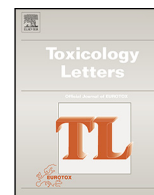
Toxicology Letters 259 (2016) 69–79

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet



Prenatal exposure to di(2-ethylhexyl) phthalate impairs development of the mouse neocortex

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HIGHLIGHTS

- We performed histologic analyses of fetal and newborn mice prenatally exposed to DEHP.
- Prenatal DEHP exposure led to the reduction of proliferation and neurogenesis, and an increase in cell death in the dorsal telencephalon of fetal mice.
- Prenatal DEHP exposure led to an abnormal neuronal distribution and reduction of neurons in the neocortex of newborn mice.

ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 27 June 2016
Accepted 16 July 2016
Available online 27 July 2016

Keywords:
DEHP
Dorsal telencephalon
Neocortex
Proliferation
Neurogenesis
Neuronal distribution

ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is currently the most commonly used phthalate for the production of flexible polyvinyl chloride. Phthalates including DEHP have been labeled as potential endocrine disruptors. The effect on the development of the neocortex, however, is unknown. To evaluate the neurodevelopmental effects of prenatal DEHP exposure at 1 and 100 mg/kg/day or 100 and 500 mg/kg/day in fetal and newborn mice, we performed a detailed histologic analysis of the developing dorsal telencephalon and neocortex. The observation of fetuses exposed to DEHP revealed reductions of proliferation and neurogenesis (1 and 100 mg/kg) and an increase in cell death (500 mg/kg). In addition, the newborns prenatally exposed to DEHP showed an abnormal neuronal distribution and a decrease in neurons. These findings suggest that prenatal DEHP exposure induces neurodevelopmental toxicity associated with the neural stem cell niche and corticogenesis.

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1. Introduction

Phthalates are industrial chemicals used primarily to increase the flexibility and durability of plastic products. Their worldwide consumption exceeds three million metric tons annually, with the majority of these chemicals used in common household products including food storage containers, children's toys, pharmaceuticals, and cosmetics (Lyche et al., 2009). Phthalates are not chemically bound to plastic polymers and are easily released into the environment (Heudorf et al., 2007; Schettler, 2006). Di(2-ethylhexyl) phthalate (DEHP) is currently the most commonly used phthalate for the production of flexible polyvinyl chloride, being present in a wide variety of consumer products (Kavlock et al., 2002).

Once phthalates enter a person's body, they are converted into breakdown products that pass out quickly into the urine. However, biomonitoring studies that measure urine metabolites in humans show widespread exposure to phthalates. Phthalates have been labeled as potential endocrine disrupting chemicals because studies have demonstrated that they interfere with hormones of the reproductive system in experimental animals.

The reproductive toxicity of exposure to phthalates early in life has been well-documented. Recent animal toxicity studies have indicated that pre- or postnatal exposure to phthalates results in

Abbreviations: DEHP, Di(2-ethylhexyl) phthalate; CNS, central nervous system; E, embryonic day; P, postnatal day; PLP, periodate lysin paraformaldehyde; PBS, phosphate-buffered saline; Tuj1, neural class III β-tubulin; BrdU, bromodeoxyuridine; CldU, chloro-deoxyuridine; IdU, iodo-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; CP, cortical plate; MHEP, mono-(2-ethylhexyl) phthalate; VZ, ventricular zone; CERHR, Center For The Evaluation Of Risk To Human Reproduction; FSCJ, Food Safety Commission of Japan; NOAEL, No Observed Adverse Effect Level; AGD, anogenital distance.

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http://dx.doi.org/10.1016/j.toxlet.2016.07.019
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severe disorders in the developing male reproductive system, including defects in the external genitalia, cryptorchidism (undescended testes), hypospadias, and other reproductive tract malformations (Andrade et al., 2006a; Christiansen et al., 2010; Swan, 2008).

Recently, public concern about the possibility that phthalates including DEHP may affect neurodevelopment and neuronal functioning has been mounting. DEHP exposure in utero and during the lactation period impaired neurons of the sexually dimorphic central nervous system (CNS) in male rats (Moore et al., 2001) and caused neurodegeneration in the rat brain (Dhanya et al., 2003). It has been demonstrated that DEHP inhibits cell proliferation and induces apoptosis via the activation of caspase-3 in a neuroblastoma cell line, Neuro-2a cells (Lin et al., 2011). In addition, Smith et al. (2011) demonstrated that acute postnatal exposure to DEHP adversely affects hippocampal development in male rats: a decreased cell density of both immature and mature neurons in the dentate gyrus and CA3, respectively. Other environmental chemicals such as polychlorinated biphenyls have been known to affect the cognitive function (Jacobson and Jacobson, 2003; Stewart et al., 2003). Therefore, phthalates distributed in the environment can disrupt the developing brain.

The objective of the present study was to examine the effect of DEHP exposure on mouse neurogenesis and neuronal distribution in the developing neocortex. To our knowledge, there have been no experimental studies examining the association of prenatal exposure to DEHP and its effects on the development of the dorsal telencephalon, proliferation, survival, neurogenesis, neuronal migration, and neuronal distribution, in the rodent neocortex. Thus, this study may be the first to immunohistologically evaluate the neurodevelopmental toxicity of maternal DEHP oral dosing.

2. Materials & methods

2.1. Animals and housing

ICR mice (8 weeks old) purchased from SLC Inc. (Osaka, Japan) were used after acclimation for 2 weeks. Mice were kept under SPF conditions and housed in polycarbonate cages in a room with a controlled temperature ($24 \pm 1\,^{\circ}C$), humidity ($55 \pm 5\%$), and light cycle (12:12-h light-dark cycle, lights on at 7 a.m.). To avoid the possibility of stressing the animals, noise levels were kept to a minimum both within the room and in the adjacent areas. Food (Certified Rodent Chow CE-2, CLEA, Japan) and drinking water were available ad libitum. Analysis of each lot of food for certification was performed by the manufacturer. The same lots of food were provided to animals in control and DEHP-treated groups and at the same times, in order to control for possible variation in its content across the groups. Water was available via glass bottles with Teflon seals during the exposure period. Pregnant females were housed individually throughout the study in polypropylene plastic tubs with stainless steel lids and corncob bedding. Mice in all experiments were humanely treated according to the guidelines of the Animal Research Committee of Kindai University. Ten- or 11-week-old mice were allowed to copulate overnight at a 1:1 male to female ratio. Females were checked the next morning for the presence of vaginal plugs, indicating copulation, and were separated from the male if a plug was present. The presence of a plug represented embryonic day (E) 0.

2.2. Test substance and treatment regimen

Di(2-ethylhexyl) phthalate (DEHP, CAS no. 117-81-7, Sigma-Aldrich, St. Louis, MO, USA) was suspended in corn oil and administered by oral gavage from E6 to E13 or from E6 to E18. The dosing volume used was 5.0 mL/kg body weight. On the basis of the previous reports (Andrade et al., 2006a,b; Christiansen et al., 2010; Smith et al., 2011), the low and high doses used in the present study were, respectively, 1 and 100 mg/kg bw/day. The dose solution was prepared once per 5 days and analyzed prior to dosing. The DEHP concentration was confirmed to be within $\pm 10\%$ of the targeted concentration by gas chromatography/mass spectrometry (HP 5860/5971A, Palo Alto, CA, USA). Administration occurred at a defined time (12:00–12:15 p.m.). Control animals received an equal volume of corn oil.

2.3. Tissue preparation of fetuses

For the histologic evaluation of fetuses, pregnant mice were euthanized by cervical dislocation and subjected to a cesarean section on E14. Fetuses were fixed in periodate lysin paraformaldehyde (PLP) for 3 h at $4\,^{\circ}C$, and washed in phosphate-buffered saline (PBS). For the evaluation of newborns, pregnant mice gave birth naturally and nursed their newborns. The day of birth was designated as postnatal day (P) 0. On P7, newborns were weighed and deeply anesthetized using sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan). Animals were perfused intracardially with PBS, and then PLP. The brains were removed and weighed. Subsequently, the brains were postfixed in PLP for 3 h at $4\,^{\circ}C$, and washed in PBS. Each sample was embedded in paraffin and sectioned at $5\,\mu m$ for histologic and immunohistochemical observation. In the present study, we used only male mice because there were no significant differences in the proliferation or neurogenesis of neural stem cells between male and female mice in our preliminary toxicological studies (data not shown).

2.4. Immunohistochemistry

The following antibodies were used: mouse monoclonal anti-neural class III β-tubulin (Tuj1, 1:500, Covance, Princeton, NJ, USA); pan-neuronal marker, rabbit monoclonal anti-Ki67 (1:200, RM9106, Thermo Fisher Scientific, Waltham, MA, USA); proliferative cell marker, rat monoclonal anti-BrdU (CldU) (1:50, BU1/75, AbD Serotec, Kidlington, UK), mouse monoclonal anti-BrdU (IdU) (1:50, clone B44; 347580, BD Biosciences, San Jose, CA, USA), and rabbit anti-caspase-3 (1:300, Cell Signaling Tec., Danver, MA, USA). Secondary antibodies were conjugated with Alexa 568 and 488 (1:200, Invitrogen, Carlsbad, CA, USA). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in mounting medium (Vector Labs, Burlingame, CA, USA). Immunohistochemistry was performed as described previously (Komada et al., 2012, 2014), and standard immunostaining procedures were used in E14 fetuses. Nine male fetuses from 3 dams in each group were used for immunostaining.

2.5. CldU and IdU incorporation

For the in vivo labeling of S-phase cells (thymidine analog incorporation), a single injection of CldU (105478, MP Biomedicals Inc., OH, USA) and IdU (I7125, Sigma Chemical Co., St.Louis, MO, USA) was administered 24 and 1 h, respectively, prior to cesarean section on E14. Fetuses were allowed to develop to E14 and then sacrificed, weighed, and processed for CldU and IdU immunohistochemistry. The quantification of positive cells and their distribution within the cortical layer were analyzed according to previously reported methods (Komada et al., 2012, 2014), with 2 anatomically matched sections from each fetus.

2.6. Analysis of cell cycle exit

Cell cycle exit was estimated from the ratio of CldU-positive cells/Ki67-negative (postmitotic and/or differentiation) cells to all

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