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Effects of six priority controlled phthalate esters with long-term low-dose integrated exposure on male reproductive toxicity in rats

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ABSTRACT

Human beings are inevitably exposed to ubiquitous phthalate esters (PEs) surroundings. The purposes of this study were to investigate the effects of long-term low-dose exposure to the mixture of six priority controlled phthalate esters (MIXPs): dimethyl phthalate (DMP), diethyl phthalate (DEP), di(*n*-butyl) phthalate (DBP), butyl benzyl phthalate (BBP), di(2-ethyhexyl) phthalate (DEHP) and di-*n*-octyl phthalate (DNOP), on male rat reproductive system and further to explore the underlying mechanisms of the reproductive toxicity. The male rats were orally exposed to either sodium carboxymethyl cellulose as controls or MIXPs at three different low-doses by gavage for 15 weeks. Testosterone and luteinizing hormone (LH) in serum were analyzed, and pathological examinations were performed for toxicity evaluation. Steroidogenic proteins (StAR, P450scc, CYP17A1 and 17β-HSD), cell cycle and apoptosis-related proteins (p53, Chk1, Cdc2, CDK6, Bcl-2 and Bax) were measured for mechanisms exploration. MIXPs with long-term low-dose exposure could cause male reproductive toxicity to the rats, including the decrease of both serum and testicular testosterone, and the constructional damage of testis. These effects were related to down-regulated steroidogenic proteins, arresting cell cycle progression and promoting apoptosis in rat testicular cells. The results indicate that MIXPs with long-term low-dose exposure may pose male reproductive toxicity in human.

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1. Introduction

Phthalate esters (PEs), environmental endocrine disruptors, including different components such as di(*n*-butyl) phthalate (DBP), butyl benzyl phthalate (BBP) and di(2-ethyhexyl) phthalate (DEHP), can cause reproductive and developmental toxicity (CHAP, 2014). High dose di-*n*-octyl phthalate (DNOP) also can cause reproductive and developmental toxicity in rats (CHAP, 2014). Dimethyl phthalate (DMP) has been recently shown to alter DNA conformation by binding to sperm DNA in herring (Chi et al., 2016). Prenatal exposure to diethyl phthalate (DEP), DBP and BBP may cause changes in anogenital distance and sperm parameters in human (Swan et al., 2005). A case-control study reported that Indian women with endometriosis showed significantly higher concentrations of DBP, BBP, DEHP and DNOP (Reddy et al., 2006) than controls. Thus, the United States Environmental Protection Agency (US EPA) has considered the six PEs, namely DMP, DEP, DBP, BBP, DEHP and DNOP, as environmental priority pollutants (US EPA, 2007). DBP, BBP, DEHP and DNOP are also in the list of priority

Abbreviations: 17β-HSD, 17 beta hydroxy steroid dehydrogenase; BBP, butyl benzyl phthalate; Cdc2, cell division cycle gene 2; CDK6, cyclin dependent kinase 6; Chk1, Checkpoint kinase 1; CYP17A, cytochrome P450 17A; DBP, di(*n*-butyl) phthalate; DEHP, di(2-ethyhexyl) phthalate; DEP, diethyl phthalate; DMP, dimethyl phthalate; DNOP, di-*n*-octyl phthalate; LH, luteinizing hormone; MIXPs, the mixture of six priority control phthalate esters; P450scc, cytochrome P450 cholesterol side-chain lyase; PAEs, phthalate esters; RfD, reference dose; StAR, Steroidogenic acute regulatory protein; TDI, tolerable daily intake.

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pollutants of the European Communities (EC, 1997). In addition, DMP, DBP and DNOP are also in the blacklist of China's water priority pollutants as identified by Chinese National Environmental Monitoring Center (CNEMC).

PEs as plasticizers are ubiquitous in our surrounding environment. They are widely detected in source waters, soil and air in China (Chen et al., 2012; Liu et al., 2014; Niu et al., 2014). PEs can migrate into foods from environment and plastic containers, causing the contamination of PEs in most foods and drinks. For examples, the total PEs concentrations in fish from Hong Kong market ranged from 1.57 to 7.10 μg/g (Cheng et al., 2013); DBP and DEHP levels in cow milk packed in polyethylene reached to 75 ng/mL and 195 ng/mL, respectively (Farajzadeh et al., 2012); DEHP was about 3.6–101 ng/mL in light alcoholic drinks and soft drinks (Russo et al., 2014). Human beings are inevitably exposed to these compounds via ingestion, inhalation and dermal exposure throughout their whole lifetimes, with ingestion as the primary exposure pathway (Chen et al., 2008, 2012; Fromme et al., 2013). The estimated total PEs exposure level of people in Yangtze River Delta area in China is about 34–159 μg/kg/d, and DEHP exposure level is about 16–116 μg/kg/d, with dietary intake accounting for over 90% of PEs' total intake (Chen et al., 2012; Guo et al., 2011; Yu et al., 2014). The reference dose (RfD) or tolerable daily intake (TDI) of DEHP is 20 μg/kg/d assessed by US EPA (2007), 37 μg/kg/d by EU Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE) (Koch et al., 2003), and 40–140 μg/kg/d by Japan's Health Department (Fujimaki et al., 2006). Therefore, the TDI range of DEHP may be about 20–140 μg/kg/d, nearly overlapping with the PEs and DEHP exposure level of the local residents in Yangtze River Delta. The residents are exposed to PEs at the critical level close to DEHP's TDI throughout the whole lifetime, which may pose safety risks to them. However, the risk of chronic low-doses exposure to mixed PEs in the range of their TDI has not raised enough concerns, and the related studies are still scarce.

The toxicological effect of each component in PEs mixture may exhibit dose-addition action, and potentially exacerbate their safety risks. Human beings are exposed to various chemicals, not limited to a single PE. Some previous studies have reported that the mixture of five PEs (BBP, DBP, DEHP, DiBP and DPP at equipotent toxicity potency, prenatal exposure from gestational day 8–18 at doses of 260–1300 mg/kg/d) inhibited fetal testicular testosterone production in SD rat in a cumulative, dose-additive manner (Howdeshell et al., 2008, 2015). The PEs mixture (BBP, DBP, DEHP, DNOP, Diisodecyle phthalate and Diisononyl phthalate at equal volumes) also induced toxicity to aquatic organisms, such as zebrafish (Chen et al., 2014). In addition, dose-addition action has also been shown in the mixture of PEs and other chemicals. For instance, the mixture of BBP (500 mg/kg/d) and linuron (75 mg/kg/d), a herbicide, (prenatal exposure from gestational day 14–18) exacerbated their reproductive toxicity in rats (Hotchkiss et al., 2004). The mixture of PE and polychlorinated biphenyls (PCBs) aggregated their toxicity on adrenal cortex and thyroid of rat (Pereira et al., 2007), and male mouse reproductive system (Fiandanese et al., 2016). Whereas in most studies, the exposure levels of PEs are much higher than the everyday human exposure dose. A single compound and a short-time exposure cannot completely simulate the real scenarios of human exposure to the complexes.

The effects of PEs exposure on male reproductive system are still inconsistent. Several studies have shown that PEs exposure led to decreased testosterone levels and infertility (Hannas et al., 2011; Noriega et al., 2009; Zheng et al., 2010), while some others reported that the exposure resulted in increased testosterone (Akingbemi et al., 2004; Kurahashi et al., 2005). These findings were obtained either under short-term exposure or at high dose of a single PE. However, it is still unclear how long-term low-dose exposure to the mixture of multiple PEs affects male reproductive system.

A number of mechanisms are reported to underlie the male reproductive toxicity of PEs exposure. Noriega et al. (2009) reported that pubertal exposure to DEHP (300- and 900 mg/kg/d) for 98 days caused the dysfunction of leydig and sertoli cells in male rat reproductive system. Li et al. (2014) reported that DEHP exposure at doses of 20–1000 mg/kg/d for 14 days induced testicular toxicity by inhibiting DNA replication, inducing the mitochondrial apoptotic pathways and leading to increased reactive oxygen species (ROS) production in rats. It has been shown that in utero PEs exposure exerted male reproductive toxicity in the fetal rats by down-regulating the expression of steroidogenic proteins, such as StAR, P450scc, CYP11, CYP17, 3β-HSD, 17β-HSD and INSL-3 (Hannas et al., 2011, 2012; Motohashi et al., 2016). The conducted studies thus far have mainly focused on high-doses PEs exposure, in utero exposure and *ex vivo* experiments. However, the mechanisms are still largely unknown, especially when rats are exposed to the mixture of PEs in a long-term low-dose model.

Well-organized cell cycle progression and apoptosis of testicular cells constitute the base of male reproductive system. Whereas the alteration of one or more related proteins can induce disordered cell cycle progression and apoptosis, consequently causing testis damages. *Ex vivo* studies of osteoblasts and ovarian antral follicles in rodents showed that PEs could cause cell cycle arrest and apoptosis (Craig et al., 2013; Sabbieti et al., 2009). Therefore, we hypothesize that chronic exposure to PEs at low-doses can induce male reproductive toxicity, which might relate to inhibited testicular cell cycle progression, induced apoptosis and altered steroidogenic proteins.

To test the hypothesis, we used the mixture of six priority controlled PEs (MIXPs) to study their reproductive toxicity and explore the related mechanisms in male rats. Organs weight, testosterone level, luteinizing hormone (LH) level, and pathological detection were performed for the toxicity evaluation. Some key steroidogenic, cell cycle and apoptosis proteins were analyzed for the mechanisms exploration.

2. Material and methods

2.1. Chemicals and reagents

The PEs used in this study were purchased form Sinopharm Chemical Reagent CO. LTD (Shanghai, China): DMP (CAS 131-11-3, purity 99.5%), DEP (CAS 84-66-2, purity 99.5%), DBP (CAS 84-74-2, purity 99%), BBP (CAS 85-68-7, purity 98%), DEHP (CAS 117-81-7, purity 99%), DNOP (CAS 117-84-0, purity 99%). Commercial assay kits of testosterone and luteinizing hormone (LH) for rats were gained from Nanjing JianCheng Bioengineering Institute, Nanjing, China. Primary antibodies: StAR, P450scc, CYP17A1, and 17β-HSD were purchased from Boster bio-engineering CO. LTD, Wuhan, China; Primary antibodies: P53, Chk1, Cdc2, CDK6, GAPDH were purchased from Santa Cruz, Texas, USA; Secondary antibodies were purchased from Beyotime, Haimen, China.

2.2. Animals

Male Sprague-Dawley rats (SPF grade, 6 weeks, weighing 200 ± 11 g) were obtained from Shanghai Jiesijie Experimental Animal CO. LTD (License Number: SCXK(HU)2013-0006). All the rats were healthy basing on physical examination and routine clinical laboratory data. Upon arrival, the animals were maintained in an environmentally controlled room (22 °C ± 2 °C, 12 h light-12 h dark cycle) with free access to standard animal feed (AIN-93M) and

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