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Determination of dibutyl phthalate neurobehavioral toxicity in mice

Vahid Farzanehfar [a], Nima Naderi [a], Farzad Kobarfard [b], Mehrdad Faizi [a, *]

[a] Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Dibutyl phthalate (DBP) is widely used as plasticizer in numerous kinds of products such as plastic packaging in food industries. There is a high risk of DBP exposure for human; it can easily migrate into the human bodies through food plastic packaging and be a potential hazard for human health. In this study the neurobehavioral effects of oral DBP for 14 days (6.25, 12.5, 25, 50, 100 and 200 mg/kg) were investigated in mice, using open field, Y-maze, elevated plus maze, passive avoidance test, rotarod and grip strength test. The results showed that DBP could reduce total distance movement, impair memory function and induce anxiety in mice. Histological analysis (haematoxylin-eosin staining) also showed significant nuclei size reduction and condensation in dentate gyrus cells of the DBP treated mice. In conclusion oral DBP administration for 14 days may cause some neurobehavioral adverse effects in mice.

1. Introduction

Phthalate esters are chemical compounds which are used as additives in different materials to make them more flexible. Numerous types of phthalate esters have been developed and there is a high risk of exposure to these chemicals for human. Dibutyl phthalate (DBP) is an ester of phthalic acid and is widely used as plasticizer in children toys, medical devices, nutritional supplements and different kinds of packaging (Blount et al., 2000). It is already shown that phthalate esters have endocrine disrupting properties and may impair locomotor activity of the amphipod *Gammarus pulex* (Sorensen, 2006; Thuren and Woin, 1991). DBP has developmental and reproductive toxicity such as reduction of estrogen binding to the specific receptors and inhibition of estrogen's transcriptional activity (Jobling et al., 1995).

A previous study has shown that DBP causes a delay in female sexual maturation and deterioration of the sperm quality in rodents (Dobrzynska et al., 2011). In another study 35 μg/l of DBP for 22 days changed nesting behavior, plasma androgen concentrations and steroidogenic gene expression in fish, suggesting that DBP has anti androgenic effects in fish (Aoki et al., 2011). DBP exposure may cause cryptorchidism (Johnson et al., 2008) and also induces hypospadias in male offsprings of rats following in utero exposure (Zhu et al., 2009).

Abdul-Ghani et al. (2012) indicated that DBP can induce teratogenic and behavioral teratogenic effects in a chick model. There are evidences suggesting that DBP in pregnant rats' diet can produce some adverse effects on the behavioral parameters and may alter cognitive abilities in their pups (Li et al., 2009). Furthermore, DBP causes a decrease in grooming behavior of the rats born from mothers which were exposed to DBP during gestation period (Hoshi and Ohtsuka, 2009).

DBP is used illegally as a clouding agent in yogurt, juices and beverages in food industries (Yen et al., 2011). Previous investigations also indicated some adverse effects caused by DBP in human. Kim et al. (2009) showed a strong positive relationship between mono butyl phthalate (major metabolite of DBP) in urine and symptoms of Attention-Deficit/hyperactivity disorder (ADHD) among children (Kim et al., 2009). In addition, Cho et al. (2010) found a significant inverse relationship between DBP metabolites in urine and children's vocabulary sub scores (Cho et al., 2010).

Although considerable investigations have been carried out in to the different aspects of DBP toxicity, but a few of them focus on the neurobehavioral toxicity of DBP in adult rodents. The aim of the present study is to evaluate the neurobehavioral toxicity of DBP using mice models.

* Corresponding author.
E-mail address: m.faizi@sbmu.ac.ir (M. Faizi).

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2. Material and methods

2.1. Animals

All experiments were carried out on male NMRI mice weighing 20–25 g, purchased from Pasteur Institute, Iran. The animals were housed under standard animal laboratory conditions in groups of ten per each cage, in a room under 12 h light-dark cycles, ventilation and controlled temperature of 22 ± 2 °C with free access to Standard mouse diet and water. The mice were used after one week adaptation and handling period. All experiments were conducted based on the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals

Dibutyl phthalate (Sigma-Aldrich, St. Louis, MO, USA); Scopolamine methyl nitrate (Sigma-Aldrich, St. Louis, MO, USA) and Midazolam (Darou Pakhsh Pharmaceutical Mfg.co, Iran) were used in our experiments. Dibutyl phthalate (6.25, 12.5, 25, 50, 100 and 200 mg/kg) was dissolved in sunflower oil as vehicle and given orally once a day for 14 days; the experiments were held on the day 15. Midazolam (1 mg/kg) and Scopolamine (1 mg/kg) were administered intraperitoneally (ip) 30min before starting passive avoidance and Y-maze tests as positive controls. The mice in control group received vehicle only.

2.3. Behavioral assessments

2.3.1. Locomotor activity

The locomotor activity test was performed in cages made of transparent Plexiglas (40 × 40 × 40 cm). After administering DBP for 14 days, on the day 15 mice were placed individually in the cage and the movement was recorded with a camera that was placed above the cage for 10min. Total distance movement, peripheral and central zone spent time for each animal were measured using video tracking software EthoVision® XT (Version 8, Noldus, Netherlands) and the data were used for the statistical analysis (Onishchenko et al., 2011).

2.3.2. Y-maze

The Y-maze is a three arm horizontal maze which is used for assessment of spontaneous alternation in rodents. The characteristics of the maze have been described previously (Huh et al., 2014). This test is based on willingness of rodents to explore new environments. Normal rodents would prefer to experience a different arm of the maze than they visited on their previous entry. The test was performed as described by Eugene et al. (2014). Each arm had the sequence like A, B and C and mice were placed in any arm. Numbers of entry to each arm were recorded manually over 10 min. A correct alternation occurred when entries into three arms were consecutive choices like ABC or BCA. After each trial the arms were cleaned with 20% ethanol to remove any residual odors. The results were expressed as described below: (Huh et al., 2014).

$$\%\ \text{Spontaneous alternation} = \left[\frac{(\text{number of Alternations})}{(\text{total arm entries} - 2)}\right] \times 100$$

2.3.3. Elevated plus maze

Elevated plus maze (EPM) was used to assess anxiety in mice. The EPM is consisted of four arms, 40 cm long, two arms were enclosed by 20 cm height walls and the other two arms had no walls. The four arms were arranged in such a way that the two arms of each type were opposite to each other. The maze was located 50 cm above the floor (Vieira et al., 2013). The possible anxiety inducing effects of DBP were assessed using the same method which was described by Komoda et al. (2008). Anxious mice naturally avoid open arms and prefer to spend more time in closed arms (Munekazu et al., 2008). The EthoVision® XT software (Version 8, Noldus, Netherlands) was used for data analysis.

2.3.4. Passive avoidance

Passive avoidance test is believed to assess the long term memory in mice (Venault et al., 1986). A two compartment step-through passive avoidance apparatus was used for the test. The apparatus was divided into bright and dark compartments (20×20 × 20 cm each) by a wall with guillotine door. The dark compartment was equipped with an electric grid floor. The test was held in two days; on the training day the mice were placed in the bright compartment and allowed to explore for 30s, after that the guillotine door was opened and the mice were allowed to enter the dark compartment freely. When the mice entered the dark compartment, the door was closed and an electrical foot shock (0.5 mA, 2 s) was given to the mice and then were gently carried to their cages. The mice which did not enter the dark compartment in 60s were excluded from the experiment. 24 h later, the mice were placed in the bright compartment and the guillotine door was raised after 30s. Latency to enter the dark part was recorded; the cut-off time was 300s (Joonki et al., 2013; Rattray et al., 2013).

2.3.5. Rotarod

Rotarod is a test of motor coordination and motor function in mice (Dunham and Miya, 1957). The latency to fall from a rod of 3 cm diameter, rotating at a speed of 6 rpm (MT 6800, Borj Sanat, Iran) was recorded over 120s. The test was conducted in two days. On the first day, before treating the mice with any drugs the animals were pre-trained and only the mice were able to remain on the rotating rod (6 rpm) for 60s were chosen for the experiment. On the test day, the mice were placed on rod and the latency to fall during 120s was recorded (Luszczki et al., 2005).

2.3.6. Grip strength

Grip strength test is used for measurement of muscular strength. The experiment was conducted using the same method described by Bachstetter et al. (2014).

Briefly a digital apparatus (GS 5000, Borj Sanat, Iran) was used to measure grip strength. The mice were held by the tail and the forelimbs were placed on the tension pad of the apparatus then the mice were pulled back gently until the forelimbs released the tension pad. Resistance force of the forelimbs was calculated automatically by the apparatus. The test was repeated 3 times for each animal and the maximum force (gram) was reported as the final grip strength (Bachstetter et al., 2014).

2.3.7. Histological analysis

At the end of the behavioral experiments, mice (received vehicle and DBP 25 and 100 mg/kg/day) were sacrificed and the brains were removed rapidly. All brains were fixed for 48 h in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded blocks were cut to 10 μm slices using a microtome device (Leica, RM 2035, Germany). Hippocampal sections were dewaxed and stained with haematoxylin-eosin staining method that was previously described by Szalai et al. (2012). The dentate gyrus was observed under light microscopy with (×100) magnification (Szalai et al., 2012) and the area of nuclei were measured using ImageJ software.