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Effects of methylmercury and alcohol exposure in *Drosophila melanogaster*: Potential risks in neurodevelopmental disorders

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ABSTRACT

Extensive evidence suggests the role of oxidative stress in autism and other neurodevelopmental disorders. In this study, we investigated whether methylmercury (MeHg) and/or alcohol exposure has deleterious effects in *Drosophila melanogaster* (fruit flies). A diet containing different concentrations of MeHg in *Drosophila* induced free radical generation and increased lipid peroxidation (markers of oxidative stress) in a dose-dependent manner. This effect of MeHg on oxidative stress was enhanced by further exposure to alcohol. It was observed that alcohol alone could also induce free radical generation in flies. After alcohol exposure, MeHg did not affect the immobilization of flies, but it increased the recovery time in a concentration-dependent manner. MeHg significantly inhibited the activity of alcohol dehydrogenase (ADH) in a dose-dependent manner. Linear regression analysis showed a significant negative correlation between ADH activity and recovery time upon alcohol exposure in the flies fed a diet with MeHg. This relationship between ADH activity and recovery time after alcohol exposure was confirmed by adding 4-methyl pyrazole (an inhibitor of ADH) to the diet for the flies. These results suggest that consumption of alcohol by pregnant mothers who are exposed to MeHg may lead to increased oxidative stress and to increased length of time for alcohol clearance, which may have a direct impact on the development of the fetus, thereby increasing the risk of neurodevelopmental disorders.

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1. Introduction

The Centers for Disease Control and Prevention (CDC) estimates the prevalence of developmental disorders such as attention deficit hyperactivity disorder, intellectual disability, cerebral palsy, autism, seizures, hearing loss, blindness, learning disorders as 1 in every 6 children in the United States (Boyle et al., 2011). Several lines of evidence point towards industrial chemicals as potential risk factors for the development of neurobehavioral disorders (Chauhan and Chauhan, 2015; Dickerson et al., 2015; Andersen et al., 2000; Grandjean and Landrigan, 2006). The U.S. National Research Council has reported that interactions between environmental factors and inherited genetic susceptibilities increase the risk of developmental disabilities (Landrigan et al., 2012). We have recently reported that bisphenol A (BPA), an environmental toxin, induces oxidative stress in human lymphoblasts (Kaur et al., 2014) and affects the behavior of *Drosophila melanogaster* (fruit flies) (Kaur et al., 2015). The human brain is more vulnerable to xenobiotics during prenatal development and in infants than in adults because the placenta does not provide effective protection against environmental toxicants (Andersen et al., 2000), and the blood-brain barrier, which does provide this protection, is not formed for up to six months after birth (Adinolfi, 1985). Therefore, exposure to environmental toxins at low levels during early fetal development can cause greater brain injury than exposure to the same levels of these environmental risk agents in adults (Grandjean and Landrigan, 2006).

Many reports suggest that heavy metals adversely affect neurodevelopment and increase the risk of autism spectrum disorders (ASDs) (Chauhan and Chauhan, 2015; Abdullah et al., 2012). Among the heavy metals, mercury (Hg) is one of the most dangerous environmental pollutants (Anderson, 2008). Some sources of Hg in the atmosphere are volcanoes, forest fires, and volatilization from the ocean. Metallic and inorganic Hg released into the atmosphere is brought down by rain, which is transformed to methylmercury (MeHg) by anaerobic organisms in soil and water. MeHg then

Abbreviations: ASDs, autism spectrum disorders; BPA, bisphenol A; MeHg, methylmercury; ADH, alcohol dehydrogenase; CDC, Centers for Disease Control and Prevention; CHARGE, Childhood Autism Risk from Genes and Environment; DCFH, 2′,7′-dichlorofluoroscein; FASD, fetal alcohol spectrum disorder; ROS, reactive oxygen species; 5-HT, 5-hydroxytryptamine.

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bioaccumulates in fish (Palmer et al., 2006; Huang et al., 2012), which are later consumed by humans.

Alcohol exposure during pregnancy is another risk factor for neurodevelopmental disorders. Intrauterine exposure of the human fetus to alcohol can cause the fetal alcohol spectrum disorder (FASD) (Jones and Smith, 1973; Chokroborty-Hoque et al., 2014). The features of FASD are neurobehavioral disturbances ranging from hyperactivity and learning disabilities to depression and psychosis (Chokroborty-Hoque et al., 2014; Coles, 2011; Hellemans et al., 2010). The brain is particularly sensitive to the neurotoxic effects of ethanol during the period of synaptogenesis (West, 1987). Alcohol dehydrogenases (ADHs) are key metabolic enzymes for ethanol and are also involved in the metabolic pathways of several neurotransmitters that are implicated in the neurobiology of neuropsychiatric disorders. Zuo et al. (2013) reported an association between common ADH gene variants and autism as well as schizophrenia.

Drosophila melanogaster is a widely used model for studies of brain disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, fragile X, and Angelman syndrome (Mackay and Anholt, 2006). Although flies and humans are distinctly different from each other, many molecular processes are conserved between them. Among 59 of the human neurological genes that have been examined, 38 have orthologs in the *Drosophila* genome (Rubin et al., 2000). The advantage of studying neurobehavioral disorders in *Drosophila* is the presence of genes that are similar to human genes for normal cognitive functions, as a result of phylogenetic conservation of these genes (Restifo, 2005). Furthermore, *Drosophila* is an attractive model for studies on environmental toxins because of its quicker generation time and easy maintenance of the cultures. We have previously reported that flies' exposure to BPA affects their behavior, including disturbances in locomotion pattern, repetitive behavior, and social interaction (Kaur et al., 2014). In this study, we analyzed the effects of MeHg and alcohol exposure (alone and in combination) using *Drosophila melanogaster* as a model.

2. Material and methods

2.1. Drosophila melanogaster

Wild-type Oregon-R *Drosophila* stocks were maintained at 25 °C on a standard cornmeal diet (Jazz-mix *Drosophila* food, Fisher Scientific, Pittsburgh, PA) under 12 h:12 h light and dark cycle.

2.2. Diet containing MeHg

A stock of 100 mg MeHg/ml dimethylsulfoxide (DMSO) was prepared. Various concentrations of MeHg were mixed with 7 ml of food for *Drosophila*. The DMSO concentration was kept constant for control and experimental samples. The flies were fed this diet for 8 days. None of the flies died that were fed 0.1 mg–0.5 mg MeHg per 7 ml in the diet for 8 days.

2.3. Alcohol (ethanol) exposure

After 8 days on the diet with MeHg, the flies were further exposed to alcohol. Fifty flies were transferred to culture vials, and the flugs of the vials were replaced with flugs soaked with 1 ml of absolute ethanol. The flies in these vials were observed to be immobilized within 8–9 min. After the flies were immobilized, fresh flugs without alcohol were placed in the vials, and the flies were allowed to recover from the effects of alcohol. The recovery time, by which all the flies started moving again, was noted.

2.4. Diet containing 4-methyl pyrazole (inhibitor of alcohol dehydrogenase, ADH)

The food for *Drosophila* was prepared and allowed to solidify. The food (0.5 g) was mixed with 498 and 996 μg of 4-methyl pyrazole and was fed to 50 flies. After 4 days, the flies were removed for the experiments.

2.5. Measurement of ADH activity

Fifty flies were homogenized in 500 μl of 50 mM Tris-HCl, pH 8.5, followed by centrifugation at 10,000*g* for 30 min. The supernatants were collected, and protein content was measured using the total protein kit from Sigma-Aldrich. ADH activity, expressed as nmol NADH released/mg protein/min, was measured in the supernatants following the method of Fibla and Gonzalez-Duarte (1993). In brief, a standard curve was obtained by serial dilution of 2 mM NADH. 50 μl of samples were added to the wells in a microtiter plate. The reaction was started by adding 150 μl of reagent solution (330 μM NAD^+, 330 μM nitroblue tetrazolium, and 8 μM phenazine methosulphate in 50 mM Tris-HCl, pH 8.5). After 10 min of incubation in the dark at room temperature, the absorbance was measured at 590 nm.

2.6. Measurement of lipid peroxidation

Malonyldialdehyde (MDA) is an end product of lipid peroxidation, and a marker for oxidative stress. The flies were fed a diet containing 0.1, 0.3, 0.5, 0.7 mg MeHg/7 ml for 8 days. Fifty flies were homogenized in 250 μl of 0.1 M potassium phosphate buffer, pH 7.4, and buffer containing 0.05% mercaptoethanol, followed by centrifugation at 10,000*g* for 10 min. MDA content in the supernatants was measured as described previously (Chauhan et al., 2004). The supernatants (100 μl) were mixed with 300 μl of thiobarbituric acid (TBA) solution containing 0.37% (w/v) TBA—15% w/v trichloroacetic acid (TCA)-0.25 M HCl. The samples were heated in a boiling water bath for 15 min and then allowed to cool to room temperature. The samples were centrifuged for 10 min, and the absorbance was measured at 535 nm. The MDA content in the samples was calculated by using the molecular coefficient for MDA at 1.56×10^5.

2.7. 2′,7′-dichlorohydrofluoroscein (DCFH) fluorescence assay for generation of reactive oxygen species (ROS), i.e., free radicals

The protein content of supernatants obtained from different samples was adjusted to a constant value, and the oxidation of DCFH as an index of free radical generation and oxidative stress was measured. In brief, the reaction mixture contained 150 μl of 0.1 M potassium phosphate buffer, pH 7.4; 40 μl of distilled water; 5 μl of DCFH-DA (200 μM stock, final concentration of 5 μM); and 5 μl of the diluted (1:2) sample. The fluorescence emission of 2′,7′-dichlorofluorescin resulting from DCFH oxidation was monitored at the excitation wavelength of 488 nm and the emission wavelength of 525 nm at 30-s intervals for 10 min.

2.8. Data analysis

The statistical analysis of the data was performed by using GraphPad Prism 5 (GraphPad Software, Inc.). The statistical significance between the experimental and control groups was examined by one-way ANOVA (Dunnette), and p values less than 0.05 were considered significant. To evaluate the correlation between ADH activity in the presence of different concentrations of MeHg or 4-methylpyrazole and recovery time after alcohol exposure, linear

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