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Hypothalamic Suppression during Adolescence Varies by Bone Envelope

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Abstract

Purpose—The purposes of this study were to suppress estradiol levels in adolescent (postpubertal rats) using gonadotropin-releasing hormone antagonist (GnRH-a) injections and to determine the changes in bone structure and mechanical strength.

Methods—In an Institutional Animal Care and Use Committee–approved study, female rats at 23 d of age were assigned to a baseline group (BL65; $n = 10$) sacrificed on day 65, a control group (Control; $n = 15$) sacrificed on day 90, or an experimental group (AMEN; $n = 9$) sacrificed on day 90 that received daily injections of GnRH-a for a 25-d period from 65 to 90 d of age ($2.5 \text{ mg} \cdot \text{kg}^{-1}$ per dose).

Results—Body weights were similar on day 65; however, the AMEN group was significantly heavier than the Control group (17%, $P = 0.001$) on day 90. In the AMEN rats relative to the Control group, plasma estradiol levels were reduced by 36% ($P = 0.0001$) and plasma insulin-like growth factor 1 levels were 24% higher ($P = 0.003$). In the femur, there was no change in periosteal bone apposition or total cross-sectional area. The marrow area increased by 13.7% ($P = 0.05$) resulting in a 7.8% decrease in relative cortical area ($P = 0.012$), and endocortical bone formation rate increased by 39.4% ($P = 0.04$). Trabecular volume and number decreased by 51.5% ($P = 0.0003$) and 49.5% ($P = 0.0003$), respectively. The absolute peak moments of the tibiae and femurs were unchanged in the AMEN group relative to the Control group, but these were reduced by 8.8% ($P = 0.03$) and 7.5% ($P = 0.09$), respectively, when normalized by body weight.

Conclusions—Suppression of estradiol by 25 d of GnRH-a administration to 65-d-old (postpubertal) rats reduced trabecular volume and number by about 50%, increased endocortical

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bone turnover, and reduced relative cortical thickness without changing tibial and femoral total area. These changes in bone structure were associated with no change in absolute mechanical strength possibly because of increases in body weight or in insulin-like growth factor 1 concentrations.

Keywords

BONE HISTOMORPHOMETRY; BIOMECHANICS; GONADOTROPIN-RELEASING HORMONE; ESTROGEN; CORTICAL BONE

Adolescence may be a key time to develop a healthy skeleton. Specifically, the timing of puberty has emerged as a crucial factor in bone strength development. Exercise studies highlight the importance of puberty and bone accrual (14). Kannus et al. (13) reported bone mass values two to four times greater in athletes who began their training before or at the age of menarche. The periosteal surface, in particular, was identified as more sensitive to loading in a study of racket sport athletes. Young starters (before menarche) had twice the bone size increases between arms compared with old starters (after menarche) and control groups (14). Furthermore, peak bone mineral accrual rate occurs at puberty, with an accrual of 26% of adult total bone mineral within 2 yr (1). Optimizing bone strength during late adolescence may represent a preventive strategy to reduce the effects of osteoporosis later in life. Exercise during this period is a key component to bone strength gains that may have a lasting effect; however, exercise is less effective in a low-estrogen environment (15,16) and may even result in injury.

A delay in the onset of puberty (primary amenorrhea) correlates with both low bone mass and an increased incidence of stress fracture (3,8,20), suggesting possible adverse effects on bone development by menstrual irregularities during adolescence. The incidence of menstrual irregularities, both primary and secondary amenorrhea, has been reported as high as 60%, with the highest incidence in younger athletes (21). Investigators have identified bone densities in young (17–35 yr) athletic women with decreased estrogen levels similar to the bone densities of 51-yr-old women (8,20). Delayed puberty and infrequent menstrual cycles that decrease estrogen levels during adolescence may affect long-term bone strength and increase the incidence of fracture during growth and at maturity.

To optimize bone mineralization, it has been hypothesized that bone must be exposed to adequate levels of estrogen (18). Estrogen regulates apoptosis of osteoclasts through estrogen receptors in bone, while also prolonging the life of osteoblasts (12,27), thus benefiting bone mass by both reducing resorption and increasing formation. Estrogens suppress endocortical resorption, reducing intracortical bone remodeling in cortical bone leading to an increase in bone mineral density (26). Therefore, amenorrhea resulting in increased endocortical resorption through suppressed estrogen levels will have a detrimental effect on bone mass accrual. However, previous studies hypothesized that females have smaller bones compared to males because increased estrogen levels inhibit periosteal apposition resulting in smaller bones (29). As a result, a suppression of estradiol during growth should result in an increased periosteal diameter potentially producing stronger bones because the resistance to bending or torsional forces is exponentially related to bone diameter. Yet in the clinic, delayed puberty and secondary amenorrhea are associated with lower bone mass (6,30) and increased stress fracture incidence (3,32) and not increased bone diameter.

Therefore, the purposes of this study were to suppress estradiol levels in adolescent (postpubertal rats) using gonadotropin-releasing hormone antagonist (GnRH-a) injections and to determine the resulting bone structure changes, bone volume, and cortical mechanical

strength. The mechanism of bone loss due to athletic amenorrhea is confounded by independent effects of hypoestrogenism and food restriction on bone (5). Therefore, the current approach (GnRH-a injections) focuses on the mechanisms resulting only from GnRH suppression and avoids confounding results from lower body weights resulting from food restriction. The withdrawal of GnRH-a restores normal hypothalamic–pituitary function allowing control of estrogen levels for a finite (transient) period, an advantage over ovariectomy surgery (OVX), which completely suppresses estradiol levels permanently. OVX surgery models also result in elevated luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, which do not replicate the response because of amenorrhea. The GnRH-a model does result in increased body weights and insulin-like growth factor 1 (IGF-1) levels, which differ from the clinical findings of athletic amenorrhea.

METHODS

Animals

At 23 d of age, 34 female Sprague-Dawley rats (Charles Rivers Laboratories, Wilmington, MA) were housed 3 per cage from 23 to 65 d of age. All animals were monitored daily for vaginal opening which is an indicator of the onset of puberty. On day 65, animals were randomly assigned into a baseline group (BL65; $n = 10$) sacrificed on day 65, an age-matched control group (Control; $n = 15$) sacrificed on day 90, and an experimental group (AMEN; $n = 9$) sacrificed on day 90 that received daily injections of GnRH-a (Zentaris GmbH, Frankfurt, Germany). Injections were given intraperitoneally for a 25-d period from the age of 65 to 90 d at a dosage of $2.5 \text{ mg} \cdot \text{kg}^{-1}$ per dose. The average age and body weight at vaginal opening were not significantly different for the Control, BL65, and AMEN groups (VO = 31.8 d of age, BW = 122.3 g). The rats received standard rat chow and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in conformance with the policy statement of the American College of Sports Medicine on research with experimental animals as published by *Medicine & Science in Sports & Exercise*®.

Body weights were measured weekly. Rats were given two calcein injections ($10 \text{ mg} \cdot \text{kg}^{-1}$), 10 and 3 d before sacrifice to quantify bone formation rates on the endocortical and periosteal bone surfaces, respectively, of the Control and AMEN groups. At 90 d of age, animals were anesthetized by intraperitoneal injection of ketamine ($80 \text{ mg} \cdot \text{kg}^{-1}$) and xylazine ($16 \text{ mg} \cdot \text{kg}^{-1}$). Blood was obtained through cardiac puncture, after which the animals were killed by overdose of sodium pentobarbital ($120 \text{ mg} \cdot \text{kg}^{-1}$ body weight). After sacrifice, uterine and ovarian tissues were harvested and weighed. The femurs and tibiae were removed, cleaned of soft tissue, and measured for length. Right femurs and tibiae were tested for mechanical strength and ashed for composition analysis. Left femurs were processed for histomorphometric analysis. Left tibiae were used for trabecular micro-computed tomography (μCT) analysis.

Blood chemistry

Serum estradiol was measured using a radioimmunoassay (3rd Generation Estradiol RIA, DSL-39100; Diagnostic Systems Laboratories, Inc., Webster, TX). Interassay coefficient of variation was less than 6% and sensitivity was $0.6 \text{ pg} \cdot \text{mL}^{-1}$. Serum IGF-1 was measured using an immunoenzymometric assay (Rat/Mouse IGF-1; Immunodiagnostic Systems, Inc., Fountain Hills, AZ). The sensitivity of the assay was $63 \text{ ng} \cdot \text{mL}^{-1}$.

Vaginal cytology

Swabs of the vaginal lining were obtained on the day of sacrifice for all animals. Cytology was examined under 40×, and the phase of the estrus cycle was determined by single observer who was blinded to the specimen identity.

Bone histomorphometry (basic and kinetic)

The left femurs were defleshed and fixed in 4% paraformaldehyde for 24 h. Each bone was sectioned in half. One half was dehydrated with ethylene glycol monoethyl (Fisher, Fair Lawn, NJ), cleared in methyl salicylate (JT Baker, Phillipsburg, NJ), and embedded in methyl methacrylate with 15% dibutyl phthalate (Fisher Scientific, Pittsburgh, PA). Undecalcified cross sections (thickness = 200 μm) were cut at the middiaphysis using an Isomet 1000 precision saw with a diamond wafering blade (Buehler, Lake Bluff, IL), polished to a final thickness of 50 μm , and coverslipped for analysis. The proximal half of the bone was decalcified and embedded in methyl methacrylate. Transverse sections (5 μm) were cut, deplasticized, and stained with a Gomori trichrome stain. Osteocyte numbers were counted on the anterior–medial and posterior–lateral surfaces on both the endocortical and the periosteal portion of the bone.

The undecalcified distal femoral diaphyses were assessed using bright field and fluorescence microscopy. A series of bright field and fluorescence images were taken of the cross section at 4× magnification and montaged into a complete cross section. Histomorphometry was performed using Bioquant Osteo II (Bioquant Image Analysis Corporation, Nashville, TN) following standard measures described by Parfitt et al. (19). All measurements were made by a single observer who was blinded to the specimens' identity. Static histomorphometric indices included total cross-sectional area (T.Ar; mm^2), cortical bone area (Ct.Ar = (T.Ar – Vd.Ar – P_o); mm^2), marrow area (Ma.Ar; mm^2), periosteal perimeter (Ps.Pm; mm), and endocortical perimeter (Ec.Pm; mm). On both the periosteal and endocortical surfaces, bone formation was assessed by measuring single-labeled surface (sL.Pm) and double-labeled surface (dL.Pm) and by calculating labeled surface (L.Pm/B.Pm, %) ($\text{L.Pm} = \text{dL.Pm} \pm \frac{1}{2} \text{sL.Pm}$), mineral apposition rate (MAR, $\mu\text{m}/\text{d}$) ($\text{MAR} = \text{Ir.L.Wi}/\text{Ir.L.t}$) (Ir.L.Wi: interlabel width; Ir.L.t: time between labeling), and bone formation rate (BFR/B.Pm, $\mu\text{m} \cdot \text{d}^{-1} \times 100$) ($\text{MAR} \times (\text{L.Pm}/\text{B.Pm})$). Increases in labeled surface (by either increased single or double labels) indicate an increase in the number of osteoblasts on a bone's surface. Increased mineral apposition rate indicates an increase in activity of the osteoblasts that are on the bone surface. Increased bone formation rates result from either an increase in the number of osteoblasts or an increase in osteoblast activity or both.

Cortical bone mechanical properties

Breaking strength of each right femur and tibia was measured under three-point bending using a material testing machine (ElectroForce Systems Group, Bose Corporation, Framingham, MA) fitted with a 1000-N load cell. To minimize the effect of shear loading, the distance between the lower support points was maximized. Femurs were placed on the loading fixture anterior side down and loaded in the anterior–posterior plane at a span length of 19.26 mm. Tibiae were placed medial surface down with a span length of 27.5 mm. Before testing, the bones were thawed in saline at room temperature to ensure hydration. The femurs and tibiae were loaded to failure at a rate of $0.05 \text{ mm} \cdot \text{s}^{-1}$, during which displacement and force were collected (100 Hz). The force and displacement values were normalized using terms derived from engineering analysis of three-point bending (28). Bending moments were calculated from the force (F) data ($M = FL/4$) (N·mm). Displacement data were divided by ($L^2/12$) ($\text{mm} \cdot \text{mm}^{-2}$), where L is the distance between the lower supports (16 mm). Whole bone mechanical properties were then determined from the moment versus normalized displacement curves including peak moment (N·mm;

ultimate load the specimen sustained), yield moment (N·mm), stiffness (N·mm²; the slope of the initial linear portion of the moment–displacement curve), postyield displacement (mm·mm^{−2}; displacement at failure minus the displacement at the yield point), and work to failure (N·mm·mm·mm^{−2}; the area under the moment–displacement curve before failure). The yield moment was calculated as the point where a 10% change in slope of the moment versus normalized displacement curve occurred.

μCT

The left tibiae ($n = 5$ per group) were imaged using an *ex vivo* μCT scanner (SkyScan 1172; SkyScan, Aartselaar, Belgium) to obtain variables measuring structural changes and bone loss in the trabecular bone sites. The Skyscan-1172 has a sealed microfocus x-ray tube that can go from 20- to 100-keV energy with 10 megapixel (4000 × 2096) 12-bit cooled CCD camera. Scanning was performed using a source setting of 60 keV·167 μA^{−1} with a 0.5-mm Al filter to minimize the beam hardening from the polychromatic nature of the sealed x-ray source. Scans were made with a rotation step of 0.40° through 180° and a pixel size of 7.92 μm. Feldkamp cone-beam reconstruction algorithm was used to reconstruct the three-dimensional cross sections along with addressing the ring artifact reduction and beam hardening correction.

The proximal tibia was scanned to analyze a 3-mm section of trabecular bone 1 mm distal to the growth plate; approximately 400 slices were analyzed. All parameters were calculated according to the American Society for Bone and Mineral Research standards (19). μCT measurements allow an assessment of the entire trabecular structure in three dimensions without having to section the bone.

Bone composition

After mechanical testing, the right tibiae and femurs were flushed with phosphate-buffered saline to discard the marrow. Dry weight of the diaphyses was determined after drying in an oven at 100°C for 12 h. Ash weight was determined after ashing the bone in a muffle furnace (Fisher Scientific) at 800°C for 24 h. Ash fraction was calculated as ash weight / dry weight (2).

Statistical analysis

Unpaired *t*-test assessed differences between the control and experimental (AMEN) groups at day 90 at a significance level of $P < 0.05$ (SPSS v17.0; SPSS Chicago, IL). One-way ANOVA was used to compare the control group, BL65, and AMEN for certain variables including estradiol and IGF-1 to evaluate maturation effects. Results are presented as mean ± SD values. Mechanical variables were normalized with a linear regression–based correction using body weight (7) because there was a significant difference in body weight between the groups at sacrifice. All variables with an R^2 level greater than 0 were normalized to avoid choosing an arbitrary R^2 value as a cutoff for normalization.

RESULTS

Uterine Weights, Body Weights, and Blood Chemistry

Suppression of the hypothalamic–pituitary–gonadal axis was confirmed by significant atrophy of the uterus (75%, $P = 0.0001$; Fig. 1). Vaginal cytology confirmed that all animals were equally dispersed among the three phases of the estrus cycle (proestrus, estrus, and meta/diestrus) in the control group before sacrifice on day 90. However, the AMEN group had 100% of the group in the meta/diestrus phase (Fig. 1) indicating menstrual irregularity.

Body weights from 23 to 65 d of age were similar for all groups, indicating similar growth status before initiating the GnRH-a injections at day 65 (Fig. 2).

Body weight at sacrifice was significantly higher in the AMEN group (319.5 ± 28.8 g) compared with the control group (273.8 ± 26.5 g, $P = 0.001$; Fig. 2).

Serum estradiol levels were significantly suppressed in the AMEN (15.5 ± 2.0 pg·mL⁻¹) group compared with the control group (24.1 ± 4.7 pg·mL⁻¹, $P = 0.0001$; Fig. 3A). IGF-1 was significantly higher in the AMEN (23.7%, $P = 0.003$) and BL65 (30%, $P = 0.0001$) groups compared with the control group (Fig. 3B).

Basic Histomorphometry

No differences in cortical bone area were found between the control and AMEN groups (Fig. 3C). A significant increase in marrow area (13.7%, $P = 0.05$) in the AMEN group and no change in total area resulted in a significant decrease (7.8%, $P = 0.012$) in the relative cortical area (Figs. 3D–F). There was a significant increase in osteocyte number (77.5%, $P = 0.05$) on the anterior–lateral periosteal surface (Table 1).

Kinetic Histomorphometry

There were no differences in the bone formation rate, mineral apposition rate, and labeled surface on the periosteal surface between the control and AMEN groups (Table 2). The endocortical surface had an increased bone formation rate in the AMEN group (395.9 ± 146.4 $\mu\text{m}\cdot\text{d}^{-1}$) compared with that in the control group (265.1 ± 160.2 $\mu\text{m}\cdot\text{d}^{-1}$, $P = 0.04$). The percent labeled surface increased by 34.6% due to a significant shift to increased double-labeled surface (57.1%, $P = 0.008$) and lower single-labeled surface (decreased 58.9%, $P = 0.04$), indicating an increase in the number of osteoblasts on the endocortical surface (Table 2). Postpubertal animals had more osteoblasts on the endocortical surface in both the control and AMEN groups indicated by bone formation rates on the endocortical surface that were, on average, twice the rates on the periosteal surface (Table 2). However, the mineral apposition rates were similar between the surfaces (Table 2). In summary, more osteoblasts were on the endocortical surface, with similar activity levels of the osteoblasts on the periosteal surface in postpubertal animals.

μCT

Trabecular—The percent trabecular bone volume in the proximal tibia significantly decreased in the AMEN group; 51.5% ($P = 0.0003$) lower than the control group and 46.8% lower than the BL65 group (Table 1). There was a significant decrease in trabecular number from 4.22 ± 0.75 (1 mm^{-1}) in the control group to 2.13 ± 0.33 (1 mm^{-1} , $P = 0.0003$) in the AMEN group (Table 1). In addition, the trabecular separation was significantly increased by 110% ($P = 0.0001$) in the AMEN group compared with that in the control group (Table 1). There were no differences in trabecular thickness. The structural model index was increased from 1.216 ± 0.311 in the control group to 2.118 ± 0.146 ($P = 0.0001$) in the AMEN group, indicating a shift toward a more rodlike trabecular structure.

Mechanical properties and composition—No differences were found in absolute mechanical strength (peak moment, stiffness, and work to failure) of either the femoral or the tibial diaphysis between the AMEN and control groups (Table 3). However, when the mechanical strength outcome measures were normalized for body weight, there was a significant decrease (8.8%, $P = 0.03$) in tibial peak moment and a decreased femoral peak moment (7.5%, $P = 0.09$) in the AMEN group (Table 3) but no change in stiffness. No differences were found in ash fraction of the tibia or femur (Table 3).

DISCUSSION

GnRH-a injections serve as a model for hypothalamic amenorrhea in postpubertal animals, independent of the cause. Many have reported suppressed estrogen during adolescence to be hypothalamic in origin (21); however, multiple factors including energy restriction may contribute to the disruption of the hypothalamus. As a result, the mechanism of bone loss may be dependent on both hypoestrogenism and food restriction (5). A study in humans investigating the interaction of energy restriction and low estrogen included a group of women with low bone mass who were estrogen deficient but were energy replete, suggesting that estrogen suppression can result from causes other than energy restriction (4). Both estrogen suppression and energy restriction affect bone loss, but the mechanisms and interactions of each factor remain elusive. Therefore, the current approach (GnRH-a injections) focuses on the mechanisms resulting only from GnRH suppression and avoids confounding results from lower body weights resulting from food restriction. In fact, the animals from the current study had increased body weight at sacrifice. Furthermore, the GnRH-a model has advantages over the traditional low-estrogen model in that the OVX surgery completely suppresses estradiol and elevates LH and FSH levels. However, the response to OVX runs contrary to what is seen clinically where amenorrhea tends to result in suppressed LH and FSH values. By comparison, the GnRH-a injection model more closely mimics the clinical data with the suppression of LH and FSH. In addition, reproductive functioning is restored to normal after the injections are stopped (24).

Before the experimental protocol, subjects from all groups displayed similar body weight and had similar ages of vaginal opening (an indicator of pubertal onset). Lower uterine weights and altered reproductive cycling (Fig. 1) confirm the effectiveness of the GnRH-a injection protocol. In addition, serum estradiol levels were significantly suppressed in the AMEN group (Fig. 3) with a significant increase in IGF-1 levels. Body weights were also significantly increased 17% compared with control animals after the GnRH-a injection protocol. Similarly, increases in body weights as well as increased IGF-1 levels have also been reported to increase after OVX (9,11,17).

Suppressed estradiol from the GnRH-a injections produced a significant decrease in the percent of trabecular bone volume in the AMEN group, a decrease below even that of the BL65 group. Data from the study included a significant decrease in trabecular number lower than that of the BL65 group and a significant increase in trabecular separation, results consistent with other models of suppressed estradiol. Previous research has shown that decreased trabecular number during adolescence remains into adulthood and beyond and may become problematic during menopausal and age-related bone loss (25). In addition, a significant change in the architecture of the trabecular bone in the AMEN group was indicated by an increase in structure model index. The trabeculae remodeled toward a more cylindrical rodlike structure compared with control that had a lower structural model index value, indicating a more platelike structure. Others have proposed that the change from a platelike to a rodlike structure to be an aging response (23). These changes in trabecular structure during postpubertal hypothalamic suppression are similar to changes found in postmenopausal bone loss and elderly populations and may have detrimental effects later in life. During menopause and postmenopause, there is an increase in the remodeling rate, producing more trabecular bone loss. Trabecular bone has more surface area per unit bone and thus is the site most affected during periods of increased resorptive activity.

Although no differences were found in cortical bone area and total cross-sectional area between the control and AMEN groups, significant increases in marrow area develop in the AMEN group. The increased marrow area over that of the BL65 group indicates that the increase in area due to bone erosion results from the GnRH-a injection protocol not just a

suppression of growth. Consequently, the relative cortical area (a surrogate measure for cortical width) was significantly lower in the AMEN group (Fig. 3F). The increased endocortical bone formation rate in the AMEN groups over that of the control supports a similar finding in OVX animals (17,22). The percentage of labeled surface was significantly greater in the AMEN group indicating an increase in the number of osteoblasts and bone turnover on the endocortical surface; a typical response to suppressed estradiol (22). Previous studies using ER knockout mice, to investigate estradiol on bone, report little effect on bone size and mass but suggest that estrogen receptors may just alter the rate of bone formation and resorption. Therefore, increased endocortical bone turnover may not significantly affect bone quantity in the short term but rather appear to be the mechanism of bone loss if suppressed estradiol conditions persisted.

Bone size is key to bone strength development because the bending strength of bone is exponentially related to bone diameter. More specifically, if the total cross-sectional area is preserved then mechanical strength may be maintained. Earlier studies have indicated that increased estradiol levels may limit periosteal expansion; one explanation for smaller bones in women. Therefore, reduced estradiol levels may result in increased periosteal surface and increased total cross-sectional area compared with control groups. However, recent data from both males and females have contested this relationship between periosteal expansion and estradiol suppression. An estrogen-deficient man with adequate androgens failed to increase bone size, yet when treated with estrogen replacement, bone size increased (31). The implication here is that for growing males, estrogen appears to stimulate rather than inhibit periosteal apposition. By comparison, estrogen may contribute to periosteal expansion during early puberty in women but to a lesser extent after puberty. Similar findings have been noted for racket sport athletes who began playing before versus after puberty (14). Previous data using the GnRH-a model in female prepubertal animals included a significantly increased periosteal labeled surface after estradiol suppression but a decrease in mechanical strength. Results from the current study found that the femoral cortical bone area was not different between the control and AMEN groups, suggesting that the quantity of bone was unchanged. Furthermore, the total cross-sectional area was also similar between groups. Additional analysis failed to detect a significant difference in periosteal bone apposition between the control and AMEN groups, indicating the formation activity on the periosteal surface was not affected by the lower estradiol levels. Previous studies that focused on bone after OVX in rats have reported similar results that include no changes to the periosteal surface. The current data indicate that suppressed estradiol has a limited effect on the periosteal surface and cortical mechanical strength in postpubertal rats, suggesting that the stage of maturation may be an underestimated variable in bone adaptation, growth, and estradiol suppression.

Mechanical strength of the diaphysis was not significantly different in the femur or tibia in the AMEN groups even with a 17.9% increase in body weight. After normalizing the strength outcome measures using a regression approach based on body weight, the peak moment at failure of the tibia of the AMEN group was significantly lower (8.8%) than that of the control group. Although peak moment at failure in the femur tended to be lower in the AMEN groups, there was no significant decrease in stiffness values or work to failure. The AMEN group did not yield any advantage in mechanical strength by their increased body weight. In contrast, data from prepubertal animals using the GnRH-a model resulted in significant deficits in mechanical strength with similar increases in body weight (33).

In summary, these data indicate a typical response to suppression of estradiol by 25 d of GnRH-a administration to 65-d-old (postpubertal) rats, reduced trabecular volume and number by about 50%, increased endocortical bone turnover, and reduced relative cortical thickness without changing tibial and femoral total area. However, there was no change in

periosteal bone formation rate in postpubertal rats with suppressed estradiol, a result similar to OVX models. Absolute bone strength was not affected by estradiol suppression. After normalization by body weight, which was significantly increased in the estradiol-suppressed group (17%), only a 7.8% decrease in the peak moment of the tibia was found, with no changes in stiffness values. Osteoporosis has been called “a pediatric disease with geriatric consequences” (10) and is no longer considered an age- or gender-dependent health issue. The potential exists that altered bone metabolism during adolescence may significantly affect bone structure. As conditions affecting bone accrual including amenorrhea and eating disorders appear to be on an increase in young athletic women, we can expect to see a rising trend in osteoporosis in this population if a proper intervention is not found.

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FIGURE 1.

A, Uterine weight was significantly lower than both the control and BL65 groups. B, Vaginal cytology confirmed that the animals in the control group were equally dispersed among the three main phases (proestrus, estrus, and meta/diestrus) of the estrus cycle. C, After the GnRH-a injection treatment, all animals in the AMEN group were in the meta/diestrus phase of the estrus cycle, indicating menstrual irregularity.

FIGURE 2.

Body weights in all groups before the GnRH-a injections were similar. AMEN body weight at sacrifice (day 90) was significantly greater than that of the control.

FIGURE 3.

A, Serum and histomorphometric measurements comparing control and AMEN groups. Estradiol levels were significantly suppressed in the AMEN group. B, IGF-1 levels were significantly higher in the control than both the BL65 and AMEN groups. C, Cortical area (Ct.Ar) was similar between the groups. D, Total Area (T.Ar) was similar between the groups. Marrow area (Ma.Ar) was significantly increased in the AMEN group (E) resulting in a significant decrease in the relative cortical area (F). * $P < 0.05$ compared with control.

TABLE 1

μ CT values for the trabecular bone in the proximal tibiae and osteocyte number per squared millimeter for femoral cross sections.

	Control	AMEN	BL65
μ CT	(n = 5)	(n = 5)	(n = 5)
Percent bone volume (%)	32.7 $\pm$ 6.3	15.8 $\pm$ 2.3 *	29.7 $\pm$ 4.1
Trabecular number per millimeter	4.2 $\pm$ 0.7	2.1 $\pm$ 0.3 *	4.1 $\pm$ 0.5
Trabecular separation, mm	0.16 $\pm$ 0.03	0.34 $\pm$ 0.04 *	0.17 $\pm$ 0.02
Structure model index	1.2 $\pm$ 0.3	2.1 $\pm$ 0.1 *	1.1 $\pm$ 0.3
Osteocyte number per squared millimeter			
Anterior lateral	(n = 15)	(n = 9)	
Periosteal	20.0 $\pm$ 5.7	35.5 $\pm$ 14.1 *	
Endocortical	13.8 $\pm$ 9.9	22.8 $\pm$ 8.3	
Posterior medial			
Periosteal	33.6 $\pm$ 12.1	20.3 $\pm$ 17.5	
Endocortical	21.0 $\pm$ 7.2	18.8 $\pm$ 10.6	

Values are mean $\pm$ SD.

* $P < 0.05$ versus control.

TABLE 2

Effect of GnRH-a injections on kinetic histomorphometry measurements at the femoral middiaphysis.

	Control (n = 15)	AMEN (n = 9)
Periosteal surface		
L.Pm/Ps.Pm (%)	40.02 ± 23.92	33.73 ± 22.17
MAR (μm·d ⁻¹)	3.30 ± 0.64	3.23 ± 0.69
BFR/Ps.Pm (μm·d ⁻¹ × 100)	124.8 ± 87.8	111.5 ± 79.2
Endocortical surface		
Ec single-labeled perimeter	2.0 ± 1.5	0.8 ± 0.6 *
Ec double-labeled perimeter	4.9 ± 2.7	7.7 ± 1.4 *
L.Pm/Ec.Pm (%)	70.18 ± 25.34	94.54 ± 12.87 *
MAR (μm·d ⁻¹)	3.61 ± 1.07	4.09 ± 1.08
BFR/Ec.Pm (μm·d ⁻¹ × 100)	284.0 ± 147.8	395.9 ± 146.4 *

Values are mean ± SD.

* $P < 0.05$ versus control.

TABLE 3

Effect of GnRH-a injections on femoral and tibial mechanics and composition.

	Control (n = 15)	AMEN (n = 9)	P
Mechanical properties			
Femur			
Peak moment (N·mm)	649.3 ± 72.8	636.5 ± 62.3	
Stiffness (N·mm ²)	48,876.0 ± 6386.2	45,860.3 ± 5286.2	
Work to failure (N·mm·mm·mm ⁻²)	8.47 ± 2.89	9.88 ± 2.96	
Tibia			
Peak moment (N·mm)	503.0 ± 55.1	505.0 ± 57.4	
Stiffness (N·mm ²)	53,334.3 ± 9576.3	56,012.8 ± 11,873.6	
Work to failure (N·mm·mm·mm ⁻²)	3.93 ± 1.04	3.30 ± 0.38	
Normalized by body weight			
Mechanical properties			
Femur			
Peak moment (N·mm)	661.1 ± 70.7	611.4 ± 56.7†	0.091
Stiffness (N·mm ²)	47,873.5 ± 6211.4	46,138.8 ± 5278.4	
Work to failure (N·mm·mm·mm ⁻²)	9.2 ± 2.7	9.4 ± 2.9	
Tibia			
Peak moment (N·mm)	525.0 ± 45.8	478.8 ± 50.6*	0.031
Stiffness (N·mm ²)	55,276.2 ± 9188.4	52,391.2 ± 11,266.7	
Work to failure (N·mm·mm·mm ⁻²)	4.00 ± 1.03	3.34 ± 0.38	
Composition			
Ash fraction (tibia) (%)	60.7 ± 2.5	60.7 ± 1.9	
Ash fraction (femur) (%)	59.3 ± 2.2	58.0 ± 1.5	

Mechanical measurements were normalized using a linear regression based correction using body weight.

Values are mean ± SD.

*
P < 0.05 compared with control.