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# Combined effects of simultaneous exposure to six phthalates and emulsifier glycerol monosterate on male reproductive system in rats

Hai-Tao Gao[a], Run Xu[a], Wei-Xin Cao[a], Qian-Nan Di[a], Rui-Xian Li[a], Lingeng Lu[b], Qian Xu[a,c,*], Shu-Qin Yu[d,e,**]

[a] Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Nanjing 210009, China
[b] Department of Chronic Disease Epidemiology, Yale School of Public Health, School of Medicine, Yale University, 60 College Street, New Haven, CT 06520-8034, USA
[c] Suzhou Key Laboratory of Environment and Biosafety, Suzhou 215123, China
[d] Jiangsu Key Laboratory for Supramolecular Medicinal Material and Applications, College of Life Sciences, Nanjing Normal University, Nanjing 210046, China
[e] Jiangsu Province Key Laboratory for Molecular and Medicinal Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210046, China

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ABSTRACT

Human beings are inevitably exposed to ubiquitous phthalate esters (PEs), and simultaneously ingesting high quantities of food emulsifiers via daily diet. Glycerin monostearate (GMS) is a widely used food emulsifier. The purposes of this study were to investigate the combined effects between the mixture of six frequently used PEs (MIXPs) and GMS on male rat reproductive system, and further to explore the underlying mechanisms. Male rats were orally administered either sodium carboxymethyl cellulose as controls or MIXPs at three different low-doses with or without GMS (200 mg/kg/d) by gavage. The 15-week exposure of MIXPs caused male reproductive toxicity in a dose- and time-dependent manner, including the decrease of serum testosterone and morphological damage of testis. Metabonomics analyses of urine and Western blotting analyses of steroidogenic proteins (StAR, P450scc, CYP17A1, 17β-HSD and P450arom) indicated that MIXPs exposure down-regulated the expression of steroidogenic proteins, and might alter androgen metabolism. The results also showed that the presence of GMS exacerbated the toxicities of MIXPs to male rat reproductive system. These findings suggest that food emulsifier GMS could enhance the toxic effects of MIXPs on male hormone biosynthesis.

## 1. Introduction

Phthalate esters (PEs), as environmental endocrine disruptors, can cause male reproductive toxicity to animals and humans, especially the six priority environmental pollutants, namely 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) (Chen et al., 2014). Based on the report of US Consumer Product Safety Commission, the no observed adverse effect levels (NOAEL) of DMP, DBP, BBP, DEHP, DNOP are 750-, 50-, 50-, 5-, and 37 mg/kg/d, respectively, by the oral route in rat (CHAP, 2014). The reference dose (RfD) of DEP is 0.8 mg/kg/d for humans (EPA, 2007), which is equal to 80 mg/kg/d NOAEL of DEP by the oral route in rat according to the species safety factor between human and animal. It was reported that DMP could interact with herring sperm DNA, change its conformation and might cause reproductive toxicity (Chi et al., 2016). Long-term low-dose DEP exposure could increase free radical production in female rats (Pereira et al., 2006). Presumably, DEP exposure might impair testicular antioxidant defense system in male rats, and enhance the reproductive toxic effect. It's reported that DEP exposure could also cause embryonic toxicity and liver toxicity (Kim et al., 2015). BBP was administered in the diet at 11,250 ppm (750 mg/kg/d) ad libitum to rats for two offspring generations, causing systemic and reproductive toxicity in adult F1 rats (Tyl et al., 2004). DBP caused decrease of testosterone and alterations of testicular structure in the male offspring following the pregnant rats exposure to 100 or 500 mg/kg/day DBP in diet from gestational day (GD) 12 to GD 19 (Struve et al., 2009). DNOP could exert reproductive toxicity to SD rats at the doses of 500 mg/kg/d for 4 weeks (Kwack et al., 2010; Kwack et al., 2009). Noriega et al. (2009) reported that pubertal administration of di(2-ethyhexyl) phthalate (DEHP) delayed puberty, suppressed testosterone production and inhibited reproductive tract development in male rats. DEHP exposure also induced sperm count reduction as well as histological abnormalities in seminiferous epithelium, concomitant with

* Correspondence to: Q. Xu, Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Nanjing 210009, China.
** Correspondence to: S.Q. Yu, Jiangsu Key Laboratory for Supramolecular Medicinal Material and Applications, College of Life Sciences, Nanjing Normal University, Nanjing 210046, China.
*E-mail addresses:* q_xu68@163.com (Q. Xu), yushuqin@njnu.edu.cn (S.-Q. Yu).

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reduction of testosterone levels and its steroidogenic gene expression (Liu et al., 2016). Both in utero and juvenile exposure to DEHP resulted in significant reductions in serum testosterone in male rats (Traore et al., 2016). Our previous study showed that the mixture of the six PEs (MIXPs) altered the histopathological structure of rat testis following 30 days' exposure at 100 mg/kg/d (Gao et al., 2016). Long-term MIXPs exposure also caused male reproductive toxicity to rats at low-doses with each single phthalate even below their respective NOAEL (no observed adverse effect level) (Gao et al., 2017). Human beings are exposed to PEs at low-doses throughout the whole lifetime. PEs and their metabolites have been detected in semen, urine, umbilical cord blood and placenta in Chinese population (Li et al., 2016; Wang et al., 2015a; You et al., 2015). The total PEs exposure level of human beings in Yangtze River Delta, Pearl River Delta, Northeast China, Taihu Lake Basin in China ranged from 34 to 159 μg/kg/d (Chen et al., 2012; Guo et al., 2011; Du and Xiao, 2012; Yu et al., 2014). However, the current studies, including our previous study, on PEs' male reproductive toxicity mainly focused on single PE and a short-time ($\leq$ 30 days) exposure at high-doses ($\geq$ 300 mg/kg/d) (Gao et al., 2016; Zhao et al., 2016; Noriega et al., 2009; Xu et al., 2007), which cannot completely simulate the real scenarios of human exposure to the complexes. Accordingly, valuable information for practical significance is very limited.

People also ingest a great deal of food emulsifiers with about 254 mg/kg/d in China (Gao et al., 2016; Gao et al., 2015), which are a group of synthetic surfactant food additives, and can reduce surface hydrophobicity. They are applied in the most frequently consumed foods with high concentrations, such as bread, noodles and cheese. Glycerin monostearate (GMS) is a widely used food emulsifier, which accounts for about 50% of the total emulsifier consumption (NHFPC, 2014). It is reported that emulsifiers could increase intestinal permeability, and elevate the absorbance of food compounds as well as potential pollutants in the food, therefore, emulsifiers could make intestines sensitive to xenobiotics (Csáki, 2011; Lugea et al., 2000). Our previous studies have shown that the absorption and bioavailability of PEs increased in rats in the presence of GMS (200 mg/kg/d), and PEs' male reproductive toxicities were exacerbated (Gao et al., 2016; Xu et al., 2016). The results indicated that the combined effects may exist between PEs and food emulsifiers. However, it is still unclear how the combined effects affect male reproductive system in the long-term low-doses exposure manner.

The study was conducted to investigate the combined toxic effects of MIXPs and GMS on male reproductive system, and to explore the underlying mechanisms. Male rats were exposed the mixture of the six PEs (MIXPs) in a long-term low-dose model with or without GMS. Testosterone levels and histopathological examination of testes were performed for the toxicity evaluation. Metabolites in urine and steroidogenic proteins were measured for the mechanism exploration.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Methanol, acetonitrile and formic acid (HPLC grade) were all bought from Tedia Inc. (USA). DMP, DEP, DBP, BBP, DEHP and DOP were purchased from Sinopharm Chemical Reagent CO. LTD (Shanghai, China). Metabolism cages were from Feng's Animal equipment company (Soochow, China). Primary antibodies: 17β-Hydroxysteroid dehydrogenases (17β-HSD), cytochrome P450 aromatase (P450arom), cytochrome P450 cholesterol side chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR) and CYP17A1 (17α-hydroxylase; 17, 20 lyase) were purchased from Abcam (Cambridge, USA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Santa Cruz (Texas, USA). IKA® T10 basic homogenizer was purchased from IKA, Germany. bicinchoninic acid (BCA) Protein Assay Kit was purchased from Beyotime Biotech Inc., China.

### 2.2. Animals

Seventy 6-week old male Sprague-Dawley (SD) rats (weighing 180–220 g) were purchased from Shanghai Jiesijie Experimental Animal CO. LTD (License Number: SCXK(Shanghai) 2013-0006). All the rats were healthy based on physical examination and routine clinical laboratory data. Prior to the experiments, the rats have been raised 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 filtered tap water for 7 days to acclimatize to their new environment at SPF grade Animal Laboratory of Southeast University. Animal experiments were conducted following the “Principles of laboratory animal care” (NIH publication 86-23, revised 1986) and the local regulations, and were approved and supervised by the Animal Care and Use Committee and the Animal Ethics Committee at Southeast University (Approval Number: 2015-0623-009).

### 2.3. Animal experimental design

Based on their body weights, seventy rats were randomized into 7 groups each with 10 rats and raised in metabolism cages. The rats in control group received 0.5% sodium carboxymethyl cellulose (CMC-Na) daily by oral gavage, while the rats in experiment groups received respectively isometrical MIXPs suspension in 0.5% CMC-Na daily at three doses either with or without GMS by oral gavage for 15 weeks. MIXPs was consisted of DMP, DEP, DBP, BBP, DEHP and DNOP at equipotent weight (DMP:DEP:DBP:BBP:DEHP:DNOP = 1:1:1:1:1:1). The MIXPs exposure levels in experiment groups were 1.6-, 16-, 160 mg/kg/day, with each PE exposure level of 0.267-, 2.67- and 26.7 mg/kg/d in the three-dose groups, respectively. The rats in the MIXPs-treated groups received MIXPs alone orally at low, medium and high dosage respectively, while the rats in MIXPs + GMS-treated groups received the corresponding equal doses of MIXPs with GMS (200 mg/kg/day).

Twenty four hours urine samples for the metabolic analyses were collected on day 0 (at the beginning of the experiment), and at the end of 2nd, 8th and 15th week, and stored in − 80 °C freezer. At the 4 time points, about 1.0 mL caudal vein blood was also collected from each rat by anesthetically cutting off the tip of its tail for serum preparation at 4 °C. The aliquoted serum was in vials were stored at − 80 °C for the measurements of testosterone. At the end of the experiment (the 15th week), all the rats were fasted overnight (12 h) and euthanized using 4% fluothane in oxygen anesthesia in an airtight container. When the rats had no-blink reflex and no autonomous breath, or no heartbeat, the left testis of each rat was taken, and then stored at − 80 °C for later analysis.

### 2.4. Histopathological observation of testis

The right testis tissues were fixed in 10% buffered formalin in phosphate buffer saline (PH 7.4), embedded in paraffin, sliced in 4 μm thick sections and stained with Hematoxylin-Eosin (HE staining). Morphological analysis was performed by a senior pathologist who was blinded to the experimental design. H·E-stained samples were observed with a TS100 inverted microscope (Nikon, Japan), and photographed under × 200 magnification.

### 2.5. Serum testosterone examination

The aliquoted serum samples were thawed at room temperature, then they were measured using a commercial ELISA kit of testosterone for rat (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). According to the manufacturer's instruction, the assay procedure including dilution and addition of standard sample, serum sample addition (10 μL), incubation for 30 min at 37 °C, washing liquid dilution (30-fold) and washing (repeat 5 times), enzyme addition, coloring for 15 min at 37 °C, stopping the reaction with 50 μL stop solution, then