Int J Clin Exp Med 2017;10(11):15775-15781
www.ijcem.com /ISSN:1940-5901/IJCEM0061849

Original Article

Short-term effects of 4-vinylcyclohexene diepoxide on ovarian follicular depletion and fertility in Sprague-Dawley rats

Fangui Zhao[1,2], Yuling Lian[1,2], Wenjun Wang[1,2]

[1]*Obstetrics and Gynecology Hospital, Fudan University, Shanghai, People's Republic of China;* [2]*Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, People's Republic of China*

Received July 19, 2017; Accepted November 1, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: The number of primordial follicles in the ovary is fixed at birth. As women age, the reserve of primordial follicles is depleted. This study aimed to explore the short-term effects of 4-vinylcyclohexene diepoxide (VCD) on ovarian follicular depletion and fertility in SD rats that were 4 weeks of age. Twenty-six 4-week-old female Sprague-Dawley (SD) rats were divided into two groups, the model and control groups. Five rats, aged 7 weeks, in each group were sacrificed to evaluate the ovarian reserve after 24 h of the final dose. The ovary weight index, histomorphological change and follicle count were determined. The serum level of AMH was assessed by ELISA, and the relative mRNA expression of AMH, ERα, ERβ, FSHR and PCNA of the ovary was detected by real-time PCR. The remaining 8 rats in each group were mated with adult male SD rats and the pregnancy time, litter size and pregnancy rate were recorded. At the age of 14 weeks, the remaining rats were sacrificed to count the follicles. The volume of the ovaries, ovary weight index and number of primordial follicles and primary follicles were reduced in rats that were 7 weeks of age in the model group compared with control group ($P < 0.05$). There was no significant difference in the numbers of secondary, antral follicles and corpus luteum; and there was no significant differrence in the serum AMH level and relative mRNA expression of AMH, ERα, ERβ, FSHR and PCNA in the ovary between the two groups ($P > 0.05$). The fertility of rats in the model group was similar to that of the control group ($P > 0.05$). The number of follicles at all stages in rats that were 14 weeks of age in the model group was lower than that of the control group ($P < 0.05$). Therefore, the short-term effects of VCD on SD rats induced the initial stage of DOR.

Keywords: Ovarian reserve, primordial follicle, 4-vinylcyclohexene diepoxide (VCD), fertility

Introduction

The number of primordial follicles in the ovary is fixed at birth. The ovarian reserve (OR) represents the stock of primordial follicles in the ovary, which is gradually depleted during a woman's reproductive lifespan, resulting in menopause. As women age, the reserve of primordial follicles is depleted, and the quality of oocytes, fertilization, and pregnancy rate are reduced [1]. Diminished ovarian reserve (DOR) is a manifestation of ovarian aging. As increasing numbers of women delay childbearing, DOR is becoming a greater challenge for providers of assisted reproductive technology (ART) [2]. In addition to reproductive concerns, DOR may also have adverse implications for women's wellbeing [3]. DOR is a complex clinical phenomenon that is influenced by age, genetics and environmental variables [4], and it is defined as the transitional period between normal OR and menopause.

According to increasing reports, the industrial chemical 4-vinylcyclohexene diepoxide (VCD) produces follicular depletion in rodents [5, 6]. VCD selectively destroys primordial and primary follicles by accelerating the natural process of follicular atresia, resulting in follicular depletion, which mimics the natural process of DOR [7, 8]. There have been reports on the long-term effects of VCD on ovarian follicular depletion in rats [9, 10]. In this study, we evaluated the short-term effects of VCD on ovarian follicular depletion and fertility in Sprague-Dawley rats.

Materials and methods

Animals and treatment

In total, twenty-six 3-week-old female Sprague-Dawley (SD) rats (35 ± 3 g weight) were provided by the Jiesijie Laboratory Animal Technology Co., Ltd. (Shanghai, China). Rats were housed in groups of 2 per cage at constant temperature

Table 1. Real time-PCR primers for mRNA

mRNA	Primer sequence (5'→3')
ERα-F	GCTTATTGACCAACCTGGCAGAC
ERα-R	AGGATCTCCAACCAGGCACAC
ERβ-F	GTACCATAGACAAGAACCGGCGTAA
ERβ-R	TCCGCACTATACGGTACCCACA
PCNA-F	GAGCTTGGCAATGGGAACA
PCNA-R	AGCTGAACTGGCTCATTCATCTCTA
FSHR-F	GCTGGATTTGGAGACCTGGAGA
FSHR-R	CATGCAACTTGGGTAGGTTGGAG
AMH-F	TGTTACAGGCTGACACAGTTGAAGA
AMH-R	ACCAGGCACAAAGGCTCAGG
Gapdh-F	GGCACAGTCAAGGCTGAGAATG
Gapdh-R	ATGGTGGTGAAGACGCCAGTA

(22 ± 1°C), humidity (50 ± 5%), and light (12 h/d) conditions with a standard pellet diet and water provided ad libitum. The animal protocol was approved by the Animal Experimental Ethical Committee of Fudan University. The rats were divided into two groups, the sesame oil treated (control, C) group (n = 13) and the VCD treated (model, M) group (n = 13). The body-weight of rats was measured every three days. After 7 days of acclimation in the animal facility, control animals were injected intraperitoneally daily with sesame oil (Sigma-Aldrich) vehicle, and model rats were injected with VCD (Sigma-Aldrich, dissolved in sesame oil) at a concentration of 80 mg/kg for 20 consecutive days. Rats were observed daily for activities, diet, fur, toxicity and mortality. At 24 h following the final dose, five rats in diestrus in each group were anesthetized by intraperitoneal injection with 7% chloral hydrate solution (0.5 ml•100 g^{-1}).

To evaluate the fertility of two groups, after 24 h of the final dose, the remaining 8 rats in each group were mated with adult male SD rats with proven fertility at a proportion of 2:1. Successful conception was defined by the presence of a vaginal plug and/or subsequent visibly growing abdomen, and pregnant female rats were separated and monitored. The number of offspring rats from each litter was counted, and the physical status was examined. The duration from the day of mating to the day of delivery was defined as the pregnancy time. Female rats that did not conceive within 1 month of mating were defined as infertile. Two months later, rats in diestrus in the two groups were anesthetized by intraperitoneal injection with 7% chloral hydrate solution (0.5 ml•100 g^{-1}).

Serum AMH measurement

After anesthesia, mid-line dermatotomy of the chest was performed, and blood was drawn via cardiocentesis. Serum was collected and stored at -80°C until use. The serum AMH level was measured using ELISA (Wuxi Donglin Science and Technology Development co., LTD, Jiangsu, China).

Histology and follicle counting

Rats were euthanized and their ovaries, hearts, livers, spleens, lungs, kidneys and thymuses were collected. The ovaries were excised, trim med of remaining fat tissue and immediately weighed. The ovary weight index was calculated by the following formula: ovary weight index = ovarian weight/body weight. The right ovaries were rapidly frozen in liquid nitrogen and stored at -80°C for use. The left ovaries, hearts, livers, spleens, lungs, kidneys and thymuses were immediately fixed with 4% paraformaldehyde for 24 h, embedded in paraffin wax, serially sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E). Three sections in the 10th, 20th and 30th sections of the largest sections of each ovary were chosen for follicle counting. Follicle classification, such as primordial, primary, secondary, and antral follicles and luteum, were counted as previously described [11].

RNA extraction and real-time PCR

Following the manufacturer's instructions, total RNA was extracted from whole ovaries by TaKaRa MiniBEST Universal RNA Extraction Kit (9767, TaKaRa, Japan). For real-time PCR experiments, total isolated RNA was reverse-transcribed into cDNA with PrimeScript™ RT Master Mix (RR036A, TaKaRa, Japan) according to the manufacturer's instructions. The experiment was performed in triplicate using SYBR®Premix Ex Taq™ II (Tli RNaseH Plus, RR820A, TaKaRa, Japan) according to the manufacturer's instructions.

Table 1 shows the primer sequences (Takara, Japan) used to amplify fragments. The data were normalized to the expression levels of the housekeeping genes GAPDH, and $2^{-\Delta\Delta Ct}$ was

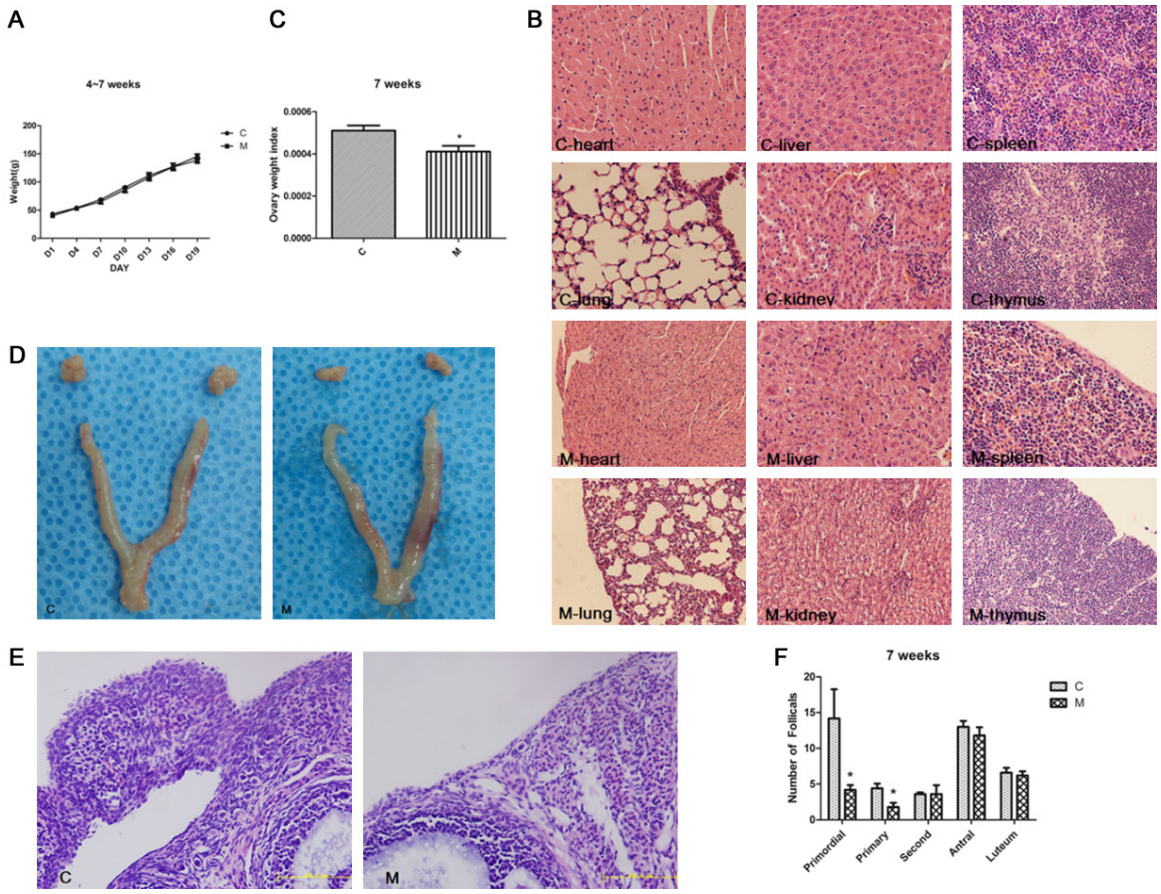

Figure 1. The physical status of rats and depletion of small follicles by VCD in SD rats (*$P < 0.05$). Female SD rats (28 days old) were treated with sesame oil or VCD for 20 days, as described in the Materials and Methods. The body weight of rats was measured every three days. Ovarian sections were stained with H&E. The follicle morphology and numbers were examined under a microscope. A. The weight gains of rats in the two groups. B. The histology of the heart, liver, spleen, lungs, kidneys and thymus (200*). C. The ovaries were smaller in group M compared to group C. D. The ovary weight index was reduced in group M. E. H&E stained sections showing the morphology of follicles between two groups with significantly reduced numbers of small follicles in group M (200*). F. Quantification of the follicle and corpus luteum numbers.

used to calculate the relative expression levels.

Statistical analysis

The data are presented as the mean ± standard deviation and the means between two groups were compared by the independent sample *t*-test with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The pregnancy rate was compared using the chi-square test. $P < 0.05$ was considered statistically significant.

Results

The effect of VCD on the physical status of rats

The growth condition of rats in the M group did not show any difference, and body weight gains paralleled the control group, as presented in **Figure 1A**. No apparent toxicity was observed in the two groups. There was no difference in the histology of the heart, liver, spleen, lungs, kidneys and thymus between the two groups (**Figure 1B**).

Depletion of ovarian primary and primordial follicles by VCD in rats that were 7 weeks of age

The SD rats that were 4 weeks of age were continuously treated with VCD at 80 mg/kg of bodyweight daily for 20 days. Contracted ovaries were observed in the VCD-treated group, which was in accordance with the reduced ovary weight index compared to the control group (**Figure 1C**, **1D**). Morphologically, the follicles and corpus luteum were similar in struc-

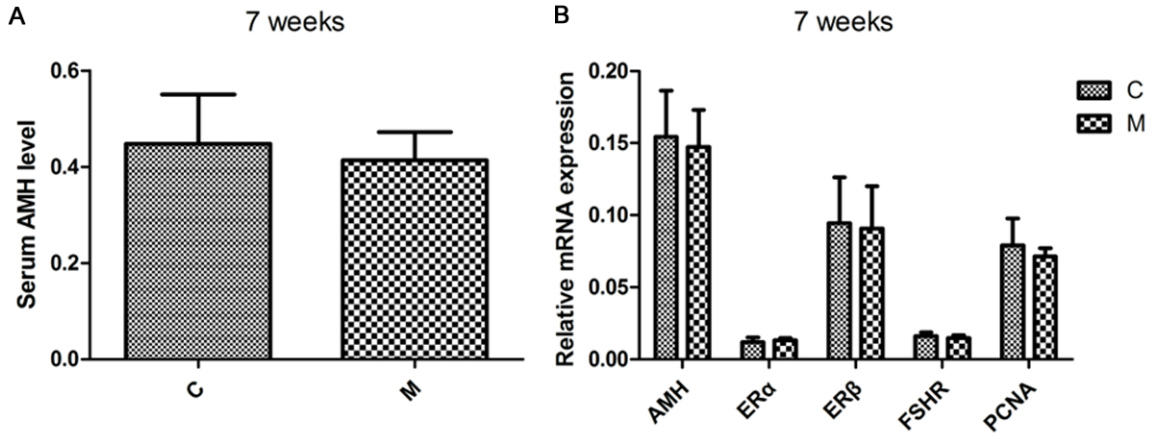

Figure 2. Short-term effects of VCD on the serum AMH level and relative mRNA expression in the ovary. There were no effects of short-term (20 days) VCD treatment on the serum AMH level and relative mRNA expression of AMH, ERα, ERβ, FSHR and PCNA in the ovary.

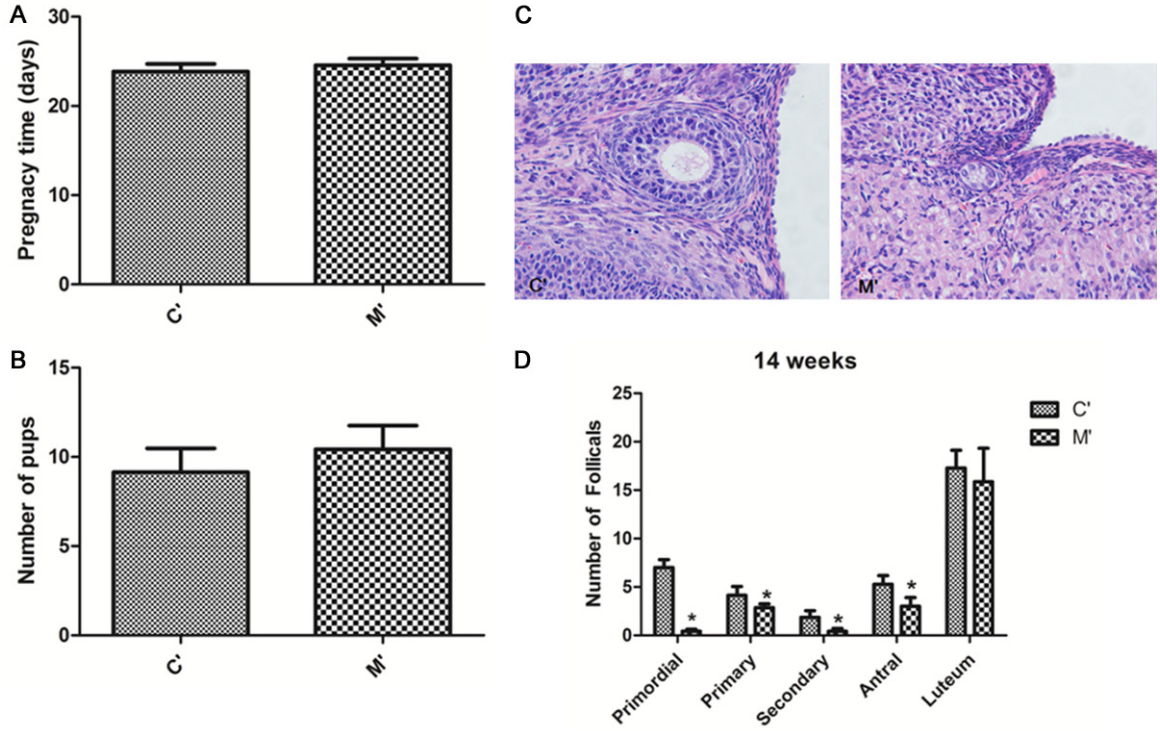

Figure 3. The fertility of the rats in both groups and depletion of ovarian follicles by VCD in SD rats that were 14 weeks of age (*P < 0.05). A. The pregnancy time was similar in both groups. B. The litter size was similar in both groups. C, D. H&E stained sections showing the morphology of follicles between two groups with significantly reduced numbers of small and large follicles in rats that were 14 weeks of age from group M' (400*).

ture between oil- and VCD-treated rats (**Figure 1E**). VCD, at this dose, reduced the primordial follicle number by 70% (4.2 ± 0.7 vs 14.2 ± 4.1) and the primary follicle number by 59% (1.8 ± 0.6 vs 4.4 ± 0.7) that of control rats, respectively. There was no significant difference in the numbers of secondary, antral follicles and corpus luteum between two groups. The data suggest that VCD selectively destroys small follicles (primordial and primary) compared with large follicles (secondary and antral) (**Figure 1F**).

Short term effects of VCD on the serum AMH level and relative mRNA expression of AMH, ERα, ERβ, FSHR and PCNA of the ovary

Serum AMH levels were examined at 7 weeks of age after treatment with VCD or sesame oil. Compared with oil controls, a small but non-significant reduction in the AMH level in the VCD-treated group was observed (**Figure2A**, $P > 0.05$). There was no difference in the relative mRNA expression of AMH, ERα, ERβ, FSHR and PCNA in the ovary between the two groups (**Figure 2B**, $P > 0.05$). Combined with the depletion of the ovarian primary and primordial follicles, the above data indicated that the situation of the rats resembled the initial stage of DOR.

The fertility of the rats in both groups and the depletion of ovarian follicles by VCD in rats that were 14 weeks of age

To evaluate the fertility of VCD-treated rats after 24 h of the final dose, the remaining 8 rats in each group (groups C' and M', respectively) were paired with healthy adult male SD rats for breeding, starting at 7 weeks of age. The pregnancy time and litter size were recorded within 1 month. **Figure 3A** and **3B** showed that there was no significant difference in the pregnancy time (23.9 ± 2.3 vs 24.6 ± 2.0, days) and litter size (9.1 ± 3.5 vs 10.1 ± 3.5) between the two groups ($P > 0.05$). The pregnancy rate was 100% in group M'. The litter size of one rat in the VCD-treated group was only one, which died when we observed it. One of the rats in group C' was infertile. In addition to a stillbirth, the size, skin color, and sucking were similar in pups in the two groups. After the offspring rats were weaned, the rats in the two groups were sacrificed following anesthesia and follicles in ovaries were observed. The numbers of primordial, primary, secondary and antral follicles in the ovaries of group M' were significantly lower than those in group C' ($P < 0.05$). There was no significant difference in the corpus luteum numbers between the two groups (**Figure 3C** and **3D**, $P > 0.05$).

Discussion

The follicle is the primary structure of the ovary and consists of oocytes located in the middle that are surrounded by different layers of granulosa and follicular cells. In the fetal stage of humans or early postpartum stage of rodents, primordial follicles consist of the oocytes formed by the division of the oogonial surrounded by a layer of flattened granulocytes. Since the oocytes in the pool of primordial follicles remain in the first meiosis, these undifferentiated primordial follicles contain all germ cells through the female life. The ovarian follicular pool undergoes a progressive decline from before birth to menopause [12]. In modern society, many factors lead to DOR in many women who are faced with reproductive health problems and other systemic problems. They would also have problems in pregnancy. The infertility caused by DOR remains a thorny problem in reproductive health. Substantial work is needed to identify the etiology of and an effective treatment for DOR. It has been reported in many studies that VCD-induced rat or mouse POF models were similar to the process of natural decline in the ovarian reserve of humans [5, 13, 14], which can replicate the entire progression from childbearing age to premenopausal age and to menopause.

In this study, the ovaries were contracted, and the ovary weight index was reduced in rats with VCD-treated for 20 days compared with the control group. The numbers of primordial follicles and primary follicles were lower in group M than in group C, which was consistent with previous reports [6, 15]. In addition to observing the ovarian reserve, the general situation of rats was observed in both groups. The results showed that there was no effect on the diet, activity, fur and weight gain in rats treated with short-term VCD. As an ovarian toxicity reagent, VCD was used to induce the decline of ovarian reserve. Kappeler et al reported that there were no obvious adverse effects on other organs in the experiment in addition to the ovarian toxicity [5]. The histological features of the heart, liver, spleen, lungs, kidneys and thymus were similar in the two groups, and no pathological changes, such as degeneration, inflammation or fibrosis, were observed. The results showed that the rats treated with short-term VCD were in the early stage of DOR. Even as other organs have not begun "aging", the ovaries age.

There was no significant difference in the serum AMH levels between the two groups in this study, which was not consistent with previous reports [16, 17], and it may be related to the shorter term effect of VCD [18]. The ovary toxic-

ity of the VCD was the depletion of ovarian primordial and primary follicles, and the secondary follicles and antral follicles had not yet been affected in short time. Therefore, the serum AMH level was not significantly changed [18]. There was no significant difference in the relative mRNA expression levels of ERα, ERβ, AMH, FSHR and PCNA in the ovary between the two groups, which indicated that the gene expression level had not been changed in the ovary of short-term VCD-treated mice in this experiment.

One of the main purposes of evaluating the ovarian reserve was to solve the problem of fertility. In this study, the remaining rats in the two groups were mated with adult male SD rats. We found that the pregnancy time, litter size and pregnancy rate were not affected by the VCD in the short term. Fertility had not been inhibited because the secondary and antral follicles had not been affected [19], which was also consistent with the situation encountered in clinical practice. The primordial follicles, primary follicles, secondary follicles and antral follicles in the ovaries of the 14-week-old group were significantly less than those in the control group, indicating that the primordial follicle reduction caused by the VCD gradually affected the development of late stage follicles. Oocyte depletion causes an irreversible change to ovarian function [20]. The depletion of follicles with VCD was not temporary.

In conclusion, the main ovary toxicity of VCD involved depletion of primordial and primary follicles. Short-term VCD treatment did not affect the serum AMH level and mRNA expression levels of AMH, ERα, ERβ, FSHR and PCNA of the ovary and fertility of rats. As time passes, the depletion of primordial follicles and primary follicles affects the late stage follicles, reducing the number of secondary and antral follicles. The short-term effects of VCD on SD rats induced the initial stage of DOR.

Acknowledgements

We acknowledge the work of technician at the Laboratory Animal Center of Fudan University. This study was supported by the Shanghai Municipal Committee of Science and Technology Project (15401931800).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wenjun Wang, Obstetrics and Gynecology Hospital, Fudan University, 419 Fangxie Road, Shanghai 200011, People's Republic of China. Tel: +86-21-33189900; Fax: +86-21-63455090; E-mail: wjwwang@aliyun.com

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