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Original Research

Effects of Impending Ovarian Failure Induced by 4-Vinylcyclohexene Diepoxide on Fertility in C57BL/6 Female Mice

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Repeated daily dosing of mice with 4-vinylcyclohexene diepoxide (VCD) causes a gradual onset of ovarian failure, providing a model for perimenopause. Because increasing numbers of women are delaying starting a family, infertility in aging women is of concern. This study was designed to determine the effects of impending ovarian failure on fertility in VCD-treated mice. Female C57BL/6J mice were dosed daily (17 d) with vehicle control or VCD (160 mg/kg, intraperitoneally) to deplete primordial follicles and then were divided into 2 groups. Group 1 was mated soon after dosing; group 2 was mated on day 20 after dosing, during impending ovarian failure. Fertility was evaluated on gestational day 16. In group 1, cycle length, pregnancy rate, and number of live fetuses did not differ between VCD-treated animals and controls, but VCD-treated mice required more matings to become pregnant and had more resorptions. In group 2, VCD-treated mice demonstrated proestrus and copulatory plugs, but only 1 animal became pregnant, and she had no viable fetuses. Ovaries from pregnant and nonpregnant controls contained similar numbers of follicles and corpora lutea. Ovaries from VCD-treated animals contained no follicles, and corpora lutea were seen only in pregnant animals. In VCD-treated mice mated soon after dosing, conception was more difficult and more resorbed fetuses were seen, whereas in those mated closer to impending ovarian failure, no successful pregnancies were achieved. These results demonstrate that VCD-treated mice can be used to model infertility in perimenopausal women.

Abbreviations: CL, corpora lutea; VCD, 4-vinylcyclohexene diepoxide

In mammalian ovaries, mature follicles grow and develop from a set number of primordial follicles that are present at birth. As a result, with age the nonrenewable pool of primordial follicles in a woman gradually becomes depleted due to repeated ovulations or loss from atresia. Once depleted of follicles, ovarian failure (menopause) occurs. Perimenopause is the 4 to 10 y that precedes menopause. During that time the follicular reserve is becoming compromised and fewer developing follicles remain. Thus, this period is associated with a gradual onset of ovarian failure.

During the perimenopausal transition, ovarian hormone secretion becomes erratic and leads to irregular menstrual patterns. Infertility issues are a growing concern in older and perimenopausal women. Age-related fertility problems increase slightly after age 35 and dramatically after age 40.[2] Under natural conditions, within 1 y 75% of women trying to conceive at age 30 will have a conception ending in a live birth, 66% at age 35, and 44% at age 40.[1] Recent studies show that the number of pregnancies in women older than 35 is dramatically increasing in western countries.[5] However, it is harder for older women to become and stay pregnant, and outcomes for both mother and child are poorer.[3,9,11,12]

Once an older woman becomes pregnant, age adversely affects the pregnancy outcome. There is an increased risk of miscarriages, ectopic pregnancies, and twinning. Fetal and chromosomal abnormalities also increase.[18] Subfertility (that is, requiring a prolonged period of time to get pregnant) is another factor greatly affected by age. Women with subfertility have a fecundability rate (ability to reproduce) of only about 3% to 5%. Therefore, the current trend among women to delay childbearing has increased the risk of infertility.[19] The biologic basis of this decline in fecundity with age in women appears to involve several factors. Possibly the greatest determinant is that the dwindling primordial follicle pool is unable to support adequate follicular development. In addition, as ovulation and atresia of follicles lead to a decline in the number of available oocytes, the quality of existing oocytes diminishes with age.[1]

Previous studies in rats and mice have shown that the occupational chemical 4-vinylcyclohexene diepoxide (VCD) specifically targets and destroys primordial and primary follicles while leaving large preantral (secondary) and antral follicles unaffected.[13,21] Mechanistic studies have determined that this selective follicle loss is due to enhancement of the natural process of atresia which occurs through apoptosis.[4,21] Therefore, VCD has been used in mice to accelerate ovarian failure and generate an animal model for peri- and postmenopause.[16] Because small preantral follicles are selectively targeted, ovarian failure results only after secondary and antral follicles (those not directly affected by VCD) have become depleted via ovulation or atresia. When compared with the ovariectomized animal more commonly used for modeling

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menopause, the VCD-induced ovarian failure model is more relevant to the study of postmenopause because the animal retains residual ovarian tissue. Furthermore, unlike the ovariectomized animal, in the VCD-treated animal onset of ovarian failure is gradual, providing a model for the perimenopausal transition as well. Therefore, the VCD model is a relevant model for women because the vast majority undergo a gradual progression to natural menopause (perimenopause) and retains residual ovarian tissue (postmenopause).

As a result of well-recognized reproductive problems in women during the perimenopausal transition, infertility is an important issue in ovarian aging research.[7] No previous studies have evaluated reproductive performance in the VCD mouse model for perimenopause during the period between primordial follicle depletion and ovarian failure (the period analogous to perimenopause in women). Therefore, the purpose of this study was to evaluate the effects of VCD-induced primordial follicle depletion on fertility during the period of impending ovarian failure in mice. The hypothesis tested was that fertility outcome would not be affected, but that as ovulations became compromised, the establishment of pregnancies would become more difficult.

Materials and Methods

Reagents. 4-Vinylcyclohexene diepoxide (lot 129 #0092) was purchased from Sigma–Aldrich (St Louis, MO).

Animals. Mice were housed in a nonsterile facility in polycarbonate cages (autoclaved periodically; 2 to 4 animals per cage) and were maintained on 12:12-h light:dark cycles in a controlled temperature of 22 ± 2 °C. Immature C57BL/6Hsd female mice were obtained from Harlan Laboratories (Indianapolis, IN). The mice were 21 d old on arrival. C57BL/6J female mice (91 d of age, optimal breeding age) and mature C57BL/6J male mice (42 to 56 d of age) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were allowed to acclimate to the animal facilities for 7 d prior to initiation of treatment. Food and water were available ad libitum. Mice were free of known adventious murine pathogens. Euthanasia was performed by CO_2 inhalation (adults) and followed by decapitation (fetuses only). All experiments and euthanasia under anesthesia were approved by the University of Arizona Animal Care and Use Committees and conformed to the *Guide for the Care and Use of Laboratory Animals*.[17]

VCD dosing. Randomly selected 28-d-old mice comprised each treatment group (n = 7 to 8). Mice were weighed and dosed daily (intraperitoneally) with VCD dissolved in sesame oil (160 mg/kg) or with sesame oil only (vehicle control) for 15, 17, 20, or 22 d. At 24 h after the final dose, animals were euthanized by CO_2 inhalation. Another group of mice that had been dosed for 17 d was maintained, and ovarian cyclicity was monitored to determine the time of ovarian failure. Beginning on the day after completion of VCD dosing (day 18), vaginal cytology was monitored daily by light microscopy to determine the stage of the estrous cycle, as described previously.[15,23] When daily vaginal cytology confirmed 15 consecutive days in metestrus/diestrus or diestrus, the animal was determined to have undergone ovarian failure. The day of the first diestrus in the 15-d series was counted as the day of ovarian failure. At the time of tissue collection (of ovaries and other tissues), ovarian failure was confirmed by histologic evaluation. To assess potential long-term effects of VCD, animals were maintained until day 180 after the onset of dosing. Ovaries and other tissues were collected and weighed. The treatment protocol using VCD to induce ovarian failure is owned by the University of Arizona (patent pending) and is according to the methods described herein as well as previous reports.[14-16] VCD-treated mice are commercially available through the Jackson Laboratory (Bar Harbor, ME).

Follicular and luteal assessment. Ovaries were collected and trimmed of surrounding fat. They were placed in Bouin fixative for 2 h and transferred to 70% ethanol. Ovaries were embedded in paraffin, and every seventh section (4 to 5 µm) was mounted and stained with hematoxylin and eosin. Numbers of oocyte-containing follicles (those with a distinct oocyte nucleus) at each developmental stage were classified and counted in every 20th section.[13] Primordial follicles were classified as an oocyte surrounded by a single layer of flattened (squamous) granulosa cells. Primary follicles were classified as an oocyte surrounded by a single layer of cuboidal granulosa cells. Secondary follicles contained multiple layers of granulosa cells, and antral follicles contained at least 2 layers of granulosa cells and a fluid-filled antral space.[10,13,20] Corpora lutea (CL) were counted in every 20th section in both ovaries of pregnant dams. CL that spanned several counted sections were only counted once.

Fertility evaluation. For fertility evaluation, more mature animals (age 91 d) were used to ensure that reproductive cyclicity had become established (as determined by vaginal cytology), whereas determinations of primordial follicle loss and time to ovarian failure were made in younger animals (age, 28 d) to enable comparison of responses with those previously determined with other strains of mice and different dosing times. Female mice were randomly selected for each treatment group, weighed, and intraperitoneally dosed daily with VCD (160 mg/kg) or sesame oil only (vehicle control) for 17 d. After dosing, the mice were moved to a breeding-specific room. To study the effects of VCD-induced impending ovarian failure on fertility, C57Bl/6 female mice were divided into groups 1 and 2 with different time points (each with control and treatment subgroups). Group 1 control (n = 5) and VCD-treated (n = 14) animals were put with mature males shortly after primordial follicle loss (to mimic early perimenopause). The females were allowed to rest for 1 cycle after dosing to give them time to acclimate to the new breeding room (individually ventilated cages) and were exposed to males on the second proestrus after dosing. There was no difference in length of the first 2 cycles after dosing between controls and VCD-treated animals. Group 2 animals (control, n = 6; VCD-treated, n = 8) were put with males at the first proestrus occurring at least 20 d after primordial follicle loss (after the end of VCD dosing) to mimic late perimenopause.

Twelve mature C57BL/6J male mice (age 8 to 12 wk) were used for breeding. They were kept in separate cages and individually identified. Each male was placed with control as well as VCD-treated mice showing proestrus. For mating, male and female mice were housed as individual pairs. Individual males were not proven breeders, but reproductive competence in individual males was assumed by the ability to produce a copulatory plug in females.

Pregnancy determination. Even though a copulatory plug can be observed without a resulting pregnancy, if one was seen the morning after being housed with a male, the female was thought to be pregnant, and this day was counted as day 0 of gestation. Vaginal cytology was continued to ensure that the female was not ovulating until pregnancy was confirmed by

a dramatic weight gain and visual inspection (usually between days 10 to 12 of gestation).

Fetal organ collection. Pregnant mice were euthanized on gestation day 16. Fetuses were evaluated for gross abnormalities, weighed, and assigned a gender after examination for the presence of testes or ovaries. Maternal tissues were collected and weighed. Maternal ovaries were fixed in Bouin's solution and prepared for histologic counting of follicles and CL. Uteri were examined for implantation sites and evidence of resorption.

Data analysis. All data collected from individual animals were averaged by group, and means ± standard error were calculated. Follicle numbers were determined in ovaries from individual animals, averaged, and the means (± standard error) in control versus treated animals in each group were calculated. Differences between groups were analyzed by 2-way analysis of variance with significance set at a P value of less than 0.05 using StatView software (SAS Institute, Cary, NC). Post-hoc tests (Fisher Protected Least Significant Difference) were used where appropriate. In fertility evaluations, when there were no significant differences between cycling control animals in groups 1 and 2, controls were evaluated as a single group to increase statistical power.

Results

Effects of daily VCD dosing. To evaluate the earliest dosing time required to destroy all primordial follicles but not damage larger secondary or antral follicles, female C57BL/6Hsd mice were dosed daily with VCD for 15 to 22 d (Figure 1 A). Primordial follicles were reduced but not completely eliminated (1.9% of control) after 15 d of dosing. Conversely, all primordial follicles were destroyed after 17, 20, and 22 d of dosing. However, secondary follicles were unaffected only on day 17; antral follicles were unaffected at any time point.

To determine the time to ovarian failure after depletion of primordial follicles, female C57BL/6Hsd mice were dosed daily for 17 d. The day of ovarian failure assigned for each VCD-treated animal by monitoring vaginal cytology was previously determined in C57BL/6Hsd mice to be 15 consecutive days of either metestrus/diestrus or diestrus.[15] The day of ovarian failure in individual animals ranged between 36 and 56 d after the onset of dosing, with an average of 44.9 ± 2.1 d (Figure 1 B).

The period between primordial follicle depletion (day 17) and ovarian failure in VCD-treated mice is considered to be analogous to perimenopause in women. During that period, age-matched control animals did not undergo ovarian failure but demonstrated regular cycles. The number of complete estrous cycles during the entire period after dosing that was evaluated varied between control (7.4 ± 0.5) and VCD-treated (2.3 ± 0.3) animals.

Body and organ weights. At the end of dosing (day 17), body weights in control C57BL/6Hsd mice (18.0 ± 0.3 g) were greater (P < 0.05) than in VCD-treated animals (16.3 ± 0.5 g). The difference did not reflect loss of body weight in VCD-treated animals but a failure to gain weight as rapidly as controls (data not shown). However, the body weights were no longer different by day 28 (11 d after the end of dosing; control, 19.4 ± 0.3 g; VCD, 18.6 ± 0.4 g). Body weights on day 180 after the onset of dosing were greater (P < 0.05) in VCD-treated (37.3 ± 2.2 g) as compared with control (26.0 ± 0.3 g) animals.

Organ weights were measured and normalized to body weight when tissues were collected on day 180. There were no significant differences between the control and VCD groups in the weights of

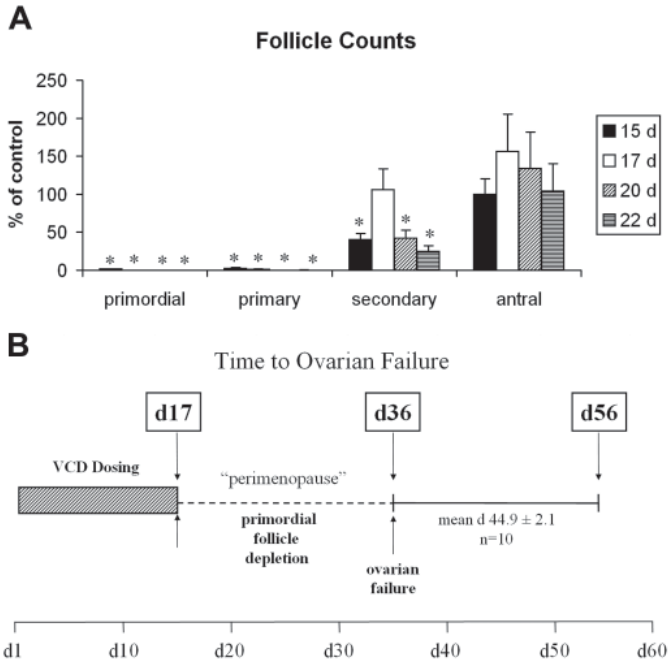

Figure 1. Follicle loss and time to ovarian failure. (A) Effect of varying days of VCD dosing on ovarian follicles. Immature female C57BL/6Hsd mice were dosed daily with vehicle control or VCD (160 mg/kg intraperitoneally) for 15, 17, 20, or 22 d. Ovaries were collected 24 h after the final dose and processed for histologic evaluation. Follicles were classified and counted as described in Materials and Methods. Values are expressed as a percentage (mean ± standard error) of the number of follicles in age-matched, cycling controls. *, P < 0.05 compared with value for controls; n = 7 to 8 mice/group. (B) Time line of dosing with VCD and ovarian failure. After dosing, ovarian cyclicity was monitored daily by vaginal cytology. Assignment of ovarian failure was 15 consecutive days of metestrus/diestrus or diestrus, as described in Materials and Methods. The range in onset of ovarian failure (day [d] 36 to day 56) in individual animals (n = 10) is indicated by the solid line; time to ovarian failure (analgous to perimenopause) is indicated by the dotted line.

kidneys, spleen, or liver. However, uterine (control, 0.296 ± 0.034; VCD, 0.083 ± 0.009 g/g body weight) and adrenal gland (control, 0.031 ± 0.003; VCD, 0.017 ± 0.009 g/g body weight) weights were lower (P < 0.05) in VCD-treated animals as compared with controls. In another study, adrenal weights in ovariectomized C57BL/6Hsd mice (not exposed to VCD) were also lower than in age-matched controls 160 d after ovariectomy.[6]

Mating efficiency. To determine the effect of impending ovarian failure on reproductive outcomes at the end of dosing (day 17), C57BL/6J female mice were divided into 2 groups (VCD-treated and controls in each group) and mated with adult males soon (group 1; early) or later (group 2; late) after VCD dosing had stopped. In group 1 controls, all mice became pregnant on the first mating attempt. In the 14 group 1 VCD-treated animals, all exhibited copulatory plugs, but 4 never became pregnant. Of the remaining 10, 5 became pregnant on the first mating attempt, whereas 5 became pregnant on the second. Group 2 VCD-treated and control animals all exhibited evidence of cyclicity and showed copulatory plugs. However, only 1 of the 8 VCD-treated compared with 4 of the 6 control animals became pregnant.

The number of times female mice were housed with males (that is, mating attempts) did not differ between control (1.44 ±

Table 1. Mating efficiency (%; mean ± standard error)

	Control (n = 13)	VCD: group 1[a] (n = 14)	VCD: group 2[b] (n = 8)
No. of plugs/no. of times with males	89.7 ± 7.9	71.3 ± 10.1	83.3 ± 12.6
No. of pregnancies/no. of times with males	64.1 ± 12.1	60.0 ± 11.1	12.5 ± 12.5[c]
No. of pregnancies/no. of copulatory plugs	57.7 ± 12.5	60.0 ± 12.1	12.5 ± 12.5[c]
Pregnancy rate	76.9 ± 12.2	73.3 ± 11.8	12.5 ± 12.5[c]

[a]Group 1 mice were mated on the second proestrus after the final dose of VCD.
[b]Group 2 mice were mated on the first proestrus at least 20 d after the final dose of VCD.
[c]$P < 0.05$ compared with value for control.

0.24) and VCD-treated (1.54 ± 0.21) females in proestrus in either group. Pregnancy outcome of mating efficiency is reported in Table 1. In group 1 VCD-treated animals, the percentage of copulatory plugs observed relative to mating attempts was not different between groups. Group 1 VCD-treated animals and controls also were similar in overall pregnancy rate. However, group 2 VCD-treated animals displayed a reduced percentage of pregnancy versus mating attempts, pregnancy versus number of plugs, and overall pregnancy rate relative to controls and group 1 VCD-treated animals.

Ovarian status. Ovarian weights in C57BL/6J dams were recorded at the time of tissue collection. Because body weights were artificially high in pregnant animals, ovarian weights were not normalized to body weight. Mean ovarian weight in control pregnant dams was 0.010 ± 0.001 g; this value was higher ($P < 0.05$) than those in VCD-treated pregnant dams (0.008 ± 0.0004 g) and VCD-treated nonpregnant females (0.003 ± 0.0004 g). Further, ovarian weights from VCD-treated pregnant dams were higher ($P < 0.05$) than from VCD-treated nonpregnant female mice. Histologic evaluation of the ovaries collected from control and VCD-treated animals in both groups (Figure 2 A) revealed numerous primordial, primary, secondary, and antral follicles in all control animals regardless of whether they had become pregnant. In contrast, there were essentially no follicles of any size in ovaries of VCD-treated animals regardless of whether they had become pregnant. The only detectable follicle population in those animals was an occasional antral follicle in animals that had become pregnant.

CL were counted in ovaries from all animals (Figure 2 B). There were no differences in numbers of CL between control animals (pregnant or nonpregnant) and VCD-treated animals that became pregnant. However, the ovaries of VCD-treated animals that did not become pregnant contained no detectable CL, suggesting that no ovulation had occurred.[8] The number of concepti (live fetuses plus resorptions) was calculated and compared with the number of CL for each animal (Figure 2 C). The ratio of numbers of concepti to CL did not differ between the control and VCD-treated pregnant animals.

Fertility outcome. Mothers and fetuses were assessed on gestational day 16 (Figure 3). The number of live fetuses did not differ significantly between control (6.44 ± 0.63) and group 1 VCD-treated (5.40 ± 0.40) dams (Figure 3 A). No live fetuses were recorded for the 1 VCD-treated dam in group 2 that became pregnant. There was a greater number of resorbed fetuses in group 1 VCD-treated (1.9 ± 0.4) mice compared with controls (0.6 ± 0.2; $P < 0.05$). This count was lower than the 5 resorbed fetuses counted in the 1 group 2 VCD-treated mouse that became pregnant (Figure 3 B).

This information was used to calculate the ratio of viable fetuses to implantation sites (Figure 3 C). Group 1 VCD-treated animals (74.7% ± 5.2%) had a smaller percentage of viable fetuses compared with controls (91.6% ± 3.6%; $P < 0.05$). The group 2 VCD-treated animal that became pregnant had no viable fetuses.

Gender distribution within litters was not different for males between controls (2.9 ± 0.6/ litter) and group 1 VCD-treated (3.1 ± 0.3/litter) dams. However, group 1 VCD-treated mice had fewer female offspring (2.3 ± 0.5) than did controls (3.6 ± 0.4; $P < 0.05$). Individual fetal weights did not differ between controls (0.55 ± 0.08 g/fetus) and group 1 VCD-treated litters (0.68 ± 0.07 g/fetus). Gross abnormalities were not noted in fetuses from any group, and no dead mature fetuses were found.

Discussion

Infertility and subfertility are prevalent problems associated with perimenopause. The further a woman progresses into perimenopause, the more difficult getting pregnant becomes. Because the VCD mouse model produces mice in a state analogous to perimenopause (cycle disruption and impending ovarian failure), it is a highly relevant model for the study of age-related infertility issues. The purpose of this study was to determine the effects of VCD-induced primordial follicle depletion on subsequent fertility in C57BL/6J female mice to determine whether fertility problems in the VCD model mimic those in aging women.

In a previous study using B6C3F1 mice, the time to ovarian failure was determined to be 135 d after the onset of 10 d of daily dosing with VCD (160 mg/kg, intraperitoneally), compared with 52 d in mice dosed for 20 d.[15] However, in that study, which used a hybrid strain of mouse, ovarian failure was not evaluated after all primordial follicles had been lost by VCD dosing, and larger follicles were still unaffected. Therefore, the period analogous to perimenopause had not been optimized. It was determined that for immature C57BL/6Hsd mice, primordial follicle depletion occurred after 17 d of daily dosing with VCD. At that time, although all primordial follicles were lost, secondary and antral follicles were unaffected. The 17-d regimen was chosen as the optimal duration of dosing for fertility evaluation. Ovarian failure occurred an average of 45 d after the onset of 17 d of dosing. Length of initial estrous cycles did not differ between VCD-treated and control animals.

Body weights in VCD-treated C57BL/6Hsd mice were lower than in controls at the end of dosing, were not different from controls on day 28, and were greater than controls by day 180 when tissues were evaluated. The lower body weight at the end of dosing did not reflect a loss of weight in VCD-treated animals but a lower rate of weight gain than that of controls. This slower weight gain in VCD-treated animals likely is due to a lower appetite in animals while they are being dosed, because those weight differ-

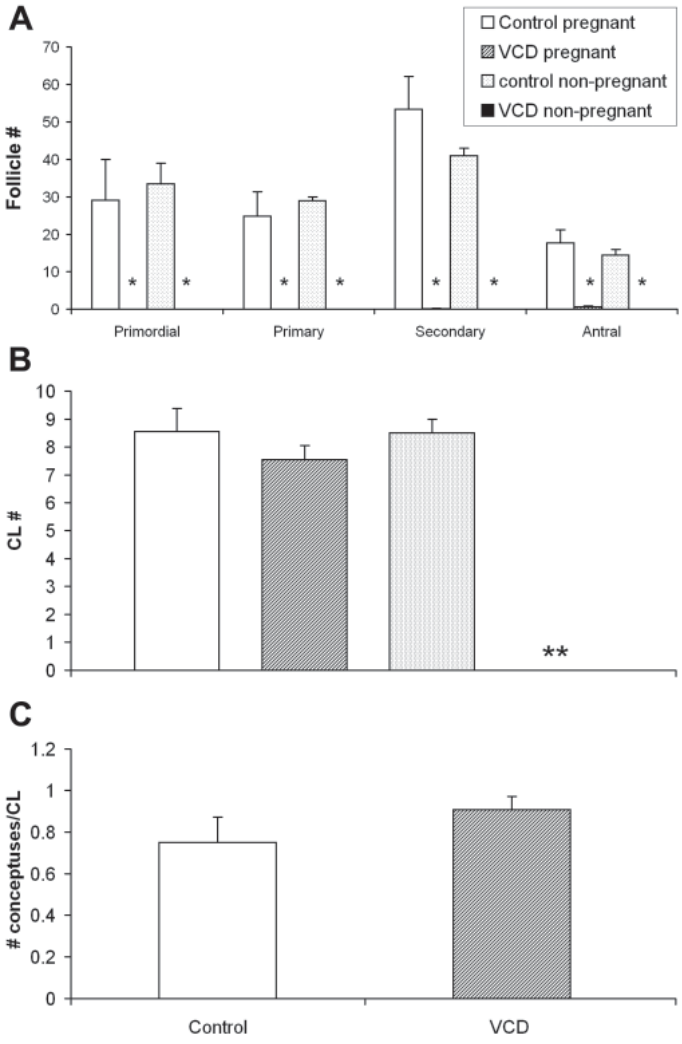

Figure 2. Ovarian morphology in pregnant versus nonpregnant mice. Mature female C57BL/6J mice were dosed with vehicle control or VCD (160 mg/kg intraperitoneally) for 17 d. Control and VCD-treated mice were mated with fertile males as described in Materials and Methods. Fertility in pregnant animals was evaluated on gestational day 16. Ovaries were prepared for histologic evaluation as described in Materials and Methods. (A) Follicles were classified and counted as described in Materials and Methods. Data are the number (mean ± standard error) of each follicle type in control pregnant (open bars; n = 9), VCD-treated pregnant (crosshatched bars; n = 10), control nonpregnant (horizontally hatched bars; n = 2), and VCD-treated nonpregnant (solid bars; n = 11) mice. (B) Corpora lutea were counted as described in Materials and Methods. Values are the number (mean ± standard error) of corpora lutea in control pregnant (open bars; n = 9), VCD-treated pregnant (crosshatched bars; n = 10), control nonpregnant (horizontally hatched bars; n = 2), and VCD nonpregnant (solid bars; n = 11) ovaries. **(C)** Ratio of concepti to ovulations. Female C57Bl/6 mice were dosed daily with vehicle control or VCD (160 mg/kg intraperitoneally) for 17 d. Control and VCD-treated mice were mated with fertile males as described in Materials and Methods. Fertility was evaluated on gestational day 16 as described in Materials and Methods. Concepti (viable plus resorbed fetuses) were counted and compared with numbers of coropa lutea determined as in Figure 2 B. Values are the ratio (mean ± standard error) of the number of concepti/number of CL; control, n = 9; VCD-treated, n = 10. *, $P < 0.05$ versus value for pregnant controls; $^{**}P < 0.003$ versus value for pregnant controls.

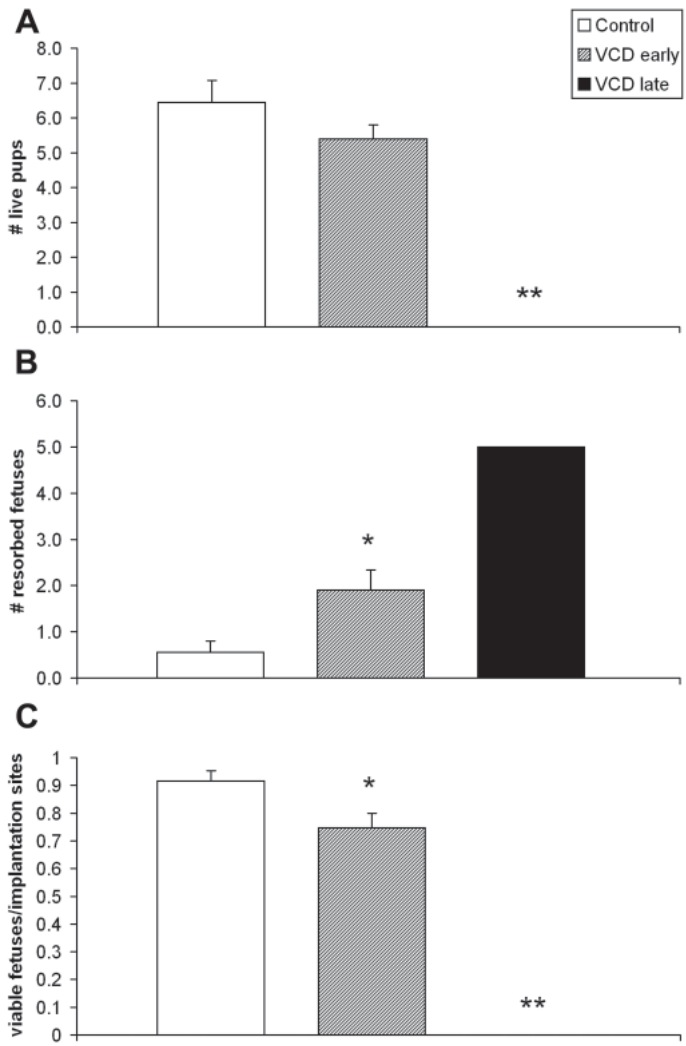

Figure 3. Effect of impending ovarian failure on fertility outcome. Mature female C57BL/6J mice were dosed daily with vehicle control or VCD (160 mg/kg intraperitoneally) for 17 d. Control and VCD-treated mice were mated with adult males as described in Materials and Methods. Fertility was evaluated on gestational day 16. Measurements in control (open bars; n = 9), group 1 VCD-treated (crosshatched bars; n = 10), and Group 2 VCD-treated (closed bars; n = 1) mice were made of (A) number of viable fetuses and (B) number of resorptions. The ratio of (C) viable fetuses versus implantation sites was calculated. Values are presented as mean ± standard error. *, $P < 0.05$ compared with value for controls; $^{**}P < 0.003$ versus value for controls.

ences resolved shortly after dosing stopped. Furthermore, following ovarian failure, the VCD-treated animals were heavier than age-matched controls.

Normalized uterine and adrenal weights on day 180 after the onset of dosing were lower in VCD-treated animals compared with controls. The lower uterine weight likely resulted from loss of the tropic effect of 17β-estradiol due to ovarian failure.[15] Why adrenal weights would be lower in VCD-treated mice is unclear but likely relates to the loss of ovarian function rather than a direct effect of exposure to VCD, because in another study, adrenal weights were lower in ovariectomized mice (with no exposure to VCD) than in controls.[6]

Group 1 control and VCD-treated C57BL/6J mice were mated shortly after primordial follicle loss in VCD-treated animals. This period in mice was assumed to be comparable to the early perimenopausal transition in women. To monitor that cyclicity was regular before mating, VCD-treated animals were observed through 1 estrous cycle prior to putting them with a mature male. All control mice and 10 of the 14 VCD-treated mice became pregnant. Of the VCD-treated mice that established pregnancies, 5 became pregnant after their first mating attempt, and the other 5 became pregnant after the second mating attempt. Therefore, on average and compared with control animals, group 1 VCD-treated animals took longer to become pregnant. In addition, 4 group 1 VCD-treated mice never became pregnant. The overall delay in conception in group 1 animals is representative of subfertility in aging women, in whom the time required to become pregnant increases.

Numbers of live fetuses did not differ between group 1 VCD-treated and control mice, but there was a greater number of resorptions in the VCD-treated group. Therefore, the conception rate was similar between groups mated soon after primordial follicle loss. There were fewer female fetuses in VCD-treated dams, although the biologic relevance of this finding is not clear. There was no difference between groups in fetal body weights.

Four of the group 1 VCD-treated animals did not become pregnant even though they displayed proestrus, and copulatory plugs were formed after mating. These mice did have longer and more irregular cycles than did those in the other groups. These 4 mice likely progressed more rapidly toward ovarian failure than the other group 1 animals. This finding supports interindividual animal variation, similar to the high variability in the onset and duration of perimenopause among women.

Compared with group 1 animals, group 2 control and VCD-treated C57BL/6J mice were mated later after primordial follicle loss (end of dosing). At that time the mice were even further in progression to ovarian failure, therefore group 2 models women later in perimenopausal transition. Although group 2 VCD-treated mice had regular cycles and displayed copulatory plugs after mating, only 1 established a pregnancy, and it did not result in any viable fetuses. Therefore, fertility clearly was impaired even though evidence of ovarian function was still present (proestrus and copulatory plugs).

Males used for mating had not previously been proven as breeders. Therefore, the subfertility in group 1 and infertility in group 2 VCD-treated mice might be due to male infertility. However, each male was mated to a female in the control as well as the VCD-treated group; therefore, differences between the groups would not have been as striking if effects were due to the males. In addition, histologic evaluation of females demonstrated a direct correspondence between ovarian status and subsequent implantation sites. These results provide strong support for VCD-induced impending ovarian failure as the cause of the impaired fertility outcome.

Histologic evaluation of ovaries was used to compare animals that became pregnant in the control and VCD-treated groups with those that did not become pregnant in the VCD-treated groups. As expected, all control animals had numerous follicles of all sizes, whereas only a few antral follicles were seen in the VCD-treated animals that had become pregnant. No antral follicles were observed in VCD-treated mice that did not become pregnant. These findings confirm that ovarian failure was approaching rapidly in VCD-treated animals. In support of that conclusion, ovarian weights were lower in VCD-treated pregnant mice than in control pregnant mice, and ovarian weights from VCD-treated nonpregnant mice were lower than in VCD-treated mice that were pregnant. Therefore, ovarian atrophy had already occurred to some extent in those VCD-treated mice that became pregnant and to an even greater extent in VCD-treated mice that did not become pregnant.

Because a corpus luteum forms at the site of ovulation, the number of CL can be used as an estimate of numbers of oocytes available for fertilization and subsequent fetal development.[8,22] Thus, correlating CL with implantations sites and fetal numbers gives an indication of reproductive efficiency in females.[8] Numbers of CL did not differ among pregnant control and VCD-treated animals and nonpregnant controls. However, no CL were observed in ovaries of VCD-treated animals that did not become pregnant even though they demonstrated cyclicity according to vaginal cytology and mating behavior according to copulatory plugs. Because no CL were seen in VCD-treated animals that did not become pregnant, these findings suggest that VCD-treated animals that became pregnant were ovulating, and those that were nonpregnant were not ovulating and had likely already undergone ovarian failure. Interestingly, numbers of CL in those mice that became pregnant related well to numbers of conceptions (live plus resorbed fetuses) in both groups. This finding suggests that prior to ovarian failure, the subfertility in VCD-treated animals that became pregnant was not the result of reduced ovulations.

It is unlikely that exposure to VCD directly caused the observed effect on fertility, because the more severe problems were seen in the second group that were mated longer after VCD dosing had been stopped. The group mated earlier still showed evidence of fertility, although slightly impaired, whereas the later group demonstrated almost complete infertility. In addition, VCD dosing did not affect weights of tissues other than ovaries, uteri, and adrenals—effects presumably resulting from loss of ovarian function.

In conclusion, VCD-treated C57BL/6J mice displayed proestrus and copulatory plugs, yet as the period of impending ovarian failure progressed, the mice demonstrated evidence of subfertility (group 1) and eventually infertility (group 2). These findings add translational relevance to the VCD mouse model, because declining reproductive function mimics that in women during the perimenopausal stage of life. Future studies will be aimed at investigating the reasons for disrupted fertility in the mouse model for perimenopause.

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