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Role of autophagy in di-2-ethylhexyl phthalate (DEHP)-induced apoptosis in mouse Leydig cells☆

Yingyin Sun [a], Jingcao Shen [a], Lin Zeng [a], Dan Yang [a], Shuxin Shao [a], Jinglei Wang [a], Jie Wei [a], Junping Xiong [c], Jiaxiang Chen [a, b, *]

[a] Department of Physiology, Medical College of Nanchang University, Nanchang, 330006, China
[b] Jiangxi Provincial Key Laboratory of Reproductive Physiology and Pathology, Nanchang, 330006, China
[c] Department of Anatomy, Medical College of Nanchang University, Nanchang, 330006, China

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) has been widely used as a plasticizer in industry. DEHP can cause testicular atrophy, yet the exact mechanism remains unclear. In this study, male mice were intragastrically (i.g.) administered with 0, 100, 200 or 400 mg DEHP/kg/day for 21 days. We found that DEHP caused disintegration of the germinal epithelium and decreased sperm density in the epididymis. Furthermore, there was a significant increase in the levels of cleaved Caspase-8, cleaved Caspase-3 and Bax proteins and a decrease in Bcl2 protein. The results indicated that DEHP could induce apoptosis of the testis tissue. Meanwhile, DEHP significantly induced autophagy in the testis tissues with increases in LC3-II, Atg5 and Beclin-1 proteins. The serum testosterone concentration decreased in the DEHP-treated group, implying that DEHP might lead to Leydig cell damage. Furthermore, oxidative stress was induced by DEHP in the testis. To further investigate the potential mechanism, mouse TM3 Leydig cells were treated with 0–80 μM DEHP for 48 h. DEHP significantly inhibited cell viability and induced cell apoptosis. Oxidative stress was involved in DEHP-induced apoptosis as N-Acetyl-L-cysteine (NAC), an inhibitor of oxidative stress, could rescue the inhibition of cell viability and induction of apoptosis by DEHP. Similar to the *in vivo* findings, DEHP could also induce cell autophagy. However, inhibition of autophagy by 3-Methyladenine (3-MA) significantly increased cell viability and inhibited apoptosis. Taken together, oxidative stress was involved in DEHP-induced apoptosis and autophagy of mouse TM3 Leydig cells, and autophagy might play a cytotoxic role in DEHP-induced cell apoptosis.

1. Introduction

Di-2-ethylhexyl phthalate (DEHP) has been widely used as a plasticizer in industry to produce items such as intravenous (IV) bags, blood bags, infusion tubing, nasogastric tubes, peritoneal dialysis bags, toys and infant products (Bourdeaux et al., 2004). DEHP can be found in air, water and soil at some amounts (Petersen and Breindahl, 2000). DEHP can also be found in meat and lipid-rich products, such as fats, at higher concentrations (≥300 μg/kg) (Serrano et al., 2014). DEHP has raised concerns pertaining to continuous exposure in animals and humans due to its ubiquity in the environment.

DEHP has been shown to induce reproductive toxicity (Fiandanese et al., 2016; Stenz et al., 2017) in addition to neurotoxicity (Du et al., 2017; Luu et al., 2017), cardiotoxicity (Posnack, 2014), immunotoxicity (Huang et al., 2015), and liver toxicity (Zhang et al., 2017). Embryonic exposure to DEHP causes testicular germ cell disorganization and impairs spermatogonial stem cells of progeny (Doyle et al., 2013). DEHP can cause testicular atrophy and decrease sperm density and motility in the epididymis of male rats (Agarwal et al., 1986). DEHP can also affect normal sexual development in male rodents by reducing testosterone synthesis (Moore et al., 2001). The primary function of testosterone, which is mainly synthesized by Leydig cells, is to maintain spermatogenesis (Tremblay, 2015). The potential mechanism by which DEHP inhibits

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* Corresponding author. Department of Physiology, Medical College of Nanchang University, 461 Bayi Road, Donghu District, Nanchang, 330006, China.
E-mail address: chenjiaxiang@ncu.edu.cn (J. Chen).

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spermatogenesis remains to be further elaborated.

Spermatogenesis consists of sequential and highly organized steps of undifferentiated spermatogonial stem cell proliferation and differentiation (Chen et al., 2011; Liu et al., 2015). Spermatogenesis is a complex process generating functional sperm in the testis and can be affected by many chemicals (Yeung et al., 2011; Chen et al., 2012). Prenatal exposure to DEHP can inhibit spermatogenesis and affect sperm DNA methylation (Prados et al., 2015). Pubertal exposure to DEHP can cause histological abnormalities in the seminiferous epithelium and reduce sperm count, which are concomitant with the reduction of testosterone levels and its steroidogenic gene expression (Liu et al., 2016a). Testosterone is mainly synthesized by Leydig cells, indicating that DEHP might affect the function of Leydig cells and decrease testosterone output. However, the actual effect of DEHP on Leydig cells and its potential mechanism remain unclear.

The aim of the present study is to investigate the potential mechanism of DEHP-induced apoptosis and autophagy of mouse Leydig cells and the role of autophagy in DEHP-induced apoptosis.

2. Materials and methods

2.1. Reagents

DEHP (Catalog No. 36735), 3-Methyladenine (3-MA) and N-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, MO, USA). Mouse Leydig cell line (TM3) was obtained from the Cell Culture Center of the Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing, China). Anti-Caspase-8 (sc-7890), anti-Caspase-3 (sc-7148), anti-Bcl-2 (sc-492), anti-Bax (sc-493), and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Atg5 (PM050), anti-LC3 (PD014), and anti-Beclin-1 (PD017) were gained from MBL Co. Ltd (Nagoya, Japan). The AnnexinV-FITC Apoptosis Kit was purchased from Invitrogen Life Technologies (Oregon, USA). Oxidation-antioxidation assay kits of superoxide dismutase (SOD) (A001-1-1), glutathione (GSH) (A006-1), malondialdehyde (MDA) (A003-1), and glutathione peroxidase (GSH-PX) (A005) were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animal administration

Male Kunming mice (8 weeks of age) were gained from the Shanghai Laboratory Animal Center at the Chinese Academy of Sciences. The male mice were intragastrically (i.g.) administered with the indicated concentrations of DEHP (0, 100, 200, or 400 mg/kg/day, diluted in coin oil) for 21 days, blood samples were collected by cardiocentesis, and the serum was then separated and stored at −20 °C. Then, the testes and the whole epididymis were quickly dissected free of fat, decapsulated and frozen in liquid nitrogen.

2.3. Histology

Male mouse epididymis and testis tissues were stained with hematoxylin and eosin (HE) according to the method described by (Matzuk et al., 1992).

2.4. Sperm count

Sperm collection was performed after the male mice were intragastrically (i.g.) administered with the indicated concentrations of DEHP (0, 100, 200, or 400 mg/kg/day) as described by Mohamed et al. (2010).

2.5. Western blotting analysis

The decapsulated and homogenized testis tissue and TM3 cells were harvested in ice-cold RIPA lysis buffer containing protease inhibitor cocktail. Equal amounts of cell lysate were utilized for western blotting analysis as described by Liu et al. (2016b).

2.6. Detection of testosterone content by enzyme-linked immunosorbent assay (ELISA)

The concentration of testosterone in the serum was detected using an ELISA kit according to the manufacturer's instructions (Shanghai Bangyi Biotechnology Co Ltd, Shanghai, China).

2.7. Oxidative stress measurement

Testis samples and TM3 cells were homogenized with a homogenizer and then centrifuged at 600 g for 10 min at 4 °C. The resultant supernatants were retained for measuring the levels of GSH and MDA, as well as the activities of GSH-PX and SOD, using the commercial kits following the manufacturer's instructions. The Bradford assay was used to determine the protein concentration.

2.8. Cell culture

TM3 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 0.1 mg/ml streptomycin, and 100 IU/ml penicillin and incubated under aerobic conditions at 37 °C in a humidified 5% CO_2/95% air. The cells were subcultured every 3–4 days to maintain cells in the logarithmic phase of growth.

2.9. MTT reduction assay

TM3 cells were seeded at 1×10^4 cells per well in a 96-well plate and treated with the indicated concentrations of DEHP in the presence or absence of NAC (5 mM), or 3-MA (1 mM) for 48 h. Cell viability was determined by adding MTT (final concentration: 0.5 mg/mL), and the absorbance of the samples was measured at a wavelength of 570 nm using a spectrophotometer (reference 660 nm).

2.10. AnnexinV-FITC/PI apoptosis assay

Cell apoptosis was observed by flow cytometry using an AnnexinV-FITC Apoptosis Kit as described previously (Liu et al., 2016b).

2.11. Transmission electron microscopy analysis

TM3 cells were treated with DMSO, 80 μM DEHP, or 5 mM NAC plus 80 μM DEHP for 48 h or were starved for 2 h in a starvation medium. The samples were observed with a transmission electron microscope as described by Liu et al. (2016b).

2.12. Statistical analysis

All values are represented as the mean ± standard deviation. Statistical differences were analyzed with a one-way ANOVA. $P < 0.05$ was considered to indicate a significant difference.