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Depletion of follicles accelerated by combined exposure to phthalates and 4-vinylcyclohexene diepoxide, leading to premature ovarian failure in rats

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ABSTRACT

Humans are at daily risk by simultaneous exposures to a broad spectrum of man-made chemicals in the commercial products. Several classes of chemicals have been shown to alter follicle development and reduce fertility, leading to premature ovarian failure (POF) in mammals. We investigate the synergistic effects of 4-vinylcyclohexene diepoxide (VCD) and phthalate, including di(2-ethylhexyl) phthalate (DEHP), butyl benzyl phthalate (BBP) and di-*n*-butyl phthalate (DBP) on POF. Combination exposure with VCD and phthalate significantly reduced the numbers of primary follicles. The expressions of *Amh* and *Sohlh2* were significantly decreased in the combination groups. Serum Amh levels were significantly lower in the combination groups. Additionally, serum levels of follicle-stimulating hormone were significantly increased in combination groups. Taken together, exposure to phthalates promotes the depletion of follicular follicles and consequently increases the risk of premature menopause, and combined exposure of phthalates and VCD to early menopausal women is likely to aggravate the POF syndrome.

1. Introduction

Currently, many humans are at daily risk of exposure to a broad spectrum of man-made chemicals, and several classes of such chemicals have similar adverse effects on human health. However, little has been reported about the human health effects of combined exposures to different chemicals on human health. Individual evaluation of a chemical's effects does not elucidate its effect when the subject is also exposed to other chemicals.

Endocrine disrupting chemicals (EDCs) are present in the environment, leading to potential daily human exposure. EDCs not only exert estrogenic effects, but they also have antiandrogenic, thyroid, peroxisome proliferator-activated receptor γ, retinoid effects, and through nuclear receptors can affect steroidogenesis [1,2]. Phthalate esters are alkyl diesters of phthalic acid and are EDCs that are widely used as plasticizers; moreover, they are present in a variety of consumer products. Phthalates have an estrogenic capacity, for example, butyl benzyl phthalate (BBP) has *in vitro* and *in vivo* estrogenic effect [3,4]. However, di-*n*-butyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) have very weak estrogenic activity; moreover, they can bind to both ERα and androgen receptors [4,5]. As a fundamental regulator of the female reproductive system, the ovary has been shown to be adversely affected by phthalate exposure. Phthalates have been shown to adversely affect two essential ovarian processes, folliculogenesis and steroidogenesis, by altering ovarian and oocyte development, accelerating primordial follicle recruitment, and inducing atresia in follicles during several stages of development [6].

The occupational chemical 4-vinylcyclohexene diepoxide (VCD) is widely used as a chemical intermediate and/or reactive diluent for diepoxides and epoxy resins [7]. In addition, VCD is reported to be ovotoxic [8] and is often used as a positive control in ovo-toxicity studies. Previous studies have suggested that both VCD and phthalates can

Abbreviations: Amh, anti-Müllerian hormone; BBP, butyl benzyl phthalate; Cyp11a1, cytochrome P450 family 11, subfamily A, polypeptide 1; DBP, di-*n*-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; EDCs, endocrine disrupting chemicals; FSH, follicle-stimulating hormone; Hsd3b1, hydroxy-delta-5-steroid dehydrogenase; POF, premature ovarian failure; Sohlh2, spermatogenesis and oogenesis helix-loop-helix 2, StAR, steroidogenic acute regulatory protein; VCD, 4-vinylcyclohexene diepoxide

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affect ovarian function. Thus, we hypothesized that a combination of exposures to VCD and phthalates can affect ovarian function through their combined effects on folliculogenesis and steroidogenesis. Moreover, we question whether combined exposures to VCD and phthalate can lead to development of the premature ovarian failure (POF) syndrome.

The POF syndrome is observed in women younger than 40 years of age. Women with POF have typical characteristics including primary or secondary amenorrhea (primary amenorrhea in 10%–28% and secondary amenorrhea in 4%–18% of POF women), hypergonadotropinism, and hypoestrogenemia. Reported causes of POF include idiopathic, genetic, autoimmune, and iatrogenic sources; moreover, POF may be the result of adverse effects from EDC exposure [9].

Early follicular growth and development are controlled by both paracrine and endocrine factors. Spermatogenesis and oogenesis helix-loop-helix 2 (*Sohlh2*) is primarily expressed in primordial and primary ovarian follicles and is undetectable in growing oocytes [10]. *Sohlh2* deficiency is reported to accelerate postnatal oocyte loss [11], produce a lack of ovarian follicle growth in the ovary, and cause female infertility. Additionally, anti-Müllerian hormone (*Amh*) has an important role in folliculogenesis and is expressed in granulosa cells from the primary to preantral follicular stages [12–15]. *Amh* acts as an inhibitory signal factor that suppresses folliculogenesis of immature follicles during the primordial-to-primary follicle transition. In mouse ovary, the absence of *Amh* has induced early depletion of primordial follicles [16]. Both *in vivo and in vitro* experiments have indicated that *Amh* deficiency enhances the follicular transition from primordial to growing, thereby leading to early exhaustion of the primordial follicle pool [17,18]. Notably, serum Amh levels have been reported to be associated with the ovarian reserve of follicles (*i.e.*, the follicle pool) [19], and it has been used as a marker for the ovarian reserve [20].

Ovarian steroidogenesis is a step-wise process involving multiple enzymes that change cholesterol into biologically active steroid hormones. Phthalates are weakly estrogenic substances that have been reported to increase steroidogenic enzyme mRNA levels of steroidogenic acute regulatory protein (*StAR*) and 3b-hydroxysteroid dehydrogenase/D5-D4 isomerase (*Hsb3b1*) in theca cells [21]. Moreover, levels of *StAR* mRNA, as well as those of cholesterol side-chain cleavage enzyme cytochrome P450scc (*Cyp11a1*) and *Hsb3b1* have been markedly increased by VCD administration [22].

In this study, we investigated the effects of phthalate and VCD treatments on adult Sprague-Dawley (SD) female rats. Adult SD rats have been reported to be sensitive to the irritant properties of VCD [23]; thus we investigated VCD and phthalate effects at low dosages over a long period. In addition, we focused on a set of “active” phthalates (DEHP, BBP, DBP) that are produced in large quantities and are commonly used in consumer products [24]. The expressions of *Amh* and *Sohlh2* were investigated to clarify the effects of phthalates and VCD on ovarian development. In addition, the expressions of steroidogenic enzymes were measured to determine the effects of phthalates and VCD on ovarian granulosa cells.

2. Methods and materials

2.1. Chemical

The 4-vinylcyclohexene diepoxide (VCD, 96%), di(2-ethylhexyl) phthalate (DEHP, 99.5%), di-*n*-butyl phthalate (DBP, 99%), and butyl benzyl phthalate (BBP, 98%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Stock solutions were diluted with corn oil (Sigma-Aldrich).

2.2. Animals and treatments

Forty-eight female SD rats at 42-days-old were purchased from Samtaco (Osan, Gyeonggi, Republic of Korea). The rats were housed in polycarbonate cages under a controlled environment with a 12-h light/dark cycle, a constant temperature of 23 °C ± 1 °C, and a relative humidity of 50% ± 10%. The rats were fed a diet of AIN-76 A and tap water. After a one week adaptation period, the 48 rats were randomly divided into eight groups (6 per group). Vehicle (VE) rats received an intraperitoneal (i.p) injection of corn oil daily for 2 weeks. The seven other groups received VCD + corn oil, DEHP or DEHP + VCD, BBP or BBP + VCD, and DBP or DBP + VCD groups. Rats in the groups treated with VCD received an i.p injection of VCD (80 mg/kg) daily for 2 weeks. After receiving the VCD or VE i.p. injection, corn oil or a phthalate were orally administered once a day for the entire 6-week study period. All treatment chemicals were dissolved in corn oil (Sigma-Aldrich). Rats were sacrificed 24 h after final administration. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Chungbuk National University.

2.3. Vaginal smear test

Vaginal smears were collected from all rats to check estrous cycle stage every day at 9 a.m since treated phthalates. After mounting the vaginal smears on slides, the slides were steeped in methanol for 5 min. Next, they were stained and imaged under a light microscope to determine estrus stage. Estrous cycle stages were classified as proestrus, estrus, metestrus, and diestrus based on the predominance of epithelial round cells, cornified cells, round cells plus leukocytes cells, or leukocytes, respectively.

2.4. Quantitative real-time PCR

Total RNA was extracted from the ovaries of all rats in each group by using Trizol reagent (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The total RNA concentration was determined by measuring absorbance at 260 nm. First-strand complementary DNA (cDNA) was prepared from 1 μg of total RNA by reverse transcription using Moloney murine leukemia virus reverse transcriptase (iNtRON Bio, Gyeonggi-do, Korea) and random primers (9-mers; TaKaRa Bio, Shiga, Japan). Quantitative real-time PCR was performed with 1 μl of the cDNA template and 2 × SYBR green (TaKaRa Bio) containing specific primers. The primer sequences are presented in Table 1. The qRT-PCR was carried out for 40 cycles using the following parameters: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity was the threshold cycle (CT). The expression of the target gene was quantified relative to that of the internal vehicle gene (18S ribosomal RNA) by comparing CTs at a constant fluorescence intensity. The 18 s rRNA levels were not significantly altered by the experimental conditions.

Table 1
Primer sequences used when performing real-time PCR.

Gene	Primer sequence (5′→ 3′)	Accession no.
Cyp11a1	F: GCTTTGCCTTTGAGTCCATC R: CATGGTCCTTCCAGGTCTTA	NM_017286.3
Hsd3b1	F: TGCCACTTGGTCACACTGTCA R: CCCTGTGCTGCTCCACTAGTGT	NM_001007719.3
StAR	F: GCGGAACATGAAAGGACTGA R: TCCTTGCTGGATGTAGGACA	NM_031558.3
Sohlh2	F: GTTGGAGATGCCACACGATA R: GCTACCTTCTTCACGTTGCT	NM_001034961.1
Amh	F: CTGGCTGAAGTGATATGGGA R: CACAGTCAGCACCAAATAGC	NM_012902.1
18 s rRNA	F: AGACTGTGTCCCTGTGGAGA R: GGACACGGACAGGATTGACA	NR_046237.1