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The effect of mono-(2-ethylhexyl) phthalate on apoptosis of rat ovarian granulosa cells in vitro

Na Li[a,b,1], *Keqiang Liu*[c,a,1], *Haitao Yuan*[a], *Jian Zhu*[a], *Guangyan Yu*[a], *Jingli Xie*[d], *Songpo Fu*[c], *Kun Guo*[a], *Lin Ye*[a,*]

[a] *Department of Occupational and Environmental Health, School of Public Health, Jilin University, Changchun, Jilin 130000, China*
[b] *The Second Hospital of Dalian Medical University, Dalian, Liaoning 116012, China*
[c] *Department of Preventive Medicine, School of Public Health, Jilin University, Changchun, Jilin 130000, China*
[d] *Department of Radiology, School of Public Health, Jilin University, Changchun, Jilin 130000, China*

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ABSTRACT

Mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite of di-(2-ethylhexyl) phthalate (DEHP), is considered to be a reproductive toxicant. This study was performed to evaluate the effect of MEHP on apoptosis of rat ovarian granulosa cells and explore potential mechanism. Granulosa cells were treated with MEHP (0, 25, 50, and 100 μmol/l). Inhibited cell viability and increased apoptosis rate were observed in 50 and 100 μmol/l groups. CASPASE3 activity and BAX expression were significantly raised in all MEHP-treated groups; BCL2 expression was elevated in 25 μmol/l group, while inhibited in 50 and 100 μmol/l groups; BAX/BCL2 ratio was increased in a typical dose–effect relationship. In conclusion, this study showed that MEHP exposure induced cell viability decrease and apoptosis, associated with increase of CASPASE3 activity and BAX/BCL2 ratio. Moreover, CASPASE3 activity showed a reversed dose-dependent effect in MEHP-treated groups, indicating there might exist other CASPASE-independent pathway involved in MEHP-induced apoptosis.

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1. Introduction

Phthalates are a class of chemicals widely used as plasticizers in many consumer and industrial products, imparting plasticity and softness to otherwise rigid materials such as polyvinyl chloride plastics (PVC) (Schettler, 2006). Among the phthalates, di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer with a production of about 2 million tons per year. As this phthalate is not chemically bound to plastic materials, over time DEHP can leach out from plastic products into air or other materials, which may result in general environmental contamination and human exposure (Halden, 2010). The exposure level of DEHP in the general population has been reported to be close to the tolerable daily intake value (TDI = 2 mg/day), however, in certain populations, the TDI for DEHP is exceeded to a considerable degree. For example, in patients undergoing hemodialysis the amount of DEHP delivered into their bodies increased up to 150 mg in 5 h, because of the contact between blood and plastic tubings (Gibson et al., 1976). Once into human body, DEHP rapidly metabolizes into mono-(2-ethyhexyl) phthalate (MEHP), which is the active metabolite

* Corresponding author. Tel.: +86 431 85619453; fax: +86 431 85619431.
E-mail address: jlyelin@163.com (L. Ye).
[1] These two authors contributed equally to this work.

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and may be more potent than its parent compound (Kavlock et al., 2002).

Recently, a growing number of epidemiologic studies has reported the reproductive toxicity of phthalates, especially DEHP and its metabolite MEHP. In males, high DEHP concentration in semen samples was associated with male infertility (Pant et al., 2011). Several recent studies pointed that human urinary concentration of MEHP was significantly associated with the decrease of sperm motility and sperm aneuploidy (Huang et al., 2014; Jurewicz et al., 2013). In females, lower rate of pregnancy and increased rate of abortion were observed in female factory workers in Russia, who had suffered high exposure level of phthalates (Aldyreva et al., 1975). Notably higher levels of DEHP and metabolite were identified in blood samples from Puerto Rican thelarche girls (Colón et al., 2000). All these phenomena indicated that DEHP and its metabolite MEHP exposure may produce reproductive toxicity.

Previous animal studies suggest that DEHP possesses endocrine disturbing properties and reproductive toxicity. For example, Erkekoglu et al. (2012) treated rat pubertal testes with DEHP (1000 mg/kg), and observed approximately 8-fold apoptosis rate of germ cells compared with the control group. In female rats, DEHP treatment (2 g/kg/day) led to prolonged estrous cycles and decreased estradiol levels (Davis et al., 1994). MEHP, as the active metabolite, is also considered to be an endocrine disruptor and reproductive toxicant. In Muczynski (2012) and colleagues' study, after cultivation with MEHP (10^{-5} mol/l) for 3 days, male germ cells displayed a significantly increased rate of apoptosis in cultured human fetal testes (Muczynski et al., 2012). Moreover, they found that MEHP also induced apoptosis in mouse fetal testes both in vitro and in vivo. Similarly, Awal et al., 2004,2005 reported that MEHP increased the percentage of apoptotic spermatogenic cells in Guinea pig prepubertal testes. Wang et al., 2012a,b reported that DEHP as well as its metabolite MEHP both inhibited growth of antral follicles in mouse ovaries through an oxidative stress pathway, however, another possible reason for the inhibition may be apoptosis of follicular cells, because lower expression of anti-apoptotic gene Bcl2 and higher expression of pro-apoptotic gene Bax were also observed in their experiments.

Females may have a greater chance of exposure to DEHP, owing to the use of cosmetics and other customer products, in which DEHP has been detected. In females, normal fertility is closely associated with follicular function, which depends largely on the health of ovarian granulosa cells. Ovaries are considered to be the target organs of DEHP for the female reproductive toxicity, and granulosa cells are likely target cells of DEHP and metabolite in ovaries (Lovekamp-Swan and Davis, 2003). Thus, studies concerning the impact of DEHP and MEHP on granulosa cells may put insight into how phthalates affect female reproductive system. In our previous studies, significantly increased granulosa cell apoptosis rate and reduced progesterone level were observed in mice treated with 500 and 2000 mg/kg DEHP (Li et al., 2012). Yet, whether DEHP itself induces granulosa cells apoptosis or through its active metabolite MEHP is unknown. Therefore, the present study was carried out to test the hypothesis that MEHP can induce apoptosis of granulosa cells, and, if so, to explore what may be the potential apoptotic induction mechanism, by measuring cell viability, cell apoptosis rate, CASPASE3 enzyme activity, and BCL2 and BAX expression of MEHP-treated rat ovarian granulosa cells.

2. Materials and methods

2.1. *Chemicals*

Mono-(2-ethylhexyl) phthalate (MEHP) (99.9%) was purchased from AccuStandard, Inc. (New Haven, USA), Dulbecco's modified Eagle's medium (DMEM)/F12 (GIBCO®) from Invitrogen (Carlsbad, CA, USA), pregnant mare's serum gonadotropin (PMSG) from Ningbo people health pharmaceutical Co., Ltd. (Ningbo, CHN), CASPASE3 activity assay kit and Annexin V-FITC/PI apoptosis detection kit from Beyotime Institute of Biotechnology (Shanghai, CHN), Dimethyl sulfoxide (DMSO) and 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) from Amresco, Inc. (Solon, USA), rabbit monoclonal anti-BCL2 and anti-BAX antibody, and horseradish peroxidase goat anti-rabbit IgG from Abcam, Ltd. (San Francisco, CA,USA), mouse anti-β-actin Monoclonal Antibody from Santa Cruz, Ltd. (California, USA). All other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, CHN).

2.2. *Animals*

Female Wistar rats (21–29 days old) were obtained from Experimental Animal Center of Jilin University (SCXK–Jilin: 2007–0003). Animals were housed under a 12:12 dark: light cycle and were given free access to food and water. This research protocol was approved by the Animal Experiment Ethics Committee of Jilin University.

2.3. *Cell culture*

Before sacrifice, the animals were intraperitoneally injected with 40 iu of PMSG (in saline) to stimulate the growth of rat ovaries. At least eight rats were used in each experiment to obtain enough granulosa cells. Forty-eight hours after doing with PMSG, the animals were sacrificed by cervical dislocation. Ovaries were collected aseptically in sterilized phosphate buffered saline (PBS) and thereafter were punctured with sterile needle to release the cells from follicles (Sirotkin et al., 2008) into Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1), supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were from multiple animals pooled. After washing twice by centrifugation at 200 g for 10 min, cells were diluted with same medium to a final concentration of 1×10^5 live cells/ml and cultured at 37 °C in a humidified atmosphere of 5% CO_2. After cultivation for about 36 h, the cells were adherent to the wall. The final cell cultures consisted of >95% granulosa cells as determined by immunocytochemical staining for follicle stimulating hormone receptor (FSHR), which is specifically expressed in granulosa cells.

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