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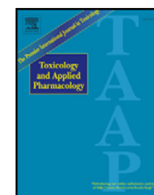

Di (2-ethylhexyl) phthalate inhibits growth of mouse ovarian antral follicles through an oxidative stress pathway

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A B S T R A C T

Di (2-ethylhexyl) phthalate (DEHP) is a plasticizer that has been shown to inhibit growth of mouse antral follicles, however, little is known about the mechanisms by which DEHP does so. Oxidative stress has been linked to follicle growth inhibition as well as phthalate-induced toxicity in non-ovarian tissues. Thus, we hypothesized that DEHP causes oxidative stress and that this leads to inhibition of the growth of antral follicles. To test this hypothesis, antral follicles isolated from CD-1 mice (age 31–35 days) were cultured with vehicle control (dimethylsulfoxide [DMSO]) or DEHP (1–100 μg/ml) ± N-acetyl cysteine (NAC, an antioxidant at 0.25–1 mM). During culture, follicles were measured daily. At the end of culture, follicles were collected and processed for in vitro reactive oxygen species (ROS) assays to measure the presence of free radicals or for measurement of the expression and activity of various key antioxidant enzymes: Cu/Zn superoxide dismutase (SOD1), glutathione peroxidase (GPX) and catalase (CAT). The results indicate that DEHP inhibits the growth of follicles compared to DMSO control and that NAC (0.25–1 mM) blocks the ability of DEHP to inhibit follicle growth. Furthermore, DEHP (10 μg/ml) significantly increases ROS levels and reduces the expression and activity of SOD1 compared to DMSO controls, whereas NAC (0.5 mM) rescues the effects of DEHP on ROS levels and SOD1. However, the expression and activity of GPX and CAT were not affected by DEHP treatment. Collectively, these data suggest that DEHP inhibits follicle growth by inducing production of ROS and by decreasing the expression and activity of SOD1.

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Introduction

Phthalates or phthalate esters (PE) are synthetic plasticizers that impart flexibility to polyvinylchloride products. They are present in a wide variety of products, including building materials, food packaging, toys, cosmetics, clothing and medical devices. More than 18 billion pounds of phthalates are used worldwide each year (Crinnion, 2010). Humans are exposed to phthalates through inhalation, ingestion and dermal absorption on a daily basis (Halden, 2010; Heudorf et al., 2007). Di (2-ethylhexyl) phthalate (DEHP) is one of the most commonly used phthalate plasticizers. Since DEHP is not covalently bound to plastic matrix, it can leach out of products and contaminate the external environment. DEHP exposure is predominantly via food and appears to be close to the tolerable daily intake of 2 mg/day in the general population (Lyche et al., 2009). However, individuals undergoing certain medical procedures may be exposed to even higher levels of DEHP via plastic medical devices (Kamrin, 2009).

DEHP is considered to be an endocrine disrupting chemical (EDC) based on its adverse effects on reproductive organs. Animal studies have shown that postnatal exposure to DEHP causes testicular atrophy and that in utero exposure to DEHP causes a number of abnormalities in male reproductive tract development in rodents (Christiansen et al., 2010; Clark and Cochrum, 2007; Martino-Andrade and Chahoud, 2009). In female rats, high dose acute exposure to DEHP reduces estradiol production and causes anovulation (Lovekamp-Swan and Davis, 2003; Lyche et al., 2009; Martino-Andrade and Chahoud, 2009). Numerous human epidemiologic studies have reported associations between chronic exposure to DEHP and various adverse reproductive outcomes, including altered male reproductive development and function (Halden, 2010; Main et al., 2006; Swan, 2008), endometriosis (Cobellis et al., 2003; Kim et al., 2010a), and increased risk of premature birth and various pregnancy complications (Latini et al., 2006; Martino-Andrade and Chahoud, 2009; Whyatt et al., 2009). However, the mechanisms by which DEHP affects the reproductive system are not fully elucidated.

One potential mechanism by which DEHP causes reproductive abnormalities may be via oxidative stress. Oxidative stress is caused by an imbalance between the production and elimination of reactive oxygen species (ROS) in the system, leading to damage to DNA, lipid peroxidation of membranes and oxidative modification of proteins (Agarwal et al., 2005). ROS are a two-edged sword: they serve as key signal molecules in various physiological processes, but also play roles in pathological processes in female reproductive organs (Agarwal et

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al., 2005). Studies have shown that ROS modulate multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy (Al-Gubory et al., 2010; Dennery, 2010; Zhang et al., 2011). However, extra accumulation of ROS leads to ovarian apoptosis, the process by which follicles undergo atresia and corpora lutea undergo regression (Agarwal et al., 2008; Lim and Luderer, 2010; Zhang et al., 2006). Excess ROS also affect angiogenesis, which is critical for follicular growth and corpus luteum formation (Agarwal et al., 2005). Epidemiology studies also have shown that oxidative stress is associated with the age-related decline in fertility, endometriosis, pre-term labor, and unexplained infertility (Agarwal et al., 2005; Al-Gubory et al., 2010; Ruder et al., 2009).

Although studies have shown that DEHP is hydrolyzed into an active metabolite mono(2-ethylhexyl) phthalate (MEHP) by lipases and esterases in the intestine (Frederiksen et al., 2007), we elected to focus on revealing the effect of DEHP on mouse antral follicles for the following reasons. First, after DEHP exposure, detectable amounts of DEHP remain in the plasma and peritoneal fluid and thus, can affect the functions of reproductive organs (Cobellis et al., 2003). Second, high plasma levels of DEHP have been correlated with various female reproductive disorders. For example, several studies have reported an association between high plasma levels of DEHP and endometriosis, indicating a possible role of DEHP in the pathogenesis of endometriosis (Cobellis et al., 2003; Kim et al., 2010a; Reddy et al., 2006a, 2006b). A study by Durmaz et al. also reported that high plasma levels of DEHP are correlated with pubertal gynecomastia (Durmaz et al., 2009). Third, DEHP has been used in different in vitro systems and has been shown to exert toxic effects on endometrial cells, granulocytes and oocytes (Kim et al., 2010b; Mlynarcikova et al., 2009; Palleschi et al., 2009).

While there is limited information on whether DEHP causes oxidative stress in the female reproductive system, oxidative stress was previously suggested to represent a common mechanism in endocrine disruptor-mediated dysfunction in reproduction (Gupta et al., 2006a, 2006b; Latchoumycandane and Mathur, 2002). Recent animal studies have shown that DEHP causes oxidative stress in male reproductive tissues, specifically in Leydig cells, Sertoli cells and germ cells by disrupting antioxidant defenses and increasing ROS (Botelho et al., 2009; Erkekoglu et al., 2010; Liu et al., 2005). Previous studies have also shown that DEHP inhibits follicle growth of antral follicles in the ovary (Gupta et al., 2010). However, the mechanisms by which DEHP inhibits growth are not fully understood. Thus, this study tested the hypothesis that DEHP inhibits antral follicle growth through an oxidative stress pathway. To test this hypothesis, we determined whether DEHP reduces ROS in the ovary and tested whether N-acetyl-cysteine (NAC, an antioxidant) rescues follicles from the toxic effects of DEHP on antral follicles. Furthermore, we investigated the mechanism by which DEHP induces oxidative stress by examining the effect of DEHP on key antioxidant enzymes: Cu/Zn superoxide dismutase (SOD1), glutathione peroxidase (GPX) and catalase (CAT).

Materials and methods

Chemicals. DEHP was purchased from Sigma (St. Louis, MO). Stock solutions of DEHP were prepared using dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) as the solvent in various concentrations (133, 13.3, and 1.33 mg/ml) that allowed an equal volume to be added to culture wells for each treatment group to control for solvent concentration. Final concentrations in culture were 1, 10, and 100 μg/ml of DEHP, which are equivalent to approximately 2.56, 25.6, and 256 μM. We chose these doses based on previous studies on the effects of DEHP on cultured cells or follicles and based on their clinical relevance (Gillum et al., 2009; Lenie and Smitz, 2009). We also chose these doses based on our preliminary dose–response experiment. N-acetyl-L-cysteine (NAC) was purchased from Sigma (St. Louis, MO). We chose NAC as opposed to other antioxidants as it is a known antioxidant, free-radical scavenger, glutathione precursor, and increases the activity of SOD and GPX enzymes (Tilly and Tilly, 1995). NAC has also been shown to protect from oxidative stress in other tissues and cell types (Gupta et al., 2006a; Hecht et al., 2002). For culture, a 100 mM stock solution of NAC was prepared using α-MEM and the final concentrations of NAC in each well of the culture were 0.25, 0.5, 1 and 2 mM.

Animals. CD-1 mice that were 31–35 days old were used for all experiments. The mice were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility under a 12:12 dark: light cycle. Food and water were provided *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Follicle culture. Female CD-1 mice were euthanized and ovaries were removed. Based on relative size (250–350 μm) and appearance, antral follicles were isolated mechanically from the ovaries and interstitial tissue was removed using fine watchmaker forceps (Gupta et al., 2006a). About 3–4 mice were used per experiment, providing approximately 25–35 follicles per mouse. The isolated follicles were randomly divided into different treatment groups (10–16 follicles per group). Doses of vehicle control (DMSO), DEHP (1, 10, and 100 μg/ml), DEHP (10 μg/ml) + NAC (0.5, 1, and 2 mM) were individually prepared in supplemented α-MEM as described previously (Gupta et al., 2006a). The final solvent concentration was 0.075%. Antral follicles were cultured for 24–96 h at 37 °C in 95% air and 5% CO_2. Non-treated controls (supplemented media only) were used in each experiment as a control for culture conditions. At the end of culture, follicles were collected, snap frozen, and stored at -80 °C for later use.

Analysis of follicle growth. Follicle size was assessed at 24 h intervals by measuring follicle diameter on perpendicular axes using an inverted microscope equipped with a calibrated ocular micrometer. Follicle diameter measurements were averaged among treatment groups and plotted to compare the effects of chemical treatments on growth over time. Data were presented as percent change over time. The statistical analyses were performed based on percentage change. At least three separate experiments were performed for each treatment group.

Gene expression analysis. Total RNA was extracted from follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. To remove any possible genomic DNA contamination, RNA was further treated with DNAse. Messenger RNA (mRNA; 200 ng) was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. The cDNA was diluted 1:4 with nuclease free water. Quantitative real-time (qPCR) was conducted using a CFX96

Table 1
Sequences of primer sets used for gene expression analysis.

Gene name	Abbreviation	Forward	Reverse
Superoxide dismutase 1	*Sod1*	5′-AAGGCCGTGTGCGTGCTGAA-3′	5′-CAGGTCTCCAACATGCCTCT-3′
Glutathione peroxidase	*Gpx*	5′-CCTCAAGTACGTCCGACCTG-3′	5′-CAATGTCGTTGCGGCACACC-3′
Catalase	*Cat*	5′-GCAGATACCTGTGAACTGTC-3′	5′-GTAGAATGTCCGCACCTGAG-3′
Actin, beta	*Actb*	5′-GGGCACAGTGTGGGTGAC-3′	5′-CTGGCACCACACCTTCTAC-3′

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