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Different effects on bone strength and cell differentiation in pre pubertal caloric restriction versus hypothalamic suppression☆☆

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Abstract

Hypothalamic amenorrhea and energy restriction during puberty affect peak bone mass accrual. One hypothesis suggests energy restriction alters hypothalamic function resulting in suppressed estradiol levels leading to bone loss. However, both positive and negative results have been reported regarding energy restriction and bone strength. Therefore, the purpose of this study was to investigate energy restriction and hypothalamic suppression during pubertal onset on bone mechanical strength and the osteogenic capacity of bone marrow-derived cells in two models: female rats treated with gonadotropin releasing hormone antagonists (GnRH-a) or 30% energy restriction. At 23 days of age, female Sprague Dawley rats were assigned to three groups: control group (C, n=10), GnRH-a group (n=10), and Energy Restriction (ER, n=12) group. GnRH-a animals received daily injections for 27 days. The animals in the ER group received 70% of the control animals' intake. After sacrifice (50 days of age), body weight, uterine and muscle weights were measured. Bone marrow-derived stromal cells were cultured and assayed for proliferation and differentiation into osteoblasts. Outcome measures included bone strength, bone histomorphometry and architecture, serum IGF-1 and osteocalcin. GnRH-a suppressed uterine weight, decreased osteoblast proliferation, bone strength, trabecular bone volume and architecture compared to control. Elevated serum IGF-1 and osteocalcin levels and body weight were found. The ER model had an increase in osteoblast proliferation compared to the GnRH-a group, similar bone strength relative to body weight and increased trabecular bone volume in the lumbar spine compared to control. The ER animals were smaller but had developed bone strength sufficient for their size. In contrast, suppressed estradiol via hypothalamic suppression resulted in bone strength deficits and trabecular bone volume loss. In summary, our results support the hypothesis that during periods of nutritional stress the increased vertebral bone volume may be an adaptive mechanism to store mineral which differs from suppressed estradiol resulting from hypothalamic suppression.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bone.2011.07.019.

Keywords

Rodent; Delayed puberty; Caloric restriction; GnRH antagonist; bone strength

Introduction

Pubertal timing is a key factor that contributes to optimal bone accrual and thus bone strength [1,2]. Bone mass doubles during the onset of puberty [3] with more than 90% of peak bone mass accrued by the end of the second decade of life [4]. However, reproductive abnormalities such as delayed puberty (primary amenorrhea) result in suboptimal bone gain and increased incidence of stress fracture [5,6].

Delayed puberty (primary amenorrhea) and/or secondary amenorrhea are highly prevalent in athletes [7–9], dancers [2,10,11] and patients with anorexia nervosa (AN) [12–14]. An association between eating disorders and reproductive abnormalities (amenorrhea) has been established [15]. Energy deficient conditions both in the long term [16,17] and short term [18–21] have been reported to affect reproductive function. Furthermore, disordered eating and menstrual irregularities are associated with decreased estrogen levels [19,22,23] and lower bone mineral density (BMD) [6,24]. A current hypothesis is that energy deficiency results in hypothalamic suppression that can then affect the reproductive axis, estrogen levels and bone accrual [25] and suboptimal bone accrual is a strong risk factor for post menopausal fracture.

An incomplete recovery of bone mass following treatment (estrogen supplementation) in young women with hypothalamic amenorrhea associated with anorexia or exercise suggests that factors other than estrogen suppression may be involved in bone loss [26–28]. Estrogen replacement therapies have been shown to result in weight gain or menses resumption, but do not result in complete bone mass recovery [29]. Nutritional therapy has been used to counter bone loss in anorexic women however in a group regaining 90% of their initial body weight there was still a sub-group of women that did not regain menses or recover their bone mass [28]. A study in humans investigating the interaction of energy restriction and low estrogen included a group of women with low bone mass that were estrogen deficient but energy replete suggesting that estrogen suppression can result from causes other than energy restriction [30]. The interaction of reproductive irregularities, energy restriction and bone accrual is complex and both estrogen dependent [19,22,23] and estrogen independent [31] mechanisms of bone loss have been hypothesized.

Models of energy restriction (ER) have not always had negative consequences on bone mass and structure. Young male rats exposed to 35% food restriction had no change in bone strength when normalized by body weight [32]. Caloric restriction in mice that were older than 1 year may in fact delay age-related bone loss and maintain the proliferative potential of certain cell types [33]. Interestingly caloric restriction in 6 month old male mice resulted in increased trabecular number and lower bone cross-sectional area, a bone phenotype analogous to juvenile mice (3 months old) [34]. Significant decreases in bone mass have resulted from energy restriction models of 40–50% but primarily in older animals ranging from 10 to 17 months of age [35].

Both estrogen suppression and energy restriction affect bone loss, but the mechanisms and interactions of each factor remain elusive. Therefore, the purpose of this study was to investigate energy restriction and estrogen suppression during pubertal onset on bone mechanical strength and the osteogenic capacity of bone marrow-derived cells in two models: female rats treated with gonadotropin releasing hormone antagonists (GnRH-a) or

30% energy restriction. Previous studies have reported that GnRH-a injections have successfully delayed the onset of puberty in female rats and have the advantage that normal hypothalamic–pituitary function was restored after cessation of injections [36–38]. In addition, the GnRH-a injections focus on the mechanisms resulting only from GnRH suppression and avoid confounding results from lower body weights resulting from food restriction. A second model used 30% energy restriction that has also been shown to delay the onset of puberty [39–43].

Materials and methods

Animals

Thirty-two female Sprague Dawley rats (21 days-of-age) were received from Charles Rivers Laboratories, Wilmington, MA, USA and housed individually. All animals received water ad libitum. A 12 h light/ dark cycle was maintained at a consistent temperature (21–23 °C) and relative humidity (58%–60%). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Temple University.

Experimental protocol

Animals were randomly assigned to one of three groups; control (C) (n=10), GnRH-a (n=10) and Energy Restriction (ER) (n=12). The control group was given daily injections (0.2 ml) of saline and food ad libitum (AIN-93G). At 23 days of age, animals in the GnRH-a group received intra-peritoneal injections of gonadotropin releasing hormone antagonist (GnRH-a) (Antide, Bachem, Torrance, Ca. USA) daily for 27 days at a dosage of 2.5 mg/kg body mass. The GnRH-a group was pair fed to the ad-lib fed control group. The ER group was fed 70% of the amount of food consumed by control animals (Open Source diet (D07100606)) (Research Diets, New Brunswick, NJ). Food intake for the ER and GnRH-a groups was adjusted daily. The food was a special formulation based on the AIN-93G chow for growing animals that reduced the cornstarch in the food but maintained normal amounts of micronutrients (vitamins and minerals).

Animals were checked daily for vaginal opening to verify the onset of puberty and the first day of estrous was confirmed by cytology analysis of vaginal cells. Body weights were taken every 4 days. Growth rates were calculated for early puberty (body weight at week 3 subtracted from body weight at week 5 divided by 2) and late puberty (body weight at week 5 subtracted from body weight at week 7 divided by 2). All animals were sacrificed on Day 50. Animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Blood was collected via cardiac puncture after which animals were then killed by overdose of pentobarbital. Ovaries, uteri, triceps surae muscle group and retro-peritoneal fat pads were dissected and weighed. The femurs and tibiae were excised, cleaned of soft tissue and measured for length. The right tibiae from six animals per group were flash frozen in liquid nitrogen at – 70 °C for RNA analysis. Bone marrow was flushed from tibiae and femurs of two animals per group using a syringe to collect the bone marrow-derived stromal cells. Left femurs and tibiae were tested for mechanical strength and ashed. Right femurs were processed for histomorphometric analysis. Lumbar vertebrae (5–7) were used for trabecular micro-CT analysis.

Blood chemistry

Serum osteocalcin was measured using an immunoassay (Rat-MID Osteocalcin EIA, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA). The sensitivity of the assay was 50 ng/ml. Serum insulinlike growth factor 1 (IGF-1) was measured using an immunoassay (Rat/Mouse IGF-1, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA). The sensitivity of the assay was 63 ng/ml.

RNA isolation, reverse transcription and PCR (qRT-PCR)

Bones were pulverized and then homogenized in Trizol and separated into an organic and aqueous layer by chloroform. RNA was recovered from the aqueous phase by isopropyl alcohol precipitation. The pellet was washed with 70% ethanol and RNA concentration was calculated using spectrophotometer. RNA integrity was evaluated by agarose gel electrophoresis on a 1% agarose/paraformaldehyde minigel stained with ethidium bromide [44]. RNA (1 µg) was reverse transcribed to cDNA and used for qPCR analysis as described previously [44]. qRT-PCR was carried out on an ABI7500 Real-Time PCR system. Reactions were run using Taqman and gene specific primer FAM probe mixes (Applied Biosystems, Foster City, CA). The PCR conditions were standard protocol of ABI Prism 7500 Sequence Detection System. The reactions were run in triplicate and the results were analyzed with SDSv1.3 software that uses the AACt method for relative quantification [45].

Bone marrow-derived osteoblast differentiation

The epiphyses of two right tibiae and two right femurs from each group were removed. Whole marrow was flushed from the diaphyses and standard culture medium consisting of alpha-MEM (Mediatech cellgro, MT10022CV; Fisher Scientific) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (SH30071.03; Hyclone Laboratories) and penicillin/streptomycin was added to the marrow. The marrow was then passed through a 70-µm filter prior to centrifugation at 1150 rpm for 10 min at +4°C. The supernatant liquid was removed, and the cell pellet was re-suspended in fresh standard medium. Red blood cells were lysed using 2.5% acetic acid. Cells were then washed with fresh standard medium, re-suspended and were counted using a hemocytometer. Cells were diluted to a concentration of 1.8×10^5 cells/ml and plated onto 12-well plastic culture plates at a concentration of 1000 cells/well. These plates were placed in a biological oxygen demand (BOD) incubator with a 5% CO₂ atmosphere at 37 °C. On the third day, non-adherent cells were discarded, and the medium was changed to a standard media supplemented with 13 mM glycerophosphate (G-9891; Sigma) and 50 mg/l ascorbic acid (A-4544; Sigma). This osteogenic medium was used throughout the rest of the culture period and was changed completely twice per week.

Cell proliferation assay

Bone marrow derived osteoblast cells were plated in 24-well plates at a density of 1240 cells/well for a period of 4 days. At the end of the culture period, cells were incubated with 50 µl/well MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) substrate for 4 h at 37 °C. Cells were then washed with PBS, 500 µl/well solubilizer (20% (w/v) SDS in 50% DMF) was added and samples were read at 570 nm using ELISA plate reader.

Alkaline phosphatase activity and staining measurement

On Day 14, cell layers from each group were rinsed with phosphate buffer saline and extracted in 150 mM Tris pH 9.0/0.1 mM ZnCl₂/0.1 mM MgCl₂(TZM buffer) with 1% Triton X-100 for 30 min at 37 °C. The cells were then scraped, transferred to eppendorf tubes, vortexed and stored at +4 °C overnight in order to extract alkaline phosphatase (AP) from the cell layer. The following day, cell assay buffer consisting of p-nitrophenol (Sigma P104) substrate in 10× TZM buffer (Sigma) was added to the tubes and the alkaline phosphatase activity was measured in duplicate. AP levels were expressed as nM of p-nitrophenol formed/ min and data were normalized for an equivalent amount of protein in each sample. Total protein content was determined from matched cultures using BCA protein assay kit (Pierce).

Alkaline phosphatase staining was performed on cultures at Day 14 using ALP staining kit (Sigma). Cells were counterstained with hematoxylin and allowed to air-dry following which the cells were examined under inverted microscope (E600 Nikon). The pictures from inverted microscope were converted from JPEG to Bioquant readable images and analyzed with Osteo II analysis system (BIOQUANT Image Analysis Corporation, Nashville TN) and quantified for Total Area, AP positive area, AP Optic Density and % positive AP area.

Bone histomorphometry

The right femurs were harvested, de-fleshed and fixed in 10% buffered formalin for 24 h then stored in 70% ethanol. The bones were dehydrated with ethylene glycol monoethyl (Fisher, Fair Lawn, NJ, USA) cleared in methyl salicylate (J.T. Baker, Phillipsburg, NJ, USA), and embedded in methyl methacrylate with 15% dibutyl phthalate (Fisher Scientific). Undecalcified cross-sections (200 μ m thickness) were cut at the mid-diaphysis using an Isomet 1000 precision saw with a diamond wafering blade (Buehler, Lake Bluff, IL, USA), polished to a final thickness of 50 μ m, and cover slipped for analysis. Histomorphometric measurements were made using the Bioquant Osteo II analysis system (BIOQUANT Image Analysis Corporation, Nashville TN) attached to an epi-flourescence microscope (Nikon E800) and were calculated according to the ASBMR standards [46]. All measurements were made by a single observer who was blinded to the specimen identity. Static histomorphometric indices included total cross-sectional area (T.Ar; mm^2), marrow area (Ma.Ar; mm^2), cortical bone area (Ct.Ar=(T.Ar–Ma.Ar); mm^2), periosteal perimeter (Ps.Pm; mm), endocortical perimeter (Ec.Pm; mm) and relative cortical area (RCA=Ct.Ar/T.Ar).

Cortical bone mechanical strength

Breaking strength of each left femur and tibia was measured under 3-point bending using a material testing machine (ElectroForce Systems Group, Bose Corporation Eden Prairie, MN, USA) fitted with a 1000 N load cell. Femurs were placed on the loading fixture anterior side down and loaded in the anterior–posterior plane at a span length of 12 mm. Tibia were placed medial surface down with a span length of 17 mm. Prior to 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 mm/s, during which displacement and force were collected (100 Hz). Displacement and force data were normalized using terms derived from engineering analysis of three-point bending [47]. Bending moments were calculated from the force (F) data ($M = FL/4$) (Nmm). Displacement data were divided by ($L^2/12$) (mm/mm^2), where L is the distance between the lower supports. Structural properties were then determined from the moment versus normalized displacement curves; peak moment (N mm), yield-moment (N mm), stiffness (N mm^2), post-yield-displacement (mm/mm^2), and energy to failure (N $\text{mm}-\text{mm}/\text{mm}^2$). The yield moment was calculated as the point where a 10% change in slope of the moment versus normalized displacement curve occurred [48]. Peak stress was calculated from mechanical and cross-sectional properties (assuming a cylindrical cross section) using the equation:

$$\text{Stress} = \text{Moment} \times c / I (\text{N}/\text{mm}^2) \times (\text{cis the distance from the neutral axis to the periosteal surface}).$$

Micro CT

Lumbar vertebrae from C, GnRH-a and ER animals (n = 5/group) were fixed in 10% buffered formalin for 24 h, stored in 70% ethanol, and scanned in an ex-vivo microCT scanner (SkyScan 1172, SkyScan, Aartselaar, Belgium). The SkyScan 1172 has a sealed micro focus X-ray tube which can go from 20 keV-100 keV energy with 10 megapixel (4000×2096) 12-bit cooled CCD-camera. Scanning was performed using a source setting of

60 keV/167 μ A 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 7.5 μ m. Feldkamp cone-beam reconstruction algorithm was used to reconstruct the 3D cross-sections along with addressing the ring artifact reduction and beam hardening correction. Approximately 400 to 500 slices in the trabecular region of the L5 vertebra were analyzed. Percent bone volume, trabecular thickness, trabecular number, trabecular separation, degree of anisotropy and structural model index (SMI) were measured and all parameters were calculated according to the ASBMR standards [49].

Data analysis

One way analysis of variance testing (ANOVA) with a significance level of 0.05 was used to assess group differences (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Tukey's honestly significant difference (HSD) post hoc analysis was conducted to determine where significant differences existed. Muscle weights and fat pad weights were calculated as a percent body weight. Mechanical variables were normalized with a linear regression-based correction using body weight [50] since there was a significant difference in body weight between the groups at sacrifice (non-normalized values are also presented). All variables with an R^2 level greater than 0 were normalized to avoid choosing an arbitrary R^2 value as a cut-off for normalization. Linear regression analyses were run for total area and body weight for each group (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Results

Suppressed IGF-1 levels in ER group and increased osteocalcin in both GnRH-a and ER groups

Blood serum levels for IGF-1 were significantly lower in the ER group (29%) and significantly higher in the GnRH-a group (13%). Osteocalcin levels were significantly higher in both groups (ER: 22%, GnRH-a: 18%) (Fig. 1).

Delay in pubertal onset in GnRH-a and ER groups

The day of VO was significantly delayed in the GnRH-a groups (48 days of age) compared to the control group (31 days of age) (Table 1). Eighty percent of the GnRH-a animals never had a vaginal opening during the protocol, therefore 50 days, the day of sacrifice, was the date of vaginal opening used for statistical analysis. The ER had a moderate delay (34 days of age) for VO. Vaginal cytology analysis illustrated variations between the control and ER groups; the percentage of animals in the estrous phase was higher in the control group (81.8%) and the percentage of animals in the pro-estrous phase was higher in the ER group (64.2%).

High growth rates in GnRH-a group and low growth rates in ER group

Pubertal growth can be divided into two phases: an early pubertal (3–5 weeks of age) and a late pubertal (5–7 weeks of age) phase [51]. The growth rate (% growth per week) of the control group was greater early puberty (week 3–5) compared to late puberty (week 5–7). No differences in growth rates were detected between GnRH and control animals during early puberty, however, the growth rate of the GnRH-a group remained elevated during late puberty (Fig. 2). Growth rate during late puberty slows down in control animals (Fig. 2). Growth rate was significantly lower in the ER group compared to the control group. In fact,

the ER groups had suppressed growth rates during both early and late puberty compared to control (Fig. 2).

ER resulted in smaller animals compared to GnRH-a with no effect on uterine weight

The GnRH-a group was pair fed to the control group and had similar body weights, muscle weights and retro-peritoneal fat weights at sacrifice (Table 1). Body weight at sacrifice was significantly lower (21%) in the ER group compared to control (Table 1). Muscle weight was significantly lower in the ER group (23%) compared to control (Table 1). After normalizing muscle weight by body weight, there were no differences between the three groups. The retro-peritoneal fat pad weight (Table 1) was significantly lower in the ER group compared to control (65%) and to the GnRH-a group (48%). Retro-peritoneal fat pad weight of the ER group remained significantly lower (55%) than control after normalizing by body weight.

Uterine weight was significantly lower in the GnRH-a group (81%) compared to control (Table 1). There was no difference in uterine weight between the control and ER groups (Table 1). However, ovarian weight (Table 1) was significantly lower in both experimental groups (GnRH-a: 83% and ER: 33%) compared to controls.

The length of both the tibiae and the femurs was not significantly different between the GnRH-a and the control groups. However, tibial length was significantly decreased (5%) in the ER group compared to both the control and GnRH-a groups. After normalization by body weight, the tibial and femoral lengths were 22% longer in the ER group compared to control. There were no significant differences in the ash fraction between groups for the tibia or femur (Table 1).

In-vitro osteoblast proliferation and differentiation differ between the GnRH-a and ER groups

Bone marrow-derived stromal cell proliferation or viability was expressed as percent of the control group (Fig. 3). Proliferation of the bone marrow-derived osteoblasts was significantly suppressed in GnRH-a group by 41% but the 15% decrease in proliferation in ER group (tibia and femur combined) was not statistically significant. Alkaline phosphatase (AP) activity was decreased by 30% in the GnRH-a group and 64% in the ER group compared to control (only the ER group was statistically significant). However, AP staining was significantly decreased in the GnRH-a group by 46% and by 66% in the ER compared to control suggesting decreased osteoblast differentiation (Fig. 3). No significant differences were found in the relative quantification (RQ) for RUNX2, a transcription factor for osteoblasts.

Body weight predicts total area for ER and control groups

Total subperiosteal area (T.Ar; mm²), cortical bone area (Ct.Ar; mm²), and relative cortical area (RCA=Ct.Ar/T.Ar; %) were not significantly different between GnRH-a, ER and control groups. The periosteal and endocortical perimeters were not significantly different between groups. However, body weight predicted total area (T.Ar) for the ER and control groups ($r^2=0.6811$) but body weight did not predict the T.Ar for the GnRH-a group ($r^2=0.2143$) (Fig. 4).

Bone strength relative to body weight is greater in the ER compared to GnRH-a group

Peak moment of the femur was significantly lower in the GnRH-a compared to the control group ($p<0.05$). When normalized using a regression protocol based on body weight, the peak moment of the femur (Fig. 5) remained significantly lower in the GnRH-a group suggesting that body weight had no effect on protecting bone strength. Stiffness (N mm²)

was significantly lower in the GnRH-a group compared to the control group for the femur (Fig. 5) and tibia. Following normalization, stiffness remained significantly lower in the GnRH-a group for the femur (28.9%) and tibia (17.5%) (Fig. 5).

Tibial and femoral peak moment values in the ER group were not significantly different from control before or after normalization (Fig. 5). Stiffness was significantly lower in the ER group compared to control in the femur by 19% but similar results were not found in the tibia. Normalizing by body weight negated any differences in stiffness in the femur and tibia between the control and the ER groups. Calculated peak stress values were significantly different between groups ($p=0.03$) in particular the GnRH-a group had a significantly lower stress compared to the ER group (Fig. 5).

ER group has increased bone volume in the lumbar spine

The percent bone volume and trabecular number were significantly lower in the GnRH-a group compared to the control group by 20% and 17% respectively (Fig. 6). Trabecular thickness and trabecular separation were not significantly different between the control and the GnRH-a groups (Fig. 6). The structural model index was significantly higher (45%) in the GnRH-a as compared to the control group suggesting an increased rod-like structure in the GnRH-a group. The percent bone volume (BV/TV) was significantly higher in the ER (18.5%) as compared to the control (Fig. 6) as was trabecular thickness (14.3%). However, trabecular number, trabecular separation and structural model index were not significantly affected in the ER group.

Discussion

Previous studies have hypothesized that energy restriction suppresses hormone levels in young female athletes [15,52]. However, energy restriction may also affect bone strength independent of estrogen suppression [31]. The current study results, using two different models to suppress pubertal development (energy restriction (ER) and hypothalamic suppression (GnRH-a)), demonstrate opposite effects on bone marrow-derived osteoblast proliferation and differentiation and bone strength in female rats. Hypothalamic suppression (GnRH-a) significantly decreased bone strength, trabecular bone volume, and bonemarrow-derived cell proliferation accompanied by elevated serum IGF-1 levels and increased body weight. In contrast, the food restriction (ER) failed to reduce bone strength and increased trabecular bone volume in the lumbar spine with decreased marrow-derived cell differentiation and serum IGF-1 levels.

We speculate that our energy restricted animals in the current study had an impaired reproductive axis indicated by a moderate delay in puberty but the impact on the reproductive axis was muted compared to the effect from the GnRH-antagonist injections. Low estradiol levels and delayed puberty are typically associated with energy restriction in a clinical population. Thus a more extreme and prolonged ER protocol including intense exercise, severe food restriction or both may affect estradiol levels resulting in increased resorption and lower trabecular bone volume similar to GnRH-a model. However, the advantage of this experimental approach was that GnRH-a injections suppress estradiol without weight loss and the 30% energy restriction did not have any micronutrient or protein deficits. Yet, the lack of directly measured leptin and bone resorption markers limits this study.

The increase in vertebral trabecular bone volume and trabecular thickness in the ER group in concert with the decrease in vertebral trabecular bone volume in the GnRH-a group provide a new perspective on the interaction of food restriction and hypothalamic suppression. Our results support the hypothesis that during periods of nutritional stress the increased vertebral

bone volume may be an adaptive mechanism to store mineral [34]. Multiple studies of caloric restriction reported increased vertebral trabecula and deficits in the trabecular bone of the femur and tibia [34,53,54]. However, the lower bone volume of the GnRH-a group was consistent with changes reported following ovariectomy surgery (OVX) a common low estrogen model of post menopausal bone loss [55,56]. Decreased serum estrogen is associated with significant decreases in trabecular architecture in both the vertebra and femur. Suppressed levels of the hormone estradiol have been reported following GnRH-a injection protocols [38,57,58] with similar uterine and ovarian tissue atrophies [38,59]. Suppressed leptin levels following energy restriction may provide an explanation for the differing vertebral bone volumes. Leptin levels may play a crucial role in pubertal timing by connecting the gonadotropic and somatotrophic axes [34,52,60] and the significantly decreased ovarian weights (33%) and IGF-1 levels in the ER group may be indirect evidence of suppressed leptin levels. Models of hypoleptinemia result in increased vertebral bone volume [61]. Studies of IGF-1 deficient mice [62,63] also reported no change or slightly improved trabecular bone volume along with increased connectivity, increased trabecular number and decreased trabecular spacing in the vertebrae. A recent study in calorie restricted young male mice reported significant decreases in bone volume in the distal femur but no changes in the vertebra [54]. Food restriction and hypothalamic suppression affect axial and appendicular trabecular bone in different ways; bone volume decreases in both weight bearing long bones (tibia and femur) and non weight bearing sites (vertebra) following estradiol suppression but caloric restriction preserves trabecular bone volume in non weight bearing sites (vertebra) with losses in the femur and tibia. In addition, trabecular bone volume and bone architecture measures in the radius, a non weight bearing bone, were similar to controls in human populations practicing long-term calorie restriction [64].

While increased serum osteocalcin was expected with suppressed estradiol levels, serum osteocalcin levels were increased in both models (27% GnRH-a and 31% ER). The unexpected increase in serum osteocalcin in the ER group does not agree with other studies of food restriction [34]. However these results do support, a recent study that showed an increase in serum osteocalcin in humans following a reduction in visceral fat mass after diet combined with exercise [65]. In that study, the authors noted the relationship between increased leg force (muscle function) and serum osteocalcin, suggesting that increased lean mass and decreased fat mass results in increased osteocalcin levels. While the current study did not manipulate exercise, lean mass was preserved. The decreased body weight in the ER group was due to loss of fat mass. The preserved muscle weight of the ER animals may be a result of the moderate level of energy restriction (30%) with a protein replete diet [66].

One could argue that the energy restricted animals may have mechanically sufficient bones for their size based on the strong relationship that exists between bone mineral density and lean mass during the linear growth and development period [67]. The body weights of both the control and ER groups were significantly related to total area in the femoral diaphysis ($r^2=.6871$). The ER group tended to have a lower body weight and total area overall compared to the control animals even though there were no significant differences between the group means (Fig. 4). In contrast, the GnRH-a animals did not have a significant relationship between body weight and total area ($r^2=.2143$). GnRH-a animals had lower total area for similar body weights in other words bone size did not increase with body size. Investigators have hypothesized that bone mass of weight bearing bones is determined by body weight [53] and our results, both femoral total area and mechanical strength, support this concept.

The significant increases in body weight and total cortical bone cross sectional area of early puberty compared to late puberty, indicate a growth spurt during early puberty [51]. While the significantly higher growth rate of the control animals during early puberty compared to

late puberty reinforced other study results [51], there was no decrease in growth rates during late puberty in the GnRH-a group. The high growth rates associated with the GnRH-a group were consistent with increased growth rates following OVX in two month old animals [68]. One could argue that increased growth rates may result in a greater amount of non-lamellar bone forming on the periosteal surface which in turn decrease material properties of the bone and thus reduce bone strength [48,69]. Furthermore, there were significantly lower growth rates in the ER group compared to the control and the GnRH-a groups yet no decreases in mechanical strength.

Evidence of the effect of growth rates on mechanical strength comes from data using chicks as a model [68]. Fast growing strains of chicks had weaker bones compared to slow growing strains replicating differences observed in mechanical strength results found between the GnRH-a and ER groups. The marrow cell proliferation rates were lower in the fast growing chick strains [68] and similar to the decrease in proliferation in the GnRH-a model with no significant changes in differentiation. The suppression of proliferation in the GnRH-a model may be due to the resulting estrogen suppression based on estrogen's previously documented ability to directly modulate the differentiation of stromal cells into the osteoblast lineage [70]. ER animals exhibited no significant change in proliferation rates compared to controls, but had significantly greater proliferation rates than the GnRH-a group. In addition, the significant suppression of marrow cell differentiation of the ER group was similar to the slow growing chicks. Although these findings may be counter intuitive to the increases in bone volume in the vertebral trabecular bone of the ER animals, the increased mineral apposition rate found in slower growing chicks offers an explanation for the increased bone volume with lower osteoblast differentiation [68,71].

In summary, ER may have an independent mechanism on bone accrual during pre-puberty that differs from suppressed estradiol resultant from hypothalamic suppression. The ER animals were smaller but had cortical bone strength sufficient for their size with an increased vertebral trabecular bone volume. The increased vertebral bone volume may be an adaptive mechanism to store mineral during periods of nutritional stress [34], whereas cortical bone in weight bearing sites is determined by body mass and thus decreases when body mass decreases due to caloric restriction [53]. In contrast, the lowered estradiol levels that follow from hypothalamic suppression reduce bone strength and trabecular bone volume compared to control potentially due to increased bone turnover. The differences during pubertal development may also be in part to growth rate differences. Fast growing chicks had different osteoblast behavior compared to slower growing chicks. Suppressed estradiol accelerates body weight gain in contrast to energy restriction which slows down body weight gain. The opposing effects on the proliferation and differentiation of bone marrow osteoblasts ultimately can affect bone strength in the long bones. Further investigation into the long-term consequences and potential catch-up growth of these models is needed as well as more data on the interaction of these models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Serum osteocalcin and IGF-1 levels in the control, GnRH-a and ER groups at 50 days of age. A. Osteocalcin levels (ng/ml) were elevated in both experimental groups (GnRH-a and ER). B. IGF-1 levels (ng/ml) were elevated in the GnRH-a group and suppressed in the ER group. * $p < 0.05$ compared to control.

Fig. 2.

Growth rate of body weight expressed as the percentage increase during early puberty (3–5 weeks of age) or late puberty (5–7 weeks of age) for the control, GnRH-a and ER groups. * $p < 0.05$ compared to control (early puberty). # $p < 0.05$ compared to control (late puberty).

Fig. 3.

Bone marrow cell proliferation and alkaline phosphatase activity and stain (Day 14). A. On Day 5 an MTT cell proliferation assay was performed. Results are presented as percent of control and are a combination of assays on the tibia and femur. B. On Day 14, alkaline phosphatase activity was measured and presented as percent of control. Results are both tibia and femur. Activity was measured in nM of p-Nitrophenol/min/ug of protein. C. Sample wells stained for alkaline phosphatase for the control, GnRH-a and ER groups. D. Quantification of the alkaline phosphatase stained percent positive area in each well for the three groups using Osteo II analysis system (BIOQUANT Image Analysis Corporation, Nashville TN). Data presented in A, B and D are the mean $\pm$ SD.

Fig. 4. Linear regression of body weight and total area (T.Ar). There was a significant relationship between body weight and total area in the control and ER groups ($r^2=.6811$). There was no significant relationship in the GnRH-a group between body weight and total area ($r^2=.2143$).

Fig. 5.

Bone strength testing using a three-point bending assay on the femur at 50 days of age in the control, GnRH-a and ER groups. Structural properties were measured from moment–displacement curves. A. Peak moment (N mm). B. Stiffness (slope of the moment–displacement curve) (N mm²). C. Peak moment (N mm) was normalized to body weight using a linear regression based method. D. Stiffness (N mm²) was normalized to body weight using a linear regression based method. E. Calculated stress $\text{Stress} = \text{Moment} \times c / I$ (N/mm²). * $p < 0.05$ compared to control.

Fig. 6. Micro-CT variables for the trabecular bone of the fifth lumbar vertebra of the control, GnRH-a and ER groups (n=5). A. Percent bone volume (%). B. Structural model index. C. Trabecular thickness (mm). D. Trabecular number (1/mm). *p<0.05 compared to control.

Table 1

Summary of group differences for body and tissue weights, femoral bone length and the day of vaginal opening. Values represent means $\pm$ standard deviation.

Variables	Control	GnRH-a	ER
Body.wt (g)	191.3 $\pm$ 17.7	185.3 $\pm$ 6.8	147.2 $\pm$ 4.6 ^a
Muscle.wt (g)	1.28 $\pm$ 0.08	1.28 $\pm$ 0.08	0.99 $\pm$ 0.07 ^a
Muscle.wt/BW (%)	0.72 $\pm$ 0.07	0.70 $\pm$ 0.04	0.70 $\pm$ 0.03
Fat.wt (g)	0.88 $\pm$ 0.37	0.73 $\pm$ 0.15	0.30 $\pm$ 0.08 ^a
Fat.wt/BW (%)	0.50 $\pm$ 0.18	0.39 $\pm$ 0.07	0.22 $\pm$ 0.06 ^a
Uterine.wt (g)	0.31 $\pm$ 0.05	0.06 $\pm$ 0.05 ^a	0.36 $\pm$ 0.14
Ovarian.wt (g)	0.12 $\pm$ 0.02	0.02 $\pm$ 0.01 ^a	0.08 $\pm$ 0.01 ^a
Tibial.L (mm)	33.1 $\pm$ 0.8	33.1 $\pm$ 1.2	31.5 $\pm$ 0.6 ^a
Tibial.L/BW (mm/g)	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01	0.22 $\pm$ 0.01 ^a
Femoral ash fraction	0.56 $\pm$ 0.06	0.56 $\pm$ 0.04	0.57 $\pm$ 0.02
Day of VO	30.07 $\pm$ 2.86	47.9 $\pm$ 7.13 ^a	34.41 $\pm$ 5.08

BW represents body weight in grams.

^aRepresents a significant difference ($p < 0.05$) vs. the control group.