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Plasma phthalate and bisphenol a levels and oxidant-antioxidant status in autistic children

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ABSTRACT

Phthalates and bisphenol A (BPA) are endocrine disruting chemicals (EDCs) that are suggested to exert neurotoxic effects. This study aimed to determine plasma phthalates and BPA levels along with oxidant/antioxidant status in autistic children [n = 51; including 12 children were diagnosed with "Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS)]. Plasma levels of BPA, di (2-ethylhexyl)-phthalate (DEHP) and its main metabolite mono (2-ethylhexyl)-phthalate (MEHP); thiobarbituric acid reactive substance (TBARS) and carbonyl groups; erythrocyte glutathione peroxidase (GPx1), thioredoxin reductase (TrxR), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) activities and glutathione (GSH) and selenium levels were measured. Plasma BPA levels of children with PDD-NOS were significantly higher than both classic autistic children and controls (n = 50). Carbonyl, selenium concentrations and GPx1, SOD and GR activities were higher ($p < 0.05$); CAT activity was markedly lower in study group. BPA exposure might be associated with PDD-NOS. Intracellular imbalance between oxidant and antioxidant status might facilitate its neurotoxicity.

1. Introduction

Prevalence of 'Autism Spectrum Disorders (ASDs)' has increased dramatically (1 in 68 in United States) (Centers for Disease Control and Prevention, 2011). However, their etiology is not well understood and it is hypothesized that both genetic and environmental factors are involved. Chemicals such as lead, methylmercury, organic solvents, endocrine disrupting chemicals (EDCs), and pesticides are suspected causes of developmental neurotoxicity (Grandjean and Landrigan, 2006), though there are no well-designed studies about the role of these chemicals in ASDs.

Endocrine disrupting chemicals are man-made chemicals that disrupt the physiological function of endogenous hormones (World Health Organization, 2012). Phthalates and bisphenol A (BPA) are the most abundant EDCs in the environment. These substances are plasticizers that are available in a large number of consumer products. Humans are exposed to these chemicals mainly by food and drink. On the other hand, dermal and indoor air exposure are also important sources (World Health Organization, 2012; Braun et al., 2013; Rubin, 2011). Phthalates are anti-androgenic chemicals that are present in many commercial products such as personal-care products, plastic materials, food packages, detergents and paints. The most abundant phthalate in food and environment is di-(2-ethylhexyl)-phthalate (DEHP) and its major metabolite is mono-(2-ethylhexyl)-phthalate (MEHP) (World Health Organization, 2012; Braun et al., 2013). BPA is a plasticizer that is used to harden plastics. It is regarded as a weak

Abbreviations: ASDs, autism spectrum disorders; BPA, bisphenol A; CAT, catalase; CV, coefficient of variation; DEHP, di-2-ethylhexyl-phthalate; DSM-V, diagnostic and statistical manual of mental disorders-V; EDCs, endocrine disrupting chemicals; GPx1, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; H_2O_2, hydrogen peroxide; LOD, limit of detection; LOQ, limit of quantification; LP, lipid peroxidation; MDA, malondialdehyde; MEHP, mono-2-ethylhexyl-phthalate; PCV, polyvinyl chloride; PDD-NOS, pervasive developmental disorder-not otherwise specified; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TNB, 5-thio-2-nitrobenzoic acid; TrxR, thioredoxin reductase; WST-1, cell proliferation reagent; XO, xanthine oxidase.

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estrogenic chemical that is available in a large number of consumer products (World Health Organization, 2012; Rubin, 2011).

Neurotoxic mechanisms of EDCs or timing of this particular toxic effect are not clearified yet. As phthalates have both anti-androgenic or weak estrogenic activity and BPA exerts weak estrogenic activity, they might interfere with hormone-sensitive periods of neural development such as neuronal differentiation, growth, and synapse formation (Fox et al., 2010; Tareen and Kamboj, 2012; Colborn, 2004). Recent studies reported that phthalates were associated with decreased mental and psychomotor index scores (Kim et al., 2011; Whyatt et al., 2012; Téllez-Rojo et al., 2013), behavior and emotional problems (Whyatt et al., 2012; Engel et al., 2010; Swan et al., 2010), social impairment (Miodovnik et al., 2011), *attention deficit hyperactivity disorder* (Kim et al., 2009). On the other hand, BPA was associated with behavioral problems (Fox et al., 2010; Braun et al., 2009; Braun et al., 2011; Perera et al., 2012). Moreover, two separate studies, which reported higher urinary concentrations of some DEHP metabolites and BPA in autistic children compared with healthy controls, were published before (Stein et al., 2015; Testa et al., 2012). Only few studies showed that these EDCs induced oxidative stress and imbalance in oxidant/antioxidant status (Kaur et al., 2014; Erkekoglu et al., 2014).

Taking into account all the available data, we aimed to evaluate plasma phthalates and BPA levels and the oxidant/antioxidant status in autistic children. To our knowledge, this is the first study investigating DEHP, MEHP and BPA exposure in autistic children along with oxidant/antioxidant status.

2. Materials and methods

2.1. Subjects

The study group comprised of 51 autistic children (mean age: 5.8 ± 2.5 years), admitted to Erciyes University Child Psychiatry Clinic in Kayseri between July 2011 and August 2012. Autistic children with associated neurologic, metabolic and genetic disorders were excluded from the study group. Since Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V) had not been published yet, all the patients with ASDs were diagnosed according to DSM-IV-Text Revision (APA, 2013) and Childhood Autism Rating Scale. Only children with classical autism and PDD-NOS were included in the study. Age and gender matched 50 healthy children (mean age: 5.6 ± 2.5 years) who were admitted to Erciyes University Children's Hospital Healthy Child Clinic comprised the control group. A questionnaire was applied to parents to determine potential exposure ways to EDCs. The study was approved by Erciyes University's Ethical Committee. Written informed consent was obtained from the parents before participation.

2.2. Preparation of plasma and erythrocyte sample

Venous blood samples were taken by a stainless steel needle from the left arm cubital vein, and the sample was allowed to drop directly into heparinized glass test tubes. The tube openings were covered by clean aluminum foil to protect the sample from contacts with plastic material. Samples were centrifuged immediately at 800*g* for 15 min to obtain plasma and erthyrocyte. Both plasma and erythrocyte samples were aliquoted and kept at −80 °C until analysis.

2.3. Chemicals, kits and equipment

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All high performance liquid chromatography (HPLC) equipments were from Agilent (Santa Clara, CA). Colorimetric assay kits for protein determination, glutathione peroxidase (GPx1), thioredoxin reductase (TrxR) and catalase (CAT) were obtained from Sigma-Aldrich (St. Louis, MO). Glucuronidase/aryl sulfatase (from *Helix pomatia*) was also from Sigma-Aldrich. Total superoxide dismutase (SOD), glutathione reductase (GR), total glutathione (GSH) and thiobarbituric acid reactive substance (TBARS) kits were obtained from Cayman Chemical Company (Ann Arbor, MI).

2.4. Measurement of di-(2-ethylhexyl)-phthalate (DEHP) and mono-(2-ethylhexyl)-phthalate (MEHP) levels in plasma

All glass tubes which venous blood samples were taken were heated in an oven at 400 °C for 4 h and all the glassware were washed with n-hexane:tetrahydrofuran (1:1, v/v) for 4 h and than dried in an incubator to after the general cleaning procedure to remove any plastic material residue.

DEHP and MEHP levels was detected by HPLC after extraction from plasma according to Paris et al. (2003) with some modifications. Briefly, 200 μl of plasma was spiked with 20 μl 20 ppm DEHP (1 ppm in the last volume) and 20 μl 20 ppm MEHP (1 ppm in the last volume). After extraction by 400 μl NaOH (1 N), 100 μl%50 H_3PO_4 and 600 μl asetonitrile, samples were vortexed for 1 min. The mixture was centrifugated at 1000*g* for 10 min. The extraction was repeated and supernatants were collected. 900 μl of the supernatant was taken into another tube and evaporated under nitrogen stream. The residues were kept at −20 °C until analysis. Later, residues were dissolved in 400 μl of 60% acetonitrile and 100 μl of these resultants were injected into our HPLC (Hewlett Packard Agilent 1200 Series with Fluorescence Detector, Vienna, Austria). The retention times for DEHP and MEHP were 39.3 min and 4.7 min, respectively. Recovery studies were performed on blank samples of plasma spiked with levels of 7.5 μg/ml of DEHP and 1.25 μg/ml of MEHP, and the average recoveries were found to be (mean ± SD) 93.41 ± 23.41 for DEHP and 82.65 ± 0.97% for MEHP on 10 occasions. Within-day precisions were (% CV) for DEHP 1.12 ± 0.56% and 4.15 ± 1.73% for MEHP. Between-run precisions were 10.31 ± 16.09% coefficient of variation (CV) for DEHP and 8.42 ± 4.42% CV for MEHP. The concentrations of DEHP and MEHP in the samples were calculated from DEHP and MEHP standards and the calibration curve of peak area was used. Limit of detections (LOD) for both DEHP and MEHP were 0.05 μg/ml, and limit of quantifications (LOQ) were for both DEHP and MEHP were 0.1 μg/ml. Determination of DEHP and MEHP concentrations was conducted by HPLC equipped with an auto sampler (Hewlett Packard Agilent 1100 Series, Vienna, Austria) using a UV detector. Spherisorb C18 ODS2 kolon (25 cm × 5 μm × 4.6 mm i.d.) column (Waters, Milford, MA) and ODS C18 precolumn (4 cm) (Waters, Milford, MA) were used for analysis. The mobile phase was 0.1% ortophosphoric acid and acetonitrile [pH 3.0, 80:20 (v/v)], and the flow rate was 1 ml/min.

2.4. Bisphenol a analysis in plasma

BPA was detected by HPLC after extraction from plasma according to Yang et al. (2003). For total BPA (conjugated plus free form), 500 μl plasma sample was spiked with 50 μl 50 ng/ml BPA (5 ng/ml spike in the last volume) and 30 μl of 2.0 M sodium acetate buffer (pH 5.0) was added to the mixture. 10 μl glucuronidase/aryl sulfatase (from *H. pomatia*) was added and mixed. The mixture was incubated 37 °C for 3 h at water bath. After incubation, 100 μl 2 N HCl was added and the mixture was extracted with 5 ml of ethyl acetate. Later, sample was centrifugated at 800*g* for 5 min 3 ml of supernatant was taken to a new glass tube, evaporated under nitrogen stream and the residues were kept at −20 °C until analysis. On the experiment days, the residues were dissolved in 300 μl of 60% acetonitrile and 100 μl of the resultants were injected into our HPLC. HPLC parameters were as follows: C18 col-