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Original Research

Modeling Perimenopause in Sprague–Dawley Rats by Chemical Manipulation of the Transition to Ovarian Failure

Jennifer B Frye,[1] Ashley L Lukefahr,[1] Laura E Wright,[1] Sam L Marion,[2] Patricia B Hoyer,[2] and Janet L Funk[1,*]

Various age-related diseases increase in incidence during perimenopause. However, our understanding of the effects of aging compared with hormonal changes of perimenopause in mediating these disease risks is incomplete, in part due to the lack of an experimental perimenopause model. We therefore aimed to determine whether manipulation of the transition to ovarian failure in rats via the use of 4-vinylcyclohexene diepoxide (VCD) could be used to model and accelerate hormonal changes characteristic of perimenopause. We examined long-term (11 to 20 mo), dose-dependent effects of VCD on reproductive function in 1- and 3-mo-old female Sprague–Dawley rats. Twenty-five daily doses of VCD (80 or 160 mg/kg daily compared with vehicle alone) depleted ovarian follicles in a dose-dependent fashion in rats of both ages, accelerated the onset of acyclicity, and caused dose-dependent increases in follicle-stimulating hormone that exceeded those naturally occurring with age in control rats but left serum levels of 17β–estradiol unchanged, with continued ovarian production of androstenedione. High-dose VCD caused considerable nonovarian toxicities in 3-mo-old Sprague–Dawley rats, making this an unsuitable model. In contrast, 1-mo-old rats had more robust dose-dependent increases in follicle-stimulating hormone without evidence of systemic toxicity in response to either VCD dose. Because perimenopause is characterized by an increase in follicle-stimulating hormone with continued secretion of ovarian steroids, VCD acceleration of an analogous hormonal milieu in 1-mo-old Sprague–Dawley rats may be useful for probing the hormonal effects of perimenopause on age-related disease risk.

Abbreviations: FSH, follicle-stimulating hormone; PND, postnatal day; VCD, 4-vinylcyclohexene diepoxide.

In women, the incidence of age-related diseases, including cardiovascular disease, metabolic syndrome, certain cancers, and osteoporosis, increases substantially across the menopausal transition, beginning during perimenpause.[17,25,26,30,31,35,38-40,44] Although the association of perimenopause with disease risks is well documented, a complete understanding of possible causal links and thus appropriate clinical management is not. Experimentally, the use of ovariectomized nonhuman primates and rodents has greatly advanced our understanding of postmenopausal hormone effects on the risk of certain age-related diseases.[5,47] However, this surgical model has certain obvious limitations in that it 1) lacks an early transitional phase of perimenopause; 2) results in the absence of any residual ovarian tissue rather than a selective loss of follicular estrogen as in natural menopause; 3) and does not allow for any graded manipulation of an intact hypothalamic–pituitary–ovarian axis.

Therefore, a novel animal model in which the normal process of ovarian follicular atresia can be accelerated via administration of an ovotoxic chemical, 4-vinylcyclohexene diepoxide (VCD) is of great interest.[14,15,27,28] The effects of VCD on ovarian follicles have been characterized thoroughly in mice and rats; VCD induces atresia of primordial and primary ovarian follicles by disrupting follicular c-Kit signaling, thus greatly depleting the follicular reserve available for subsequent maturation.[2,6,9,15,19,28,43] In mice, the reproductive sequelae of VCD-induced follicular atresia is an early increase in follicle-stimulating hormone (FSH) and irregular cyclicity, followed within 2 mo by ovarian failure (persistent diestrus) with low or undetectable estradiol, continued ovarian production of androstenedione, and persistent elevations in FSH.[22,23,27,46] Therefore in mice, VCD induces a rapid transition to an acyclic estrogen-deplete state analogous to natural human menopause.[1] In rats, the reproductive sequelae of VCD-induced follicular atresia have not been characterized. Unlike mice, rats normally experience a prolonged acyclic but estrogen-replete phase (persistent estrus) prior to frank ovarian failure.[16,33,45] During persistent estrus, FSH levels are elevated, and 17β-estradiol levels are normal, a hormonal milieu similar to human perimenopause.[3,36] This scenario suggests that differential dosing of VCD in rats could be used to manipulate the timing, magnitude, and duration of hormonal changes that are analogous to human perimenopause. This hypothesis has not been explored previously, given that the only study to date of long-term effects of VCD in rats focused on changes in ovarian follicles rather than reproductive function.[28]

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Departments of [1]Medicine, [2]Physiology, College of Medicine, The University of Arizona, Tucson, Arizona.
[*]Corresponding author. Email: jfunk@u.arizona.edu

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To fill these knowledge gaps with the goal of determining whether VCD-treatment in rats can be used to model hormonal changes in human perimenopause, we undertook studies to characterize the long-term, dose-dependent effects of VCD on reproductive function, including screening for possible nonovarian effects, in female Sprague–Dawley rats (*Rattus norvegicus*), a strain that has been a mainstay of menopausal research probing effects of ovariectomy on cardiovascular function, cancer, bone, and cognition.[4,12,18,42] Here we administered VCD to 28-d-old rats, the age at which the follicular effects of VCD have been best described,[15] and 3-mo-old rats, whose disease 'target organs' (for example, bone, breast) are more mature, an important consideration when seeking to elucidate the hormonal effects on disease risk at these sites (that is, osteoporosis, breast cancer). Two doses of VCD were tested with the goal of identifying a treatment that would induce persistent estrus within months of treatment in Sprague–Dawley rats: 80 mg/kg daily, the only dose previously examined longitudinally in rats (Fischer 344), in which changes in cyclicity were documented at 1 y but not at 4 mo;[28] and 160 mg/kg daily, which induces follicular atresia without toxicity in Fischer 344 rats with unknown long-term sequelae with respect to reproductive function.[13]

Materials and Methods

Animal procedures. Female Sprague–Dawley rats (Harlan, Indianapolis, IN) were housed in clear polycarbonate cages with Sani-chip bedding (Harlan Teklad) and maintained on 12:12-h light:dark cycles at 22 ± 2 °C with ad libitum access to rodent diet (Harlan Teklad 8604) and reverse-osmosis–treated water. Rats were allowed to acclimate to the animal facility 1 wk prior to the start of intraperitoneal administration indicated dosing regimens of VCD or vehicle beginning at either 1 mo (peripubertal 'juveniles') or 3 mo ('adults') of age. During VCD treatment, rats were monitored daily for assessment of body weight and general appearance, including but not limited to respiratory rate, ambulation, and grooming, and were euthanized if moribund. Blood was collected from all rats at harvest immediately after treatment or at the end of experiments to assess effects of VCD on immune, renal, and hepatic function. A veterinary pathologist blinded to treatment groups performed necropsies, including histologic assessment of vital organs, on sentinel animals (n = 1 to 2 per treatment group) immediately after completion of VCD treatment. All studies were performed in accordance with institutional guidelines and approved by the IACUC of The University of Arizona.

Administration of VCD to Sprague–Dawley rats. For all studies, VCD (Sigma-Aldrich, St Louis, MO) was dissolved in tissue-culture–grade DMSO (Sigma-Aldrich) in a sterile fume hood at a concentration of 128.5 mg/mL and stored in sterile glass vials at −20 °C until use. In one set of experiments, 3-mo-old female adult rats (postnatal day [PND] 94) were assigned randomly to the following treatment groups: vehicle (DMSO; n = 38), VCD at 80 mg/kg daily (low-dose VCD; n = 11), and VCD at 160 mg/kg daily (high-dose VCD; n = 40). Vehicle or VCD was administered intraperitoneally (total volume, 0.25 to 0.35 mL) for a total of 25 doses, either on consecutive days or 5 times each week. Because ovotoxicity immediately after completion of VCD treatment was the same for both protocols (data not shown), these data were combined. In a second set of experiments, 1-mo-old female juvenile rats (PND 34) were assigned randomly to the following treatment groups: vehicle (DMSO; n = 24), 80 mg/kg VCD daily (n = 18), and 160 mg/kg daily VCD (n = 29). Dosing was administered intraperitoneally (total volume, 0.1 to 0.3 mL) 5 times each week for a total of 25 doses. The VCD dosing regimens used were known to deplete follicles without toxicity in 28-d-old Fischer rats.[15,13]

Tissue collection. Rats were anesthetized by intraperitoneal administration of 1 µL/g body weight of a mixture of 11 mg/mL xylazine, 33 mg/mL ketamine, and 1.3 mg/mL acepromazine ['rabbit mix']) prior to euthanasia either at the end of VCD dosing (PND 120 for adults or PND 68 for juveniles) or the end of the experiment. Because of unanticipated toxic effects of VCD when administered to 3-mo-old (adult) rats, these studies were terminated after 1 y (12 mo [PND 355]). Because VCD was without evidence of toxicity when administered to 1-mo-old rats (juveniles), these studies were continued for almost 2 y (20 mo [PND 604]). Kidneys, spleen, liver, and uterus were collected and weighed at time of euthanasia. Ovaries were removed, weighed, and fixed in Bouin solution (15 mL picric acid solution [1.3%], 5 mL formaldehyde [37%], and 1 mL glacial acetic acid) for 4 h before being transferred to 70% ethanol.[28]

Hematopoietic and chemical blood analyses. Blood was collected by using a vacuum phlebotomy tube butterfly catheter (BD, San Jose, CA) from the inferior vena cava of a subset of rats immediately after VCD dosing and from remaining rats at the end of experiment. WBC counts were determined in EDTA-treated blood by using an automated analyzer (Hemavet 880, CDC Technologies, Oxford, CT), and cell differentials were determined by manual counting.[10] Heparinized whole-blood samples were kept at 4 °C for at least 30 min before centrifugation, with aliquots of these plasma samples stored at 4 °C for less than 24 h prior to assay of creatinine and ALT by using an automated analyzer (Endocheck Plus Chemistry Analyzer, Hemagen Diagnostics, Columbia, MD).[10]

Histologic assessment of ovaries. Fixed ovaries were embedded in paraffin and sectioned serially (5-µm sections). Every 40th section was stained with hematoxylin and eosin for follicular classification and counting.[28] Follicles were categorized as primordial (oocyte surrounded by a single layer of flattened granulosa cells), small primary (oocyte surrounded by a single layer of 8 to 10 round granulosa cells), large primary (oocyte surrounded by a single layer of more than 10 round granulosa cells), secondary (oocyte surrounded by 2 or more layers of round granulosa cells) or antral (an epithelial cavity surrounding the oocyte), by using standard criteria.[7] The number of each type of follicle per treatment group are reported as mean ± SEM of the total number of follicles identified per ovary, after counting of every 40th 5-µm section.

Vaginal cytology. To determine the effects of VCD on onset and duration of persistent estrus, reproductive status was monitored at approximately monthly intervals by daily assessment of vaginal cytology during 7- to 10-d periods by using standard techniques.[11] Vaginal smears were considered to be in 1 of 4 stages: proestrus, estrus, metestrus, or diestrus. The onset of persistent estrus was assigned when an animal displayed more than 75% of days in the epithelial phase (proestrus or estrus).[21] Data are expressed as incidence of persistent estrus per treatment group.

Immunoassays of reproductive hormones. Whole-blood samples were collected in the afternoon from either the tail vein or inferior vena cava (at time of euthanasia) immediately after completion VCD treatment and at approximately monthly intervals thereafter,

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beginning 3 to 4 mo after the start of VCD dosing. Blood was allowed to clot for 15 min at room temperature before centrifugation and storage of serum aliquots at −80 °C prior to assay. Serum 17β-estradiol, androstenedione, and FSH concentrations were determined by radioimmunoassay immediately after completion of VCD treatment, at various times throughout the experiments, and at final harvest. Androstenedione and 17β-estradiol levels were measured by using a direct androstenedione competitive radioimmunoassay (sensitivity, 75 pg/mL) or an estradiol double-antibody radioimmunoassay (sensitivity, 2.5 pg/mL), respectively, according to manufacturer's (Siemens Medical Solutions Diagnostics, Los Angeles, CA) protocol.[22] Serum levels of FSH were determined by competitive radioimmunoassay (sensitivity, 500 pg/mL) using FSH standards and primary antibody obtained from AF Parlow of the National Hormone and Peptide Program.[28]

Statistical analyses. Values are presented as the mean ± SEM except where indicated. Statistical significance was determined by ANOVA with post hoc testing or unpaired t test by using InStat software (GraphPad Software, San Diego, CA) or log–rank testing for indicated survival curves and ANOVA with Newman–Keuls post testing for weight curves by using Prism software (GraphPad Software).

Results

Immediate and delayed effects of VCD on survival and body weight. During the dosing period, acute dose-dependent effects of VCD (80 or 160 mg/kg daily) on body weight and mortality in juvenile (1 mo; Figure 1 A) and adult (3 mo; Figure 1 B) female Sprague–Dawley rats and compared with those of vehicle only. All juvenile rats survived VCD dosing (Figure 1 A), and average body weights for all groups increased overtime without any significant difference between groups (Figure 1 A, inset). In contrast, adult rats treated with either dose of VCD weighed less ($P < 0.001$) than did vehicle-treated controls throughout the dosing period (Figure 1 B, inset), with a final weight loss at end of dosing of less than 20% in high-dose VCD rats. Adult animals surviving high-dose VCD lost weight relative to baseline weights ($P < 0.01$), as compared with continued weight increases in vehicle-treated and low-dose VCD rats during the same period. Moreover, a significant ($P < 0.001$) number of high-dose VCD adult rats ($n = 10$) died or were euthanized ($n = 1$) during treatment (76% survivorship) as compared with no deaths in the vehicle or low-dose VCD groups (Figure 1 B). The cause of death in the 3-mo-old high-dose VCD rats was not immediately evident on gross inspection, given that these rats were indistinguishable from survivors, displaying weight loss without other evidence of moribund behavior prior to death.

During the 19-mo follow-up after the VCD dosing period, survival and body weights in juvenile rats treated with either dose of VCD remained the same as those in vehicle-treated rats (Figure 1 C and inset). When adult rats that survived the VCD dosing period were followed for an additional 9 mo, low-dose VCD did not significantly alter survival as compared with vehicle only (Figure 1D). Final survivorship in rats treated with high-dose VCD was 51% as compared with 100% in controls ($P < 0.001$; Figure 1 D). Average body weights of adult Sprague–Dawley rats that survived treatment with low- or high-dose VCD rose quickly to match those of vehicle-treated animals (Figure 1 D, inset).

Immediate and delayed effects of VCD on hematopoietic, hepatic, and renal function. No adverse hematopoietic effects were noted in VCD-treated juvenile rats acutely or at the end of the experiment (Table 1). In contrast, acute adverse effects on immune cells were present in adult rats that survived 25 d of high-dose VCD; WBC counts dropped ($P < 0.01$) due to lymphopenia ($P < 0.01$; Table 1) in association with decreased ($P < 0.01$) spleen weight as compared with that in controls (Table 2). In addition, a small but significant ($P < 0.05$) decrease in lymphocytes occurred acutely in adults treated with low-dose VCD (Table 1), with no change in spleen weight (Table 2). At end of experiment, however, no adverse immune effects were noted in adult rats surviving VCD treatment (Tables 1 and 2).

Liver weight was increased in juvenile rats treated with high-dose VCD, both acutely and at the end of the experiment (Table 2). However, liver function, as assessed by serum levels of ALT, was normal at all times and doses in VCD-treated juvenile rats (Table 1). Liver function and weight also were normal in adult rats treated with low-dose VCD (Tables 1 and 2). In contrast, adults surviving treatment with high-dose VCD rats had increased ($P < 0.001$) serum levels of ALT (Table 1) and increased ($P < 0.001$) liver weight (Table 2) immediately after completion of VCD treatment, as compared with controls. Histologic assessment of livers obtained immediately after treatment with high-dose VCD in adult rats revealed normal hepatic parenchyma in a rat with normal ALT, but an architecturally abnormal liver with vacuolar changes in hepatocytes and sinusoidal inflammatory infiltrates consistent with the documented increase in ALT in the other rat examined. However, at end of experiment, ALT levels were normal in adult survivors of high-dose VCD (Table 1).

Renal function, as assessed by serum creatinine levels, was normal at all times in VCD-treated rats of either age (Table 1). Kidney weights were also unchanged with VCD treatment, with the exception of increased ($P < 0.05$) kidney weight in high-dose VCD adult rats immediately after completion of treatment (Table 2).

Immediate and delayed effects of VCD on depletion of ovarian follicles. Immediately after completion of VCD treatment in rats of either age, total follicle counts were significantly ($P < 0.05$) decreased in a dose-dependent fashion as compared with those in age-matched controls; high-dose VCD decreased the total follicular count by 91% in juveniles (Figure 2 A) and 93% in adults (Figure 2 B), whereas low-dose VCD decreased the total follicular count by 71% and 82% in juveniles and adults, respectively (Figure 2 A and B). At this early time point, follicles of each type were decreased ($P < 0.05$) in all VCD-treated rats, with the exception of secondary follicles in low-dose VCD juveniles (Figure 2 A). In addition, ovarian weights in VCD-treated rats immediately after treatment were no different than those of age-matched vehicle-treated controls (Table 2).

When adult rats surviving VCD treatment were followed for an additional 236 d (until 12 mo of age [PND 355]), total follicle counts were further decreased as compared with those in age-matched controls (low-dose VCD: –97%, $P < 0.001$; high-dose VCD: –99%, $P < 0.001$), with the number of follicles of each type lower ($P < 0.05$) than those in vehicle-treated controls (Figure 2 D). Of note, during this period, the total number of follicles in control rats decreased ($P < 0.001$) by 64% with aging. Ovarian weights at end of experiment in VCD-treated adults were significantly ($P < 0.05$) decreased relative to those in age-matched controls

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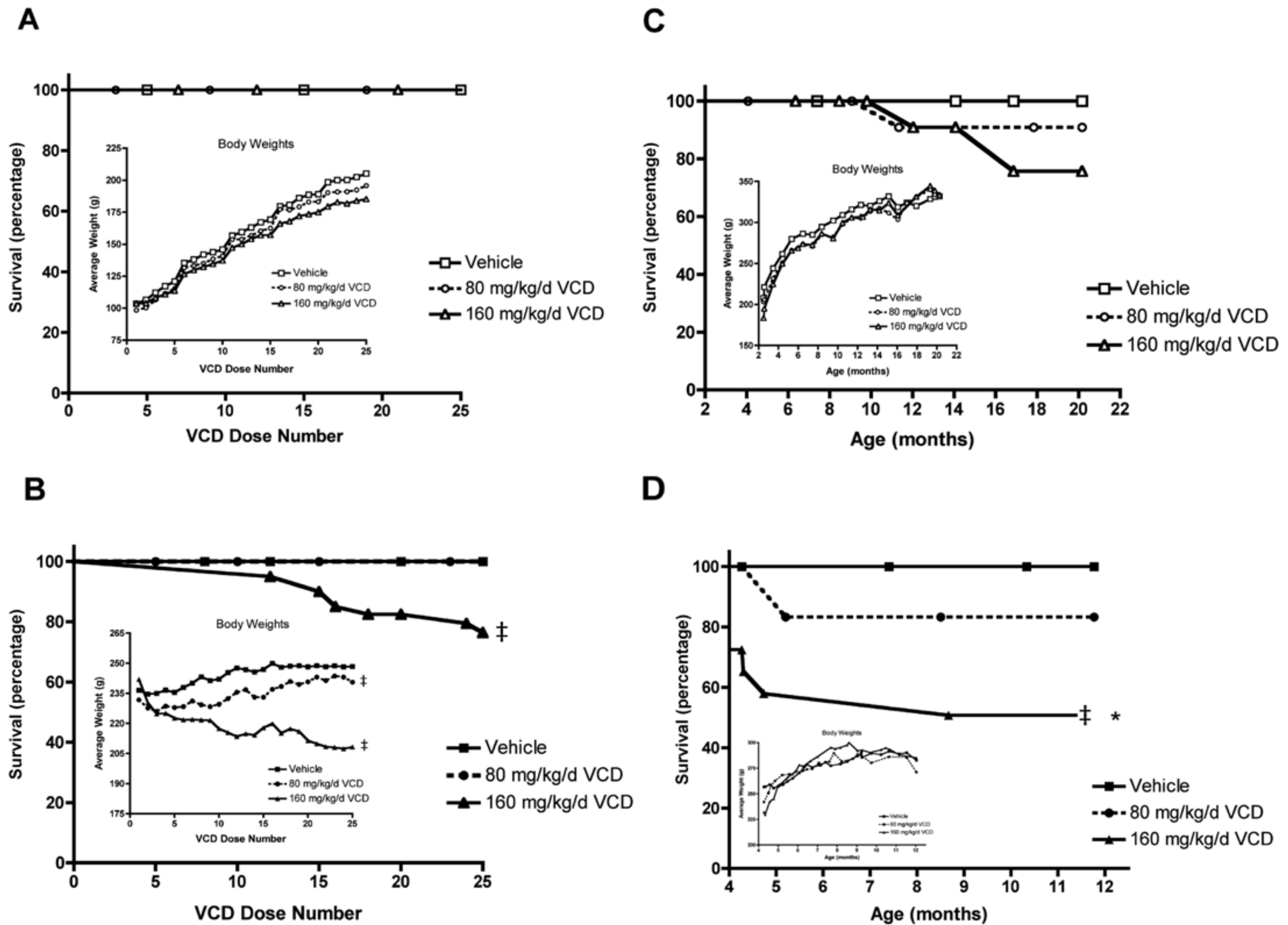

Figure 1. Mortality and body weights of juvenile and adult Sprague–Dawley rats treated with VCD. (A) Kaplan–Meier survival curve of juvenile rats during injection with vehicle (n = 24), low-dose VCD (n = 18), or high-dose VCD (n = 29). There were no significant differences between groups. Inset, Average body weights of juvenile rats. No significant differences between groups by ANOVA. (B) Kaplan–Meier survival curve of adult rats during injection with vehicle (n = 38), low-dose VCD (n = 11), or high-dose VCD (n = 40). ‡, $P < 0.001$ compared with control by log-rank testing. Inset, Average body weights of adult rats. ‡, $P < 0.001$ compared with controls over time by ANOVA with Newman–Keuls testing. (C) Kaplan–Meier survival curve of juvenile rats after completion of treatment with vehicle (n = 10), low-dose VCD (n = 12), or high-dose VCD (n = 12). There were no significant differences between groups. Inset, Average body weights of juvenile rats. There were no significant differences between groups by ANOVA. (D) Kaplan–Meier survival curve of adult rats after completion of treatment with vehicle (n = 17), low-dose VCD (n = 5), or high-dose VCD (n = 10). ‡, $P < 0.001$ compared with controls by log-rank testing. Inset, Average body weights of adult rats in same treatment groups. No significant differences between groups by ANOVA.

(Table 2). Similar long-term effects of VCD on ovarian follicles were documented in juvenile rats, which were followed for an even longer period of time than adults (534 d after the last VCD dose, until 20 mo of age [PND 604]; Figure 2 C). With the exception of antral follicles in low dose VCD rats, follicles of each type were completely depleted in VCD-treated juvenile rats (Figure 2 C), such that total follicle counts were decreased ($P < 0.0001$) by 97% and 100% in low- or high-dose VCD animals, respectively, as compared with age-matched controls. At the end of experiments, ovarian weights, which had decreased over time in controls, were no different in VCD-treated compared with vehicle-treated rats (Table 2).

Dose-dependent effects of VCD on cyclicity. Although low-dose VCD treatment of rats of either age significantly ($P < 0.05$) depleted follicles, it had little effect on the onset or duration of persistent estrus. In contrast, high-dose VCD treatment at either age accelerated persistent estrus as compared with that in normal aging controls, speeding its onset or peak incidence or both (Figures 3 A and 4 A).

Dose-dependent effects of VCD on serum FSH. VCD treatment of Sprague–Dawley rats of either age caused dose-dependent increases ($P < 0.05$) in FSH as compared with that in vehicle-treated control animals (Figure 3 B, juveniles; Figure 4 B, adults). In response to high-dose VCD, the highest documented increase in FSH (Figure 3 B and 4 B) coincided with peak incidence of accelerated persistent estrus in both juvenile and adult rats (5.2 and 8.6 mo of age, respectively; Figures 3 A and 4 A). FSH levels in rats of either age treated with low-dose VCD were significantly ($P < 0.05$)

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Table 1. Effects of VCD treatment on hematopoietic, hepatic, and renal function

		Immediately after VCD			At end of experiment		
		Vehicle	VCD 80 mg/kg/d	VCD 160 mg/kg/d	Vehicle	VCD 80 mg/kg/d	VCD 160 mg/kg/d
Juvenile rats							
	WBC (× 10^3/μL)	9.55 ± 1.06	8.32 ± 0.30	8.54 ± 0.56	3.95 ± 0.06	5.02 ± 0.40	7.24 ± 2.67
	Lymphocytes (× 10^3/mL)	7.15 ± 0.73	6.35 ± 0.29	6.09 ± 0.26	2.93 ± 0.22	3.31 ± 0.23	3.26 ± 0.33
	Neutrophils (× 10^3/mL)	2.07 ± 0.46	1.67 ± 0.18	2.102 ± 0.47	0.93 ± 0.18	1.59 ± 0.22	3.63 ± 2.12
	Hematocrit (%)	41.24 ± 1.73	50.1 ± 1.00[c]	42.76 ± 1.91	37.47 ± 3.27	41.1 ± 0.64	36.74 ± 1.25
	ALT (U/L)	38.27 ± 1.92	35.51 ± 1.31	43.47 ± 3.24	37.93 ± 8.27	29.61 ± 4.31	23.52 ± 6.11
	Creatinine (mg/dL)	0.20 ± 0.02	0.18 ± 0.03	0.2 ± 0.02	0.23 ± 0.12	0.34 ± 0.02	0.32 ± 0.02
Adult rats							
	WBC (× 10^3/μL)	10.79 ± 0.64	9.092 ± 1.35	5.44 ± 0.45[b]	6.16 ± 0.39	5.82 ± 0.24	7.35 ± 0.89
	Lymphocytes (× 10^3/mL)	7.85 ± 0.19	6.01 ± 0.45[a]	2.99 ± 0.30[b]	4.61 ± 0.28	3.87 ± 0.20	4.9 ± 0.49
	Neutrophils (× 10^3/mL)	2.72 ± 0.45	2.73 ± 0.95	2.78 ± 0.67	1.58 ± 0.43	1.59 ± 0.09	2.12 ± 0.48
	Hematocrit (%)	44.04 ± 0.76	42.56 ± 0.81	40.49 ± 1.43	45.69 ± 1.25	44.80 ± 2.39	45.09 ± 1.43
	ALT (U/L)	26.13 ± 1.56	21.10 ± 4.16	55.63 ± 4.65[b]	18.78 ± 2.93	21.10 ± 3.91	24.27 ± 6.15
	Creatinine (mg/dL)	0.24 ± 0.02	0.28 ± 0.04	0.27 ± 0.02	0.29 ± 0.02	0.36 ± 0.02	0.34 ± 0.02

Data are expressed as mean ± SEM in animals immediately after 25 d of treatment (n = 5 to 17 per group; juvenile rats, PND 68; adult rats, PND 120) and at the termination of the experiment (n = 3 to 10 per group; juvenile rats, 20 mo of age; adult rats, 12 mo of age). Statistical significance was determined by ANOVA.

[a] $P < 0.05$ compared with value for vehicle-only group.

[b] $P < 0.05$ compared with values for rats receiving vehicle or 80 mg/kg VCD daily.

[c] $P < 0.05$ compared with values for rats receiving vehicle or 160 mg/kg VCD daily.

Table 2. Effects of VCD treatment on organ weights (% of body weight)

		Immediately after VCD			End of experiment		
		Vehicle	VCD 80 mg/kg/d	VCD 160 mg/kg/d	Vehicle	VCD 80 mg/kg/d	VCD 160 mg/kg/d
Juvenile rats							
	Body weight (g)	205.3 ± 2.55	195.8 ± 3.29	185.3 ± 2.82	332 ± 20.78	333.5 ± 3.9	333.6 ± 11.5
	Spleen	0.38 ± 0.01	nd	0.37 ± 0.01	0.30 ± 0.05	0.21 ± 0.01	0.30 ± 0.05
	Liver	3.54 ± 0.10	nd	4.34 ± 0.10[a]	2.56 ± 0.15	2.55 ± 0.08	3.00 ± 0.14[b]
	Kidney	0.34 ± 0.04	nd	0.42 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	0.34 ± 0.01
	Ovary	0.02 ± 0.001	nd	0.02 ± 0.002	0.01 ± 0.001	0.01 ± 0.002	0.01 ± 0.001
	Uterus	0.19 ± 0.02	nd	0.18 ± 0.02	0.28 ± 0.05	0.32 ± 0.03	0.34 ± 0.02
Adult rats							
	Body weight (g)	248.4 ± 1.84	240.5 ± 2.58	208.3 ± 8.19[b]	280.8 ± 5.42	271 ± 9.3	285 ± 5.35
	Spleen	0.34 ± 0.01	0.36 ± 0.01	0.28 ± 0.02[b]	0.31 ± 0.03	0.34 ± 0.02	0.30 ± 0.02
	Liver	3.29 ± 0.07	3.62 ± 0.15	4.26 ± 0.14[b]	2.55 ± 0.11	2.55 ± 0.11	2.90 ± 0.10
	Kidney	0.36 ± 0.01	0.37 ± 0.02	0.39 ± 0.01[a]	nd	nd	nd
	Ovary	0.02 ± 0.002	0.02 ± 0.012	0.02 ± 0.002	0.02 ± 0.001	0.01 ± 0.001[a]	0.01 ± 0.001[a]
	Uterus	0.23 ± 0.02	0.17 + 0.02	0.16 ± 0.01[a]	0.34 ± 0.08	0.37 ± 0.04	0.26 ± 0.02

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Tissue weights are expressed as percentages (mean ± SEM) of total body weight in animals immediately after 25 d of treatment (n = 5 to 17 per group; juvenile rats, PND 68; adult rats, PND 120) and at the termination of the experiment (n = 3 to 10 per group; juvenile rats, 20 mo of age; adult rats, 12 mo of age). Statistical significance was determined by ANOVA or unpaired t test.

[a] $P < 0.05$ compared with value for vehicle group.

[b] $P < 0.05$ compared with values for rats given vehicle or 80 mg/kg VCD daily.

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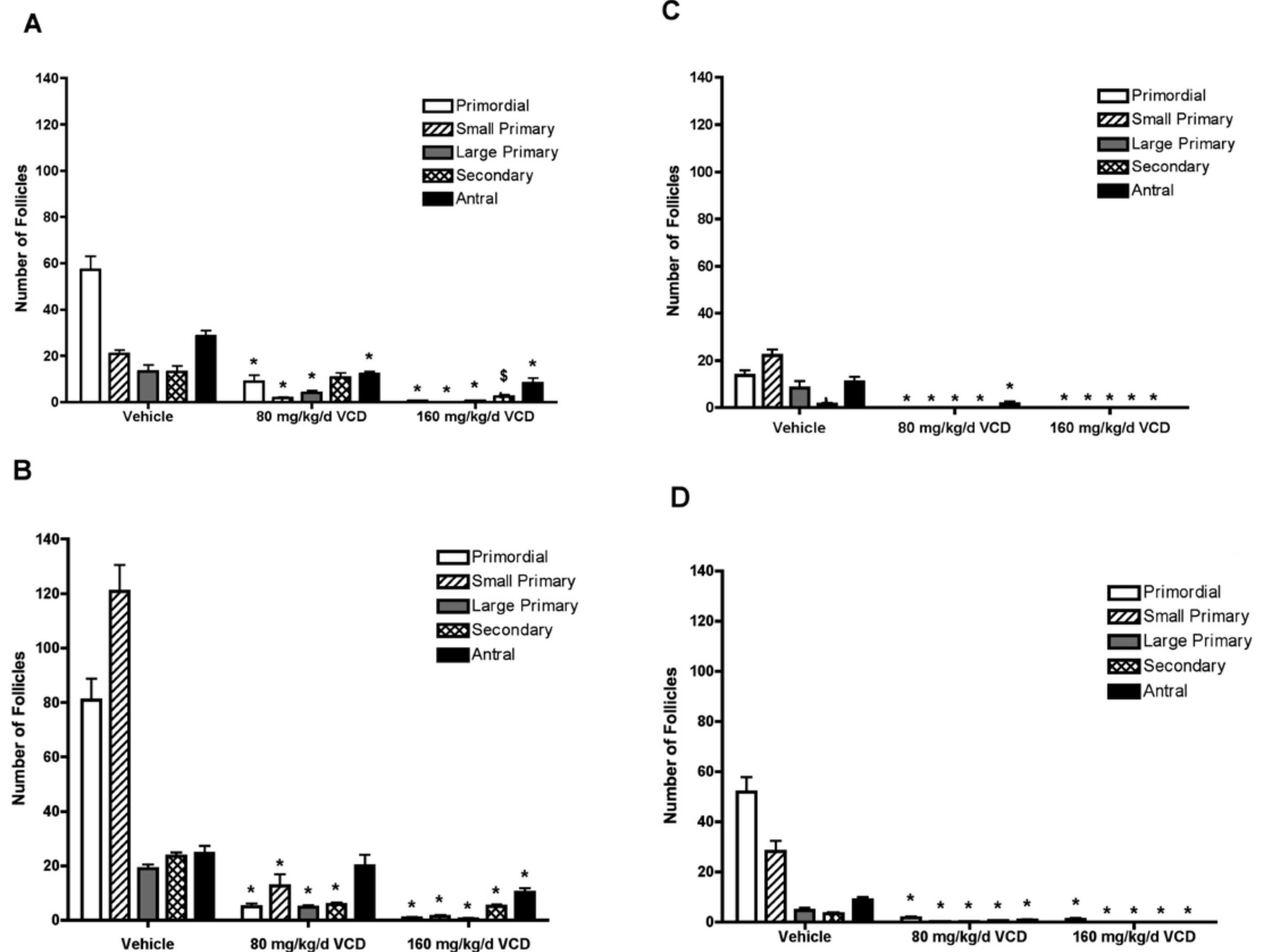

Figure 2. Dose-dependent depletion of ovarian follicles by VCD in juvenile (1 mo) and adult (3 mo) female Sprague–Dawley rats. Follicle number per ovary (mean ± SEM) are reported for end of VCD treatment or end of the experiment. (A) Ovarian follicle counts in juvenile rats immediately posttreatment with vehicle ($n = 5$), low-dose VCD ($n = 6$), or high-dose VCD ($n = 6$). *, $P < 0.05$ compared with vehicle; $, $P < 0.05$ compared with vehicle or 80 mg/kg/d VCD. (B) Ovarian follicle counts in adult rats immediately after treatment with vehicle ($n = 17$), low-dose VCD ($n = 5$), or high-dose VCD ($n = 17$). *, $P < 0.05$ compared with vehicle. (C) Ovarian follicle counts in 20-mo-old juvenile rats, 18 mo after completion of VCD treatment with vehicle ($n = 3$), low-dose VCD ($n = 4$), or high-dose VCD ($n = 4$). *, $P < 0.05$ compared with vehicle. (D) Ovarian follicle counts in 12-mo-old adult rats at 8 mo after completion of VCD treatment with vehicle ($n = 15$), low-dose VCD ($n = 5$), or high-dose VCD ($n = 7$). *, $P < 0.05$ compared with vehicle.

increased as compared with those of age-matched controls (Figures 3 B and 4 B), despite the lack of a marked change in cyclicity (Figures 3 A and 4 A).

Effects of VCD on serum levels of ovarian steroid hormones. Serum 17β-estradiol levels in VCD-treated rats of either age were no different than those of age-matched controls and were unchanged over time within each treatment group, including controls (Figures 3 C and 4 C). Consistent with this lack of change in estradiol levels, uterine weights did not change over time in control rats and remained unaltered at 12 or 20 mo of age in adult or juvenile VCD-treated rats, respectively, as compared with age-matched controls (Table 2). As with estradiol, androstenedione levels in vehicle-treated controls were unchanged over time (Figures 3 D and 4 D). In VCD-treated juveniles, androstenedione levels, although highly variable, were not statistically different from those in controls throughout the experiment (Figure 3D). In VCD-treated adults (Figure 4 D), androstenedione levels after high-dose VCD were increased ($P < 0.001$) immediately after treatment, as compared with those in control or low-dose VCD rats, but were no different than levels in vehicle-treated controls or low-dose VCD rats by the end of the experiment.

Discussion

In the fields of osteoporosis, cardiovascular disease, and cancer, our 'estrogen-centric' view of diseases that increase in incidence at the start of the menopausal transition is increasingly being challenged as roles for additional reproductive hormones in these disease processes are uncovered.[20,32,34,37] This shift is particularly true for perimenopause, given that human studies over

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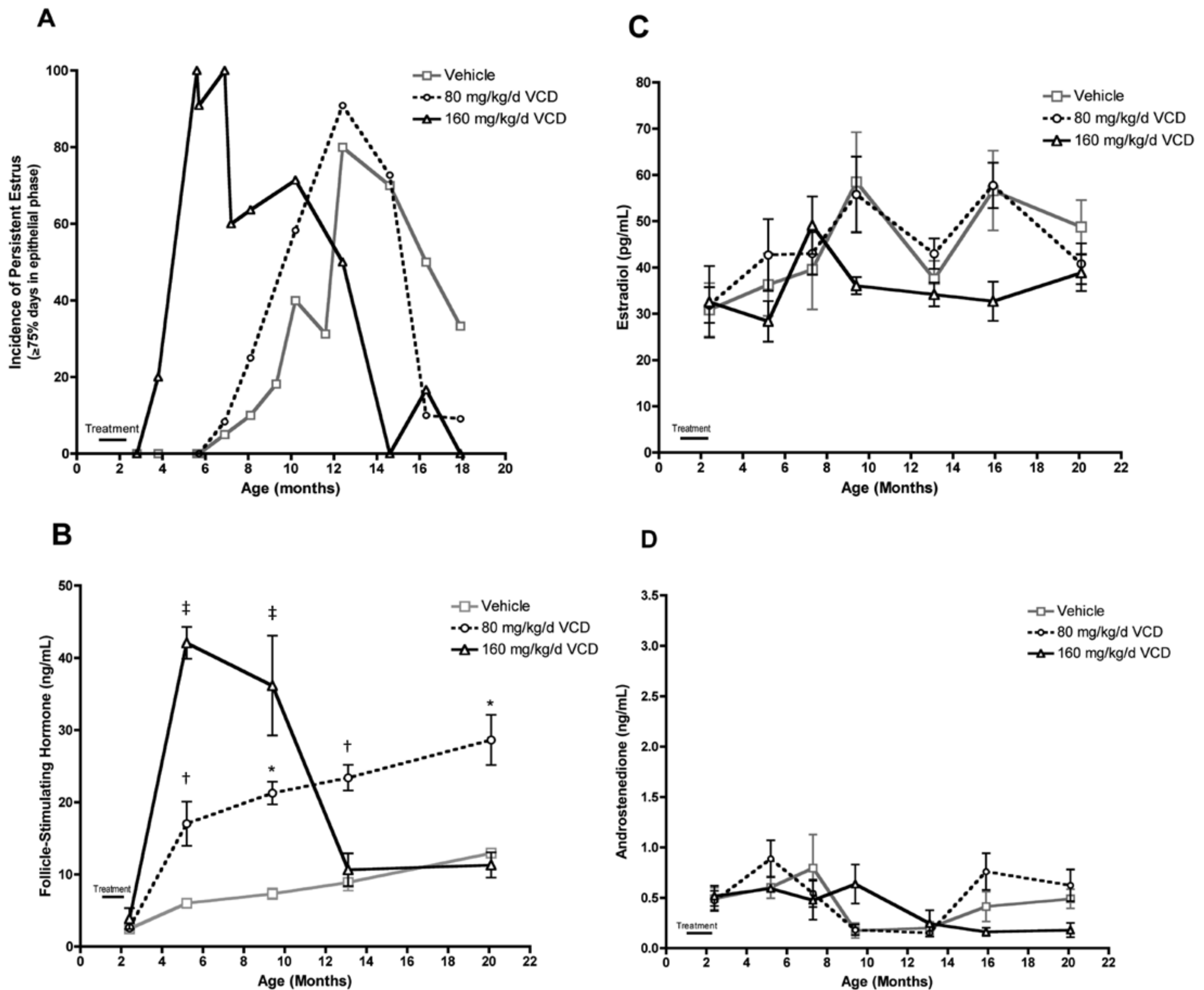

Figure 3. Effects of VCD on reproductive function and hormones when administered to 1-mo-old Sprague–Dawley rats. (A) Cyclicity in juvenile rats treated with vehicle ($n = 3$ to 17), low-dose VCD ($n = 10$ to 12), or high-dose VCD ($n = 4$ to 12), expressed as the incidence of persistent estrus (at least 75% of days in epithelial phase determined by days in proestrus and estrus). (B) Serum FSH levels in rats treated as juveniles with vehicle ($n = 3$ to 10), low-dose VCD ($n = 5$ to 12), or high-dose VCD ($n = 4$ to 12). Within each treatment group, FSH levels in aging rats were significantly ($P < 0.05$) increased over baseline (2.4 mo), except for high-dose VCD rats at 13 and 20 mo. *, $P < 0.05$ compared with vehicle; †, $P < 0.01$ compared with vehicle; ‡, $P < 0.001$ compared with vehicle. (C) Serum 17β-estradiol levels in rats treated as juveniles with vehicle ($n = 3$ to 10), low-dose VCD ($n = 5$ to 10), or high-dose VCD ($n = 4$ to 6). No significant differences were observed between treatment groups by ANOVA or unpaired t test as appropriate, nor were significant differences detected within any treatment group as compared with baseline (2.4 mo cycling animals). (D) Serum androstenedione levels in rats treated as juveniles with vehicle ($n = 3$ to 10), low-dose VCD ($n = 5$ to 10), or high-dose VCD ($n = 4$ to 6). No significant difference was observed between or within groups.

the last decade have revealed that this period is not characterized by declining estradiol levels, as previously thought, but rather is an estrogen-replete state.[3,36] Therefore, unlike those during menopause, disease risks during perimenopause cannot be attributed to a lack of estrogen. This difference raises important questions about the hormonal pathogenesis of disease processes specific to this transitional period, such as the onset of osteoporotic bone loss that begins in perimenopause and accounts for 40% of a woman's lifetime loss of trabecular bone.[31,38] However, preclinical studies assessing disease risks during perimenopause are limited by the lack of a rodent model.

The studies presented here, which are unique in their detailed examination of longitudinal dose-dependent changes in reproductive hormones in response to VCD in rats, provide the first evidence that this treatment can be used to model and manipulate a hormonal milieu analogous to human perimenopause. The current studies demonstrate that this experimental model can reproduce important characteristics of the perimenopausal tran-

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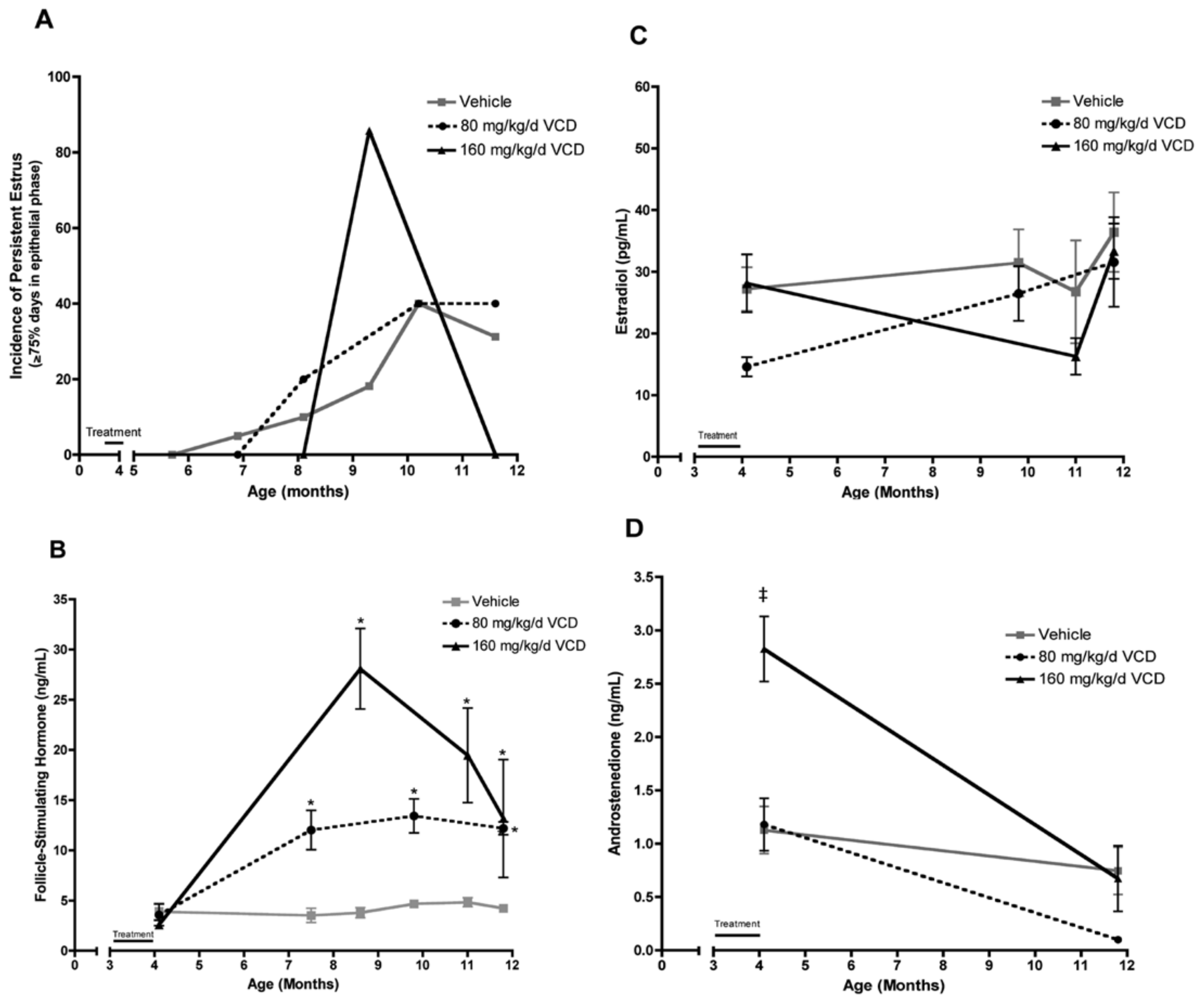

Figure 4. Effects of VCD on reproductive function and hormones when administered to 3-mo-old Sprague–Dawley rats. (A) Cyclicity in adult rats treated with vehicle (n = 3 to 17), low-dose VCD (n = 5), or high-dose VCD (n = 7 to 10), expressed as incidence of persistent estrus. (B) Serum FSH levels in rats treated as adults with vehicle (n = 5 to 11), low-dose VCD (n = 5), or high-dose VCD (n = 5 to 8). In control rats, FSH levels did not change over time (compared with 4.1 mo), as assessed by ANOVA. *, $P < 0.05$ compared with vehicle. (C) Serum 17β-estradiol (E_2) levels in rats treated as adults with vehicle (n = 5 to 15), low dose VCD (n = 5), or high dose VCD (n = 3 to 10). No significant differences were observed between treatment groups, nor were significant differences detected within any treatment group as compared with initial values at 4.1 mo. (D) Serum androstenedione levels in rats treated as adults with vehicle (n = 5 to 17), low-dose VCD (n = 5), or high-dose VCD (n = 7 to 10). In control rats, androstenedione levels did not change over time (compared with levels at 4.1 mo), as assessed by Student t test. Within each VCD treatment group, androstenedione levels declined at the later time point (12 mo, $P < 0.01$) but were no different than those in age-matched controls. At 4.1 mo, androstenedione levels in high-dose VCD rats were increased as compared with age-matched controls. ‡, $P < 0.001$.

sition; that is, a phase of persistently elevated FSH occurring in estrogen- and androgen-replete women who are not yet oocyte-deplete.[1] Therefore, VCD acceleration of follicular depletion in Sprague–Dawley rats appears to be a uniquely appropriate experimental model of human perimenopause, a critical phase in a women's reproductive life for which, to our knowledge, no other rodent model exists. VCD effects in Sprague–Dawley rats can be contrasted to those previously documented in mice.[22,23,27,46] Within 2 mo of initiating VCD treatment, mice are in persistent diestrus and are estrogen-deplete but with continued androstenedione production, thus modeling a rapid transition to a state analogous to natural (compared with surgical) human menopause.[3] The prolonged estrogen-replete phase of persistent estrus normally occurring in Sprague–Dawley rats, but not mice,[45] is undoubtedly a critical factor accounting for the contrasting ability of VCD to modulate the onset and magnitude of prolonged periods of increased FSH in estrogen-replete Sprague–Dawley rats, thus modeling perimenopause rather than natural menopause. Fur-

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thermore, the increase in FSH that occurs with VCD acceleration of persistent estrus is unique as compared with other experimental means of inducing persistent estrus in rats, such as exposure to constant light, where the FSH level remains unchanged.[41]

Acute depletion of over 90% of ovarian follicles in both peripubertal and adult rats with high-dose VCD led to early increases in FSH and an early onset of persistent estrus. In contrast, acute depletion of only 70% to 80% of ovarian follicles by low-dose VCD, although it similarly caused early and marked increases in FSH, had little (if any) effect on the onset of persistent estrus. VCD-induced changes in FSH were dose-dependent in Sprague–Dawley rats of both ages, in that the higher VCD dose led to FSH increases that were greater in magnitude as compared with those after low-dose VCD. The magnitude of the VCD-induced FSH increases was greater in juvenile Sprague–Dawley rats than in adults, suggesting that VCD administration to 1-mo-old Sprague–Dawley rats, which caused no signs of toxicity, may be a particularly robust way to explore dose-dependent effects of FSH on disease risks during the transition to ovarian failure. Indeed, we recently used VCD treatment of 1-mo-old rats to model the hormonal milieu of perimenopause to examine correlations between bone loss and nonestrogenic hormones.[24]

The source of continued normal levels of estradiol in control and VCD-treated rats throughout the 1- to 2-y study duration was not examined in these current experiments; however, abnormal structures that remained in the ovaries of follicle-deplete VCD-treated rats may have contributed to continued estradiol secretion.[8] Indeed, during persistent estrus in normal Sprague–Dawley rats, estradiol is produced by remaining follicles that are devoid of oocytes but retain steroidogenic granulosa cells.[33] In addition, consistent with older reports,[45] normal Sprague–Dawley rats at the end of persistent estrus were not uniformly in diestrus, and estradiol levels did not plummet.[33] Therefore, even with aging, normal or VCD-treated rats cannot be used to model the hormonal milieu of menopause.

The current studies also clearly revealed practical limitations to the use of VCD in Sprague–Dawley rats to model perimenopause. Most notable are the acute toxic effects of high-dose (160 mg/kg daily for 25 doses) VCD when administered to 3-mo-old Sprague–Dawley rats, a regimen that resulted in significant weight loss and 24% mortality, with lymphopenia and hepatotoxicity documented in survivors. Clearly, the acute toxicity of VCD in adult Sprague–Dawley rats, which replicates other recent findings after examination of the acute ovarian effects of VCD in Sprague–Dawley rats,[29] limit the dosing range for VCD when used to model perimenopause in mature rats. Indeed, evidence of adverse effects of VCD in adults, including death, led to our decision to terminate data collection in these animals at 12 mo of age (compared with 20 mo of age in juvenile rats) after comparative effects on FSH and cyclicity had been determined. In contrast, but consistent with prior studies examining ovarian effects of VCD in peripubertal Fischer 344 rats,[15,13] this same high-dose of VCD lacked toxicity in young Sprague–Dawley rats. The reason for this apparent age-related toxicity cannot be determined from the current experiments. However, in addition to possible effects of developmental stage on toxicity (that is, increased vulnerability of mature immune system and liver to VCD toxicity), the larger absolute amount of VCD administered to adult rats, given that dose was based on body weight, might also have been a contributing factor. Alternative dosing strategies (that, is fewer total days of a high or intermediate VCD dose) may extend the dosing range of VCD in adult Sprague–Dawley rats to examine, for example, concentration-dependent effects of increased FSH on a disease or disease risk in more developmentally mature animals.

Because, as demonstrated here, VCD can be used to manipulate the age at onset of hormonal changes analogous to human perimenopause, this model could be used to examine the relative roles of aging itself compared with hormonal shifts in contributing to disease risks associated with the menopausal transition. Similarly, the perimenopausal hormone profile induced by VCD in Sprague–Dawley rats might serve as a useful model for probing key clinical questions such as the relative risk of cardiovascular disease associated with hormone replacement therapy in the perimenopausal compared with late postmenopausal periods, a dilemma raised by results of the Women Health Initiative.[39] The potential use of this rat model to explore cardiovascular disease risk is of particular interest in light of emerging evidence that nonsteroidal ovarian hormones (for example, activins) may play a role in atherosclerosis.[20,32] Similarly, a recent intriguing report of specific expression of functional FSH receptors in the neovasculature of human breast carcinomas[37] points to the potential experimental importance of an estrogen-replete model of elevated FSH (that is, perimenopause) as we continue to explore the role of reproductive hormones in the pathogenesis of diseases that peak in incidence during the menopausal transition. Lastly, because VCD dosing can be used to modulate the magnitude of elevations in key reproductive hormones, such as FSH, dose-dependent effects of these hormones can now be investigated in animals whose hypothalamic–pituitary–ovarian axis remains essentially intact. Therefore, the VCD model in Sprague–Dawley rats appears to provide a unique tool, when used alone or in comparison to the well-studied ovariectomized Sprague–Dawley rat model, to extend our knowledge of the interplay of hormones, aging, and disease risk in women across the menopausal transition.

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References

1. **Al-Azzawi F, Palacios S.** 2009. Hormonal changes during menopause. Maturitas **63:**135–137.
2. **Borman SM, Christian PJ, Sipes IG, Hoyer PB.** 2000. Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to 3 polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. Toxicol Appl Pharmacol **167:**191–198.
3. **Burger H.** 2008. The menopausal transition—endocrinology. J Sex Med **5:**2266–2273.
4. **Couillard S, Gutman M, Labrie C, Bélanger A, Candas B, Labrie F.** 1998. Comparison of the effects of the antiestrogens EM800 and tamoxifen on the growth of human breast ZR-75-1 cancer xenografts in nude mice. Cancer Res **58:**60–64.
5. **Danilovich N, Ram Sairam M.** 2006. Recent female mouse models displaying advanced reproductive aging. Exp Gerontol **41:**117–122.
6. **Dasgupta A, Rehman HU.** 2006. Neuroendocrinology of menopause. Minerva Ginecol **58:**25–33.
7. **Devine PJ, Hoyer PB, Keating AF.** 2009. Current methods in investigating the development of the female reproductive system. Methods Mol Biol **550:**137–157.

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-25

8. **Doerr JK, Hooser SB, Smith BJ, Sipes IG.** 1995. Ovarian toxicity of 4-vinylcyclohexene and related olefins in $B6C3F_1$ mice: role of diepoxides. Chem Res Toxicol **8:**963–969.
9. **Fernandez SM, Keating AF, Christian PJ, Sen N, Hoying JB, Brooks HL, Hoyer PB.** 2008. Involvement of the KIT–KITL signaling pathway in 4-vinylcyclohexene diepoxide-induced ovarian follicle loss in rats. Biol Reprod **79:**318–327.
10. **Funk JL, Frye JB, Oyarzo JN, Kuscuoglu N, Wilson J, McCaffrey G, Stafford G, Chen G, Lantz RC, Jolad SD, Sólyom AM, Kiela PR, Timmermann BN.** 2006. Efficacy and mechanism of action of turmeric supplements in the treatment of experimental arthritis. Arthritis Rheum **54:**3452–3464.
11. **Goldman JM, Murr AS, Cooper RL.** 2007. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol **80:**84–97.
12. **Hamilton KL, Lin L, Wang Y, Knowlton AA.** 2008. Effect of ovariectomy on cardiac gene expression: inflammation and changes in SOCS gene expression. Physiol Genomics **32:**254–263.
13. **Hariri LP, Liebmann ER, Marion SL, Hoyer PB, Davis JR, Brewer MA, Barton JK.** 2010. Simultaneous optical coherence tomography and laser induced fluorescence imaging in rat model of ovarian carcinogenesis. Cancer Biol Ther **10:**438–447.
14. **Hoyer PB, Devine PJ, Hu X, Thompson KE, Sipes IG.** 2001. Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model. Toxicol Pathol **29:**91–99.
15. **Hoyer PB, Sipes IG.** 2007. Development of an animal model for ovotoxicity using 4-vinylcyclohexene: a case study. Birth Defects Res B Dev Reprod Toxicol **80:**113–125.
16. **Huang HH, Meites J.** 1975. Reproductive capacity of aging female rats. Neuroendocrinology **17:**289–295.
17. **Janssen I, Powell LH, Crawford S, Lasley B, Sutton-Tyrrell K.** 2008. Menopause and the metabolic syndrome: the Study of Women's Health Across the Nation. Arch Intern Med **168:**1568–1575.
18. **Kalu DN.** 1991. The ovariectomized rat model of postmenopausal bone loss. Bone Miner **15:**175–191.
19. **Kao SW, Sipes IG, Hoyer PB.** 1999. Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. Reprod Toxicol **13:**67–75.
20. **Kozaki K, Ouchi Y.** 1998. Activin–follistatin and atherosclerosis—a review. J Atheroscler Thromb **5:**36–40.
21. **LeFevre J, McClintock MK.** 1988. Reproductive senescence in female rats: a longitudinal study of 490 individual differences in estrous cycles and behavior. Biol Reprod **38:**780–789.
22. **Lohff JC, Christian PJ, Marion SL, Arrandale A, Hoyer PB.** 2005. Characterization of cyclicity and hormonal profile with impending ovarian failure in a novel chemical-induced mouse model of perimenopause. Comp Med **55:**523–527.
23. **Lohff JC, Christian PJ, Marion SL, Hoyer PB.** 2006. Effect of duration of dosing on onset of ovarian failure in a chemical-induced mouse model of perimenopause. Menopause **13:**482–488.
24. **Lukefahr AL, Frye JB, Wright LE, Marion SL, Hoyer PB, Funk JL**. 2012. Decreased bone mineral density in rats rendered follicle-deplete by an ovotoxic chemical correlates with changes in follicle-stimulating hormone and inhibin A. Calcif Tissue Int **90:**239–349.
25. **Mastorakos G, Valsamakis G, Paltoglou G, Creatsas G.** 2010. Management of obesity in menopause: diet, exercise, pharmacotherapy, and bariatric surgery. Maturitas **65:**219–224.
26. **Matthews KA, Crawford SL, Chae CU, Everson-Rose SA, Sowers MF, Sternfeld B, Sutton-Tyrrell K.** 2009. Are changes in cardiovascular disease risk factors in midlife women due to chronological aging or to the menopausal transition? J Am Coll Cardiol **54:**2366–2373.
27. **Mayer LP, Devine PJ, Dyer CA, Hoyer PB.** 2004. The follicle-deplete mouse ovary produces androgen. Biol Reprod **71:**130–138.
28. **Mayer LP, Pearsall NA, Christian PJ, Devine PJ, Payne CM, McCuskey MK, Marion SL, Sipes IG, Hoyer PB.** 2002. Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. Reprod Toxicol **16:**775–781.
29. **Muhammad FS, Goode AK, Kock ND, Arifin EA, Cline JM, Adams MR, Hoyer PB, Christian PJ, Isom S, Kaplan JR, Appt SE.** 2009. Effects of 4-vinylcyclohexene diepoxide on peripubertal and adult Sprague–Dawley rats: ovarian, clinical, and pathologic outcomes. Comp Med **59:**46–59.
30. **National Cancer Institute.** [Internet]. DCCPS, Surveillance Research Program, Cancer Statistics Branch, Surveillance, Epidemiology, and End Results (SEER) Program. SEER*Stat Database: 2004-2008. [Cited 09 March 2012]. Available at: http://www.seer.cancer.gov/statfacts/html/breast.html.
31. **Neer RM, SWAN Investigators.** 2010. Bone loss across the menopausal transition. Ann N Y Acad Sci **1192:**66–71.
32. **Oklu R, Hesketh R, Wicky S, Metcalfe J.** 2011. TGFβ–activin signaling pathway activation in intimal hyperplasia and atherosclerosis. Diagn Interv Radiol **17:**290–296. .
33. **Peluso JJ, Steger RW, Huang H, Meites J.** 1979. Pattern of follicular growth and steroidogenesis in the ovary of aging cycling rats. Exp Aging Res **5:**319–333.
34. **Perrien DS, Achenbach SJ, Bledsoe SE, Walser B, Suva LJ, Khosla S, Gaddy D.** 2006. Bone turnover across the menopause transition: correlations with inhibins and follicle-stimulating hormone. J Clin Endocrinol Metab **91:**1848–1854.
35. **Pietschmann P, Rauner M, Sipos W, Kerschan-Schindl K.** 2009. Osteoporosis: an age-related and gender-specific disease—a mini-review. Gerontology **55:**3–12.
36. **Prior JC, Hitchcock CL.** 2011. The endocrinology of perimenopause: need for a paradigm shift. Front Biosci (Schol Ed) **3:**474–486.
37. **Radu A, Pichon C, Camparo P, Antoine M, Allory Y, Couvelard A, Fromont G, Hai MTV, Ghinea N.** 2010. Expression of follicle-stimulating hormone receptor in tumor blood vessels. N Engl J Med **363:**1621–1630.
38. **Riggs BL, Melton LJ, Robb RA, Camp JJ, Atkinson EJ, McDaniel L, Amin S, Rouleau PA, Khosla S.** 2008. A population-based assessment of rates of bone loss at multiple skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. J Bone Miner Res **23:**205–214.
39. **Rossouw JE.** 2006. Implications of recent clinical trials of postmenopausal hormone therapy for management of cardiovascular disease. Ann N Y Acad Sci **1089:**444–453.
40. **Salehi F, Dunfield L, Phillips KP, Krewski D, Vanderhyden BC.** 2008. Risk factors for ovarian cancer: an overview with emphasis on hormonal factors. J Toxicol Environ Health B Crit Rev **11:**301–321.
41. **Singh KB.** 2005. Persistent estrus rat models of polycystic ovary disease: an update. Fertil Steril **84:**1228–1234.
42. **Singh M, Meyer EM, Simpkins JW.** 1995. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague–Dawley rats. Endocrinology **136:**2320–2324.
43. **Thompson KE, Sipes IG, Greenstein BD, Hoyer PB.** 2002. 17β-Estradiol affords protection against 4-vinylcyclohexene diepoxide-induced ovarian follicle loss in Fischer 344 rats. Endocrinology **143:**1058–1065.
44. **Vitale C, Miceli M, Rosano GM.** 2007. Gender-specific characteristics of atherosclerosis in menopausal women: risk factors, clinical course, and strategies for prevention. Climacteric **10 Suppl 2:**16–20.
45. **Vom Saal FS, Finch CE, Nelson JF.** 1994. Natural history and mechanisms of reproductive aging in humans, laboratory rodents, and other selected vertebrates, p 1213–1314. In: Knobil K, Neill JD, editors. The physiology of reproduction, 2nd ed. New York (NY): Raven Press.
46. **Wright LE, Christian PJ, Rivera Z, Van Alstine WG, Funk JL, Bouxsein ML, Hoyer PB.** 2008. Comparison of skeletal effects of ovariectomy versus chemically induced ovarian failure in mice. J Bone Miner Res **23:**1296–1303.
47. **Wu JM, Zelinski MB, Ingram DK, Ottinger MA.** 2005. Ovarian aging and menopause: current theories, hypotheses, and research models. Exp Biol Med (Maywood) **230:**818–828.

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