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Characterization of Cyclicity and Hormonal Profile with Impending Ovarian Failure in a Novel Chemical-Induced Mouse Model of Perimenopause

Jessica C. Lohff, Patricia J. Christian, Samuel L. Marion, Anthony Arrandale, and Patricia B. Hoyer*

4-Vinylcyclohexene diepoxide (VCD) causes early, gradual ovarian failure in mice because it specifically targets small pre-antral ovarian follicles. The period between loss of these follicles and ovarian failure is analogous to perimenopause in women. We sought to characterize the period of onset of ovarian failure in VCD-treated mice in regard to estrous cycle length and hormonal changes. Female C57Bl/6 mice (age, 28 days) were dosed daily for 15 days with VCD (160 mg/kg intraperitoneally) to cause early ovarian failure or with vehicle only (control animals). Cycle length was monitored by vaginal cytology. Plasma levels of 17β-estradiol (E2), progesterone (P4), and follicle-stimulating hormone (FSH) in control and VCD-treated animals were measured during proestrus of cycles 1 through 12. Cycle length (mean, 5.8 days) did not differ between groups for cycles 1 through 4. In contrast, cycle length during cycles 5 through 12 was increased (mean length, 10.9 days; $P < 0.05$ versus control) in VCD-treated animals, which also showed an apparent increase in plasma FSH levels. Plasma E2 and P4 at proestrus did not differ between groups during any cycle. Ovarian failure in VCD-treated mice was confirmed by histological evaluation on day 156 after onset of dosing, whereas control animals were still cycling. Therefore, despite compromised cycle length in VCD-treated mice, peak ovarian steroid production in preovulatory follicles at proestrus is adequate. These results demonstrate that the VCD-treated mouse can serve as an appropriate model to mimic hormonal changes during the perimenopausal transition in women.

17β-Estradiol (E2) and progesterone (P4) are steroids secreted by preovulatory follicles and corpora lutea, respectively, in the ovary. During estrous cycling E2 and P4 exert negative feedback on secretion of the gonadotropic hormones (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) at the level of the hypothalamus and anterior pituitary. Ovaries also produce peptide hormones called inhibins (A and B), which selectively inhibit FSH secretion from the pituitary (10, 17).

During menopause, women undergo ovarian failure due to depletion of healthy ovarian follicles required for development and ovulation. Because large antral follicles and corpora lutea cannot form, secretion of E2, inhibin, and P4 dramatically decreases (10). Due to loss of negative feedback by these ovarian hormones on the anterior pituitary, there is a significant increase in plasma LH and FSH (10). The loss of ovarian hormones, particularly E2, after menopause is thought to contribute to physical symptoms, such as loss of vaginal secretions, night sweats, episodes of depression, and other various long-term consequences affecting women's health (3, 6, 10). The traditional view has been that hormone replacement therapy (HRT) after menopause will alleviate these symptoms and protect against such health risks as cardiovascular disease and osteoporosis (12, 18). However, in light of results from the Women's Health Initiative (WHI) in 2002, the benefit of estrogen used as HRT has been questioned (5). Thus, the relative benefits versus risks of HRT have become a controversial issue that remains unresolved (5).

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The Department of Physiology, College of Medicine, The University of Arizona, Tucson, Arizona.
**Corresponding author.*

In the years just prior to menopause, menstrual periods often become irregular due to fluctuating hormone levels. This period is termed perimenopause. Studies in nonhuman primates have demonstrated that timing of the initiation of HRT is crucial as to whether the therapy will have beneficial versus adverse cardioprotective effects (1, 5, 12). From these studies, it has been predicted that HRT is more likely to cause protective effects if it is started prior to complete ovarian failure. As a result, detecting early perimenopause in women may be a crucial issue for instituting HRT at the optimally beneficial time. Therefore, development of a relevant animal model for perimenopause would be of tremendous value.

Prospective longitudinal studies in women have shown that the earliest hormonal changes that accompany perimenopause are a decline in inhibins A and B, followed by increases in FSH and LH. However, it has been difficult to characterize specific changes in circulating E2 during this time. Although in some studies there was a decline in E2, other studies report that E2 levels may even be elevated, relative to premenopausal levels in women (4, 17). These inconsistent findings are, in part, likely due to an inability to predict the preovulatory peak of E2 in women experiencing wide fluctuations in cyclicity. This situation prompts the question as to whether the unpredictable fluctuations seen in perimenopausal women are due to a lack of sampling uniformity or to differences in steroidogenic capacity of preovulatory follicles as ovarian function begins to decline.

4-Vinylcyclohexene (VCH) and its diepoxide metabolite VCD are chemicals used in the manufacture of flame-retardants, insecticides, and rubber tires (2). Previous studies have determined that repeated daily dosing of rats and mice with VCD selectively

destroys ovarian primordial and primary (small pre-antral) follicles by accelerating the natural process of atresia (apoptosis; 7-9, 19, 20). The ability of VCD to selectively target small pre-antral follicles, therefore, has been used to cause premature ovarian failure in mice (13). These studies have demonstrated that, aside from loss of small pre-antral follicles, no effects of VCD on larger follicles or other tissues could be demonstrated. Therefore, VCD under these conditions did not cause any observable generalized toxicity (13). Because only primordial and primary follicles are targeted by VCD, larger pre-antral and antral follicles continue to develop and ovulate until no pool of smaller follicles is available for recruitment. This novel mouse model is analogous to human peri- and postmenopause because it causes chemical-induced gradual ovarian failure and the animal retains residual ovarian tissue (13).

The present study has been designed to follow cyclicity, E2, P4, and FSH levels in the VCD mouse model as gradual ovarian failure progresses to establish a relevant model for evaluating hormonal changes at this time (i.e., a model for perimenopause). The hypothesis being tested is that peak E2 and P4 production by large preovulatory antral follicles at proestrus is not compromised as cycles become irregular and impending ovarian failure progresses.

Materials and Methods

Animals. Immature female C57Bl/6 mice (age, 21 days) were obtained from Harlan Laboratories (Indianapolis, Ind.), housed in high-temperature polycarbonate cages with isolator lids, and maintained on a 12:12-h light:dark cycle at 22 ± 2°C. Prior to initiation of treatment, animals were allowed to acclimate to the animal facilities for 7 days. Food and water were available ad libitum. 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* (14). Results of pathogen monitoring were negative in all animal groups.

Treatment. The treatment protocol using VCD to induce ovarian failure is owned by the University of Arizona (patent pending), and VCD-treated mice are commercially available from the Jackson Laboratory (Bar Harbor, Maine). At 28 days of age, mice were randomly selected for each treatment group, weighed, and dosed intraperitoneally daily with VCD (160 mg/kg; n = 15) or sesame oil (vehicle control, n = 15) for 15 days. All reagents were obtained from Sigma–Aldrich (St. Louis, Mo.). Animals within treatment groups were sorted into three separate groups (n = 3 to 6 per group) in order to stagger blood sample collection such that no animal was bled more often than every 2 weeks. Blood was collected on the day of proestrus by retro-orbital puncture of each animal in the appropriate subgroups, with one subgroup representing every cycle. Blood sampling was begun in the first group with the first proestrus seen after the onset of dosing. Thus, some samples were collected during the dosing period. On day 156 after the onset of VCD dosing, age-matched control and VCD-treated animals were killed by CO_2 inhalation. Organs were collected and weighed. Ovaries were processed for histologic evaluation to confirm follicle depletion.

Cyclicity. Estrous cycles of each animal were monitored daily by microscopic examination of vaginal cytology beginning at the vaginal opening (average age, 38 days). The cycle length was defined by the number of days between two successive demonstrations of proestrus. On each day of proestrus, blood samples were collected from one subgroup of animals (n = 3 to 6) by retro-orbital puncture, and plasma was separated and stored at –20°C for hormone assay.

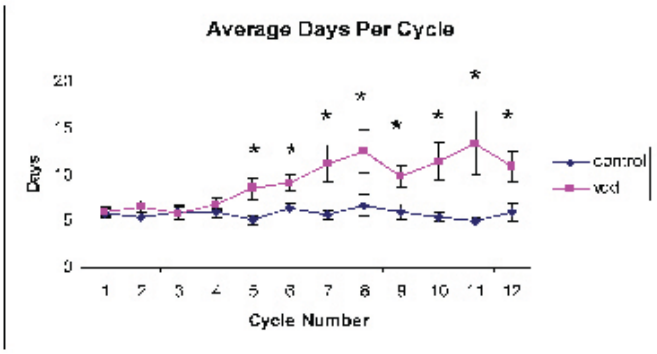

Figure 1. Female C57Bl/6 mice were dosed daily with VCD or sesame oil for 15 days. Cycle length was determined by counting the days between periods of proestrus as defined by vaginal cytology. Data are presented as mean ± SE (n = 6); *, $P < 0.05$ compared with control.

Hormone assays. Plasma E2 and P4 levels were measured by radioimmunoassay (RIA) with kits from Diagnostic Products Corp. (Los Angeles, Calif.) according to the manufacturer's instructions. Plasma FSH was measured by RIA using reagents supplied by Dr. A. F. Parlow (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md.). The sensitivities of the assays for E2 and P4 were 2.5 pg/ml and 100 pg/ml, respectively; that for FSH was 100 pg/ml. The coefficient of intra-assay variation was 1.95% (E2), 6.57% (P4), and 2.60% (FSH). The results of all RIAs were calculated by four-parameter logistic analysis using the software AssayZap (BioSoft, Ferguson, Mo.).

Data analysis. Differences between groups in length of cycle, and E2 and P4 levels were analyzed by two-way analysis of variance (ANOVA) with significance set at $P < 0.05$ using StatView software (SAS Inst., Cary, N.C.). Post-hoc tests (Fisher's Protected Least Significant Difference) were used where appropriate. Because of insufficient plasma sample volumes, FSH levels were measured in pooled samples from all animals (n = 2 to 6) at each time point.

Results

Effect of VCD on cycle length. For cycles 1 through 4, there was no significant difference in the mean number of days per estrous cycle between VCD-treated (6.3 days) and control (5.8 days) mice. However, cycles were significantly ($P < 0.01$) longer in VCD-treated (8.5 days) than control (5.1 days) animals during cycle 5 (mean, 28.1 days after the onset of VCD dosing) and remained so throughout the study (Fig. 1). Because of irregular cycles, the time to cycle 12 was longer ($P < 0.0001$) in VCD-treated (114.6 ± 9.4 days after onset of VCD dosing) than control (72.6 ± 2.8 days) animals.

Plasma E2 and P4 levels. Plasma levels of E2 and P4 were measured on the day of proestrus in every cycle. There was no difference in plasma E2 at the time of proestrus between controls and VCD-treated mice during any cycle (Fig. 2A). During cycles 9 and 11, P4 was higher ($P < 0.05$) in VCD-treated animals than in controls. However, the overall trend of P4 levels in proestrus did not show consistent differences (Fig. 2B). The E2:P4 ratio did not differ between control and VCD-treated mice during any cycle (Fig. 2C).

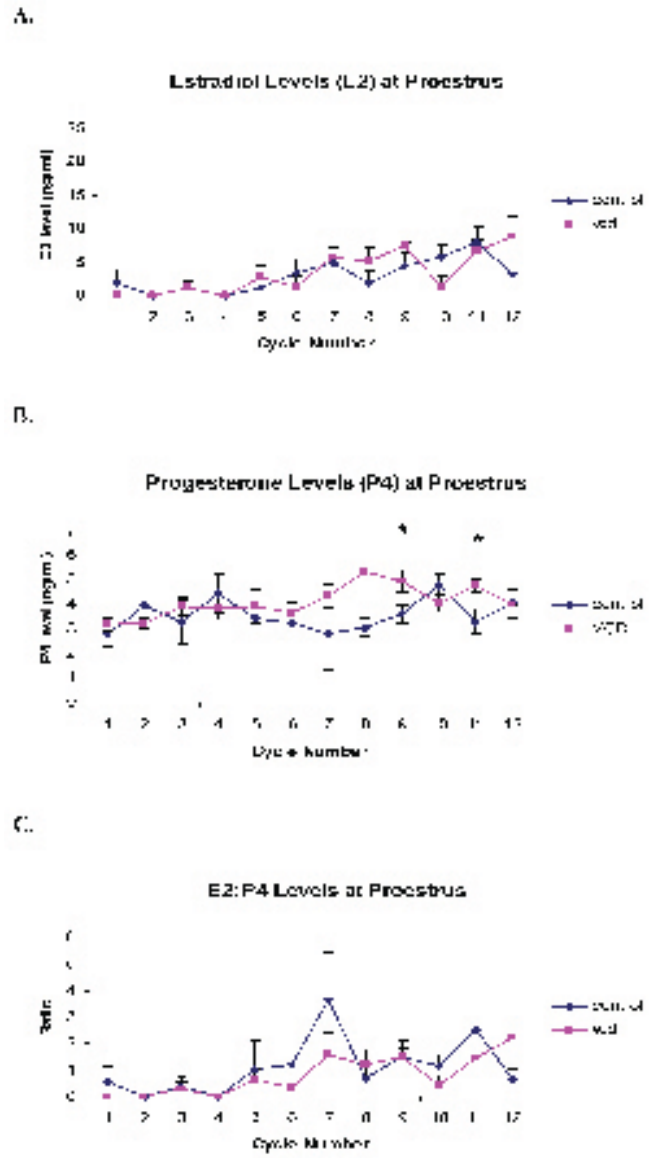

Figure 2. Female C57Bl/6 mice were dosed daily with VCD or sesame oil for 15 days. Blood was collected on the day of proestrus during each cycle. Plasma levels of (A) E2 and (B) P4 were measured by radioimmunoassay. In light of the sensitivity of the assay, steroid concentrations < 2.50 pg/ml are reported as zero. (C) The E2:P4 ratio was calculated for each cycle. Data are presented as mean ± SE (n = 15); *, $P < 0.05$ compared with control.

Plasma FSH levels. There was no apparent difference in FSH levels during cycles 1 to 4 between control and VCD-treated animals. However, during cycles 5 through 12 there was an apparent increase in FSH levels in the pooled plasma samples in VCD-treated mice as compared with control mice (Fig. 3). Statistical evaluation could not be made due to insufficient plasma sample volumes. For each time point, FSH was measured from a single pooled sample pool representing two to six animals.

Organ weights and ovarian failure. Organs were collected from all animals on day 156 after the onset of dosing. There was no difference in body weight between control and VCD-treated animals or in normalized uterine, spleen, adrenal, or liver weights. However, normalized ovarian weights were significantly ($P < 0.0001$) lower in VCD-treated (0.008 ± 0.001 g ovaries/g body weight) as compared with control mice (0.028 ± 0.002 g ovaries/g body weight). Histologic evaluation confirmed that no follicles remained in ovaries of VCD-treated mice at the time of euthanasia, whereas follicles and corpora lutea were present in ovaries from control animals (Fig. 4). Mean time of ovarian failure was 85 ± 6 days after the onset of dosing with VCD (range, 59 to 133 days; n = 15). After ovarian failure, circulating E2 levels in VCD-treated animals dropped to undetectable levels (data not shown).

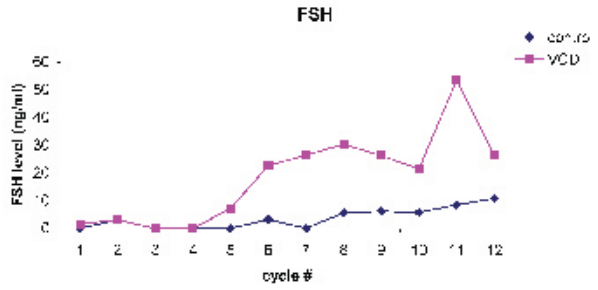

Figure 3. Female C57Bl/6 mice were dosed daily with VCD or sesame oil for 15 days. Blood was collected on the day of proestrus during each cycle, and plasma levels of FSH were measured by radioimmunoassay.

Discussion

VCD selectively targets ovarian primordial and primary follicles (2, 11, 20). Subsequently, because of lack of precursor follicle populations for recruitment, larger follicles are also lost, cyclicity becomes irregular, and eventually, no ovarian follicles remain (ovarian failure). The gradual onset of ovarian failure in our VCD-treated mice occurs during the time between the loss of primordial follicles and loss of large antral follicles. This period is analogous to perimenopause in women. Irregular cyclicity during this time can be monitored using vaginal cytology. The present study characterizes the onset of irregular cyclicity and hormone levels during impending ovarian failure in VCD-treated mice. Compared with controls, VCD-treated mice showed no difference in cycle length during cycles 1 through 4. However, beginning with cycle 5, the length became extended in VCD-treated mice. This pattern was in contrast to that in control animals, which demonstrated regular cycles of 5.8 days throughout the study. These results are similar to those of previous studies in middle-aged (10 to 12 months) C57Bl/6 mice, in which loss of regular ovulatory cyclicity corresponded to loss of regular ovarian function in some animals as a result of aging (15, 16).

Because of the extended cycle length in VCD-treated mice, we investigated whether peak ovarian steroid production by preovulatory follicles (i.e., on the day of proestrus) changes as ovarian function becomes compromised. Fewer days of proestrus occurred in VCD-treated mice as cycles lengthened. However, there was no difference in peak E2 levels of any cycle when proestrus was seen in VCD-treated mice as compared with controls. Presumably overall E2 production would become erratic at this time, as is the case with perimenopausal women (4, 17). During cycles 9 and 11, the level of P4 in VCD-treated mice was significantly ($P < 0.05$) greater than that in control mice, but

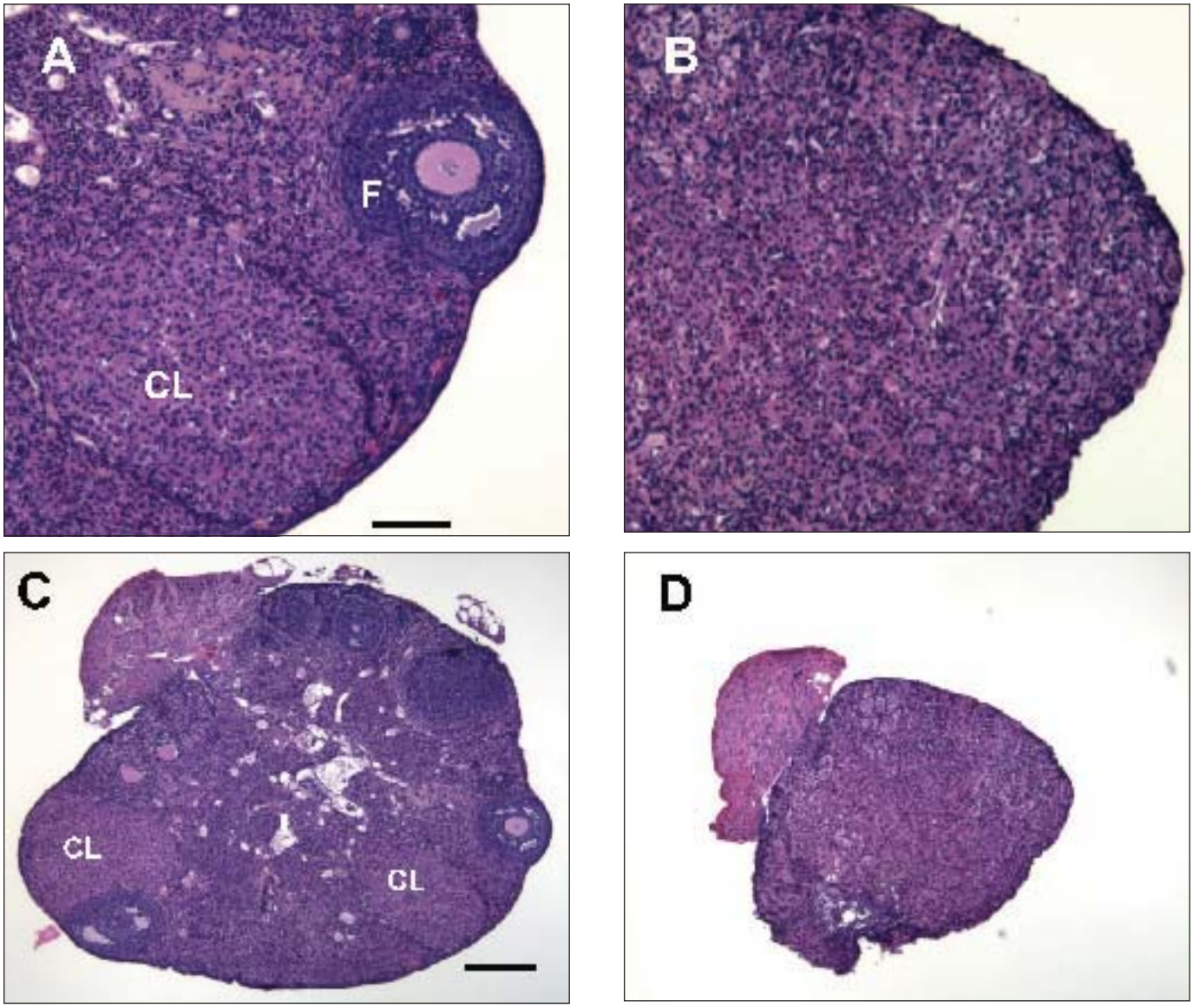

Figure 4. Micrographs of ovaries collected on day 156 after the onset of 4-vinylcyclohexene diepoxide (VCD) dosing in age-matched cycling controls (A, C) or VCD-treated (B, D) animals. Micrographs from the control animals (A, C) show evidence of active ovarian function, but no follicles (F) or corpora lutea (CL) are present in those of the VCD-treated animals (B, D), which have undergone ovarian failure. Low-magnification images reveal ovarian atrophy in the VCD-treated animal (D) when compared with the cycling control (C). Magnification, ×10 (A, B) or ×4 (C, D). Bars, 100 μm (A, B) or 250 μm (C, D).

these differences were not part of a consistent trend and seem to be due to animal variability or assay sensitivity rather than represent a finding of biological significance. E2:P4 ratios showed no difference between groups in any cycle. Therefore, regardless of the irregular and extended cycle lengths, follicular production of E2 and P4 in VCD-treated mice at proestrus in each cycle was comparable to that in control animals. This result supports the hypothesis that widely fluctuating levels of E2 in perimenopausal women reflect disruptions in cycle length rather than impairment of steroidogenic capacity in those follicles capable of ovulation (4, 17).

Increased circulating FSH levels are seen in perimenopause in women and are used as an early predictor that precedes the onset of menopause (10). We found that during cycles 1 through 4, FSH levels in pooled plasma samples of VCD-treated mice at proestrus were not different from those of controls in cycles 1 to 4. However, unlike ovarian steroids, FSH showed an apparent increase in the VCD-treated mice, beginning in cycle 5. This trend in VCD-treated mice began 33.8 days after onset of dosing, on average. Our finding corresponds with previously reported increases ($P < 0.05$) in FSH levels measured in VCD-treated mice on day 37 after the onset of dosing (13). This apparent increase also coincides with the onset of irregular cycle lengths.

These observations support the finding that, although irregular cycles develop with impending ovarian failure, E2 and P4 levels at proestrus are not compromised. This pattern in our mouse model contrasts with that in women in perimenopause, in whom circulating E2 levels fluctuate widely and are not a reli-

able marker of impending menopause (4, 17). One possible reason that E2 levels in women are so unpredictable at this time is that correlating blood sample collection with the menstrual cycle is problematic because periods are irregular and the preovulatory peak cannot be identified accurately according to menstrual bleeding. Therefore, samples collected during the preovulatory period would be interpreted as elevated, whereas those collected during the prolonged period between preovulatory peaks would be interpreted as low (10). The VCD mouse model of perimenopause supports this conclusion by demonstrating that peak E2 levels prior to ovulation (proestrus) are not different from those of control animals, even though cycle length and numbers have become irregular. Because multiple blood samples could not be obtained, animals were not tested for relative E2 levels in the extended periods between ovulations (diestrus). The loss of negative feedback on the hypothalamus and pituitary (increased FSH) that we observed with impending ovarian failure in the VCD-treated mice likely resulted from prolonged time between peak steroid production, decreased inhibin production, or both. These findings demonstrate that monitoring of FSH levels in the VCD-treated mouse model closely mimics events occurring in perimenopausal women.

Animals had completed cycle 12 by day 156 after the onset of VCD dosing. Therefore, organs were collected and weighed at that time. The only difference between groups was lower ovarian weights in VCD-treated mice. This finding supports the previous report that VCD dosing does not cause direct, generalized toxicity (13). In addition, the loss of ovarian weight resulted from an indirect effect of VCD—atrophy due to ovarian failure—which was confirmed by histologic evaluation.

Because of the substantial number of menopause-related health risks, it is important to develop relevant animal models that mimic the natural progression (perimenopause) experienced by most women that leads to menopause (1, 5, 12). In addition, after the natural onset of menopause, women retain residual (follicle-deplete) ovarian tissue. The VCD-treated mouse exhibits both of these traits, is highly relevant as a model for peri- and postmenopause-related studies, and should be characterized in detail.

In summary, the results presented here show the characterization of cycle length and hormonal fluctuations in the VCD-treated mouse during the period preceding ovarian failure (a period modeling perimenopause). The results demonstrate that, because cyclicity in individual animals can be accurately monitored in mice, blood samples could be obtained at the time of proestrus (analogous to the preovulatory E2 peak in women). Taking this approach enabled us to test the hypothesis that preovulatory follicles in mice exhibiting irregular cycles and impending ovarian failure retain similar steroidogenic capacity to those in age-matched cycling mice. Therefore, VCD-treated mice display hormonal and cyclic changes (unpredictable changes in E2 levels and increased FSH) known to be a hallmark of perimenopause in women, and these mice will be useful for elucidating those events in a controlled animal model system.

Acknowledgments

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