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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 ER α 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 ovoidotoxic [8] and is often used as a positive control in ovoidotoxicity 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 hypoeestrogenemia. 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 3 β -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 \pm 1 °C, and a relative humidity of 50% \pm 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 μ g 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 μ l of the cDNA template and 2 \times 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.
<i>Cyp11a1</i>	F: GCTTTCCTTTGAGTCCATC R: CATGGTCCTTCCAGGTCCTTA	NM_017286.3
<i>Hsb3b1</i>	F: TGCCACTTGGTCACACTGTCA R: CCCTGTGTGTCCACTAGTGT	NM_001007719.3
<i>StAR</i>	F: GCGGAACATGAAAGGACTGA R: TCCTTGCTGGATGTAGGACA	NM_031558.3
<i>Sohlh2</i>	F: GTTGGAGATGCCACACGATA R: GCTACCTTCITCAGCTTGCT	NM_001034961.1
<i>Amh</i>	F: CTGGCTGAAGTGATATGGGA R: CACAGTCAGCACCAAATAGC	NM_012902.1
18 s rRNA	F: AGACTGTGTCCCTGTGGAGA R: GGACACGGACAGGATTGACA	NR_046237.1

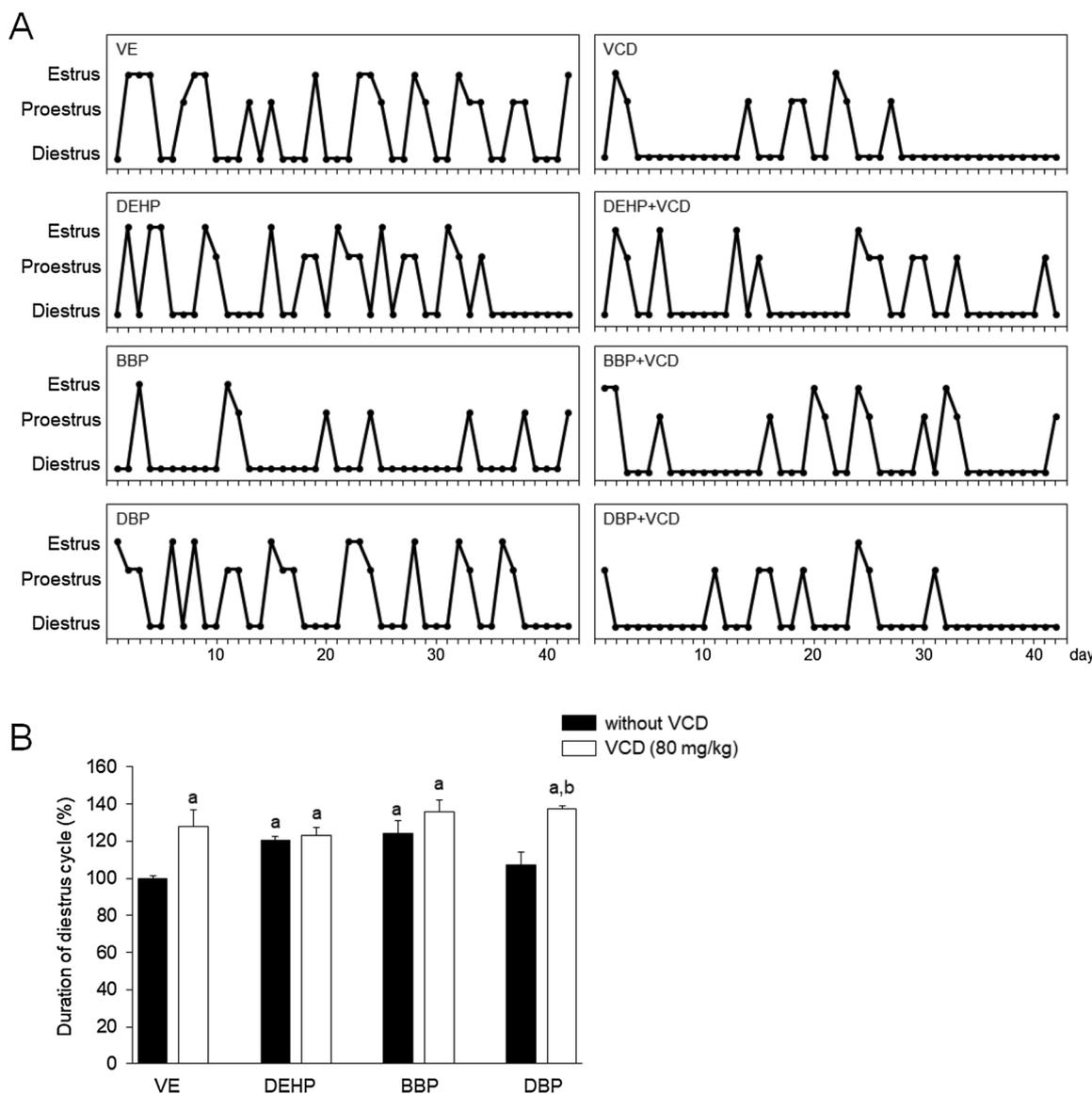


Fig. 1. Effects of phthalates and VCD on rat estrus cycle.

(A) The estrus cycles of rats in each group were monitored by examination of vaginal smears. Each stage of the estrus cycle was determined by the predominance of lymphocytes, nucleated epithelial cells, or keratinocytes. (B) The diestrus stage was prolonged by treatment with DEHP only, BBP only, and VCD with or without phthalates (6 rats per group). Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a $p < 0.05$ vs. Vehicle (VE), ^b $p < 0.05$ vs. phthalate. VE, corn oil; VCD, 80 mg/kg/day; DEHP, 25 mg/kg/day; BBP, 250 mg/kg/day; DBP, 250 mg/kg/day.

2.5. Western-blot analysis

Three random ovaries of euthanized rats in each group were rapidly excised and washed in cold sterile 0.9% NaCl solution. Proteins were extracted by using Pro-prep solution (InTron, Seoul, Republic of Korea) according to the manufacturer's protocol. Cytosolic proteins (20 μ g) were separated by performing 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the product was transferred to a polyvinylidene fluoride (PVDF) membrane (Perkin Elmer, Wellesley, MA, USA) in a TransBlot Cell (TE-22, Hoefer, CA, USA) according to the manufacturer's protocol. The resulting blot was blocked in TBS (Tris-buffered saline) containing 5% skim milk for 60 min, then incubated at room temperature in primary antibody SOHLH2 (Origene, catalog TA330852, diluted 1:1000) for 4 h or α -Tubulin (Cell Signaling Technology, catalog No. #2144, diluted 1:2000) for 60 min. After washing with TBS-T buffer, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:2000, Santa Cruz) for 1 h at

room temperature. Then, the membrane was washed four times for 10 min each with TBS-T. Enhanced chemiluminescence reagent (Santa Cruz Biotechnology) with a charge-coupled device was used to detect antibody binding. Western blots were assessed by using the ChemiDoc (Bio-Rad) or GenGnome 5 (Syngene, Cambridge, UK) imaging systems. The optical density of the target band was analyzed by using Image J (NIH, Bethesda, MD, USA).

2.6. Hematoxylin and eosin staining

Three random ovaries from rats in each group were used for histological analysis. Initially, the ovary was fixed in 10% neutral-buffered formalin, dehydrated at room temperature for 24 h, and embedded in paraffin. The ovary was then cut across the mid-region of the ovary into slices with a thickness of 4 μ m, and the slices mounted on slides. The sections were then stained with hematoxylin and eosin and imaged by using light microscopy (BX51; Olympus, Tokyo, Japan) to determine follicle numbers and stages. To quantify the number of follicles, follicles

from six sections of each ovary were counted and the numbers averaged. Follicle stages were classified as primordial, primary, and secondary depending on the shape and number of layers of granulosa cells and the presence or absence of an antral cavity.

2.7. Serum FSH and Amh analysis

Blood samples were collected from rats at 24 h after the final treatment administration. Serum was separated and stored at -70°C until required for serum follicle-stimulating hormone (FSH) and anti-Müllerian hormone (Amh) analysis. The serum levels of FSH and Amh were determined by using commercial ELISA kits (FSH ELISA Kit, Cusabio, MD, USA and AMH ELISA Kit, MyBioSource, San Diego, USA, respectively). Quantitative determination of hormone concentrations was performed as per the manufacturers' protocols.

2.8. Statistical analyses

Statistical significance was determined by using one-way ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons. Real-time PCR and western-blot experiments consisted of three separate trials. Data were analyzed by using GraphPad Prism (v.4.0; GraphPad Software, La Jolla, CA, USA) and are presented as mean \pm SEM values. The *p* values for each comparison are described in the figure legends.

3. Results

3.1. Effects of phthalates and VCD on estrous cycle

The estrous cycles of the subject rats were checked daily by performing a vaginal smear test. Both phthalates and VCD have been reported to disrupt the estrous cycles in rodents [25,26]. The typical rat estrous cycle is 4–5 days long (proestrus = 1 ± 0.5 day, estrus = 1 day, metestrus = 1 day, diestrus = 1 ± 0.5 day). In each group, cycle stages were determined daily and their durations were monitored (Fig. 1A). The estrous cycle of the VE group had a regular repeating cycle, whereas the diestrus phases in the DEHP and BBP groups were drawn out and were particularly prolonged in the VCD and phthalate combination groups (Fig. 1B). However, DBP treatment did not affect the estrous cycle pattern. There were no significant differences in estrous cycles between the VCD group and the VCD + phthalate combination groups.

3.2. Effects of phthalates and VCD on the number of primordial, primary, and secondary follicles

In all groups, follicles were counted in the largest cross-section of the ovary and were classified into primordial, primary, and secondary stages based on the histological morphologies of the granulosa cells and the oocytes. Compared to the VE group, the numbers of primordial and primary follicles were significantly lower in all groups, except the BBP group (Fig. 2A–2B). The number of primordial follicles was low in the BBP group but not significantly low. Notably, the VCD + phthalate combination treatments markedly reduced the numbers of primary follicles, even showing a complete absence of primary follicles in some ovaries in the BBP + VCD and DBP + VCD groups (Fig. 2B). Additionally, there were marked decreases in primordial and primary follicles in the combination groups compared to the numbers in the DEHP-, BBP-, DBP-, or VCD-alone treatments. The number of secondary follicles was significantly lower in the VCD + phthalate combination groups than those in the VE and phthalate-only groups (Fig. 2C). There were no significant differences in numbers of Graafian follicles among the study groups (data not shown). These results suggest that a combination of phthalates and VCD may result in mature ovarian failure.

3.3. Effects of phthalates and VCD on steroidogenesis in rat ovary

The mRNA expressions of ovarian steroidogenesis (cholesterol metabolism)-associated genes such as *StAR*, *Cyp11a1*, and *Hsd3b1* were determined by performing real-time PCR. The expressions of *StAR* mRNA expression were significantly higher in the DEHP and DBP groups than in the VE group (Fig. 3A). Interestingly, treatment of DBP alone produced a lower expression of *StAR* mRNA than those in the VCD and DBP + VCD groups. The expressions of *Cyp11a1* and *Hsd3b1* mRNA were not significantly different among the various groups (Figs. 3B and 3C, respectively). These results indicate that only *StAR* expression was affected by the phthalate treatment and that VCD + phthalate combination treatments did not significantly affect steroidogenesis.

3.4. Effect of phthalates and VCD on *Sohlh2* mRNA and protein expression in rat ovary

In order to elucidate the mechanism by which the combination of phthalate and VCD may affect the formation of primordial follicles, we investigated the effects of phthalate exposure on *Sohlh2* mRNA and protein expressions (Fig. 4A). DEHP, BBP, and DBP administered individually had no significant effect on the expression of *Sohlh2* mRNA. However, the DEHP + VCD, BBP + VCD, and DBP + VCD combination treatments reduced *Sohlh2* mRNA expression by approximately 94%, 85%, and 86%, respectively, from that in the VE group. Further, *Sohlh2* mRNA levels in the DEHP + VCD was significantly lower than that in the VCD-alone group. Similarly, compared to the VE group, *Sohlh2* protein expressions were markedly lower in all groups, except the DEHP group. Notably, *Sohlh2* protein expression was also lower in the DEHP + VCD group than in the DEHP group (Fig. 4B). These results suggest that VCD + phthalate combination exposure can impair primordial follicle development.

3.5. Effect of phthalates and VCD on *Amh* mRNA expression in rat ovary and on serum *Amh* level

To investigate whether phthalates and VCD affect folliculogenesis, we assessed the expression of *Amh* mRNA in ovary (Fig. 5A). The mRNA expression of *Amh* was significantly lower (approximately 80% lower) in the VCD + phthalate combination groups than that in the VE group. There were no differences in *Amh* mRNA expressions between the VE group and the VCD-alone or phthalate-alone treatment groups. Treatment with DEHP or BBP produced higher *Amh* mRNA expressions than that in the VCD group. The *Amh* mRNA expression levels were significantly lower in the VCD + phthalate combination groups than that in the VCD-alone group. Moreover, *Amh* mRNA expressions in the combination groups were significantly lower than those in the DEHP-, BBP-, and DBP-alone groups. These results suggest that the combined treatment of phthalates and VCD significantly inhibits the expression of *Amh* mRNA, producing *Amh* mRNA levels lower than those obtained from DEHP-, BBP-, DBP-, or VCD-alone administrations.

Serum Amh level has been used as a marker for the ovarian reserve, and 3.5 ng/ml of serum Amh is considered a cut-off; value below which there is a high risk of ovarian hyperstimulation syndrome in humans [27]. In this study, compared to that in the VE group (VE = 12.53 ± 1.75 ng/mL), serum Amh levels were not significantly lower in the individual phthalate groups (DEHP = 8.20 ± 3.25 ng/mL; BBP = 7.60 ± 3.09 ng/mL; DBP = 7.85 ± 2.58 ng/mL) (Fig. 5B). Serum Amh levels were approximately 6 times lower in the DEHP + VCD (2.33 ± 1.09 ng/mL) and DBP + VCD (2.08 ± 1.71 ng/mL) groups than in the VE group, approximately 4 times lower in the BBP + VCD group (BBP + VCD = 3.41 ± 1.73 ng/mL) than in the VE group, and approximately 2 times lower in the VCD-alone group (VCD = 5.45 ± 1.33 ng/mL) than in the VE group (Fig. 5B). Moreover, the VCD + phthalate combination treatment results were significantly

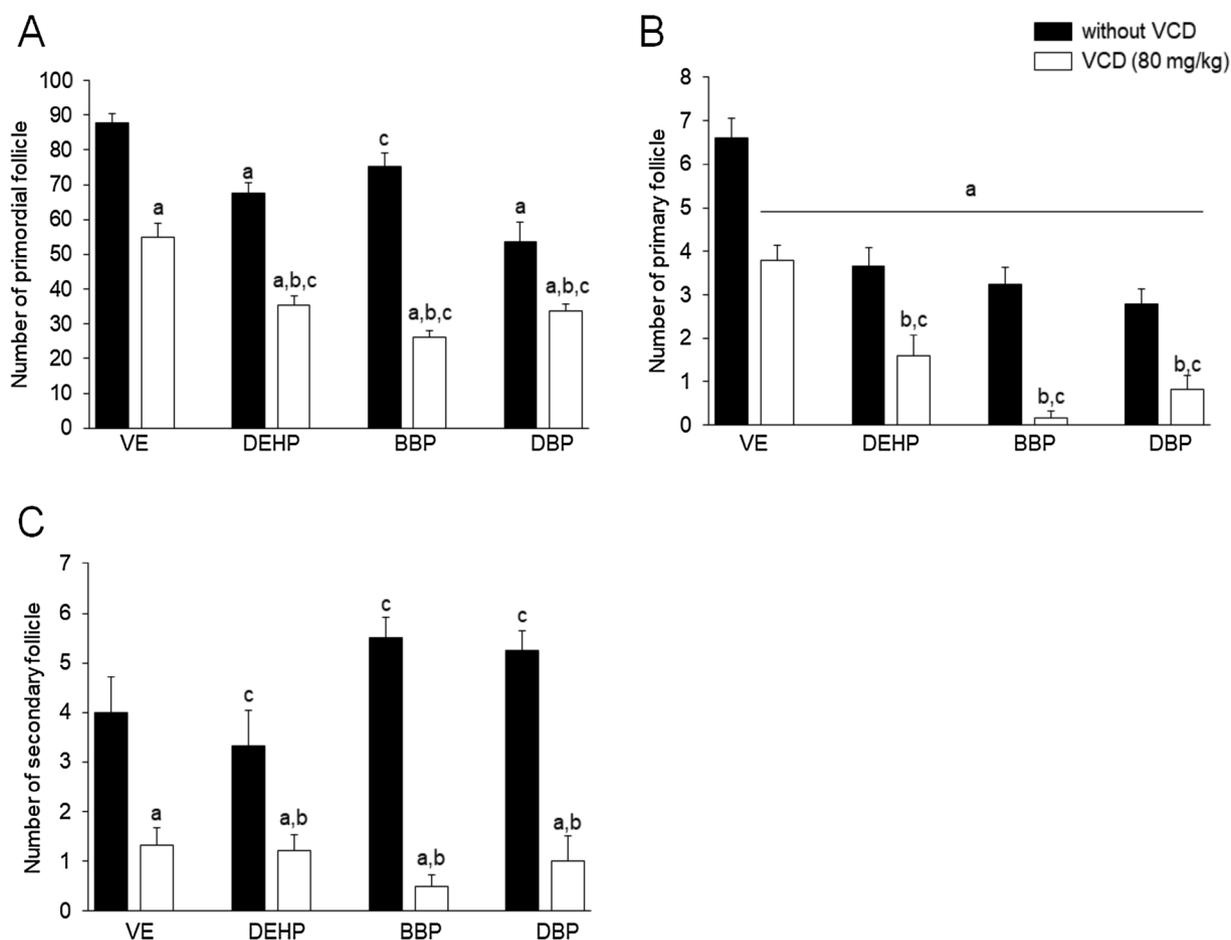


Fig. 2. The number of ovarian is decreased in phthalate and VCD treatment.

The sections from the ovary region with the greatest cross-sectional area were hematoxylin and eosin stained for histological analysis. Quantification of primordial (A), primary (B), and secondary (C) follicles were characterized in each ovary. The number of primordial and primary follicles were significantly decreased by both phthalate and VCD treatments with marked reductions in the VCD + phthalate combination groups. The number of secondary follicles was significantly different between the VCD-only group and the combination BBP + VCD group. $N = 3$ ovaries per group. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a $p < 0.05$ vs. VE, ^b $p < 0.05$ vs. phthalate, ^c $p < 0.05$ vs. VCD.

lower than those from the single phthalate or VCD treatments. Taken together, our results suggest that combined exposure to VCD and phthalates can affect follicle development by reducing the expression of *Amh* in ovary.

3.6. Change in serum FSH levels

A rise in serum FSH concentration has been shown to indicate an accelerated loss of the ovarian reserve, and POF can be diagnosed on the basis of serum FSH concentration (*i.e.*, POF presence if $FSH \geq 40$ mIU/mL). Compared to the VE group ($VE = 14.17 \pm 0.38$ mIU/mL), serum FSH levels were approximately 1.5 times higher in the single treatment phthalate groups ($DEHP = 20.62 \pm 1.06$ mIU/mL; $BBP = 21.09 \pm 0.68$ mIU/mL; $DBP = 20.05 \pm 1.02$ mIU/mL) (Fig. 6). Notably, serum FSH levels were approximately 4.5 times higher in the DEHP + VCD (63.87 ± 1.06 mIU/mL), BBP + VCD (62.85 ± 3.32 mIU/mL), and DBP + VCD (62.70 ± 3.01 mIU/mL) combination groups than in the VE group and was approximately 4 times higher in the VCD group ($VCD = 55.29 \pm 3.5$ mIU/mL) than in the VE group (Fig. 6). Moreover, the VCD + phthalate combination treatment results were approximately 3 times higher than those from the phthalate-only treatment groups. In addition, the serum FSH levels in the VCD + phthalate treatment groups were significantly higher than that in the VCD group. These results show that a combined exposure to VCD and phthalates can lead to a substantial reduction in oogenesis.

4. Discussion

The purpose of this study was to assess the effects of combined phthalate and VCD exposures on ovarian function. The study hypothesis was that exposure to both VCD and phthalates would disrupt the growth and development of ovarian follicles, affect the biosynthesis of ovarian steroids, and lead to the POF syndrome.

At birth, the ovaries of female neonates contain a limited number of primordial follicles. Folliculogenesis is an irreversible process, in which a recruited primordial follicle grows and develops into primary follicle before becoming a preantral follicle and, ultimately, a mature antral follicle. Ovarian steroid production has important roles in the maintenance of standard ovarian development and function.

Exposure to high doses (1000 mg/kg/day) of DEHP is reported to result in a significant decrease in the number of primordial follicles and an increase in the numbers of primary, secondary, and antral follicles, thereby shortening reproductive lifespan *via* earlier entry into ovarian failure [28]. Moreover, DEHP exposure can directly disrupt the estrous cycle and significantly decrease primordial follicle abundance in ovary after administration for 10 days [29]. DBP has been shown to significantly suppress the growth of antral follicles and induce follicular atresia [30]. Short-term exposure to DBP decreases the serum estradiol level and the number of antral follicles but increases the serum FSH level [31]. Additionally, exposure to DBP is reported to significantly increase serum levels of estradiol and progesterone [32]. Exposure to

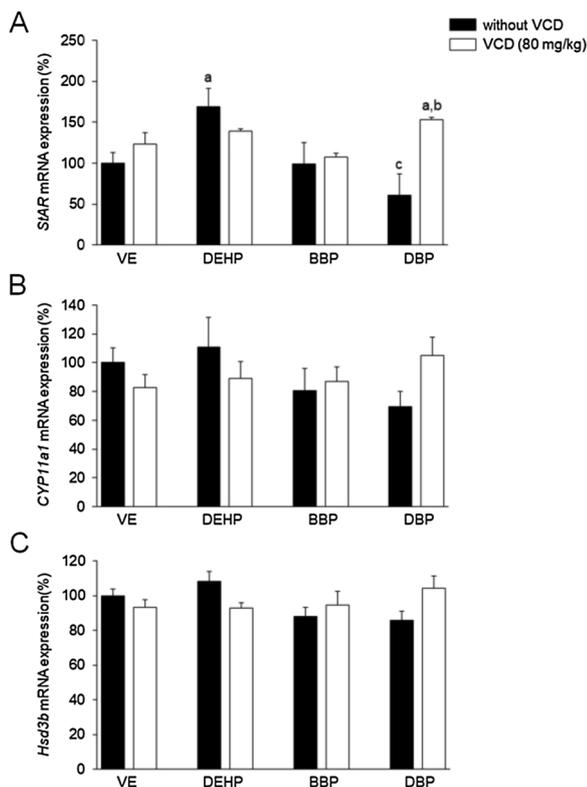


Fig. 3. Expression of steroidogenesis-related genes.

The mRNA levels of steroidogenesis-related genes were assessed by using real-time PCR in rat ovary tissues. A: Expression of *StAR* mRNA was significantly high in the DEHP and DBP + VCD groups. B, C: Expressions of *Cyp11a1* and *Hsd3b* mRNA were not significantly different between the VE group and all other groups. Results were internally confirmed by the comparative cycle threshold method against 18S as the standard gene. N = 6 rats per group. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a*p* < 0.05 vs. VE, ^b*p* < 0.05 vs. phthalate, ^c*p* < 0.05 vs. VCD.

VCD results in selective destruction of primordial and primary follicles in both rat and mouse following *in vivo* or *in vitro* exposure [33–37].

In this study, the numbers of primordial and primary follicles were significantly decreased in all groups treated with VCD and/or phthalates from those in the VE group. The decreases in primordial and primary follicles after VCD + phthalate combination treatments were markedly greater than those after individual VCD or individual phthalate treatments. Compare to the VE group, the number of secondary follicles was significantly lower in the VCD and VCD + phthalate groups. These results suggest that combined exposure to VCD and phthalates can reduce the number of primordial and primary follicles. Moreover, VCD + phthalate combination exposure has a greater effect on primordial and primary follicles than the effects from individual exposures by VCD, DBP, BBP, or DEHP.

Ovarian steroidogenesis is a step-wise process involving multiple enzymes, including *StAR*, *Cyp11a1*, and *Hsd3b1*, that process cholesterol into biologically active steroid hormones. The *StAR*, *Cyp11a1*, and *Hsd3b1* mRNA expressions were shown to increase at a VCD treatment concentration of 160 mg/kg/day at 20 days after such exposure [22]. In addition, phthalates have been reported to increase the expressions of the *StAR*, *Cyp11a1*, and *Hsd3b1* genes [21]. In this study, the *Cyp11a1*, and *Hsd3b1* mRNA expressions were not significantly changed by the administration of phthalates and VCD in either individual or combination treatments; however, there were changes in *StAR* mRNA expressions. The contrast in results between [22] and this study may be due to differences in concentrations and administration times; therefore, we will focus on assessing both VCD and phthalate concentrations and exposure times in future studies.

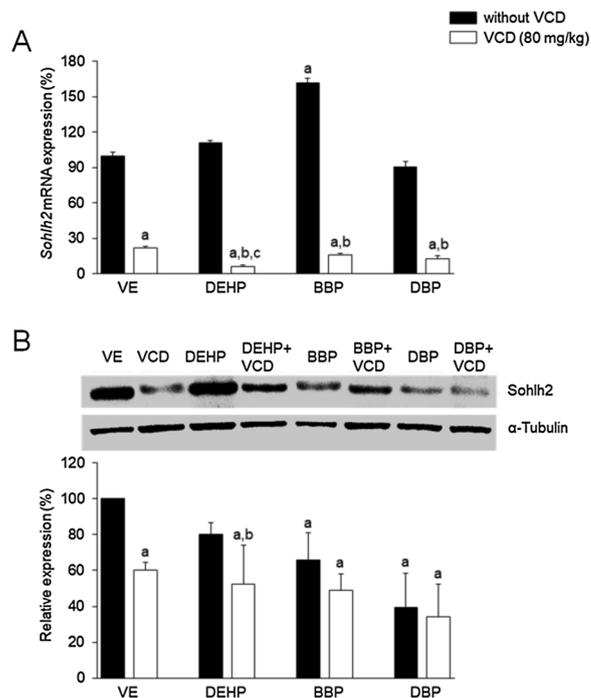


Fig. 4. Expression of *Sohlh2* in rat ovaries.

The mRNA and protein expressions of *Sohlh2* in rat ovary were decreased by phthalate and VCD treatments. A: The mRNA levels of *Sohlh2* were assessed by performing real-time PCR on rat ovary tissues. Results were internally confirmed by the comparative cycle threshold method against 18S as a standard gene. N = 6 rats per group. B: Expression of *Sohlh2* protein was investigated by western blotting. The band intensity of the *Sohlh2* protein was normalized to the α -tubulin level. Histograms indicate quantification of blots. N = 3 rats per group. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a*p* < 0.05 vs. VE, ^b*p* < 0.05 vs. phthalate, ^c*p* < 0.05 vs. VCD.

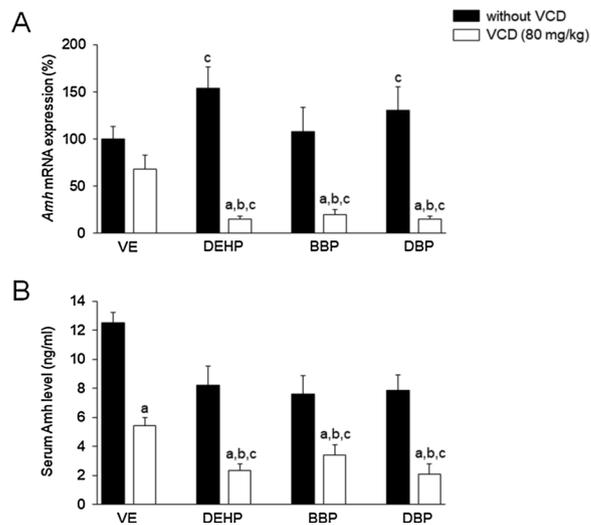


Fig. 5. Change in mRNA expression of *Amh* in rat ovaries and serum Amh level.

A: The mRNA levels of the *Amh* gene were assessed by performing real-time PCR on rat ovary tissues. The *Amh* mRNA levels were significantly low in the VCD + phthalate combination groups. Results were internally confirmed by the comparative cycle threshold method against the 18S standard gene. B: The serum Amh levels were measured at the end of the six week treatment period by using ELISA kits. The serum Amh levels were markedly low in the VCD + phthalate combination groups. N = 6 rats per group. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a*p* < 0.05 vs. VE, ^b*p* < 0.05 vs. phthalate, ^c*p* < 0.05 vs. VCD.

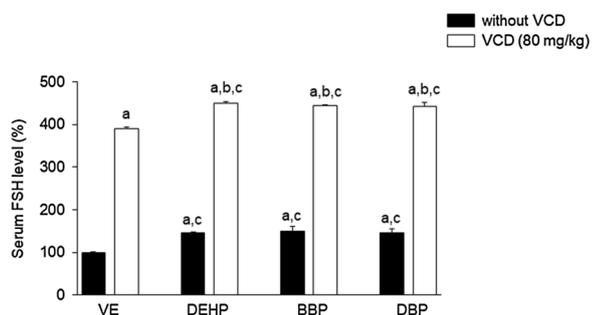


Fig. 6. Effects of phthalates and VCD on serum FSH levels.

The serum FSH levels were measured at the end of the six week treatment period by using ELISA kits. The serum FSH levels were significantly high in the single treatment groups and markedly high in the VCD + phthalate combination groups. $N = 6$ rats per group. Statistical significance was determined by one-way ANOVA with the Bonferroni correction test. ^a $p < 0.05$ vs. VE, ^b $p < 0.05$ vs. phthalate, ^c $p < 0.05$ vs. VCD.

Among the folliculogenesis related genes, *Sohlh2*, a spermatogenesis- and oogenesis-specific basic helix-loop-helix transcription factor, is primarily expressed in primordial and primary ovarian follicles but is undetectable in growing oocytes [10]. Previous study results have suggested that *Sohlh2* deficiency is related to disruption of the transition from primordial follicle to primary follicle. Moreover, DEHP exposure can reduce the expression of *Sohlh2* mRNA *in vitro* [38]. In this study, the mRNA expression of *Sohlh2* was significantly lower in the DEHP + VCD, BBP + VCD, and DBP + VCD groups than in the VE group. Further, *Sohlh2* mRNA levels in the DEHP + VCD and BBP + VCD groups were significantly lower than that in the VCD-only group. *Sohlh2* protein expressions in the DEHP and VE groups were not significantly different, but *Sohlh2* expressions in all other groups were markedly lower than that in the VE group. Recently, variants in the *Sohlh2* gene have been shown to contribute to human POF in different ethnicities [39]. The results of this study suggest that combined exposure to VCD and some phthalates may result in premature exhaustion of the available oocyte supply *via* the reduction of *Sohlh2* expression.

Amh is a member of the TGF- β family of growth factors and has important roles during the initial and cyclic recruitment of ovarian follicles. Amh is produced by the pool of growing follicles and acts as a feedback signal to inhibit the recruitment of primordial follicles [40]. From the primary stage onward, all follicles express Amh; as well, Amh is secreted by the granulosa cells of maturing follicles. This may explain the marked decrease in *Amh* mRNA expression and serum Amh level when loss of primary follicles occurs. In previous studies, the serum Amh level was significantly decreased in response to a high-dose VCD treatment (240 mg/kg/day) [41]. Additionally, VCD has been shown to decrease the ovarian expression of *Amh* mRNA in PND4 rat ovary culture [42]. The effect of phthalates on *Amh* expression has not been fully elucidated. However, in the present study, a combination of exposures to VCD and phthalates resulted in greater loss of primary follicles than that from individual VCD or phthalate treatments. In addition, *Amh* mRNA expression and serum Amh levels were also significantly reduced. The mRNA expressions of *Amh* were significantly lower (by approximately 80%) in the VCD + phthalate groups than in the VE and VCD-only groups. However, there were no differences in *Amh* mRNA expressions in the individual phthalate groups or in the VCD-only group from that in the VE group. Moreover, *Amh* mRNA expressions were significantly lower in the combination groups than those in the individual phthalate and VCD groups. Compared to the VE group, serum Amh levels were markedly lower in the DEHP + VCD and DBP + VCD groups, less so in the BBP + VCD group, and even lower in the VCD group. Further, serum Amh level was not significantly lower in the individual phthalate and VCD groups than in the VE group. Overall, the serum Amh levels in the VCD + phthalate groups were approximately

2.5 times lower than those in the single treatment groups. As mentioned, the serum Amh level (less than 3.5 ng/ml) can be used to indicate a risk of ovarian hyperstimulation syndrome [27], and a reduced Amh level can be used as a marker of fertility decline, such as that associated with the POF syndrome [43]. These results suggest that exposure to a combination of VCD and phthalates can induce the loss of primary follicles by strongly inhibiting the mRNA expression of *Amh* and decreasing the serum Amh level.

Moreover, folliculogenesis in the ovary is mediated by endocrine factors of the pituitary-gonadotropin axis such as FSH and LH. Recently, serum FSH concentration has been used as a marker of the ovarian reserve. In a previous study, DEHP orally treated at 2 g/kg/day decreased the serum estradiol level in adult rats [44], leading to a secondary rise in FSH. Moreover, DBP exposure has increased serum FSH levels in mice treated with DBP at 0.1 and 1000 mg/kg [31], whereas, VCD-treated mice had increased levels of FSH at dose levels of 80–320 mg/kg [22,45]. On the basis of serum FSH concentration, POF is currently diagnosed when the serum FSH concentration is ≥ 40 mIU/mL. In the present study, serum FSH levels were approximately 1.5 times higher in the single treatment groups than in the VE group. Notably, serum FSH levels were markedly higher (approximately 4.5 times) than that in the VE group in the DEHP + VCD, BBP + VCD, and DBP + VCD groups and approximately 4 times higher than the VE level in the VCD-only group. In addition, the VCD + phthalate combination treatments produced FSH levels that were 3 times higher than those in the single treatment phthalate groups. Moreover, serum FSH levels in the combination groups were significantly higher than that in the VCD group. Previous studies have shown that an accelerated loss of ovarian reserve is reflected by a rise in serum concentration of FSH. Taken together, the results of this study suggest that exposure to VCD and phthalate, separately or in combination, can lead to POF.

5. Conclusion

POF is biochemically characterized by high level of FSH and a decrease of serum Amh level [43]. In the present study, treatment with VCD alone or VCD treatment combined with DEHP, BBP, or DBP treatments elevated serum FSH levels and reduced serum Amh levels. Additionally, the results indicate that a combined exposure to phthalates and VCD can result in POF. The combined effects of phthalate and VCD exposures produced a greater level of harmful effects than those from the individual phthalate and VCD treatments. Further, the results indicate that exposure to phthalates promotes the depletion of follicles and, as a consequence, can increase the risk of premature menopause. Moreover, combined exposure to phthalates and VCD in early menopausal women is likely to exacerbate the potential for POF syndrome development.

Author contributions

Dinh Nam Tran, Changhwan Ahn, and Eui-Bae Jeung designed the experiments; Dinh Nam Tran performed the experiments; Eui-Man Jung, Changhwan Ahn, and Hee Young Kang analyzed the data; Dinh Nam Tran wrote the paper. Eui-Man Jung, Yeong-Min Yoo, and Changhwan Ahn revised the manuscript; Eui-Bae Jeung conceived and supervised the study.

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