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Induction of *hsp70*, *hsp60*, *hsp83* and *hsp26* and oxidative stress markers in benzene, toluene and xylene exposed *Drosophila melanogaster*: Role of ROS generation

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ABSTRACT

Exposure to benzene, toluene and xylene in the human population may pose a health risk. We tested a working hypothesis that these test chemicals cause cellular toxicity to a non-target organism, *Drosophila melanogaster*. Third instar larvae of *D. melanogaster* transgenic for *hsp70*, *hsp83* and *hsp26* and Oregon R^+ strain were exposed to 1.0–100.0 mM benzene, toluene and xylene for 2–48 h to examine the heat shock proteins (hsps), ROS generation, anti-oxidant stress markers and developmental end points. The test chemicals elicited a concentration- and time-dependent significant ($p<0.01$) induction of the hsps in the exposed organism in the order of *hsp70*>*hsp83*≥*hsp26* as evident by β-galactosidase activity after 24 h. RT-PCR amplification studies in Oregon R^+ larvae revealed a similar induction pattern of these genes along with *hsp60* in the order of *hsp70*>*hsp60*>*hsp26*≥*hsp83*. Under similar experimental conditions, a significant induction of ROS generation and oxidative stress markers viz. superoxide dismutase, catalase, glutathione S-transferase, thioredoxin reductase, glutathione, malondialdehyde and protein carbonyl content was observed. Sub-organismal response was propagated towards organismal response i.e., a delay in the emergence of flies and their reproductive performance. While *hsp70* was predominantly induced in the organism till 24 h of treatment with the test chemicals, a significant or insignificant regression of Hsp70 after 48 h was concurrent with a significant induction ($p<0.01$) of *hsp60*>*hsp83*≥*hsp26* in comparison to the former. A significant positive correlation was observed between ROS generation and these hsps in the exposed organism till 24 h and a negative correlation between ROS generation and hsp70 in them after 48 h indicating a modulatory role of ROS in the induction of hsps. The study suggests that among the tested hsps, *hsp70* may be used as an early bioindicator of cellular toxicity against benzene, toluene and xylene and *D. melanogaster* as an alternative animal model for screening the risk posed by environmental chemicals.

Introduction

A variety of chemicals are used in pharmaceutical and chemical industries, and among them monocyclic aromatic hydrocarbons like benzene, toluene and *o*-, *m*- and *p*-xylene isomers are the major ones. These aromatic hydrocarbons are present in gasoline (3–15%), paints, rubber products, plastics, detergents, dyes, pesticides, adhesives and several other household products (Derwent et al., 2000; Kim and Kim, 2002; Chang et al., 2007). Thus benzene, toluene and xylene (collectively known as BTX) are harmful substances in occupational and non-occupation environment and humans are frequently exposed to low levels of these chemicals for prolonged periods (McDermott et al., 2008). In recent years, they acquired great relevance as one of the major pollutants of the outdoor and indoor environment (Lee et al., 2002; Srivastava and Devotta, 2007). Benzene is a well known carcinogen that has been causally linked to leukemia (IARC) (US-EPA, 1996; ATSDR, 2005). However, toluene and xylene are not currently listed as carcinogens (Gallegos et al., 2007). Benzene, toluene and xylene have been reported to induce oxidative stress, an imbalance of the anti-oxidant system. In this context, alteration of the anti-oxidant system such as SOD, GPx, GSH and MDA content in petrochemical workers was reported (Croute et al., 2002; Georgieva et al., 2002).

Stress response and anti-oxidant defense system comprising of stress proteins (also termed as heat shock proteins; Hsps) and anti-oxidants (both enzymatic and non-enzymatic), respectively, are the primary protective responses that are highly conserved components of cellular stress responses found in all phyla from bacteria to man (Lindquist and Craig 1988). Originally discovered as inducible proteins against heat shock, the hsps counter proteotoxic effects and play various roles in a cell including chaperoning proteins

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during synthesis, folding, assembly and degradation (Morimoto, 1993). Other than heat shock, diverse agents including amino acid analogues, transition metal ions, sulphydryl reagents, glucose deprivation, hypoxia etc. have been shown to induce heat shock genes (Nover, 1991). Among the stress genes family, *hsp70* is one of the highly conserved genes and first to be induced in *Drosophila* (Ritossa, 1962, Feder et al., 1992), mammalian cell lines (Farzaneh et al., 2005), parasite and free living protozoa (PeÂrez-Serrano et al., 2000), isopoda (Kohler et al., 1999), freshwater plant species (Ireland et al., 2004), marine teleost (Maradonna and Carnevali, 2007) against various physical (Laubitz et al., 2006), physiological and chemical stressors (Franzellitti and Fabbri, 2005, Lynes et al., 2007). Apart from *hsp70*, other hsps also participate in stress response in organisms exposed to various stresses. Earlier studies have shown that *Drosophila* Hsp83 (homologue of Hsp90 in mammals) and Hsp60 work as chaperone protein refolding system in coordination with or without Hsp70 (Pratt and Toft, 2003; Young et al., 2004). A study by Bagchi et al. (1996) showed that Hsp89a and Hsp89b were significantly induced in PC-12 cells following exposure to selected pesticides. A significant induction of both *hsp70* and *hsp60* in nematodes and isopods and mites after exposure to heavy metals both under field and laboratory conditions respectively were shown indicating their role in biomarker evaluation (Arts et al., 2004; Kohler et al., 2005). A study in *Trichinella spiralis* muscle larvae after exposure to elevated temperature and hydrogen peroxide showed significantly elevated expression of Hsp70, Hsp60 and Hsp90 (Martinez and Rodrıguez-Caabeiro, 1999). Timofeyev et al. (2004) showed that natural organic matter isolated from eutrophic Sanctuary Pond has an adverse impact on a number of amphipod species tested as evidenced by increased Hsp70 expression. Earlier, small hsps like *hsp26* were found to be significantly induced by a chlorinated pesticide, hexachlorocyclohexane in *D. melanogaster* (Kar Chowdhuri et al., 1999) and by a herbicide 2,4-dichlorophenoxyacetic acid in *Saccharomyces cerevisiae* (Simoes et al., 2003). The upregulation of these stress genes in human population after exposure to various environmental stresses was shown by Wu and Tanguay (2006).

Molecular oxygen is the key to aerobic life but is also converted into cytotoxic byproducts referred to as reactive oxygen species (ROS). In addition to their involvement in the normal metabolic activities, ROS have been reported to play a major role in the toxicity of several xenobiotics including metals and pesticides (Fortunato et al., 2006). Aerobic organisms have therefore developed multiple defense mechanisms where the cellular anti-oxidant molecules or the anti-oxidant enzymes remove ROS to rescue cells from oxidative stress (Ahmad et al., 2000). The detection of oxidatively modified molecules such as protein carbonyl (PC) and malondialdehyde (MDA) believed to be specific products of protein and lipid oxidation, respectively have been considered to be reliable markers of oxidative stress (Pragasam et al., 2006). Glutathione (γ-glutamyl cysteinyl glycine) is a highly versatile component of the anti-oxidative system and can directly react with a variety of ROS including superoxide anion, hydroxyl radical and several organic radicals (Hegde and Verma, 2004). Thus evaluation of anti-oxidant stress markers together with stress genes will give an account of the chemical stress caused to the exposed organism.

The present study was aimed to examine the cellular toxicity of benzene, toluene and xylene as individual chemicals per se in *D. melanogaster* with an objective to understand the role of selected stress genes expression (*hsp70*, *hsp83*, *hsp60* and *hsp26*) and anti-oxidant defense systems. The study was further extended to examine the organismal (emergence of the exposed organisms vis-a-vis reproduction) effect of these compounds.

Drosophila as an experimental model has been chosen for its worldwide acceptability due to its defined genetics and molecular biology. The model raises few ethical concerns and is approved by European Centre for the Validation of Alternative Methods (ECVAM) (Festing et al., 1998). Its genome can be easily manipulated by P-element mediated germ line transformation for studying a particular gene of interest under a defined condition (Lis et al., 1983; O'Kane and Gehring, 1987; Adams et al., 2000).

Materials and methods

Fly strains. Transgenic *Drosophila melanogaster* strains viz. (*hsp70-lacZ*) Bg^9 (Lis et al., 1983), (*hsp83-lacZ*) *83Z-880* (Xiao and Lis, 1989) and (*hsp26-lacZ*) *351-94A* (Glaser and Lis, 1990) that express bacterial β-galactosidase as a response to stress and wild type *D. melanogaster* (Oregon R^+) were used in the study. Flies and larvae were reared at 24±1 °C on standard *Drosophila* diet containing agar-agar, maize powder, sugar, yeast, nepagin (methyl-*p*-hydroxy benzoate) and propionic acid. Additional yeast suspension was provided for healthy growth of the organism.

Treatment schedule. Four different concentrations (1.0, 10.0, 50.0 and 100.0 mM) of technical grade benzene (99.7% from Ranbaxy Pvt Ltd., India), toluene (99.5%, SRL Pvt Ltd., Mumbai, India) and xylene (mixture of *o*, *m* and *p*, 99.8%, SRL Pvt Ltd., Mumbai, India) corresponding to different fractions of the LC_{50} (48 h) value of benzene and toluene and xylene in third instar larvae of both Oregon R^+ and transgenic *D. melanogaster* were used during the present study. Larvae were grown on standard *Drosophila* diet, contaminated with or without different concentrations of three test chemicals in DMSO (99.5%, SRL, Mumbai, India) with final concentration of 0.3% (~42.0 mM) in food (Nazir et al., 2003) for 2–48 h. Three control groups were included in the study viz., negative (without any treatment), positive (temperature shock) and DMSO control (0.3% DMSO).

Quantitative determination of benzene, toluene and xylene by GCMS (SPME-HS). Oregon R^+, Bg^9 and *83Z-880* third instar larvae were grown on food contaminated with 100.0 mM benzene, toluene and xylene for 48 h. Three biological replicates (control and treated) were run for each chemical. A Perkin Elmer gas chromatography (USA) coupled with mass selection detector was used for the analysis.

The injection port was fitted with Solid phase micro-extraction (SPME) inlet guide (Supelco, USA) and pre-drilled thermogreen LB-2 septa to secure the SPME fibre holder. A special inlet linear for SPME was used in this study (Supelco, San Diego, USA). Desorption of SPME fibre in GC injection port was 5 min at 250 °C with purge gas off. After sample desorption, the fibre was further heated in the injection port for an additional 30 min at 250 °C with purge gas turned on to remove trace residue in the fibre. The fibre was then retracted into the protection needle and the needle was inserted into a clean vial ready for the next sample extraction.

Headspace extraction procedure was followed by using a 2 ml larval homogenate (10% w/v) prepared in deionized water by placing it in a 10 ml headspace vial. The vials were heated at 65 °C for 30 min with magnetic agitation. After the equilibrium, septa were pierced with SPME needle and the SPME polydimethyl siloxane (PDMS) fibre was exposed to headspace for 15 min to affect the adsorption of the test chemicals in the sample. SPME fibre was collected and inserted directly into the GC injection at 200 °C. Total time of this chromatographic analysis was 27 min. Blank analysis was carried out to avoid any carry over phenomena and/or external contamination between analyses of samples (Alegretti et al., 2004).

Emergence of flies. Newly eclosed first instar larvae of Oregon R^+, Bg^9, *83Z-880* and *351-94A* (±0.5 h) were transferred to normal food, food containing extracting solvent and to food containing different concentrations of test chemicals (50 larvae per vial and 5 vials per