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Decreased Bone Mineral Density in Rats Rendered Follicle-Deplete by an Ovotoxic Chemical Correlates with Changes in Follicle-Stimulating Hormone and Inhibin A

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Abstract

Bone loss during perimenopause, an estrogen-sufficient period, correlates with elevated serum follicle-stimulating hormone (FSH) and decreased inhibins A and B. Utilizing a recently described ovotoxin-induced animal model of perimenopause characterized by a prolonged estrogen-replete period of elevated FSH, we examined longitudinal changes in bone mineral density (BMD) and their association with FSH. Additionally, serum inhibin levels were assessed to determine whether elevated FSH occurred secondary to decreased ovarian inhibin production and, if so, whether inhibins also correlated with BMD. BMD of the distal femur was assessed using dual-energy X-ray absorptiometry (DXA) over 19 months in Sprague-Dawley rats treated at 1 month with vehicle or 4-vinylcyclohexene diepoxide (VCD, 80 or 160 mg/kg daily). Serum FSH, inhibins A and B, and 17-ß estradiol (E_2) were assayed and estrus cyclicity was assessed. VCD caused dose-dependent increases in FSH that exceeded values occurring with natural senescence, hastening the onset and prolonging the duration of persistent estrus, an acyclic but E_2-replete period. VCD decreased serum inhibins A and B, which were inversely correlated with FSH (r^2 = 0.30 and 0.12, respectively). In VCD rats, significant decreases in BMD (5–13%) occurred during periods of increased FSH and decreased inhibins, while BMD was unchanged in controls. In skeletally mature rats, FSH (r^2 = 0.13) and inhibin A (r^2 = 0.15) correlated with BMD, while inhibin B and E_2 did not. Thus, for the first time, both the hormonal milieu of perimenopause and the association

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of dynamic perimenopausal changes in FSH and inhibin A with decreased BMD have been reproduced in an animal model.

Keywords

4-Vinylcyclohexene diepoxide; Follicle-stimulating hormone; Inhibin; Bone; Estradiol; Osteoporosis; Perimenopause

Decreased bone mineral density (BMD) at the lumbar spine and proximal femur has been identified in women categorized as perimenopausal by changes in bleeding patterns [1–7]. This perimenopausal decrease in BMD and an associated increase in bone resorption correlate with the two major hormonal changes marking this transitional but estrogen-replete period [8–11]: (1) diminished inhibin production by the follicle-depleted ovary [12, 13] and (2) increased pituitary follicle-stimulating hormone (FSH) secretion secondary to loss of negative feedback by inhibin [1, 3–7, 12, 13]. FSH, the best studied of these two hormones with respect to perimenopausal bone loss, correlates with [1, 3–6] and is also predictive of [4] BMD loss during the menopausal transition; significant decreases in areal BMD begin to occur during later stages of perimenopause after FSH levels have increased five- to sixfold relative to premenopausal values [7]. Decreased inhibins A and B have also been demonstrated to correlate with increased bone resorption and decreased BMD during perimenopause [12, 13]. FSH and inhibins are each reported to have direct skeletal effects, having opposing catabolic vs. anabolic actions, respectively [13–16]. However, causal effects of either of these hormones in driving bone loss in perimenopause remains unproven, leaving open the question of whether these hormonal changes are simply markers versus mediators of perimenopausal bone loss [17–21].

Exploration of possible causal links between these perimenopausal hormonal shifts and bone loss has been limited, in part, by the absence of an experimental model of perimenopause as manipulation of hormones in animals with an intact hypothalamic–pituitary–ovarian (HPO) axis and normal ovarian function causes secondary changes in other skeletally active hormones that do not reproduce the perimenopausal hormonal milieu. For example, the partial reversal of trabecular bone loss in OVX animals in response to FSH inhibition is suggestive of in vivo catabolic bone effects of elevated FSH [22]. However, conversely, bone mass in intact mice overexpressing FSH is also increased [23]. In the latter case, however, the anabolic bone phenotype occurs secondary to FSH-driven ovarian overproduction of inhibin, estradiol, and androgens, all of which are anabolic to bone [23].

We have recently described a new experimental animal model of perimenopause wherein the normal process of ovarian failure in Sprague-Dawley rats is accelerated by administration of an ovotoxic chemical, 4-vinylcyclohexene diepoxide (VCD), that induces atresia of primordial and primary follicles, thus depleting the pool of ovarian follicles available for subsequent recruitment and maturation [24]. The transition to ovarian failure in rats is significantly different from that in women as rats become acyclic prior to oocyte depletion, entering a transitional phase of “persistent estrus” during which FSH is elevated but 17-β estradiol (E_2) levels remain normal as maturating follicles degenerate into granulosa cell–containing “cysts” with retained steroidogenic capacity [25–28]. VCD administration hastens the onset of persistent estrus in Sprague-Dawley rats, causing dose-dependent increases in serum FSH levels in animals that remain estrogen-replete, as determined by bioassay (uterine weights) and serum estradiol levels [24]. The magnitude of VCD-induced elevations in FSH exceeds that occurring with normal aging in rats but is similar to that occurring in late perimenopause in women when BMD decreases occur [7, 10]. We therefore postulated that VCD-induced increases in FSH in Sprague-Dawley rats

could similarly be associated with decreased BMD. To test this hypothesis, the prospective studies described here were undertaken to assess areal BMD of the distal femur in VCD-treated rats in comparison with vehicle-treated control rats and to correlate any changes in BMD with VCD-induced elevations in FSH. Furthermore, effects of VCD on ovarian production of inhibins A and B were also explored to determine (1) whether previously documented dose-dependent increases in FSH in this model occur secondary to VCD-induced changes in ovarian inhibin production and, if so, (2) whether decreased inhibins also correlate with bone loss, thus completely reproducing key hormonal changes of perimenopause and their associations with bone loss.

Materials and Methods

Animals

All experiments involving animals were approved by The University of Arizona Institutional Animal Care and Use Committee and conformed to the *Guide for the Care and Use of Experimental Animals*. One-month-old female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were housed in plastic pans (four animals per pan) with Sani-Chips bedding (P.J. Murphy, Montville, NJ) and maintained on a 12-h light/dark schedule at 22 ± 2°C. Water (reverse osmosis) and pelleted feed (Teklad Global Diet 2018S, Harlan Laboratories; 1% calcium, 0.7% phosphorus) were available ad libitum. Animals were allowed to acclimate to the animal facility for 1 week prior to random assignment to treatment groups at 1 month of age. Rat age is either expressed as postnatal days (PNDs) or as 30-day months.

Animal Procedures

VCD (≥96% purity) was obtained from Sigma (St. Louis, MO), stored at −20°C, and handled to avoid skin contact as it is a putative human carcinogen to which latex or PVC disposable gloves are both permeable [29, 30]. VCD dosing was begun at 1 month of age (PND 34). Rats were administered 25 daily intraperitoneal (ip) doses of 80 mg/kg VCD (low-dose VCD, $n = 18$), 160 mg/kg VCD (high-dose VCD, $n = 29$), or vehicle (control, 1.25 μl DMSO/g body weight, $n = 24$) for 5 days per week for 5 weeks [24]. These VCD doses have previously been demonstrated to deplete ovarian follicles without evidence of systemic toxicity, as demonstrated by mortality, body weight, and hematopoietic, renal, and liver function [24]. A subset of rats in each treatment group was killed immediately after 25 days of treatment to verify acute follicle-depleting effects of VCD, using standard histologic criteria [24]. The remaining rats were killed 19 months (570 days) after the onset of VCD treatment at 20 months of age. To confirm that the skeleton of aged control rats remained responsive to hormonal changes, a subset of vehicle-treated rats ($n = 5$) underwent bilateral ovariectomy (OVX) at 15 months (PND 450), an age when vehicle-treated rats had already displayed persistent increases in FSH for 5 months and had been in persistent estrus for 3 months. In all experiments, serum samples were collected via the tail vein at monthly intervals or via the inferior vena cava at the time of death under mixed anesthesia (1 μl/g body weight of a mixture of 11 mg/ml xylazine, 33 mg/ml ketamine, 1.3 mg/ml acepromazine [“rabbit mix”]) and then stored at −80°C prior to assay.

Hormone Assays

FSH was measured by a competitive radioimmunoassay (RIA), using standards and primary antibodies obtained from A.F. Parlow (Los Angeles, CA), with a sensitivity of 500 pg/ml [31]. Circulating E_2 was measured per the manufacturer's guidelines using an RIA kit (Siemens, Los Angeles, CA) with a sensitivity of 2.5 pg/ml [32]. Inhibin A and inhibin B were measured using human RIAs (Diagnostic Systems Laboratories, Webster, TX) validated for use in rats [33] with sensitivities of 13 and 20 pg/ml, respectively, by The

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Estrus Cyclicity Staging

Temporal changes in ovarian function were monitored for 7 consecutive days at approximately monthly intervals by examination of vaginal cytology, which was classified as proestrus, estrus, metestrus, or diestrus using standard techniques [34]. The onset of persistent estrus, a prolonged acyclic period that precedes ovarian failure in rats [25–28], was assigned when an animal displayed ≥75% of days in the epithelial phase (proestrus or estrus) [35, 36]. The duration of persistent estrus, thus defined, for each treatment group is reported as the period when a majority of animals (>50%) were in persistent estrus. For the purposes of this study, “ovarian senescence” refers to all age-related declines in ovarian function, while “ovarian failure” is considered the final stage of ovarian senescence when rats are estrogen-deplete and in persistent diestrus [25].

Dual-Energy X-Ray Absorptiometry Assessment of BMD

In vivo femoral BMD was determined in rabbit mix–anesthetized animals using a PIXImus densitometer (GE Lunar, Madison, WI) calibrated with a phantom of defined density using the manufacturer's software (version 1.4x). BMD of the distal 25% of the femur, a site estimated to contain 20% trabecular bone [37], was analyzed prospectively as OVX-induced, estrogen-reversible bone loss is greatest in this region [37, 38]. The short-term coefficient of variation for in vivo measurement of BMD at this site was 1.19%. BMD was analyzed at baseline (day-4 relative to the onset of VCD treatment), at the end of VCD dosing, and serially thereafter at approximately monthly intervals.

Statistics

All data are expressed as mean ± SEM. As appropriate, statistical significance, defined as $P < 0.05$, was determined by unpaired Student's t test, one-way analysis of variance (ANOVA) with Student–Newman–Keuls posttest, or paired ANOVA with post hoc testing (InStat software v. 2.01; GraphPad, San Diego, CA). Linear regressions were computed by least squares analyses, with associations and their significance determined by the coefficient of determination (r^2) and/or the unadjusted Pearson's correlation coefficient (r) with $\alpha = 0.05$, using Prism 4.0 software (GraphPad).

Results

FSH, E_2, Estrus Cyclicity, and BMD in Normal Aging Female Sprague-Dawley Rats

Age-related changes in reproductive function were assessed in control rats undergoing natural ovarian senescence and compared with longitudinal changes in BMD. When vehicle-treated Sprague-Dawley rats were followed until 20 months of age, increased FSH (approximately twofold) relative to 2.4-month-old normally cycling rats was first documented at 5 months of age (Fig. 1a, solid line [n = 3–10/time point]). By ~14 months of age, FSH levels were more markedly increased (3.5-fold) and remained at this level for the duration of the experiment (Fig. 1a). The onset of persistent estrus, a period of acyclicity that precedes ovarian failure in rats [25–28], occurred prior to maximal elevations in FSH, with onset at 12 months of age and termination by 16 months (Fig. 1a). Circulating E_2 levels throughout the experimental course (Fig. 1a, broken line) were unchanged relative to baseline E_2 levels in 2.4-month-old cycling rats (Fig. 1a, with E_2 levels [mean ± SD] in young cycling rats indicated by gray bar). A plateau in areal BMD, reflective of skeletal maturation, was achieved at 5 months, the age when FSH increases were first documented (Fig. 1b, closed squares [n = 10/group]). During the entire 15-month period of two- to

fourfold FSH elevations that followed skeletal maturation (age 5–20 months), BMD of the distal femur remained unchanged from peak values achieved at 5 months of age (Fig. 1b, closed squares).

To assess whether the lack of skeletal response, as determined by areal BMD, to approximately fourfold increases in FSH in rats >14 months of age could be attributable to intrinsic effects of skeletal aging, OVX was performed in 15-month-old rats, with skeletal and reproductive effects assessed over the following 5 months compared to age-matched controls. By 5 months post-OVX, OVX had led to a significant 16% decrease in BMD (Fig. 1b, open squares [$n = 5$]) compared to age-matched controls. FSH levels in OVX rats (37.6 ± 2.4 ng/ml) were increased 12-fold relative to young cycling controls compared with the less than fourfold increase in FSH in age-matched controls during this period. Consistent with prior studies [39–43], E_2 levels were not completely suppressed in OVX rats (17.1 ± 3.2 pg/ml) but were significantly decreased compared to age-matched controls (−65%, $P < 0.001$) and uterine weights were decreased (Table 1).

Dose-Dependent Effects of VCD on FSH, E_2, Estrus Cyclicity, and BMD in Female Sprague-Dawley Rats

As anticipated, by the end of the VCD dosing period (2.4 months of age), antral follicles were present in ovaries from all treatment groups (data not shown) but the pool of primordial and primary follicles available for subsequent recruitment was dose-dependently depleted in VCD-treated versus control rats (−83% for low dose VCD, −99% for high dose VCD; $P < 0.001$). Consistent with the presence of mature follicles at this early time point (2.4 months), serum levels of FSH and E_2 (low-dose VCD Fig. 2a [$n = 5$–12/group] and high-dose VCD Fig. 2b [$n = 4$–12/group]) were no different from age-matched controls (Fig. 1a, 2.4 months) and uterine weights were unchanged (Table 1). Three months later (5 months of age), dose-dependent increases in FSH, relative to young cycling age-matched controls, were documented in VCD-treated animals (Fig. 2a, b) coincident with the time of skeletal maturation in age-matched controls (Fig. 1b). Maximal FSH increases in response to either VCD dose (Fig. 2a, b) were greater than those occurring from 5 to 20 months of age with natural senescence (Fig. 1a). In low-dose VCD rats, FSH was five- to eightfold increased compared to normal cycling animals during a 15 month period (5–20 months of age) (Fig. 2a), while high-dose VCD caused an early >12-fold increase in FSH relative to young cycling controls as early as 5 months that persisted for 8 months before returning at 13–20 months to levels documented in age-matched controls (Fig. 2b). VCD also dose-dependently hastened the onset and increased the duration of persistent estrus, which lasted for 5.5 months in low-dose VCD animals (age 9.7–15.2 months) (Fig. 2a) and 8 months in high-dose VCD animals (age 4.5–12.4 months) (Fig. 2b) compared to 4 months in normal aging controls (age 11.9–16.3 months) (Fig. 1a). As in normal aging controls (Fig. 1a), VCD-treated rats remained estrogen-replete throughout the experimental period as E_2 levels did not differ between groups at any time (Fig. 2a, b), with mean values remaining within or above the range of E_2 values documented in normally cycling animals (gray box, Fig. 2a, b). Consistent with unchanged E_2 levels, uterine weights in VCD animals at the end of the experiment were no different from controls (Table 1). Immediately after completion of VCD dosing at 2.4 months of age when animals were still normally cycling, BMD of the distal femur was unchanged in rats treated with low-dose VCD or high-dose VCD compared to age-matched controls (Fig. 2c [$n = 10$–18] and 2d [$n = 5$–11], 2.4 months). However, 10 months later, at 12 months of age, BMD in rats treated with low-dose VCD began to decrease compared to age-matched controls, with 7–13% decreases in BMD documented over a ~4-month period that roughly correlated with persistent estrus (Fig. 2c). In rats treated with high-dose VCD, which caused an earlier and more profound elevation in FSH as well as an earlier onset and longer duration of persistent estrus, significant decreases in BMD

compared to age-matched controls were documented as early as 3.3 months of age (Fig. 2d). Decreased BMD in the high-dose VCD group achieved statistical significance (−5 to 13%, $P < 0.05$) (Fig. 2d) at varied times over a 13-month period (~3–16 months) that generally coincided with persistent estrus.

Inhibins A and B and Their Associations with FSH

Because dose-dependent effects of VCD on serum FSH levels were suggestive of VCD-induced decreases in ovarian inhibins in these estrogen-replete rats, effects of VCD on serum levels of inhibin A (Fig. 3) and inhibin B (Fig. 4), which like estradiol are major hormonal inhibitors of FSH [9, 25], were assessed and compared to controls. In control rats (Fig. 3a, $n = 3–10$/group), inhibin A levels did not decline with age but rather were statistically increased at 5 and 14 months of age relative to baseline values at 2.4 months. In low-dose VCD-treated rats (Fig. 3b, $n = 4–11$/group), inhibin A levels were significantly lower than in age-matched controls from 9.4–20 months of age, displaying longitudinal changes suggestive of an inverse relationship between inhibin A and FSH. In high-dose VCD rats (Fig. 3c, $n = 4–11$/group), inhibin A levels were significantly decreased relative to both control and low-dose VCD animals at early times (5.2–9.4 months of age) but returned to age-matched control values by 13–20 months. As with low-dose VCD, time-dependent changes in inhibin A and FSH in high-dose VCD animals suggested an inverse relationship (Fig. 3c). Linear regression analysis examining the association of inhibin A with FSH in all rats (control and VCD-treated) documented a statistically significant and inverse correlation between inhibin A and FSH (Fig. 3d) ($r^2 = 0.30$, $r = −0.55$, $P < 0.0001$).

Serum inhibin B levels decreased in both control (Fig. 4a, $n = 3–6$/group) and low-dose VCD rats (Fig. 4b, $n = 5–9$/group) with aging, suggestive of an inverse correlation with FSH, and were not statistically different between groups. In high-dose VCD animals, inhibin B levels were statistically decreased compared to age-matched controls at 7.3 months of age (Fig. 4c, $n = 4–12$/group) but then rebounded and exceeded control levels at later times, again suggesting an inverse relationship with FSH. Linear regression analysis documented a statistically significant and inverse association between inhibin B and FSH (Fig. 4d) ($r^2 = 0.12$, $r = −0.37$, $P < 0.01$), a correlation that was weaker than that of inhibin A. Elimination of young cycling animals (2.4 months) from inhibin A and inhibin B regression analyses improved the strength of the association of inhibin A with FSH ($r^2 = 0.45$, $r = −0.67$, $P < 0.0001$) but further weakened that of inhibin B ($r^2 = 0.07$, $P = 0.08$).

Associations Between Reproductive Hormones and BMD

Given the temporal association of decreased BMD in VCD-treated rats with coincident supraphysiologic changes in FSH and inhibins, the relationships between individual reproductive hormones and absolute values of BMD were further assessed in animals from all treatment groups across the experimental period. First, regression analyses and correlations of FSH, inhibin A, inhibin B, or E_2 with BMD were assessed in young 2.4-month-old cycling animals that had not yet achieved peak BMD; no statistically significant relationships were found (data not shown). Next, regression analyses and correlations between these same hormones and BMD were assessed in animals ≥5 months of age after peak BMD had been achieved and reproductive function began to decline. Serum FSH was significantly and negatively correlated with BMD (Fig. 5a) ($r^2 = 0.13$, $r = −0.36$, $P = 0.0006$, $n = 86$). Serum inhibin A was significantly and positively correlated with BMD (Fig. 5b) ($r^2 = 0.15$, $r = + 0.39$, $P = 0.0002$, $n = 83$), while inhibin B was not associated with BMD (Fig. 5c) ($r^2 < 0.0001$, $P = 0.95$, $n = 47$). When inhibin B levels were added to inhibin A, total inhibin levels did not correlate with BMD ($r^2 = 0.05$, $P = 0.23$). No correlation between serum E_2 levels and BMD was found (Fig. 5d) ($r^2 = 0.008$, $P = 0.35$, $n = 110$). When regression analyses and correlations between reproductive hormones and BMD were

limited to those periods when a majority of animals were in persistent estrus (Figs. 1a, 2a, b), the associations of FSH or inhibin A with BMD were even stronger ($r^2 = 0.24$ and $r^2 = 0.33$, respectively; $P < 0.002$); a significant and positive, albeit relatively weak, association between E_2 and BMD was detected ($r^2 = 0.11$, $r = 0.33$, $P < 0.05$); and inhibin B and BMD remained uncorrelated ($r^2 = 0.01$, $P = 0.66$).

Discussion

Evaluation and manipulation of the normal, gradual transition to ovarian failure in rats, an estrogen-replete period that mimics the hormonal changes of perimenopause, provides a unique tool for exploring the association of nonestrogenic reproductive hormones with BMD loss during the menopausal transition. Our finding that longitudinal changes in FSH were statistically associated with BMD loss at trabecular-enriched sites during the transition to ovarian failure recapitulates in rats the clinical findings of the Study of Women's Health Across the Nation, a large, longitudinal, community-based study demonstrating a correlation between FSH and decreased BMD of the spine and hip across the perimenopausal transition [3–6]. The correlation between FSH and decreased BMD documented here is also consistent with previous reports of a positive correlation between FSH and bone resorption in perimenopausal women [1, 12, 13]. Thus, our finding of a positive association between FSH and BMD loss in an experimental model of perimenopause reproduces epidemiologic data in perimenopausal women, including the documentation of significant losses in BMD only when FSH is increased five- to sixfold or more over normal cycling levels [7].

Decreased ovarian production of inhibins during the transition to ovarian failure is thought to be a primary driver of increased pituitary secretion of FSH in women [9]. Our data would suggest that supraphysiologic FSH increases in VCD-treated rats are similarly driven by decreases in ovarian production of inhibins A and B in these follicle-depleted rats, with inhibin A being most strongly correlated. Having thus reproduced a second important hormonal feature of perimenopause, these studies further documented a significant correlation of decreased inhibin A with BMD loss in animals transitioning to ovarian failure, an association that was stronger than that of FSH. As inhibin A (vs. inhibin B) has been reported to correlate most strongly with bone resorption in perimenopausal women [13], these findings in the rat model would thus appear to reproduce the association of decreased inhibin A with bone loss in women during perimenopause.

In contrast to the significant correlations of FSH and inhibin A with bone loss demonstrated in these studies but consistent with previous findings in perimenopausal women [1, 3], serum levels of estradiol either did not correlate with (in skeletally mature rats) or only weakly correlated with (during persistent estrus) BMD. Because the periods of decreased BMD in VCD-treated animals generally coincided with persistent estrus, one could postulate that loss of cyclic variations in E_2 levels during this period could be detrimental to bone. However, the well-documented normalization of BMD in OVX rats in response to continuous E_2 replacement would seem to run counter to this theory [40, 41, 44, 45].

VCD effects on ovarian inhibin production have not previously been reported, nor to our knowledge have dynamic changes in serum inhibin A versus B levels been characterized over time in normal aging rats. It is thus intriguing to note a divergence between inhibin dynamics in premenopausal women versus normal aging Sprague-Dawley rats, as reported here. Inhibins A and B are both decreased during perimenopause in women [9], while in normal aging rats inhibin A levels actually increased with age while serum inhibin B levels decreased. It is notable, therefore, that inhibin A levels were uniquely suppressed in VCD-treated animals coincident with the onset of persistent estrus, the period when bone loss was most pronounced. While the design of the studies reported here does not allow for analysis

of the ovarian sources of inhibins A and B, in normal rats inhibin beta B-subunits are reported to be preferentially expressed in less mature follicles (secondary and early antral), while inhibin beta A is preferentially expressed in more mature antral follicles [46]. Additionally, and unique to persistent estrus, inhibin beta A is localized to cystic follicles present during persistent estrus, a period during which the inhibin alpha-subunit is produced by ovarian follicles and the ovarian stroma [47]. Therefore, it is possible that inhibin B serves as a bio-marker of maturing ovarian follicles in cycling animals and, thus, decreased in all treatment groups with aging, albeit at different rates. In contrast, increasing inhibin A levels with aging in normal rats may reflect an increasing pool of cystic follicles associated with persistent estrus. Future studies will be required to determine whether decreases in inhibin A during persistent estrus in VCD-treated animals occur secondary to changes in follicular number vs. follicular (or stromal) expression of inhibin alpha and beta A.

This study has several limitations. First and foremost is the relative insensitivity of dual-energy X-ray absorptiometry (DXA), the method available to us for tracking skeletal changes in these animals, for assessing trabecular bone loss. The distal femur, while enriched for metabolically active trabecular bone, is comprised of only 20% cancellous bone [37]. Thus, BMD loss in OVX animals at this site is <20%, as reported here and by others [37, 38, 41], while methods that can specifically assess trabecular (vs. cortical) bone, such as histomorphometry or microCT, can detect losses of >50% in the trabecular compartment of the distal femur in response to OVX [37, 41, 45, 48]. Also, while FSH and inhibin A levels significantly correlated with BMD in our studies, accounting for up to 33% of the variation in BMD during persistent estrus, extreme changes in these hormones occurred only with VCD treatment. Thus, the possibility that direct adverse skeletal effects of VCD, unrelated to hormonal changes, mediated and/or contributed to BMD changes cannot be ruled out. However, it should be noted that the delay in BMD loss relative to the time of VCD exposure, particularly in low-dose VCD rats where these events were separated by a 10-month gap, would argue against this possibility.

In summary, these studies provide novel evidence demonstrating that depletion of ovarian follicular reserves in Sprague-Dawley rats by VCD administration can be used as an experimental tool to reproduce and manipulate key hormonal changes of human perimenopause in animals that retain an intact HPO axis. In this model, dose-dependent decreases in ovarian production of inhibins A and B likely result in supraphysiologic increases in FSH, while serum estradiol levels, as in human perimenopause [8, 9], remain normal to high despite a loss of regular estrus cyclicity. Using this model as a tool, the association of perimenopausal changes in FSH and inhibin A with BMD loss at cancellous-enriched bone sites has been reproduced for the first time in an animal model. Because inhibin A was most strongly correlated with BMD loss in these studies and normalization of FSH in postmenopausal women has previously been demonstrated to have no effect on bone turnover [18], inhibin A remains a likely candidate in the search for hormonal mediators of perimenopausal bone loss. As all known biological effects of the inhibins can be attributed to their interference with receptor binding of activin [49], a hormone reported to be selectively increased in women during perimenopause [50], further examination of inhibin/activin signaling pathways in bone may prove fruitful in future studies probing the mechanistic basis of perimenopausal bone loss.

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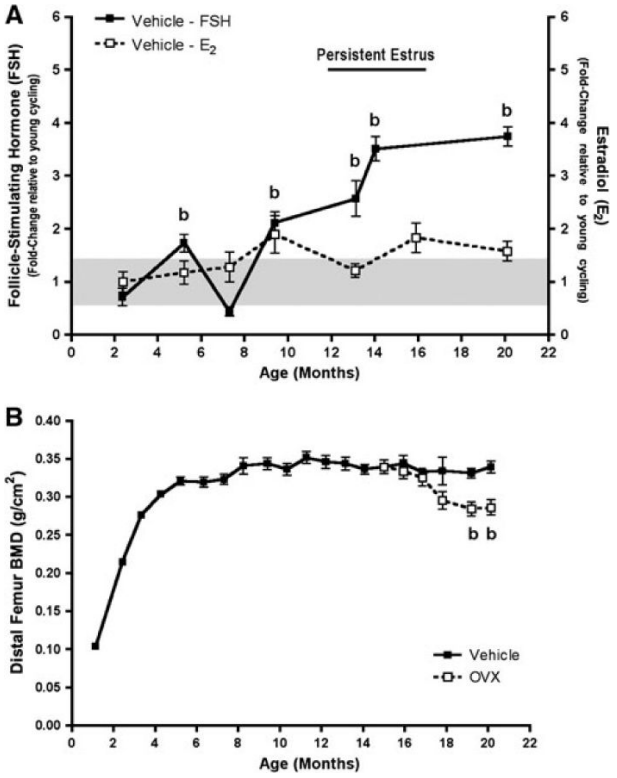

Fig. 1.

Longitudinal assessment of FSH, E_2, estrus cyclicity, and BMD in aging female Sprague-Dawley rats. All results are expressed as mean ± SEM, with statistical significance determined by ANOVA. **a** Serum levels of FSH and E_2 (n = 3–10/time point) are reported as fold-change relative to young (2 months) cycling animals where FSH was 3.46 ± 0.60 ng/ml and E_2 was 30.9 ± 5.8 pg/ml. The temporal relationship of persistent estrus, assessed by vaginal cytology, to hormonal changes is represented as a *black bar*. $^{b}P < 0.01$ versus baseline values at 2.4 months. **b** BMD of the distal femur of vehicle-treated rats (n = 10) was assessed longitudinally by DXA. Peak BMD was achieved at 5 months, as assessed by ANOVA with post hoc analysis. A subset of control animals underwent OVX at 15 months of age (n = 5). $^{b}P < 0.01$ versus controls

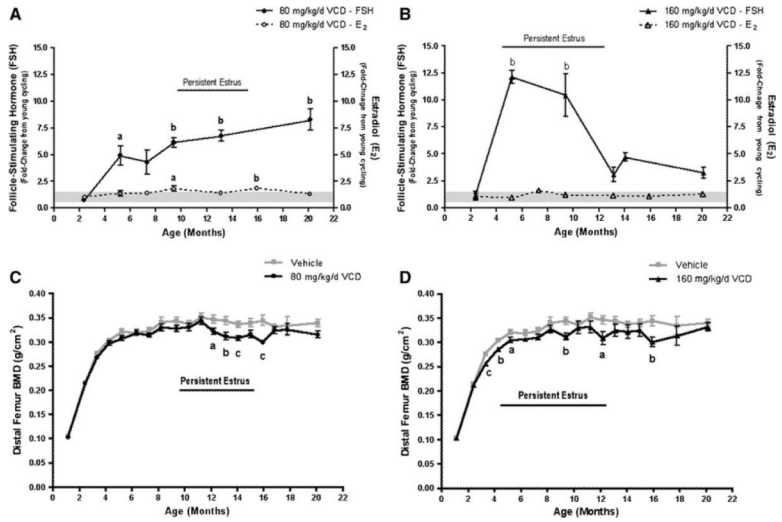

Fig. 2.

Longitudinal assessment of FSH, E_2, estrus cyclicity, and BMD following VCD treatment of female Sprague-Dawley rats at 1 month of age. Results are expressed as mean ± SEM, with statistical significance determined by ANOVA with post hoc testing. **a** Serum FSH and E_2, reported as fold-change relative to young (2 month) cycling animals where FSH was 3.46 ± 0.60 ng/ml and E_2 was 30.9 ± 5.8 pg/ml, were assessed longitudinally in animals treated with low-dose VCD (80 mg/kg daily for 25 days, $n = 5–12$/group). $^{a}P < 0.05$ and $^{b}P < 0.01$ versus baseline values at 2.4 months. FSH levels for all ages >2.4 months were also increased relative to age-matched controls ($P < 0.05$). *Black bar* represents the period when a majority of animals were in persistent estrus, as assessed by vaginal cytology. **b** Serum FSH and E_2 were assessed longitudinally in animals administered high-dose VCD (160 mg/kg daily for 25 days, $n = 4–12$/time point), with the *black bar* indicating persistent estrus, as determined by vaginal cytology. $^{b}P < 0.01$ versus baseline values at 2.4 months. FSH levels at 5 and 9 months were also increased relative to age-matched controls ($P < 0.001$). **c** BMD of the distal femur was assessed longitudinally by DXA in rats treated at 1 month with low-dose VCD ($n = 10–18$). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ versus age-matched controls. **d** BMD of the distal femur was assessed longitudinally by DXA in rats treated at 1 month with high-dose VCD ($n = 5–11$). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ versus age-matched controls

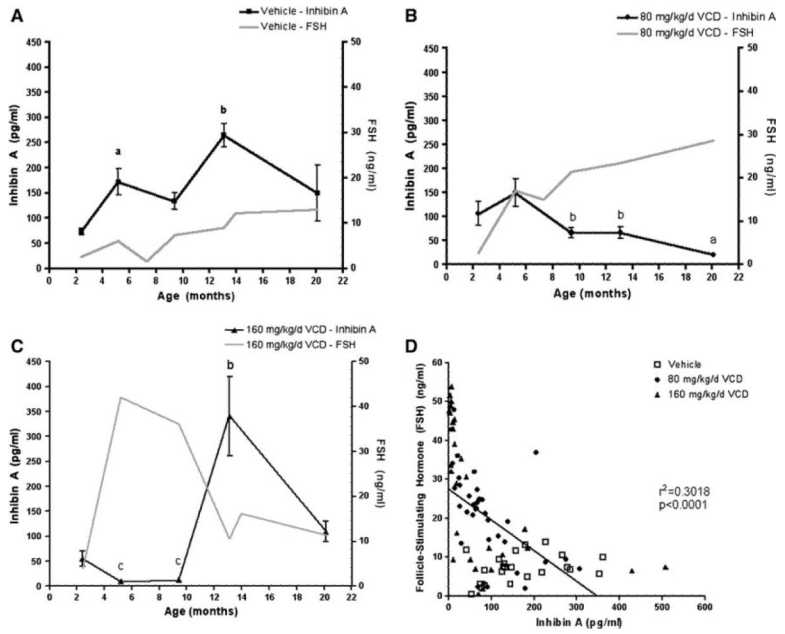

Fig. 3.

Longitudinal assessment of inhibin A and its relationship to FSH following dosing of 1-month female Sprague-Dawley rats with vehicle, 80 mg/kg daily VCD, or 160 mg/kg daily VCD. **a** Inhibin A serum levels (average ± SEM, n = 3–10/time point) in vehicle-treated control animals (*black line*). FSH data, previously presented with statistical analyses in Fig. 1, are indicated in *gray* for comparison. $^{a}P < 0.05$ and $^{b}P < 0.01$ versus baseline in 2.4-month-old rats. **b** Inhibin A serum levels in low-dose VCD animals (n = 4–11, *black line*). FSH data, previously presented in Fig. 2, are indicated in *gray* for comparison. $^{a}P < 0.05$ and $^{b}P < 0.01$ versus age-matched controls. **c** Inhibin A serum levels in high-dose VCD animals (n = 4–11, *black line*) with FSH data indicated in *gray* for comparison. $^{c}P < 0.001$ vs. age-matched controls. **d** Scatter plot and regression analysis of data from all ages and treatments to assess an association of inhibin A with FSH. Linear regression was done by least squares fit, and correlations are reported as the unadjusted Pearson correlation coefficient (r) or Pearson coefficient of determination (r^2), with significance determined with $\alpha = 0.05$

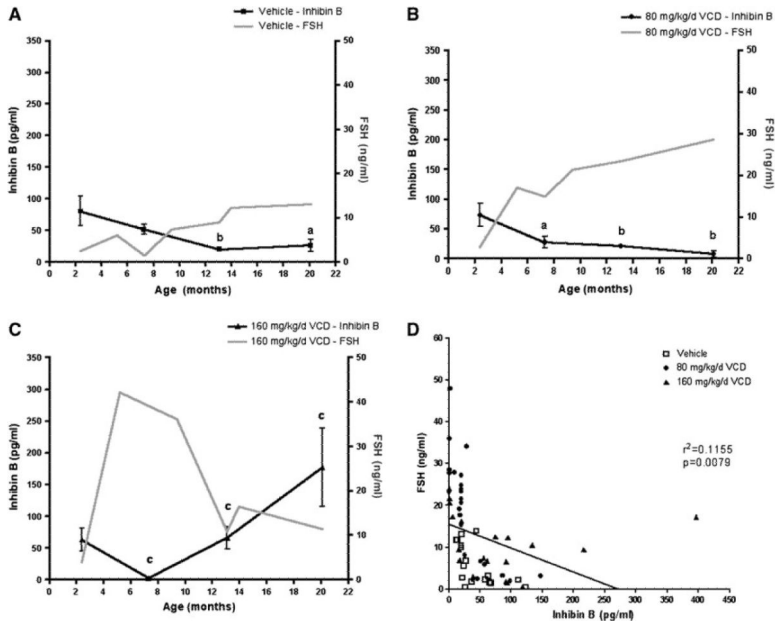

Fig. 4.

Longitudinal assessment of inhibin B and its relationship to FSH following dosing of 1-month female Sprague-Dawley rats with vehicle, 80 mg/kg daily VCD, or 160 mg/kg daily VCD. **a** Inhibin B serum levels (average ± SEM, $n = 3–6$) in vehicle-treated control animals (*black line*). FSH data, previously presented in detail in Fig. 1, are indicated in *gray* for comparison. $^{a}P < 0.05$ and $^{b}P < 0.01$ versus baseline in 2.4-month-old rats. **b** Inhibin B serum levels in low-dose VCD animals ($n = 5–9$, *black line*) with FSH data, previously presented in detail in Fig. 2, indicated in *gray* for comparison. $^{a}P < 0.05$ and $^{b}P < 0.01$ versus baseline at 2.4 months of age. Inhibin B levels at all ages were no different from age-matched controls ($P > 0.05$). **c** Inhibin B serum levels in high-dose VCD animals ($n = 4–12$, *black line*) with FSH data indicated in *gray* for comparison. $^{c}P < 0.001$ versus age-matched controls. **d** Scatter plot and regression analysis of data from all ages and treatments to assess an association of inhibin B with FSH. Linear regression was done by least squares fit, and correlations are reported as the unadjusted Pearson correlation coefficient (r) or Pearson coefficient of determination (r^2), with significance determined with $\alpha = 0.05$

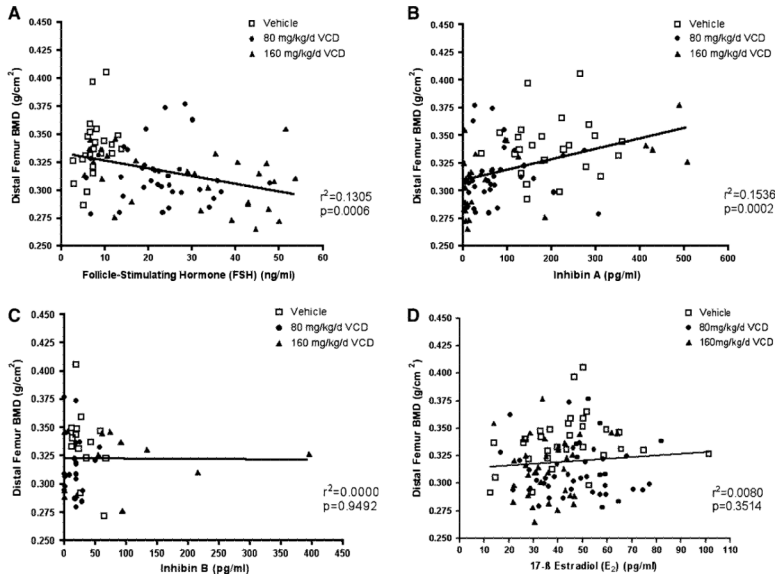

Fig. 5.

Relationships of reproductive hormones with BMD of the distal femur across the transition to ovarian failure in skeletally mature (≥5 months) female Sprague-Dawley rats. One-month animals were administered 25 daily doses of vehicle or VCD (80 or 160 mg/kg) and assessed longitudinally for changes in serum hormones and/or BMD. Data are presented as scatter plots with linear regression by least squares fit. Correlations are reported as the unadjusted Pearson correlation coefficient (r) or Pearson coefficient of determination (r^2), with significance determined with $\alpha = 0.05$. **a** Correlation of FSH with BMD ($n = 86$). **b** Correlation of inhibin A with BMD (n = 83). **c** Correlation of inhibin B with BMD ($n = 47$). **d** Correlation of E_2 with BMD ($n = 110$)

Table 1

Body and uterine weights

	Vehicle	VCD 80 mg/kg	VCD 160 mg/kg	OVX
Body weight (g)				
Immediately post-VCD	210.8 ± 3.6	196.8 ± 6.6	203.2 ± 5.1	–
End of experiment	332.0 ± 20.7	339.6 ± 11.5	333.5 ± 3.9	419.0 ± 16.0*
Uterine weight (% of body weight)				
Immediately post-VCD	0.20 ± 0.04	–	0.18 ± 0.04	–
End of experiment	0.28 ± 0.08	0.32 ± 0.08	0.34 ± 0.03	0.08 ± 0.03**

Sprague-Dawley rats were treated as indicated in Materials and Methods with vehicle or VCD. A subset of animals from each treatment group were harvested immediately post-VCD (postnatal day 71), while the remainder were followed for 20 months. At 15 months a subset of control rats were ovariectomized. OVX rats were not pair-fed. Body weights and uterine weights at each time point are indicated as mean ± SEM (n = 5–10/group)

* $P<0.001$ versus vehicle control

** $P<0.01$ versus vehicle control