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

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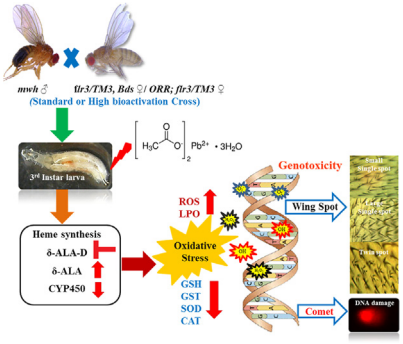
HIGHLIGHTS

- 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*.

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.