones, N: number of wings examined for each treatment. C: the number of cells scored in each wing (approx. 24,400) (Frei et al., 1992).
[e] Balancer chromosome *TM3* does not carry the *flr3* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

determining the relative contribution of mutational or recombinational events in induction of mutant spots. Weak and inconclusive spots were categorised in flies of BH genotype in the ST or HB cross (Tables 1 and 2). In the wings of BH flies, only *mwh* single spots were noticed and large single spots are negligible. These spots are due to mutational events (Valadares et al., 2008). In the present study PbAc was incapable of inducing somatic recombination in flies of BH genotype (Table 2). Here only mutational events lead to wing spots because all somatic recombination events are eliminated owing to multiple inversions present on the *TM3* balancer chromosome and its structurally normal homologue is non-viable in flies of BH genotype (Andrade et al., 2004).

It is a well-known phenomenon that the total *mwh* clones in BH wings are always smaller than those in MH wings, which may be due to clones with induced segmental aneuploidy that show reduced proliferation capacity (Frei et al., 1992). This is best demonstrated in the present study by the values of the total *mwh* clone frequencies in the different concentrations of PbAc tested in MH and BH genotypes of ST or HB crosses (Tables 1 and 2). For example in the ST cross at the concentration of 0.8 mM PbAc induction of total *mwh* clone frequency was 1.39 and 0.44 in MH and BH genotypes respectively. This observation is in agreement with Carmona et al. (2011), who reported that different chemical forms of Pb are an aneugenic compounds. From the frequencies of total *mwh* clones at the higher concentrations in the small and large single spots phenotypes, it can be concluded that PbAc most probably has mutagenic activity. This conclusion is further supported by the fact that no positive results were obtained for the

Table 2
Frequency of wing mutant spots observed in the marker trans-heterozygous descendants (MH; *mwh/flr3*) and balancer heterozygotes (BH; *mwh/TM3*) of *D. melanogaster*, derived from the high bioactivation cross (HB) treated with different PbAc concentrations or positive control(EMS) or negative control.

Genotype	Treatment compound	Con. (mM)	Number of wings (N)	Spots per wing (no. of spots) statistical diagnosis[a]				Total mwh clones[c] (n)	Mean mwh clone size class[b] (î)	Frequency of clone formation per 10^5 cells[d]	
				Small single[b] spots (1–2 cells) (m = 2)	Large single[b] spots (>2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)			Without clone size correction $f_h = (n/NC) \times 10^5$	With clone size correction $f'_h = (2^{(\hat{i}-2)}) \times f_h$
MH; mwh/flr3	NC-without PbAc	0.0	80	0.30(24)	0.05(4)	0.00(0)	0.35(28)	0.29(23)	1.30	1.18	1.91
	PC: EMS	0.1	80	1.69(135)+	0.75(60)+	0.50(40)+	2.94 (235)+	2.78(222)	2.74	11.37	18.98
	PbAc	0.2	80	0.33(26)w	0.05(4)−	0.04(3)i	0.41(33)−	0.35(28)	1.70	1.69	2.08
		0.4	80	0.35(28)w	0.05(4)−	0.05(4)i	0.45(36)i	0.41(33)	1.57	1.69	2.28
		0.6	80	0.40(32)+	0.23(18)i	0.05(4)i	0.68(54)i	0.60(48)	2.03	2.46	2.52
		0.8	80	0.53(42)+	0.28(22)+	0.08(6)i	0.88(70)i	0.80(64)	2.12	3.28	3.57
BH; mwh/TM3	NC-without PbAc	0.0	80	0.23(18)	0.01(1)	[e]	0.24(19)	0.23(18)	1.06	0.92	1.77
	PC: EMS	0.1	80	1.44(115)+	0.48(38)+		1.91(153)+	1.88(150)	2.02	7.68	7.81
	PbAc	0.2	80	0.21(17)w	0.03(2)i		0.24(19)−	0.24(19)	1.28	0.97	1.61
		0.4	80	0.20(16)w	0.03(2)i		0.23(18)−	0.23(18)	1.12	0.92	1.70
		0.6	80	0.24(19)w	0.01(1)i		0.25(20)−	0.25(20)	2.50	1.02	1.45
		0.8	80	0.24(19)w	0.03(2)i		0.26(21)−	0.26 (21)	2.67	1.08	1.71

NC-without PbAc: Negative control; PC: Positive control; EMS: Ethyl methanesulfonate.
[a] Statistical diagnoses following Frei and Wurgler (1988): +, positive; −, negative; i: inconclusive, w: weak positive; m: multiplication factor; significance levels $\alpha = \beta = 0.05$.
[b] Including rare *flr3* single spots.
[c] Considering *mwh* clones from *mwh* single and twin spots.
[d] Frequency of clone formation per 10^5 cells = $(n/NC) \times 10^5$; n: the number of *mwh* clones, N: number of wings examined for each treatment. C: the number of cells scored in each wing (approx. 24,400) (Frei et al., 1992).
[e] Balancer chromosome *TM3* does not carry the *flr3* mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

Table 3
Standardized *mwh* clone induction frequencies per millimolar (mM) PbAc concentration in the *Drosophila* food media and the prevalence of recombination events[a].

Crosses	*mwh/flr3* marker heterozygotes (MH)				*mwh/TM3* balancer heterozygotes (BH)				Recombination (%)	
	Standardized frequency[b] (*mwh* clones per10^5 cells) (f_t)	Mean clone size class ($\hat{\imath}_t$)	Geometric mean of clone size[c] ($2^{\hat{\imath}t-1}$)	Standardized frequency[d] Corrected for clone size ($f'_t = (2^{(\hat{\imath}t-2)}) \times f_t$)	Standardized frequency[b] (*mwh* clones per10^5 cells) (f_h)	Mean clone size class ($\hat{\imath}_h$)	Geometric mean of clone size[c] ($2^{\hat{\imath}h-1}$)	Standardized frequency[d] Corrected for clone size ($f'_h = (2^{(\hat{\imath}h-2)}) \times f_h$)	Without clone size correction	With clone size correction[d]
Standard (ST)	6.16	7.33	2.89	7.33	1.87	3.94	5.66	4.14	76.69	44.92
High bioactivation (HB)	3.55	2.34	2.41	3.60	1.09	3.06	3.63	1.56	79.46	62.89

[a] All values are control corrected. Frequencies in MH and BH are calculated with or without clone size correction.
[b] Clone frequencies per wing divided by the number of cells examined per wing (24400) estimate frequencies per cell and per cell division (Frei and Wurgler, 1988).
[c] Geometric mean and.
[d] Corrections calculated according to Frei et al. (1992).

induction of twin spots (Tables 1 and 2), which are exclusively due to somatic recombination.

The positive results for small and large single spots in MH genotype flies in the ST or HB cross are observed at higher concentrations of PbAc treated groups (Tables 1 and 2), indicating higher concentrations of PbAc is effective in inducing their genotoxicity. The mean *mwh* clone size class in MH and BH genotypes of ST and HB crosses flies were in the range of 1.75 to 3.33 (Tables 1 and 2) revealing an effect not at the end but at the beginning of the treatment, inhibiting more cell division and as a consequence, larger spots formation is limited. PbAc produced a positive small single spot in higher concentrations of 0.6 and 0.8 mM PbAc and significant positive large single spots in 0.8 mM PbAc in flies of MH genotype and weakly positive small single spots and inconclusive large single spots in the flies of BH genotype in HB cross (Table 2) because its constitutively expressed CYP450s eliminated the Pb by hydroxylation before it could exert any inducing or genotoxic activity.

Positive results in the small and large single spot at the higher PbAc concentrations tested and none of the PbAc concentrations induced

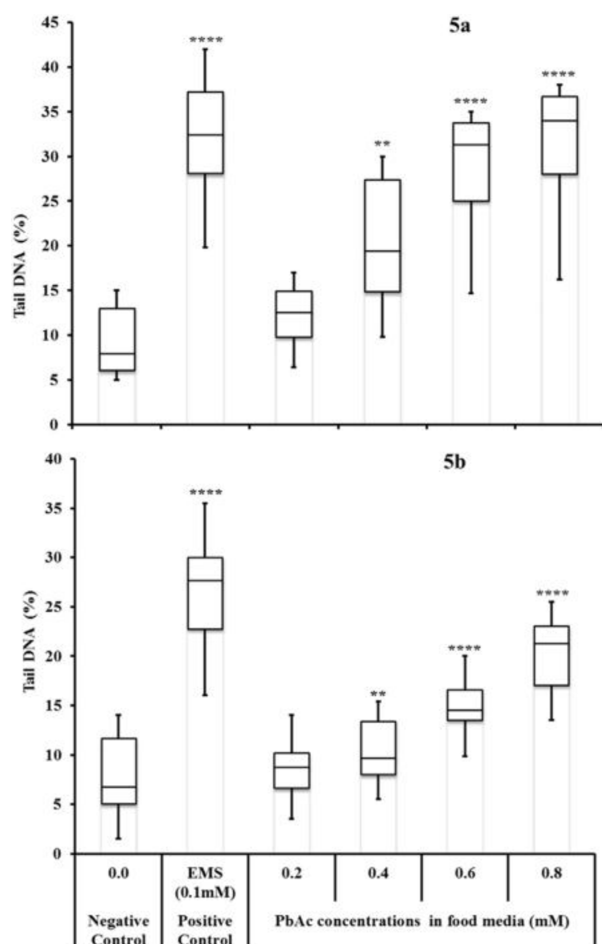

Fig. 5. Pb induced DNA damage measured as percent tail DNA comet parameter using the alkaline (a) and neutral comet (b) assays in third instar larvae (96 ± 4 h) derived from ST crosses reared in the control or PbAc treated groups. Each box plot reflects distribution of data indicating 25th and 75th percentile as box, median as a line within the box, maximum and minimum values as whiskers. Box plot without the asterisk (*) are not significant compared to control at $p > 0.05$; and significant at ** $p < 0.01$; **** $p < 0.0001$ level.

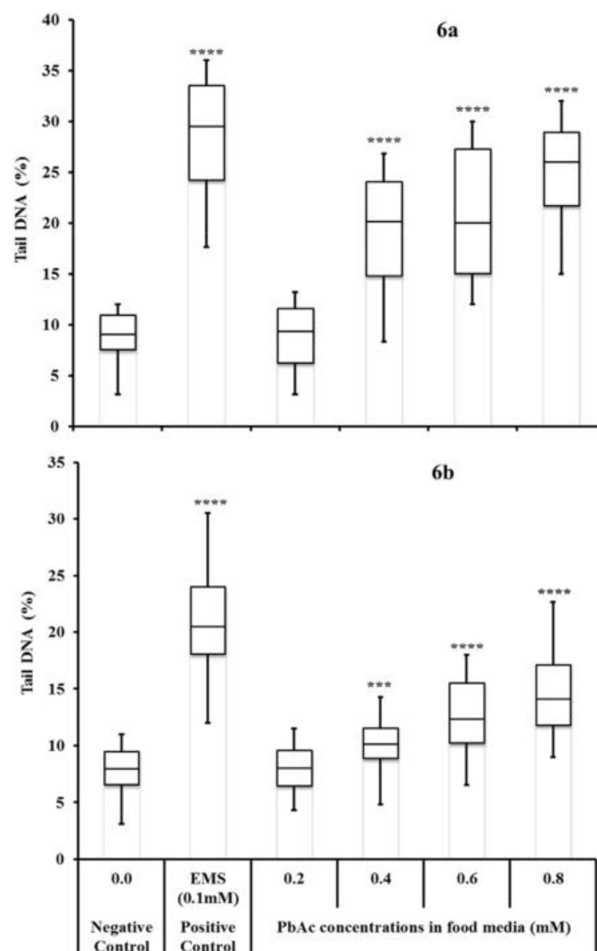

Fig. 6. Pb induced DNA damage measured as percent tail DNA comet parameter using the alkaline (a) and neutral comet (b) assays in third instar larvae (96 ± 4 h) derived from HB crosses reared in the control or PbAc treated groups. Each box plot reflects distribution of data indicating 25th and 75th percentile as box, median as a line within the box, maximum and minimum values as whiskers. Box plot without the asterisk (*) are not significant compared to control at $p > 0.05$; and significant at *** $p < 0.001$; **** $p < 0.0001$ level.

statistically significant positive twin spots or total spots in HB cross compared with that of ST cross data, indicating that cytochrome P450 enzyme is not involved in PbAc biotransformation in *D. melanogaster*.

In view of the positive results for small single spots in MH genotype and weak positive results in small single spots or inconclusive results in large single spots in BH genotype of both ST and HB crosses (Tables 1 and 2); it is difficult to assess the PbAc induced genotoxicity. Moreover, from the current data it is difficult to determine whether mutation (gene and chromosome mutation) or somatic recombination or both events contribute for the induction of mutant spots. Thus, following the method described by Frei and Wurgler (1995) the mutagenic and recombinagenic events were calculated and standardized clone induction frequency per mM PbAc in the *Drosophila* food media was also established. PbAc produced 6.16 and 3.55 standardized *mwh* clone formation frequencies per 10^5 cells per mM in MH genotype of ST and HB crosses respectively (Table 3). From these results it can be concluded that PbAc is a genotoxic compound; it is well established that clone formation frequency per 10^5 cells higher than 2.0 are indicative of genotoxic effect of the particular chemical (Graf et al., 1984). Thus, the clone formation frequencies for both MH and BH genotypes allowed the quantification of recombinagenic action of PbAc. Most genotoxins so far studied for recombinogenicity in the wing spot test show values of ~70% in the range ~ 50–90% (Marcelo et al., 2002; Liliane et al., 2017). Thus, the values obtained in the current study is 45% in ST cross and 63% in HB cross related to the somatic recombination action of PbAc, these values are clearly in the lower range of so far studied genotoxins. This suggests that PbAc may be considered as a mutagenic and weak recombinogenic.

Oxidative stress has been proposed as one of the mechanisms involved in the genotoxicity of Pb (Dobrakowski et al., 2017). This mechanism appears to be the principal cause for the generation of mutant spots. The Pb is capable of creating variety of free radicals per se and by the modulation of heme synthesis pathway. The production of these free radicals acts directly on the mitotic proliferation of imaginal disc cells thereby generating mutant clones. Previous work of Gaivao et al. (1999) has clearly demonstrated the utility of using SMART assay for the evaluation of oxidative stress inducers in generating the wing spot, which supports the current study.

4.5. Pb induced DNA damage in hemocytes

In the present study significantly increased percent tail DNA was observed in PbAc treated groups compared to negative controls indicating Pb induced DNA damage (Figs. 5 and 6). These results provide evidence that DNA damage observed after PbAc exposure could be a consequence of free radical attack to DNA. Since elevated expression of ROS and LPO indicated the generation of oxidative stress by the Pb modulated heme synthesis pathway (Fig. 3a and b), one can presume that the genotoxicity in this might be a product of such oxidative stress. Significantly elevated levels of LPO in workers exposed to low concentrations of Pb have observed oxidative DNA damage (Mohammad et al., 2008; Pawlas et al., 2017). In Pb-exposed workers, a positive significant correlation between MDA and ROS production and DNA breakage was observed (Siddarth et al., 2018).

Generation of ROS and level of LPO was shown to cause chemical modification and alteration in DNA including base modification and strand break (Valko et al., 2006). Evidences indicated that Pb ions take part in fenton reaction generating oxidative free radicals and causing DNA damage (Saima and Arif, 2017). Impairment of antioxidant defenses is another mechanism of oxidative stress induced DNA damage (Gonenc et al., 2012). Pb modulated δ-ALA-D and accumulation of its substrate δ-ALA (Fig. 2a and b) that can be rapidly oxidized to generate free radicals in the present experimental conditions. The final oxidation product of δ-ALA, 4, 5-dioxovaleric acid is an alkylating agent potential to induce DNA damage (Mitra et al., 2017) in the present study.

The formations of DNA single strand breaks (SSBs) are considered as oxidative DNA damage marker induced by oxidative free radical (Trzeciak et al., 2012). The possible mechanism for the occurrence of DNA SSBs in the PbAc treatment could be due to increase in rate of free radical formation. Significant percent tail DNA in alkaline comet as compared with neutral comet (Figs. 5 and 6) suggests that SSBs are formed. As suggested by Trzeciak et al. (2012), in the present study Pb induced DNA SSBs may be due to ROS production which is formed by modulated heme synthesis.

In the current study, Pb is inducing more DNA damage in hemocytes of ST cross larvae compared to HB cross larvae (Figs. 5 and 6) suggested that xenobiotic metabolism in the HB cross with high levels of CYP450 decreases DNA damage. The difference in the regulated and highly constitutive synthesis of CYP450 enzymes in the ST and HB crosses respectively, could result in a high ability to metabolize PbAc faster and thus avoid DNA damage in the HB cross but not in the ST cross. This evidence supports the idea that enzymatic differences may be responsible for the presence or absence of PbAc induced DNA damage. This study in *Drosophila* supports the hypothesis that the magnitude of genotoxicity of Pb could be related to quantitative level of CYP450 hemeprotein enzyme (Fig. 2c).

5. Conclusion

In summary, third instar *Drosophila* larvae derived from the ST or HB cross reared in different sub lethal concentrations of PbAc treated food media showed Pb toxicity. Pb was readily taken up and accumulated in the ST or HB cross larvae. Pb modulated heme synthesis by significant reduction of δ-ALA-D and CYP450 and increased accumulation of δ-ALA. The results have also demonstrated that Pb induced oxidative stress by overproducing ROS and LPO and depleting the antioxidant enzymes such as SOD, CAT, GSH and GST. The positive small and large single spots, negative or inconclusive total spots and twin spots per wing frequencies were observed in the ST and HB crosses of MH genotype flies indicating PbAc is mutagenic. PbAc per mM produced 6.16 and 3.55 standardized *mwh* clone formation frequencies per 10^5 cells in MH genotype of ST and HB crosses respectively showing that PbAc is a genotoxic. The quantification of recombinogencity of PbAc per mM is 45% in ST cross and 63% in HB cross suggests that PbAc is a weak recombinogenic. There was an increased percent of tail DNA in alkaline comet compared to that of neutral comet revealing the DNA SSBs which were the products of Pb modulated heme synthesis pathway induced oxidative free radicals. The level of effect was more pronounced in ST cross-compared to HB cross indicating the difference in the regulated and high constitutive synthesis of CYP450 enzyme. Present study is the first report systematically demonstrated the mechanism of Pb modulated heme synthesis pathway inducing oxidative stress that mediates the genotoxicity. Taken together, current results offer new perspectives on the mechanism of Pb toxicity using *D. melanogaster* as a model organism.

Conflict of interests

None declared.

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