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Experimental Induction of Reduced Ovarian Reserve in a Nonhuman Primate Model (*Macaca fascicularis*)

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Chronic diseases including coronary heart disease and osteoporosis represent a substantial health burden to postmenopausal women, yet the initiation of these conditions and their relationships with reproductive aging remain poorly understood. This situation is due, in part, to the lack of animal models reflecting ovarian and hormonal characteristics of peri- and postmenopausal women. Ovaries of women approaching menopause are nearly depleted of primordial follicles but retain a pool of larger developing follicles and androgen-producing stroma, a condition known as reduced ovarian reserve (ROR). The long-term goal of the research presented here was to create a monkey model of reproductive aging, beginning with ROR and progressing to perimenopause and finally postmenopause. Here we sought to develop a method to reduce primordial follicles in cynomolgus monkeys (*Macaca fascicularis*) and document hormonal changes associated with follicle reduction or ROR. At 30 d after surgical placement of a biodegradable fiber containing approximately 200 mg of 4-vinlycyclohexene diepoxide (VCD) next to one ovary in each of 8 monkeys, primordial follicles were reduced by approximately 70%, with a corresponding decrease (83%) in antimüllerian hormone (AMH, a serum marker of ovarian follicle numbers). At 4 mo after VCD-treatment of both ovaries in 29 monkeys (approximately 200 mg VCD per ovary), AMH was reduced 56% from baseline, testosterone was unchanged, and follicular phase estradiol was slightly increased. These data indicate that VCD treatment markedly reduced primordial follicles while preserving larger estradiol- and testosterone-producing follicles and ovarian stroma, a condition that mimics ROR in women.

Abbreviations: AMH, antimüllerian hormone; ROR, reduced ovarian reserve; OVX, ovariectomy or ovariectomized; VCD, 4-vinylcyclohexene diepoxide.

The numbers of postmenopausal women are increasing worldwide (more than 1.1 billion are expected by 2025), and those women are surviving longer than their predecessors. For example, the average life expectancy of women in developed countries is projected to increase from 79 to 82 y.[35] Chronic conditions such as coronary heart disease, osteoporosis, metabolic syndrome, and cognitive decline comprise a substantial part of the health burden of postmenopausal women. Despite considerable epidemiologic and clinical research, the initiation and trajectory of these conditions, as well as their relationship with reproductive aging, remain poorly understood. For example, whether perimenopause (a several-year period of hormone dysregulation leading to the final menstrual period [or menopause]) is a time of accelerated disease progression is unknown. Similarly, intriguing evidence suggests that pathobiologic changes that have accumulated by the time of menopause could establish the trajectory of postmenopausal disease outcomes, irrespective of postmenopausal intervention.[9,27]

The lack of models that reflect the ovarian and hormonal characteristics of peri- and postmenopausal women has impeded translational research investigating the effect of reproductive aging on risk for chronic disease. Although ovariectomized (OVX) animals have provided valuable insights with respect to the consequences of estrogen depletion,[1,14,15,26,27] such models do not provide the opportunity to assess the pathophysiologic changes that coincide with declining follicle numbers (reduced ovarian reserve, ROR) characteristic of late-reproductive-aged women,[36] the persistent hormonal disruption characteristic of the perimenopausal transition,[32] or the continued production of hormones (androgens) by the follicle-depleted, stroma-intact postmenopausal ovary.[7,8]

Accumulating data have made the need for a nonhuman primate model of reproductive aging even more urgent. For example, premenopausal women younger than 41 with ROR (a reproductive stage that occurs prior to the onset of clinical signs associated with the perimenopausal transition) were almost twice as likely to have low bone mineral density, compared with their counterparts with normal ovarian reserve.[36] In addition, significant associations were present between markers of ovarian reserve (hormones and ovarian size) and markers of increased bone turnover. Taken together, these data indicate that young women with ROR may be on a trajectory for bone loss similar to that seen in women at more advanced stages of reproductive aging. Fur-

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thermore, adverse lipid profiles in normally cycling premenopausal women with ROR have been reported,[36] suggesting that adverse changes in plasma lipids may occur along a continuum of ovarian decline. Therefore, the identification of women with markers of increased cardiovascular risk at the onset of ovarian decline may provide a rational basis for interventions that could prevent cardiovascular disease in the postmenopausal years. A monkey model of ROR could confirm the degree of bone loss and increased cardiovascular risks associated with this condition and allow interventions to be tested.

The long-term goal of our research is to create a monkey model of reproductive aging (beginning with ROR and progressing to perimenopause and finally postmenopause). Here we describe 2 experiments using nonhuman primates to model the first stage of reproductive aging in women, ROR. To mimic the experience of women, the ROR monkey model should be characterized by ovaries that are nearly depleted of primordial follicles but retain a pool of larger, developing follicles and an intact, androgen-producing stroma. The chemical 4-vinylcyclohexene diepoxide (VCD) has been shown to selectively destroy primordial and primary follicles in rodents while leaving the stroma intact.[19-24,44,45] Our research has focused on translating this rodent model to nonhuman primates (macaques),[2,6] which resemble women physiologically (similar menstrual and reproductive hormone patterns) and develop many of the same chronic diseases (for example, cardiovascular disease and osteoporosis) when fed diets similar to those typically consumed by American women.[25,30,31,39,52-56]

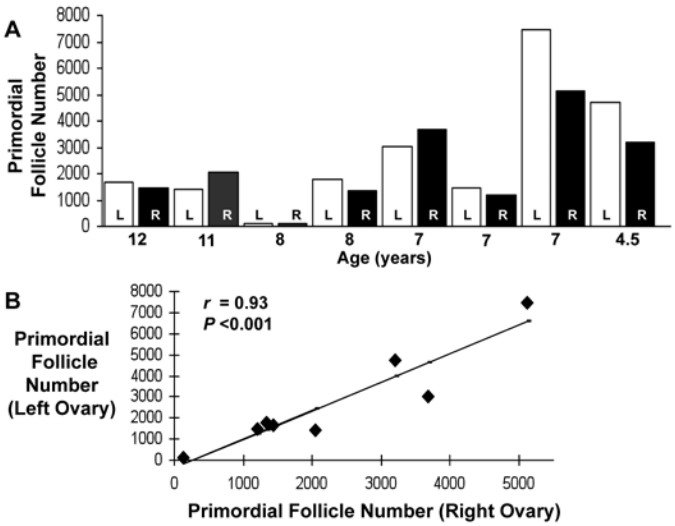

Figure 1. (A) Interindividual variation in ovarian primordial follicle numbers between adult cynomolgus monkeys (age, 4.5 to 12 y) and between left (L, open bars) and right (R, closed bars) ovaries of each monkey (intraindividual variation). (B) Correlation of primordial follicle counts between left and right ovaries in individual monkeys. Data presented in this figure were collected from untreated monkeys in which ovaries were collected at baseline from studies unrelated to experiments 1 and 2. Data are presented as the number of follicles counted per ovary after serial sectioning and counting of every 50th section.[3]

Materials and Methods

Experiment 1. ***Study design.*** The objective of the first experiment was to determine the acute effects of VCD on follicle number and antimüllerian hormone (AMH) concentration. Female cynomolgus macaques (*Macaca fascicularis*; *n* = 8; Covance Primates, Alice, TX, and Institut Pertanian, Bogor, Indonesia) with a mean age of 9 y (range, 6 to 18 y) and a mean weight of 3.4 kg were used for this experiment. At baseline, an ovary was removed to serve as the untreated control and then a poly-L-lactic-co-glycolic acid fiber containing 200 mg VCD was placed around the remaining ovary. After 30 d, a second laparotomy was done to remove the remaining ovary and VCD fiber, and follicles were counted in both ovaries. Serum AMH concentration was measured at baseline and 14 and 30 d after placement of the VCD-containing fiber. Justification for using one ovary as the untreated control stems from data collected in previous studies[3] unrelated to the present study. Those studies demonstrated that there was large interindividual variation in primordial follicle numbers between cynomolgus monkeys, even at similar ages (Figure 1 A)[3], and therefore large numbers of monkeys would be required to detect an effect of VCD on follicle number. However, data from those same preliminary studies indicated that the intraindividual variation in primordial follicle counts between left and right ovaries is small and that the correlation between ovaries is high (r = 0.93, $P < 0.001$; Figure 1 B)[3], thus allowing one ovary to serve as the untreated comparator.

VCD fibers. Two of the authors (JTP and NKV) impregnated a poly-L-lactic-co-glycolic acid (50:50) biodegradable fiber with VCD (99% purity, Pfaltz and Bauer, Waterbury, CT). Each fiber contained 20% to 25% VCD by weight (approximately 200 mg VCD). To determine the amount of VCD released over time from the fiber, 1-mm sections of VCD-containing fibers (approximately 10 mg each) were placed in separate vials of PBS at 37 °C in a shaker bath. The amount of VCD remaining at 0.5, 1, 2, 4, and 24 h and 3, 4 , 7, 14, and 21 d was determined. At each time point, one of the 10-mg samples was collected, and the PBS was replaced with 2 mL dichloromethane. The samples were extracted for 2 h at room temperature, after which the dichloromethane was transferred to autosampler vials and analyzed by gas chromatography (GC 8000, Fisons Instruments, Milan, Italy)–flame ionization detection (Carlo Erba, Fisons Instruments) with a capillary column of 30 m × 0.25 (0.25-µm film thickness; RTX-624, Restek, Bellefonte, PA). A 10.103 mg/mL working standard was prepared by weighing 252.57 mg VCD and adding dichloromethane to a final volume of 25 mL. Serial dilutions were made from the working solution to prepare a 6-point calibration curve (0.10, 0.51, 1.01, 2.53, 5.05, and 10.103 mg/mL) to determine the amount of VCD remaining in the fiber. Linearity was demonstrated.

Surgery. Monkeys were premedicated with atropine (0.03 mg/kg IM) 10 min prior to sedation with ketamine HCl (15 mg/kg IM). Ketoprofen analgesic (5 mg/kg IM) was given, and animals were prepared aseptically for a midline abdominal incision. Monkeys were intubated and anesthetized with isoflurane gas, and intraoperative respiratory rate (15 to 45/min), heart rate (100 to 220 beats/min), PO_2 (> 95%), and body temperature (> 97°F) were recorded every 10 min. A recirculating water blanket (Bair Hugger, Arizant, Eden Prairie, MN) was used to maintain core body temperature during surgery and recovery. A standard midline laparotomy was done, and either the right or the left ovary (randomly assigned) was identified and removed after ligatures were placed on both the ovarian and uterine vessels. A VCD-impregnated fiber (containing approximately 200 mg VCD in the fiber, which was coiled such that its dimensions were approximately 2 cm × 2 cm) was placed around the remaining ovary and held in

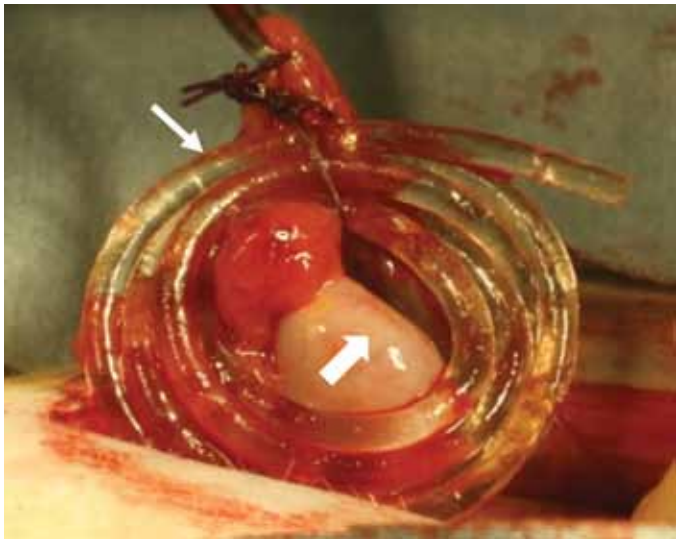

Figure 2. Placement of poly-L-lactic-co-glycolic acid (VCD-releasing) fiber (small arrow) around the ovary (large arrow) during laparotomy.

place with absorbable suture attached to the ovarian pedicle fat (Figure 2). The remaining ovary and the VCD-containing fiber were removed during a second laparotomy, 30 d after placement of the fiber. After both laparotomy surgeries, macaques received a subcutaneous injection of lactated Ringers solution (100 to 200 mL) and were monitored postoperatively according to approved guidelines. Pain medication (ketoprofen, 5 mg/kg IM) was given once daily for 3 d. Monkeys generally were climbing in their cages within 30 to 45 min after surgery. An experienced veterinary surgeon (SEA) conducted all surgical procedures and supervised the postsurgical recovery. In addition, complete postoperative records were kept and reviewed by the veterinary staff.

Ovarian follicle number and histopathology. After removal, both the baseline and VCD-treated ovaries were trimmed of fat, fixed in Bouin solution (75 mL picric acid solution [1.3%], 25 mL formaldehyde [37%], and 5 mL glacial acetic acid) for 24 h, and transferred to 70% ethanol. Both the baseline and treated ovaries were transferred to the University of Arizona for follicle counting (PBH, NS). The ovaries were embedded in paraffin, processed routinely for histology and sectioned serially (every 4 to 5 µm). Every 50th section was saved, placed in order of sectioning on glass slides, and stained with hematoxylin and eosin. The total number of sections per ovary varied from 8 to 14, depending on individual differences in ovarian size and the presence or absence of corpora lutea. As published previously,[6] follicles were classified and counted in every 100th section, and only follicles with an oocyte nucleus were counted. Data are reported as total follicles counted per ovary (that is, the sum of all follicles in each section counted per ovary). Follicles were classified as primordial (oocytes surrounded by a single layer of flattened granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells), and secondary (oocytes surrounded by 2 or more layers of cuboidal granulosa cells).[38] A previous comparison between counting follicles in every 100th section compared with every 50th section[6] demonstrated that the rank order of monkeys on the basis of their primordial follicle count did not differ whether every 100th section or twice that number (every 50th section) was counted. In addition, the relative effect of treatment with VCD was identical whether follicles were counted every 50th or 100th section. The same relationships were true for primary and secondary follicles. A board-certified veterinary pathologist (NDK) systematically evaluated all control and VCD-treated ovaries for signs of adverse effects of VCD (other than follicle destruction) on the ovaries.

AMH measurement. AMH determinations were done at baseline, 2 wk after VCD treatment, and at removal of the VCD-treated ovary (approximately 30 d). Blood (serum) for AMH measurement was collected between 0800 and 1300 h from overnight-fasted macaques. Blood samples were stored at –70ºC until assayed. AMH was assayed by using ELISA (Diagnostics Systems Laboratories, Webster, TX). Consistent with previous findings that human AMH ELISA recognizes cynomolgus macaque AMH,[29] we encountered no problems of cross reactivity in using a human-reagent ELISA. The intra-assay coefficient of variation was 4.53% at 13.62 ng/mL; inter-assay coefficients of variation were 19.75% at 0.23 ng/mL, 11.42% at 9.61 ng/mL, and 11.27% at 1.89 ng/mL. Due to sampling error, serum AMH was not measured in 2 monkeys.

Experiment 2. *Study design.* Experiment 2 is part of a larger, ongoing investigation of the role of ROR in the pathogenesis of chronic diseases including atherosclerosis, bone loss, and metabolic syndrome. The data presented herein represent the first 4 mo of the larger, ongoing multiyear study. The objective of the data presented here was to demonstrate the early effects of bilateral ovarian treatment with VCD on AMH, estradiol, and testosterone.

Female cynomolgus macaques (mean age, 15 y [range, 7 to 21 y]; mean weight, 2.88 kg; Institut Pertanian Bogor, West Java, Indonesia) were used for this experiment. Monkeys in the VCD treatment group (n = 29) underwent a single laparotomy (as described for experiment 1) to place VCD fibers around both ovaries (200 mg VCD per ovary). These fibers were left in place, and the ovaries were not removed. Both ovaries were removed in an additional 20 monkeys so that they could serve as OVX comparators, and 20 monkeys were anesthetized but did not undergo laparotomy (premenopausal controls). All pre- and postsurgical care was done as described for experiment 1.

Hormone concentrations. AMH and testosterone determinations were done at baseline and approximately 4 mo after treatment. Follicular-phase (cycle day 1 to 5) estradiol was measured once before treatment and at 3 mo thereafter. Because the ovaries were not removed during this experiment, AMH concentrations were used as a marker of follicle count. AMH is highly correlated with primordial, primary, and secondary follicles ($r \geq 0.66$, $P < 0.001$ for all follicle types) in cynomolgus monkeys (n = 29),[4] and this correlation has been observed in groups as small as 12 monkeys (primordial: $r = 0.66$, $P = 0.01$; secondary: $r = 0.59$, $P = 0.04$)[3] AMH was collected and analyzed as described experiment 1. Estradiol was measured from serum by using a modification[37] of a commercially available radioimmunoassay from Diagnostic Products Corporation (Los Angeles, CA). Intra-assay and inter-assay coefficients of variation were less than 4% and 10%, respectively. Testosterone was measured from serum by using a commercially available kit (Diagnostics Systems Laboratories). The inter-assay coefficients of variation for testosterone were 5.95% at 0.68 ng/mL and 4.14% at 5.67 ng/mL.

Hematology and clinical chemistry measures. Hematology and clinical chemistry measures were done at baseline and 2 wk after

VCD fiber implantation. Between 0800 and 1200 h, overnight-fasted monkeys were sedated with ketamine (15 mg/kg IM), and blood samples were collected from the femoral vein into EDTA-containing tubes for CBC and serum-separator tubes for blood chemistry. Hematology measures (Hemavet model 950, Drew Scientific, Oxford, CT) included WBC count, platelet count, hemoglobin concentration, RBC count, hematocrit, and segmented neutrophil and lymphocyte counts. Blood chemistry measures (DxC 800 Clinical Chemistry System, Beckman Coulter, Brea, CA) included quantitation of electrolytes (Na^+, K^+, Cl^-), glucose, Ca^{2+}, albumin, total protein, blood urea nitrogen, creatinine, total bilirubin, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase.

Diet and housing. Monkeys in experiment 1 were fed a semipurified diet (Wake Forest University Primate Center Diet Laboratory) that was formulated to contain 0.02 mg cholesterol per calorie and to derive 20% of its energy from protein (casein and lactalbumin), approximately 28% from fat, and approximately 52% from carbohydrates. The monkeys were fed 120 kcal of diet per kilogram of body weight daily. Because experiment 2 was part of a larger, ongoing investigation of the effects of hormonal changes on atherosclerosis progression, these monkeys were fed a diet higher in cholesterol (0.20 mg cholesterol per calorie) and fat (approximately 35%) than were those in experiment 1, but the diets were similar with respect to protein (19%, from casein and lactalbumin for the diet used in experiment 2), carbohydrates (approximately 47% in experiment 2), and amount fed daily (120 kcal diet per kilogram body weight in both experiments).

For both experiments, monkeys were housed indoors under conditions of controlled temperature (68 to 84 ºF [20.0 to 28.9 °C]) and humidity (greater than 30%) in social groups of 2 to 4 monkeys per pen. Each pen is equipped with perches and at least the minimum amount of floor space required by federal rules, regulations, and guidelines. All monkeys are monitored daily by veterinary staff for potential health problems, and blood is collected yearly for complete blood counts and serum chemistry analysis, which are evaluated by a staff veterinarian. All monkeys in both experiments were clinically healthy. All animal procedures were done in accordance with federal, state, and institutional guidelines, and all studies were approved by the Institutional Animal Care and Use Committee of Wake Forest University. Wake Forest University is AAALAC-accredited.

Data analysis. All data were analyzed by using a statistical software package (JMP7, SAS Institute, Cary, NC); significance was defined as a *P* value of less than 0.05.

Experiment 1. Because primordial and primary follicle counts and AMH concentration data were not normally distributed and because neither log nor square-root transformation improved normality, a nonparametric Wilcoxon test was used to determine significance of treatment effect. Medians are reported for these variables. Paired Student *t* tests were used to determine treatment effect on secondary follicles, and data are reported as mean ± SE.

Experiment 2. AMH and testosterone concentration data were not normally distributed and were not improved significantly with transformation; therefore, a nonparametric Kruskal–Wallis test was used to determine differences among groups (OVX, VCD, premenopause) at baseline and 4 mo after treatment. A Wilcoxon test was used to determine whether AMH in VCD-treated monkeys was significantly reduced from baseline. One-way ANOVA was used to determine differences in estradiol among the 3 groups at baseline and after treatment. For health measures, we compared baseline with posttreatment data for each treatment condition (OVX, VCD, premenopause). Paired *t* tests were used to determine treatment effects for variables that were normally distributed or log transformed (aspartate transaminase and alanine transaminase), and a nonparametric Wilcoxon test was used for those variables that were not normally distributed (creatinine, glucose, albumin, Na^+, Cl^-, K^+, alkaline phosphatase).

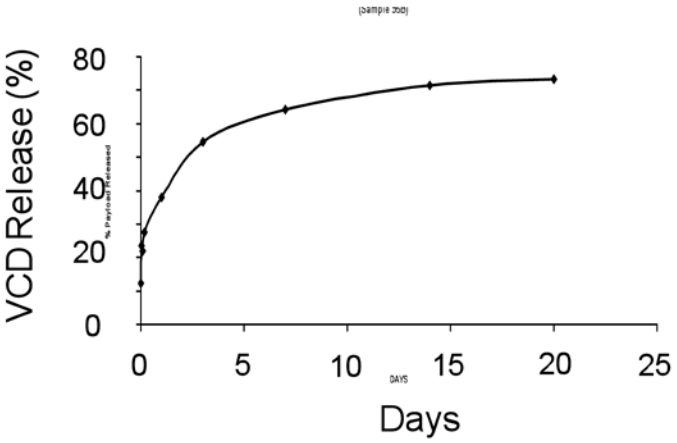

Figure 3. Percentage release of 4-vinylcyclohexene diepoxide (VCD) from poly-L-lactic-co-glycolic acid fiber over time (in vitro).

Results

Experiment 1. ***VCD release over time*** In vitro data indicated that VCD was released slowly from the fiber over approximately 21 d (Figure 3). An initial burst release of 12% was observed at 30 min and approximately 38% of the VCD was released at 24 h, observed followed by a slower, controlled release, which was monitored for 21 d.

Ovarian follicle counts and histopathology. VCD treatment for 30 d reduced primordial follicle number in cynomolgus macaques by approximately 70% (baseline median, 924; post-VCD median, 269; $P = 0.008$), primary follicles by approximately 45% (baseline median, 29; post-VCD median, 16; $P = 0.04$), and secondary follicles by 46% (baseline mean [± SE], 26 ± 3.7; post-VCD mean, 14 ± 3.7; $P = 0.02$. Importantly, despite the marked effect of VCD on follicle number, neither gross nor histologic examination revealed any adverse effects of VCD on the ovary (Figure 4). A mild inflammatory reaction was present in the tissues adjacent to the ovary (ovarian and mesenteric fat) and characterized by lymphocytes and multinucleated giant cells infiltrated with occasional crystalline material presumed to be remnant fiber or suture. These changes were typical of inflammatory responses that occur in the presence of a foreign body (that is, a VCD fiber); however, no inflammatory response was observed in the ovary proper (capsule, stroma).

AMH. Serum AMH concentration declined significantly (83%; $P = 0.004$) within 14 d of VCD treatment (5.9 ± 1.0 ng/mL to 1.0 ± 0.2 ng/mL; Figure 5). At 30 d after VCD treatment, AMH was still below baseline (1.25 ± 0.98 ng/mL, $P = 0.01$). Because AMH is produced only by the ovaries, the removal of one ovary was expected to result in a 50% reduction in AMH. Given that the reduction in AMH was 83%, the additional reduction (approximately 33%) likely was due to VCD treatment.

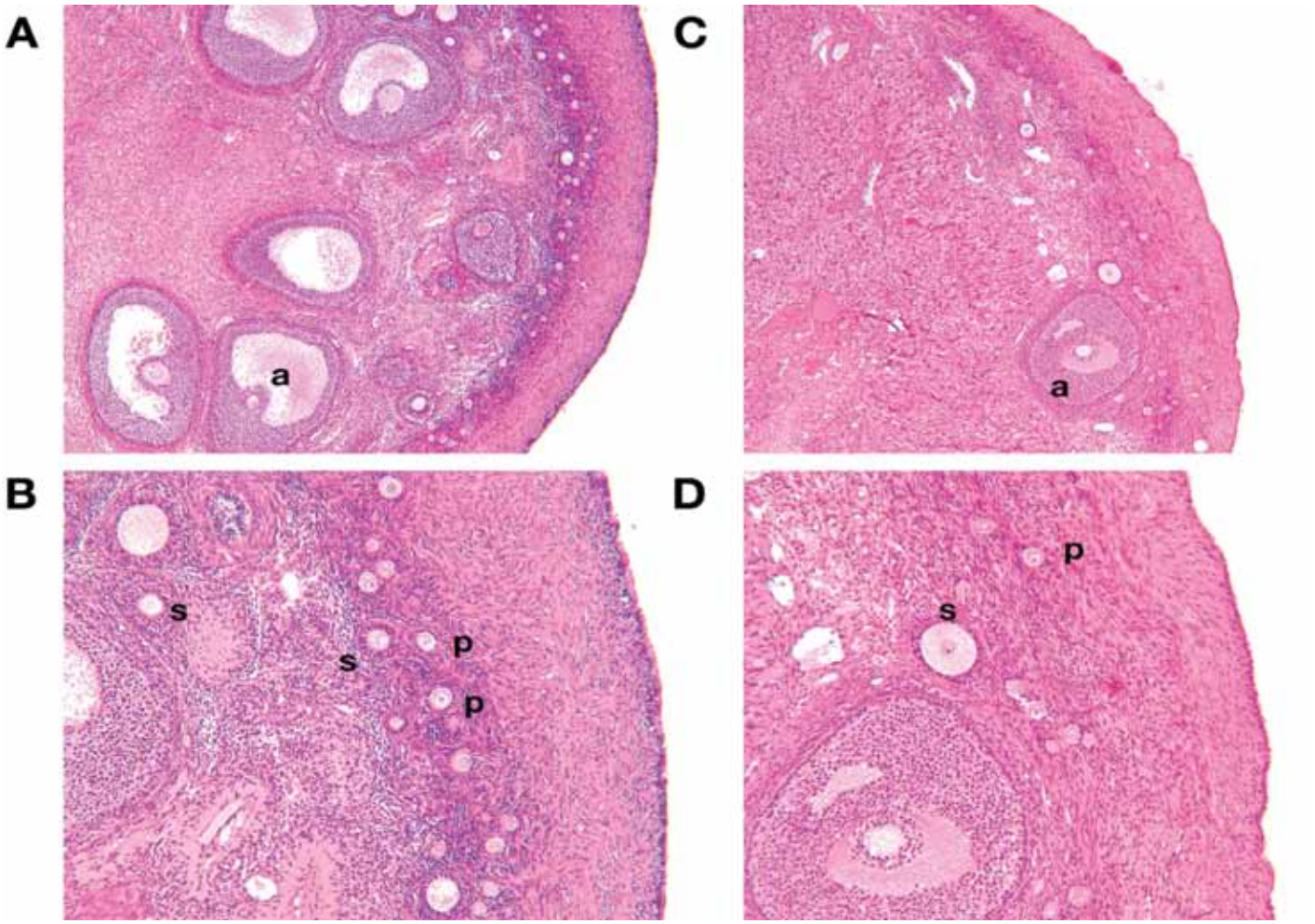

Figure 4. Histologic images of (A and B) an untreated ovary and (C and D) the contralateral ovary 30 d after VCD treatment. Note (A and B) the numerous primordial–primary (p), secondary (s) and antral (a) follicles in the untreated ovary and (C and D) decreased numbers of follicles in the treated ovary. Magnification, ×40 (A and C); ×400 (B and D).

Experiment 2. *AMH*. As noted in the Methods, ovaries were not removed for follicle counting; therefore, AMH served as a surrogate marker of follicle number. AMH concentrations at baseline did not differ ($P = 0.61$) among monkeys assigned to the premenopausal (11.4 ± 2.1 ng/mL), VCD-treated (14.1 ± 2.0 ng/mL), and OVX (10.4 ± 1.1 ng/mL) conditions (Figure 6). Consistent with findings from experiment 1, marked reductions in AMH were present after VCD fibers were placed adjacent to both ovaries (baseline, 14.1 ± 2.0 ng/mL; after VCD treatment, 6.24 ± 1.1 ng/mL; $P = 0.001$), with 14 of 29 monkeys having concentrations less than 3 ng/mL. Furthermore, AMH concentrations were lower ($P = 0.004$) in VCD-treated monkeys (6.24 ± 1.1 ng/mL) than premenopausal controls (12.26 ± 2.3 ng/mL) at 4 mo after treatment. Finally, removal of both ovaries (OVX group) resulted in significant ($P < 0.0001$) decrease in AMH to below the level of detection of the assay.

Testosterone. Testosterone at baseline did not differ ($P = 0.25$) among monkeys assigned to the premenopausal (0.54 ± 0.09 ng/mL), VCD-treated (0.60 ± 0.05 ng/mL), or OVX (0.63 ± 0.07 ng/mL) groups. Similarly, VCD-treated monkeys did not differ ($P = 0.25$) from premenopausal controls with respect to testosterone concentration at 4 mo after treatment (VCD, 0.50 ± 0.03 ng/mL; premenopausal, 0.56 ± 0.04 ng/mL), whereas OVX monkeys had significantly ($P = 0.01$) lower testosterone concentrations (0.37 ± 0.07 ng/mL) than did the other 2 groups (Figure 7). This finding in the OVX group was expected because, as in women, approximately 40% of total androgens in female macaques are produced by the ovaries, and the adrenal gland is the primary remaining source for androgen production after ovariectomy.

Estradiol. Follicular-phase estradiol concentrations at baseline did not differ ($P = 0.7$) among monkeys assigned to the premenopausal (16.5 ± 1.9 pg/mL), VCD (18.4 ± 1.6 pg/mL), or OVX (16.8 ± 1.9 pg/mL) conditions. VCD treatment led to slightly higher ($P = 0.05$) follicular-phase estradiol concentrations (18.8 ± 1.1 pg/mL) than those in the premenopausal control monkeys (14.6 ± 1.4 pg/mL); as expected, estradiol was at or below the level of detection for OVX monkeys.

Health measures. Hematology and serum chemistry measures remained within the normal ranges for this species[11,42] at both baseline and 2 wk after surgical implantation of the VCD fibers. When compared with baseline values, differences in hematology parameters were seen after treatment in the OVX and VCD groups but not in the premenopausal control monkeys. OVX monkeys had slight increases in total WBC count (baseline, 8.4

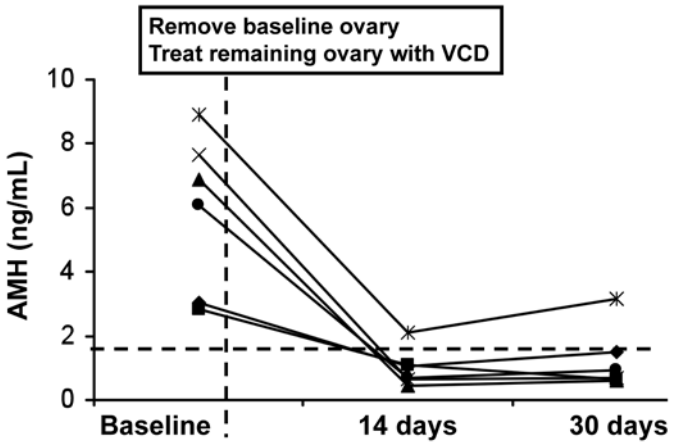

Figure 5. Serum concentrations of antimüllerian hormone (AMH) measured before the removal of one ovary (baseline) and 14 and 30 d after treatment of the remaining ovary with approximately 200 mg VCD. Data are shown as AMH concentration (ng/mL) per monkey (n = 6).

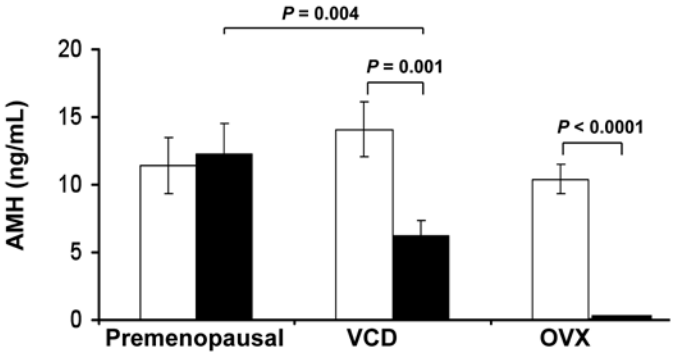

Figure 6. The effect of sham surgery (premenopausal), placement of VCD fibers next to both ovaries (VCD), or ovariectomy (OVX) on serum antimüllerian hormone (AMH) concentrations (mean ± SE) of adult cynomolgus monkeys. Open bars, before treatment (baseline); closed bars, 4 mo after treatment. Serum AMH concentrations for OVX monkeys were below the level of detection for the assay.

$\times 10^3/\mu L$; after treatment, $10.1 \times 10^3/\mu L$; $P = 0.006$) and lymphocyte count (baseline, $3.7 \times 10^3/\mu L$; after treatment, $5.2 \times 10^3/\mu L$; $P = 0.0003$) and a decrease in hematocrit (baseline, 35.2%; after treatment, 32.6%; $P = 0.05$). VCD-treated monkeys experienced an increase in number of lymphocytes (baseline, $3.65 \times 10^3/\mu L$; after treatment, $4.74 \times 10^3/\mu L$; $P = 0.002$) and mild decreases in hematocrit (baseline, 37.2%; after treatment, 33.8%; $P = 0.005$) and hemoglobin concentration (baseline, 11.37 g/dL; after treatment, 10.7 g/dL; $P = 0.02$). No noteworthy changes in serum chemistry measures, including enzymes indicative of liver function (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), were present 2 wk after VCD fiber placement, OVX, or sham surgery (premenopausal controls). Total bilirubin was decreased significantly ($P \leq 0.03$ for all) in all treatment groups, but the change was not clinically relevant. Similarly, serum creatinine and blood urea nitrogen were reduced significantly ($P < 0.01$) in premenopausal control monkeys but remained within normal range.

Discussion

The data reported here provide evidence that treatment of cynomolgus monkey ovaries with a slow-release VCD-containing fiber markedly reduces the number of primordial follicles. Importantly, measures of testosterone and estradiol indicate that larger, estradiol- and testosterone-producing follicles and ovarian stroma remain functional after VCD treatment. Furthermore, supporting our previous findings,[4] the reduction in follicle number can be detected reliably and noninvasively by measuring concentrations of AMH in serum. Finally, gross and histologic examination of VCD-treated ovaries and measurements of general health indicate that VCD does not have direct adverse health effects in cynomolgus monkeys.

The development of a monkey model of reproductive aging (beginning with ROR and progressing to perimenopause and finally postmenopause) has the potential to improve understanding of the processes by which postmenopausal women are affected by progressing osteoporosis, cardiovascular disease, metabolic syndrome, and other degenerative conditions. Such diseases are speculated to be exacerbated markedly by changes in the hormonal milieu accompanying the menopausal transition and menopause. However, despite considerable research, the relationship between reproductive hormonal changes and the development of chronic disease remains poorly understood, due in part to the lack of availability of large numbers of nonhuman primate models of reproductive aging and to the lack of control of confounding factors inherent in large-scale epidemiologic studies in women.

The challenge in using nonhuman primates to model reproductive aging in women largely reflects 3 features present in women: 1) a several-year period characterized by a subclinical exponential decline of primordial follicles (ROR); 2) irregular menstrual cycles and fluctuating hormonal milieu with or without vasomotor symptoms (perimenopause); and 3) follicle-depleted residual ovarian tissue (stromal and hilar cells) capable of producing androstenedione and testosterone (postmenopause).[7,8] Although natural menopause has been reported to occur in numerous anthropoid species[5] and although the hormonal and menstrual profiles of naturally perimenopausal and postmenopausal macaques have many similarities to those of women,[5,10] these monkeys are generally at an advanced age by the time they reach menopause, making them comparatively considerably older than women at the time of menopause. In addition, their numbers in domestic breeding colonies are low, making it difficult to acquire the large numbers of monkeys needed for investigation of the perimenopausal or naturally menopausal life stages.

The data presented here were generated by using cynomolgus monkeys and provide evidence that, within 30 d of VCD treatment, monkeys have reduced primordial follicle numbers (that is, ROR). Furthermore, despite significantly reduced numbers of primordial follicles, testosterone and follicular-phase estradiol concentrations were unchanged 4 mo after treatment—a finding consistent with reports from studies of women in this reproductive stage. Therefore, this model provides a window of time between ROR and the onset of the perimenopausal transition during which the potential health effects of ROR can be studied. Furthermore, this model could be applied to monkeys of any age group, thus removing any potential confounding age-associated factors.

An important requirement for the monkey model of reproductive aging is the ability to identify each reproductive stage as

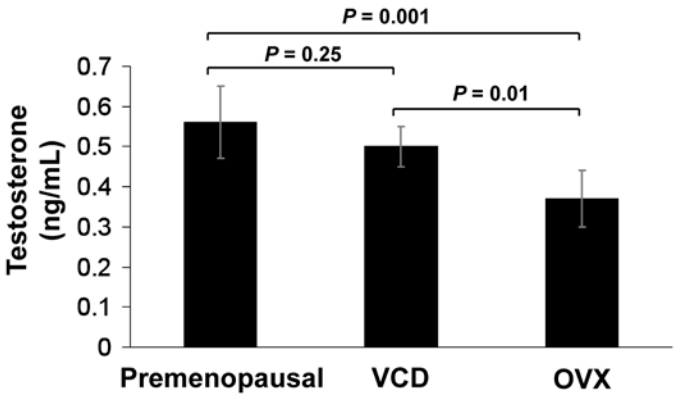

Figure 7. Serum testosterone concentrations (mean ± SE) of adult cynomolgus monkeys 4 mo after sham surgery (premenopausal), placement of VCD fibers next to both ovaries (VCD), or ovariectomy (OVX).

monkeys transition from ROR to perimenopause to menopause. In women, the large variability in hormonal patterns and the need for 'timed' sample collections during the menstrual cycle make it extremely difficult to predict accurately the onset of the perimenopausal transition by using a single hormonal marker. However, increasingly prominent as a marker of ovarian reserve (follicle number) is AMH concentration. AMH is produced by granulosa cells of small growing (primary, preantral, and small antral) follicles in the ovaries of rodents, monkeys, and women,[33,41,47,51] where it acts to inhibit the recruitment of primordial follicles and the responsiveness of growing follicles to follicle-stimulating hormone.[50] Several studies suggest that AMH concentrations reflect the size and possibly the quality of the primordial follicle pool and that AMH may be the best single marker for predicting ovarian response to gonadotropin stimulation (that is, oocytes retrieved).[16,34,43,50,51] Furthermore, AMH concentration is relatively stable across the menstrual cycle,[18,28,46] and a decline in serial serum AMH concentration occurs in women as they transition to menopause.[12,13,40,48,49] Importantly, AMH declines significantly (nearly 9-fold) during the early stages of the menopausal transition and may predict changes in ovarian reserve earlier than do increases in follicle-stimulating hormone, which do not occur until just prior to the onset of menopause.[17] Finally, recent data from a large Dutch study (the EPIC trial) suggest that AMH might be used to predict the time to menopause.[48]

We have published previously that the relationship between AMH and follicle number in cynomolgus macaques is nearly identical to the aforementioned relationships in women,[4] and the acute reduction in AMH after VCD treatment in our model further supports that finding. As we continue to develop our nonhuman primate model of reproductive aging in women, repeated measures of AMH and menstrual cyclicity will be made to determine when monkeys with ROR enter the perimenopausal transition. Monkeys will be characterized as perimenopausal when significant variability in cycle lengths and bleeding patterns, as well as declining AMH and increasing follicle-stimulating hormone concentrations, are observed. Menopause will be defined as complete cessation of menstrual cycles and the inability to detect circulating concentrations of AMH.

The studies presented here have some potential limitations. In experiment 1, an ovary was removed at baseline to serve as the control for the VCD-treated ovary. The loss in follicles observed after VCD treatment of a single ovary and the corresponding reduction in AMH were used as the basis for the second study (experiment 2). We cannot rule out the possibility that the removal of one ovary prior to VCD treatment could have affected the response of the remaining ovary to VCD. However, the short duration (30 d) of the experiment suggests that an acute compensatory response by the remaining ovary that resulted in marked follicle loss is unlikely. In experiment 2, we saw considerable variation among monkeys in the response to VCD treatment (as indicated by variability in AMH reductions). This variability may have been due to anatomic differences among monkeys and their responses to the surgical procedure. We expect that continued refinement of the technique will reduce this variation. For both experiments, it would have been informative to determine whether measurable concentrations of VCD were present in serum after local administration of VCD near the ovary. Unfortunately, due to the reactive chemical properties of VCD and its rapid metabolism by the liver into inactive tetrols, there are no validated methods available at present for measurement of VCD in serum from monkeys. Although it is unlikely that VCD was present in high concentrations in the systemic circulation after local administration of a slow-release fiber, we acknowledge that the research community may still harbor concerns regarding potential effects of VCD on organ systems other than the ovary. A previous publication[5] reviewed the extensive toxicologic studies that have been done in mice and rats treated with VCD intraperitoneally, as well as necropsy evaluations from a previous monkey study in which VCD was administered intramuscularly. Taken together with the histologic and health data from the work we present here, these studies indicate an absence of irreversible toxic effects on major organ systems after exposure to VCD.

In conclusion, the data we report here indicate that treatment of cynomolgus monkeys with VCD significantly decreased the number of primordial follicles while larger, estradiol- and testosterone-producing follicles, as well as ovarian stroma, remained functional. Therefore, direct application of a VCD-containing fiber to an ovary is a feasible method of inducing ROR in monkeys, a stage of the menopausal transition in women that is not modeled in OVX animals.

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