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Potential of casein as a nutrient intervention to alleviate lead (Pb) acetate-mediated oxidative stress and neurotoxicity: First evidence in *Drosophila melanogaster*

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ABSTRACT

Understanding the interaction between dietary protein deficits and neurotoxicants such as lead (Pb) is critical since oxidative stress is a common denominator under such conditions. The *Drosophila* system is an extensively used model to investigate the interaction between nutrients and environmental toxicants. Accordingly, we have examined the hypothesis that casein (CSN) enrichment has the propensity to attenuate Pb-associated phenotype, oxidative stress and neurotoxicity in *Drosophila melanogaster*. Exposure of young (2–3 d) and adult flies (10–12 d old) to Pb acetate (0–20 mM, 7 d) in the medium resulted in a concentration dependent mortality and the survivors exhibited a hyperactive phenotype. While males showed higher susceptibility to Pb among both age groups, young flies were relatively more susceptible than adults. Pb exposure (5–10 mM, 5 d) among young flies caused robust oxidative stress as evidenced by markedly elevated levels of reactive oxygen species with concomitant perturbations in the activities of antioxidant enzymes (diminished SOD and elevated thioredoxin reductase) and altered redox state. Further, Pb caused significant elevation in the activity of acetylcholinesterase and dopamine levels. In a satellite study, we assessed the modulatory effect of CSN-enriched diet (1–2%) on Pb intoxication in terms of lethality, hyperactivity, oxidative stress and neurotoxicity. CSN markedly offset Pb-induced lethality and diminished the hyperactivity response. While CSN enrichment among Pb (5 mM) treated flies caused further elevation in ROS levels and thioredoxin reductase activity, the SOD levels were restored to normalcy. Further, CSN improved the activity levels of complex I–III and restored the dopamine levels. Our data suggest that Pb-induced toxicity in the *Drosophila* system may be predominantly mediated through oxidative stress mechanisms and the propensity of casein-enriched diet to abrogate such responses. Hence, we propose that enrichment of diet with protein such as casein may be a useful approach to alleviate Pb associated adverse effects in children.

1. Introduction

Lead (Pb) is a ubiquitous, persistent and non-essential toxic heavy metal which can be detected in almost all areas of the environment and biological systems (Xu et al., 2008). The role of Pb as a neurological toxicant is well established and its exposure has been associated with reductions in cognitive function, hearing loss, hyperactivity, shortened concentration spans and poor school performance in children (Tong et al., 2000). Epidemiological evidence suggests Pb to be a high environmental risk factor for the development of attention-deficit hyperactivity disorder (ADHD) (Froehlich et al., 2009; Luo et al., 2014). Several surveys have also indicated higher blood Pb levels among children in cities in India (Kumar and Scott Clark, 2009) and its correlation with increased neurobehavioral deficits/ADHD. A recent review has illustrated the scenario of Pb exposure in South Africa and concluded that not only children, but a large numbers of people are at risk of Pb exposure and concomitant health, neuro-developmental and social effects (Mathee, 2014).

Lead (Pb) exposure has been demonstrated to cause generation of excessive amount of ROS and perturbations of antioxidant defense systems in animals (Adonaylo and Oteiza, 1999; Bokara et al., 2008; Neal and Guilarte, 2010; Prasanthi et al., 2010) and in occupationally exposed workers (Gurer-Orhan et al., 2004).

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Substantial experimental evidence has shown that Pb is capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules. Hence there is general consensus that oxidative stress mechanisms play a predominant role in the observed adverse effects of Pb such as impaired learning ability in children. A recent study in rats has shown that pre- and neonatal exposure that results in Pb concentrations below the level currently thought to be safe (10 μg/dL) in offspring blood resulted in disruption of the pro/antioxidant balance as well as down regulation of mRNA and protein expression especially in the hippocampus (Baranowska-Bosiacka et al., 2012).

To alleviate Pb associated neurotoxic effects, various strategies such as chelators, antioxidants, their combination and phytoconstituents (e.g. phenolic compounds) have been attempted (Liu et al., 2013; Reckziegel et al., 2011; Khalaf et al., 2012; Kumar and Muralidhara, 2014). It is well known that Pb exposure is often greater among children of low socioeconomic status (SES) (Toscano and Guilarte, 2005) and low SES is a powerful predictor of neurodevelopment (Tong et al., 2007). Besides, experimental and epidemiological data suggest that SES might also modify Pb neurotoxicity (Bellinger, 2008). Current therapeutic approaches in the treatment of childhood Pb intoxication are not effective in reversing learning deficits once they have occurred and application of the conventional chelators in children is somewhat prohibited by adverse health effects. Hence, researchers have focused on the use of selected nutrients such as methionine and choline, to prevent Pb-induced cognitive impairment (Fan et al., 2010). Low protein levels in the diet and the consequent decrease in essential amino acids are known to significantly alter the antioxidant system and the redox state in the hippocampus (Bonatto et al., 2005; Feoli et al., 2006; Tatli et al., 2007). A recent study demonstrated that postnatal protein malnutrition (PMN) induces several neurochemical alterations leading to behavioral deficits in rats (Adebayo et al., 2014).

Chronic exposure to environmentally relevant levels of Pb during early life is shown to alter morphology and neurogenesis in the hippocampus of young rats (Verina et al., 2007). Recent evidence in rats has convincingly demonstrated that pre- and neonatal exposure to Pb induces ultrastructural/molecular alterations in the hippocampus and alters postnatal cholinergic and aminergic systems (Baranowska-Bosiacka et al., 2012; Basha et al., 2012). Epidemiological evidence have shown that malnutrition (MN), a worldwide problem affecting millions of unborn/young children during the most vulnerable stages of their brain development results in behavioral abnormalities, cognitive dysfunctions and impaired learning/memory (Morgane et al., 2002; Lister et al., 2005; Cardoso et al., 2013). Further, pre and early postnatal MN produces behavioral impairments, memory deficits (Lukoyanov and Andrade, 2000; Almeida and De Araújo, 2001; Valadares and de Sousa Almeida, 2005) and deleterious effects on the population of GABA neurons in the dentate gyrus and cornu ammonis of the dorsal hippocampus (Díaz-Cintra et al., 2007). In the area of neuroprotection, currently, nutrition intervention strategies are being considered as therapeutic since they may play a preventive or suppressive role (Virmani et al., 2013). Hence it may be important to investigate whether dietary protein (quantity and quality) has the potential to influence the adverse effects of Pb in animal models. It is in this context that we have employed *Drosophila* as a model to examine the ameliorative potential of casein-enriched diet under Pb exposure.

Drosophila melanogaster has been extensively used to understand the pathophysiology and genetics of several human neurodegenerative diseases (Botella et al., 2009; Feany, 2010). *Drosophila* has distinct advantages with respect to life cycle duration, laboratory expenses, genetic manipulability, efficiency of screening methods and conservation with higher organisms (Rand, 2010). In our laboratory, we have successfully employed the *Drosophila* system to obtain insights into the neuromodulatory propensity of spice bioactives and phytochemicals (Prasad and Muralidhara, 2012; Girish and Muralidhara, 2012; Hosamani and Muralidhara, 2009). Some researchers consider *Drosophila* an excellent animal model for studying the neurotoxicology of lead (Hirsch et al., 2003, 2012). Substantial evidence suggests that protein is the major dietary component affecting oxidative stress and longevity in flies (Bruce et al., 2013; Pichaud et al., 2013). To the best of our knowledge no attempts have been made to determine if casein-enrichment can alleviate Pb-induced adverse effects in animal models. Hence, we hypothesized that casein enrichment is likely to modulate the susceptibility of flies to Pb exposure and may provide a dietary approach to alleviate low-level Pb exposure in children in general and those subjected to protein deficiency conditions, in particular. With this objective, we determined toxicity profiles for Pb acetate in two different age groups of *D. melanogaster*. The induction of oxidative stress, mitochondrial dysfunction and neurotoxicity was assessed in young male flies. Subsequently, the potential of casein enrichment to alleviate Pb-induced oxidative stress and neurotoxicity was investigated.

2. Materials and methods

2.1. Chemicals

Lead (Pb) acetate trihydrate, reduced glutathione, 5-aminolevulinic acid hydrochloride, NADH, dopamine and DCFHDA were procured from Sigma Chemical Co., St. Louis, USA. Purified casein was procured from Himedia. 2,4-Dinitrophenylhydrazine (DNPH), Nicotinamide Adenine Dinucleotide reduced (NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitro benzene (CDNB) and other chemicals used were of analytical grade.

2.2. Drosophila culture and husbandry

D. melanogaster, wild (Oregon K) were originally procured from the National Stock Facility, Manasagangothri, University of Mysore, Karnataka, India. Flies were maintained and cultured at the fly laboratory of our research institute under standard conditions (22 ± 1 °C, 70–80% relative humidity) on a standard wheat flour-agar diet with yeast granules as the protein source (Hosamani and Muralidhara, 2009). For all the studies, age-synchronized young (2–3 d old) or adult (10–12 d old) male flies (50/replicate; 3 replicates per dose) were introduced into glass vials with 2 mL synthetic medium containing the test compounds.

2.3. Recapitulation of lead (Pb)-induced adverse effects in flies

2.3.1. Susceptibility pattern of young and adult flies to Pb and flying speed phenotype

In the first set of experiments, both young and adult male flies maintained on media containing sucrose-agar diet (Good and Tatar, 2001) were exposed to varying concentrations (1, 5, 10 and 20 mM) of Pb acetate in a 7 day treatment protocol in order to assess their susceptibility pattern in terms of lethality. In a second set of experiments, young flies were exposed to Pb at three concentrations (1, 5 and 10 mM) in the diet for 5 days to determine hyperactivity phenotype.

2.3.2. Effect of Pb on biochemical markers in young flies

In the third set of experiments, young flies were subjected to Pb exposure (1, 5 and 10 mM, 5 d) and induction of oxidative stress and neurotoxicity were assessed. Since Pb is known to disrupt the pro- and antioxidant balance of the developing hippocampus, we