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Delayed pubertal development by hypothalamic suppression causes an increase in periosteal modeling but a reduction in bone strength in growing female rats

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Abstract

The timing of the pubertal growth is a critical event in skeletal development. A delay in the onset of puberty has been correlated with increased stress fracture incidence in young women and as a result, suboptimal skeletal development may affect long-term bone strength. Gonadotropin releasing hormone antagonist (GnRH-a) injections were used to delay the onset of puberty in growing female rats. Twenty-three-day-old female rats were injected with a GnRH-antagonist at 2 dosage levels ($n = 15/\text{group}$). The low dose group (1.25 mg/kg/dose) received daily injections for 27 days (sacrifice 49 days). The high dose received (5.0mg/kg/dose) only 5 days per week over a 26 day period (sacrifice 48 days). Calcein injections measured bone formation activity on the periosteal and endocortical surfaces. Standard histomorphometric and biomechanical analyses were performed on the femora and ash content was measured on the tibiae of all animals. Serum estradiol and insulin-like growth factor (IGF)-1 levels were assayed. Significant delays in pubertal development occurred in the two GnRH-a groups as evidenced by delayed vaginal openings, decreased uterine and ovarian weights and suppressed estradiol levels compared to control. Femoral lengths were significantly shorter in the experimental groups and serum IGF-1 levels were higher than control. Bone strength and stiffness were significantly lower in the GnRH-a groups. Cortical bone area was decreased and total area was not different between groups. There was a significant decrease in % Ct.Ar/T.Ar. The decreased bone strength may have resulted from a decrease in the amount and distribution of bone, however, stress and Young's modulus were also decreased. There was a different response between endocortical formation indices and periosteal formation indices to the GnRH-a protocol. Endocortical bone formation rates decreased and there was an increase in periosteal labeled surface. A dose response between bone strength and GnRH-a dosage was found. The data suggest that hypothalamic

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suppression during pubertal development resulted in decreased bone strength which may result in fracture development.

Keywords

puberty; hormones; bone histomorphometry; biomechanics; modeling

Introduction

Osteoporosis has been called “a pediatric disease with geriatric consequences”(1). Suboptimal skeletal development may affect long-term bone strength and increase the incidence of fracture during growth and at maturity. Young girls with childhood fracture had lower bone mineral content compared to a non-fracture group and also had lower bone mass at pubertal maturity (2). Simply stated, suboptimal skeletal development in childhood and adolescence may result in decreased bone strength and an increase in lifetime fracture incidence. Stress fracture incidence in young women is on the rise due to a particularly devastating problem, the female athlete triad, an interrelation between energy availability, menstrual function and bone mineral density (BMD) (3–7). The incidence of menstrual irregularities, both primary and secondary amenorrhea, has been reported as high as 60% with the highest incidence in younger athletes suggesting possible adverse affects on bone development (8,9). The mechanisms of reduced bone strength due to amenorrhea, the recovery of bone strength following amenorrhea and the effects of the timing, duration and severity of amenorrhea as well as the effect of recurrent episodes have not been clarified.

The timing of puberty has emerged as a crucial factor in bone strength development. Peak bone mineral accrual rate occurs at puberty (10), with an accrual of 26% of total adult bone mineral within 2 years (11). However, a delay in the onset of puberty (primary amenorrhea) correlates with both low bone mass and an increased incidence of stress fracture in young women (12). In a comparison of fracture versus non-fractured elite female athletes, bone mineral density (BMD) was not different between groups, however there was a significantly later age at menarche (puberty) in the fractured group (13). The question remains whether this low bone mass and increased stress fracture incidence develops into fracture problems later in life. Animal models of delayed puberty have suggested the existence of “catch-up” growth (14) or a recovery of bone strength. Yingling et al., 2006 (15) reported a transient decrease in bone strength but a full recovery of bone strength at 6 months of age. However, the mechanism of this recovery remains elusive (16) and data from human studies suggest a sustained bone deficit. Warren et al. (2003) (17) treated amenorrheic dancers for 2 years with hormone replacement therapy and found no difference in BMD between treated and placebo groups. To sum up, animal studies suggest that one negative perturbation during bone development may not have a long term effect on bone strength but clinically women with a history of amenorrhea have sustained bone loss (12,17). Other factors such as the severity of exposure, age of onset, and duration of amenorrhea must be investigated.

Bone strength is based on the cellular activity of both the endocortical and periosteal surfaces. The relative cellular activity on the periosteal and endocortical bone surfaces specifically during puberty affects bone size, a critical element of bone strength (18). Increased estrogen levels during puberty have been hypothesized to inhibit periosteal modeling in females (19) and thus result in smaller and weaker bones compared to males. As a result, a delay in puberty should result in an increased periosteal diameter potentially producing stronger bones since the resistance to bending or torsional forces is exponentially related to bone diameter. However in the clinic, delayed puberty is associated with lower bone mass (20–22) and increased stress

fracture incidence (12,13) and furthermore an animal model of delayed puberty has reported short-term decreases in peak moment and stiffness without changes in total area (15).

Recruitment and function of osteoblasts and osteoclasts on the periosteal and endocortical surfaces has not been thoroughly investigated during delayed pubertal growth. Therefore, a clear understanding of the spatial and temporal relationship of endocortical and periosteal modeling during the pubertal years is necessary.

Young growing rats are useful models for studying factors that modulate the relative cellular activity on the periosteal and endocortical bone envelopes and the resultant bone strength. Animal models using ovariectomy represent only the most extreme condition of delayed reproductive development with the complete cessation of estrogen. However, gonadotropin releasing hormone antagonists (GnRH-a) have successfully delayed the onset of puberty in female rats as determined by delayed vaginal opening, lower ovarian weights and lower serum estradiol levels (15,23–25). Furthermore, the withdrawal of GnRH antagonists restores normal hypothalamic-pituitary function allowing control of estrogen levels for a finite (transient) period of time. This model offers an opportunity to reproduce the environment of delayed pubertal development to investigate adaptation mechanisms, both positive and negative, on bone structure and ultimately bone strength. However, a dose response between GnRH-a injections and bone strength has not been investigated.

Therefore the purpose of this study was two-fold; first to determine the bone formation activity on the periosteal and endocortical surfaces and the resultant bone strength in female rats with delayed puberty and second to investigate the severity of primary amenorrhea by evaluating two doses of GnRH-antagonist.

Materials and methods

Animals

Forty-five female Sprague-Dawley rats (23 days-of-age) (Charles Rivers Laboratories, Wilmington, MA, USA) were housed 3 per cage in a 12 hour light-dark cycle. They received standard rat chow and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Brooklyn College (City University of New York).

The animals were randomly assigned into a control group (C) (n=15) and two experimental groups (n=15/group) that received injections of gonadotropin releasing hormone antagonist (GnRH-a). Injections (0.2 ml) of either saline or the GnRH-a (Zentaris GmbH) were given intraperitoneally. The experimental groups were separated into a group that injected 1.25 mg/kg dose daily (7 days per week) (Low Dose) and 5.0 mg/kg/dose, 5 days per week (High Dose). All animals were monitored daily for vaginal opening an indicator of pubertal onset and vaginal swabs were taken to confirm the day of the estrous phase of the cycle. Body weights were measured weekly. Approximately 7 weeks of age (C: day 49, Low Dose: day 49, High Dose: day 48), an age that the growth curve begins to plateau suggesting a decrease in the rate of bone mass accumulation(26), animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg)). Blood was taken through cardiac puncture, after which the animals were killed by overdose of pentobarbital. 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 were tested for mechanical strength and left femurs were processed for histomorphometric analysis. Right tibiae were used for bone composition analysis.

Blood chemistry

Serum estradiol was measured using a radioimmunoassay (3rd Generation Estradiol RIA, DSL-39100, Diagnostic Systems Laboratories, Inc. Webster, TX, USA). Inter-assay coefficient of variation was less than 6% and sensitivity was 0.6 pg/mL. Serum insulin-like growth factor 1 (IGF-1) was measured using an immunoenzymometric assay (Rat/Mouse IGF-1, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA). The sensitivity of the assay was 63 ng/mL. A positive control test to verify the IGF-1 assay results was completed on animals that were 23 days of age and 49 days of age.

Bone histomorphometry (static and kinetic)

Bone labeling with intraperitoneal injections of calcein (10mg/kg, Sigma) was performed 10 and 2 days prior to sacrifice for C and Low Dose and 11 and 3 days prior to sacrifice for High Dose. Left femurs were fixed in 10% buffered formalin for 24–48 hours and thereafter kept in 70% ethanol until processing. The bones were bulk stained with a Villanueva mineralized bone stain (Arizona Histology & Histomorphometry Services, Phoenix, AZ, USA) for 7 days. Following staining the femurs 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 100 μ m, and cover slipped for analysis.

Cortical bone changes were assessed using bright field and fluorescence microscopy. Histomorphometry was performed using the OsteoMeasure system (Osteometrics, Atlanta, GA, USA) following standard measures described by Parfitt et al. (1987) (27). All measurements were made by a single observer who was blinded to the specimen identity. Static histomorphometric indices included total subperiosteal area (T.Ar; mm²), cortical area (Ct.Ar = (T.Ar – Vd.Ar – P_o); mm²), marrow area (Ma.Ar; mm²), periosteal perimeter (Ps.Pm; mm), endocortical perimeter (Ec.Pm; mm). Porosity within the intracortical envelope was calculated as (Po/Ct.Ar, %) ((Vd.Ar – Ma.Ar)/Ct.Ar $\times$ 100). The intracortical porosity occurred very near the endocortical surface and was contiguous with the marrow space in some animals and not in others. There for a measure of void area (Vd.Ar, mm²) (marrow area and porosity area) and void perimeter (Vd. Pm, mm) were used to express the void space in the endocortical region. On both the periosteal and endocortical surfaces bone formation was assessed by measuring single labeled surface (sL.Pm), double labeled surface (dL.Pm) and calculating labeled surface (L.Pm/B.Pm, %) (L.Pm = dL.Pm + $\frac{1}{2}$ sL.Pm), mineral apposition rate (MAR, μ m/day) (MAR = Ir.L.Wi / Ir.L.t) (Ir.L.Wi: interlabel width; Ir.L.t: time between labeling) and bone formation rate (BFR/B.Pm, μ m/day $\times$ 100) (MAR * (L.Pm/B.Pm)).

The medial-lateral and anterior-posterior moments of inertia (I_{ml}; mm⁴, I_{ap}; mm⁴) were measured from mid-diaphysis femoral cross-sections using image processing software (Image J, NIH Image) and a macro (Momentmacro, C. Ruff, Ph.D, Johns Hopkins University School of Medicine). The mean polar moment of inertia (J_o; mm⁴) was then calculated. The cortical widths (Ct.Wi; μ m) were measured on the cortex along the principal axes as well as an additional 4 points between the principle axes.

Cortical bone mechanical properties

Breaking strength of each femur was measured under 3-point bending using a materials testing machine (Instron, Canton, MA, U.S.A.) fitted with a 1000 N load cell. Femurs were placed on the loading fixture anterior side down and loaded in the anterior-posterior plane. In order to minimize the effect of shear loading the distance between the lower support points was maximized at 16 mm. Prior to testing, the right femurs were thawed in saline at room

temperature to ensure hydration. The femurs were loaded to failure at a rate of 0.05 mm/s, 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 ($M=FL/4$) (Nmm). Displacement data were divided by ($L^2/12$) (mm/mm²), 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 (Nmm) (ultimate load the specimen sustained), yield moment (Nmm), stiffness (Nmm²) (the slope of the initial linear portion of the moment-displacement curve), post yield displacement (mm/mm²) (displacement at failure minus the displacement at the yield point), and work to failure (Nmm-mm/mm²) (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 (29). Peak stress was calculated from structural and cross-sectional properties using the equation (30):

$$\text{Stress} = \text{Moment} * c / I_{ml} \text{ (N/mm}^2\text{)} \text{ (c is the distance from the neutral axis to the cortex)}$$

$$\text{Young's Modulus} = \text{Stiffness} / I_{ml} \text{ (GPa)}$$

Bone composition

The distal right tibia (below the fibular insertion) and proximal tibial plateau were removed and the marrow flushed with phosphate buffered saline. Dry weight of the diaphyses was determined after drying in an oven at 100°C for 12 hours. 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 (30).

Statistical analysis

A One-way ANOVA and a LSD post hoc test assessed differences between the control and experimental groups at a significance level of $p < 0.05$ (Sigma Stat 3.0, SPSS Chicago, IL, U.S.A.). If the normality or equal variance assumptions were violated, a Kruskal-Wallis one-way ANOVA on ranks with a Dunnett T3 Method post hoc analysis assessed group differences (estradiol, uterine weights, Stiffness, T.Ar, T.Pm, Stress, Young's Modulus). Prior to statistical evaluations, the majority of outcome measures were found to scale with body weight and were normalized with a linear regression-based correction (31). 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. The correction decreased the variability in the data. The relationship of body weight to serum IGF-1 levels was determined by regression analysis ($p < 0.05$). The relationship of Vd.Ar to T.Ar was determined by regression analysis ($p < 0.05$). The dose response of bone strength measures to GnRH-a dose level was assessed using linear regression ($p < 0.05$). Results are presented as mean (SD) values. Trends were indicated when group differences were $2x$ the SEM value.

Results

Delayed puberty model and blood chemistry

A delay in the timing of puberty through GnRH-a injections was confirmed by the delay in the day of vaginal opening of the experimental groups compared to control. 50% of the animals in the Low Dose group did not reach puberty prior to sacrifice. 93% of the animals in the High Dose group did not reach puberty (Figure 1). The significant decrease ($p = 0.001$) in uterine and ovarian weights by 62.5–75.5% was a further indicator of the efficacy of the protocol to delay pubertal development (Table 1).

The experimental animals were significantly heavier (18%) compared to the control group at sacrifice but there was no difference between the Low and High Dose groups (Table 1). Femoral length was significantly ($p = 0.001$) shorter after the injection protocol, and the High Dose group was significantly shorter than the Low Dose group (Table 1).

Estrogen levels were 24% and 27% lower in the Low and High Dose experimental groups respectively after the GnRH-a injections ($p = 0.001$) (Figure 2). IGF-1 levels were 32% higher in the experimental groups ($p = 0.003$) and post hoc analysis did not find differences between the Low and High Dose groups (Figure 2). A positive control test to verify the assay results was completed on animals that were 23 days of age and 49 days of age. There was an expected increase in IGF-1 levels from 23 to 49 days of age and the results from the 49 day old group were not different from the control animals from the current study who were 50 days of age (Figure 2).

Mechanical properties

The delay of pubertal onset by 4–5 estrus cycles resulted in a deficit in mechanical strength of the cortical diaphysis, specifically peak moment was significantly ($p = 0.001$) less in the experimental animals compared to control animals by -13% and -17% respectively for Low and High Dose groups (Table 2). Stiffness was -18% and -23% lower than control animals in the Low and High Dose groups respectively ($p < 0.001$). The yield moment in the High Dose group was significantly lower than both the control and Low Dose groups ($p = 0.002$). However, the post-yield displacement in the High Dose group was significantly higher compared to the Low Dose group and control. Work to failure decreased in the Low Dose group ($p = 0.018$) compared to control and the High Dose groups. Calculated peak stress and Young's modulus were significantly lower in both experimental groups ($p = 0.001$), indicating that the bone in the Low and High Dose groups may have lower quality from a delayed puberty compared to control animals. Linear regression illustrated a significant dose response between GnRH-a dose and mechanical strength with the coefficient of determination (r^2) ranging between ($0.027 - 0.464$) (Table 2).

Structure, Geometry and Composition

Cortical bone area was significantly lower in the Low and High dose groups compared to control (Table 2). The percentage of cortical area per total area (Ct.Ar/T.Ar, %) was significantly decreased in the High dose group ($p = 0.001$) with a trend in the Low Dose group ($p = 0.079$) (Table 2). Total subperiosteal area (T.Ar) was unchanged by the delay in puberty although there was a significant increase in the moment of inertia in the medial-lateral plane (I_{ml}) in the High dose compared to Low dose ($p = 0.031$). Cortical width measured on the medial surface along the primary axis (Med.Ct.Wi) was significantly decreased in both the Low and High dose groups (Table 2). The lateral cortical width measure was at least 50% larger than the medial cortex (Table 2). However, the average cortical width (based on 8 measurements per bone) were not significantly different (Table 2). The ash fraction remained constant at approximately 69–70% across control and experimental groups ($p = 0.646$) (Table 2).

Kinetic Histomorphometry

Modeling results in large size and morphology changes in growing bone. In the current study, the bones drifted in the posterior-lateral direction (Figure 3) similar to previous studies (32). Periosteal labeled surface (L.Pm/Ps.Pm), an indicator of osteoblast number, was higher in the Low and High dose groups ($p = 0.007$). However the modeling activity was primarily on the posterior-lateral periosteal surface (Figure 3) with minimal labeled surface detected on the anterior-medial surface. On the endocortical surface, both formation and resorption occurred. Double labels indicating bone formation were measured predominately on one half of the

endocortical surface, specifically the anterior-medial surface (Figure 3). Endocortical labeled surface (L.Pm/Ec.Pm) decreased 25% in the High dose compared to control, but the result was not statistically significant ($p = 0.09$) (Table 3). The percentage of labeled surface from double labels decreased from 98.5% in the control group to 76% in the GnRH-a group. Bone formation rates (BFR) were significantly lower in the High dose group ($p = 0.044$) with a trend in the Low dose group. However, the MAR an indicator of osteoblast activity was not different between the groups. The posterior-lateral endocortical surface had porosity a possible indicator of increased bone resorption on that surface, however eroded surfaces were not directly measured.

Discussion

Delayed pubertal development by suppressing gonadotropin releasing hormone from the hypothalamus significantly diminishes bone strength development. Previous studies using GnRH-a injections to model delayed puberty successfully delayed the onset of puberty and reported effects on both cortical and trabecular bone but some studies did not directly measure bone strength or bone formation on the endocortical and periosteal surfaces and they did not assess the effect of increased dosage. Rakover et al. (33) reported a suppression of estradiol after 4 weeks of GnRH antagonist injections at a dosage of 125 ug/day. Femoral BMD, bone mineral content (BMC), trabecular density and cortical width were all lower after the 4-week injection period in the experimental group compared to the control group. However, Rakover et al. (33) did not measure bone strength and began the GnRH antagonist injections at 42 days of age which may have been after the onset of puberty. In our laboratory, the average day of vaginal opening is 31 days of age suggesting that a delay in the onset of puberty was not achieved by Rakover et al. (33) but a suppression of estrogen levels post-puberty (secondary amenorrhea) was achieved. Roth et al. (2005) (25) did successfully delay the onset of puberty in their relatively short protocol duration of 4 or 12 days. Significant reductions in cortical area related to body weight and periosteal circumference related to body weight were found. We have previously reported a transient decrease in bone strength at a low dosage of GnRH-a and long term effects on trabecular architecture measured by wavelet analysis (15,23,34). The current data illustrate that an increase in GnRH-a dose, modeling an increase in the severity of the delay in puberty, results in decreased strength values compared to the Low dose. The existence and mechanism of catch-up growth from this increased dosage remains to be investigated.

Delayed pubertal development resulted in a significant suppression of bone strength of the femoral diaphysis even with increases in body weight in the experimental groups. Multiple factors contribute to bone strength including the amount of bone, its distribution and the quality of the bone tissue. The amount of bone as measured by cortical bone area (Ct.Ar) decreased 8–10% in the Low and High dose groups. Furthermore, the cortical bone percent per total subperiosteal area also decreased with no significant change in total subperiosteal area, indicative of the trend towards an increase in marrow area (Table 2). This is also supported with the decrease in medial cortical width values by 10–18%. The cortical width may have been affected by the decreased bone formation rates on the endocortical surface, although the average cortical width was not different between groups. Decreasing bone formation on the endocortical surface would diminish the contraction of the marrow area and decrease the cortical width since total area was not significantly changed in the current study. However, cortical width measurements in growing bone with a modeling drift should be measured and interpreted cautiously. The polar moment of inertia (J) was not significantly different between groups. However there was a 6% increase in I_{ml} although there were no changes in total subperiosteal area or periosteal perimeter. The preservation of the total subperiosteal area could have maintained the bone strength; however, the calculated stress and Young's modulus values were significantly lower in the experimental groups. The ash fraction of the tibial diaphysis,

a measure of mineral content in bone was not different between groups; not a surprising result as Price et al. (2005) (18) reported that the variability in ash content is established early in life and thus is not a major accommodation strategy to alter stiffness during pubertal delay. Factors such as collagen cross-linking, mineral crystal size, collagen fiber orientation and microstructure organization may affect bone strength (35). Specific measures include changes in the number and size of lacunar and vascular porosity as well as proportions of woven versus lamellar bone (36).

In the current study, as the bones grew they also drifted in the posterior-lateral direction. The attainment of cortical area and moment of inertia during growth is based on the relative expansion of the periosteal and endocortical surfaces (18). However, it cannot be assumed that the periosteal surface only undergoes formation and the endocortical surface only resorption. Figure 3 illustrates that the endocortical surface has both formation and resorption activity. Therefore, systemic hormonal changes such as a suppression of estradiol may affect each surface differently. Percent labeled surface was greater in groups treated with the GnRH-a injection protocol but did not result in a significantly greater total subperiosteal area. The endocortical bone formation rate was lower, illustrating divergent responses of bone formation on the periosteal and endocortical surfaces. Systemic responses may affect bone formation and resorption by their ability to modulate local mediators of bone development (37), but local mediators such as local growth factors, bone marrow microenvironments and bone stress concentrations need to be elucidated. Direct measurements of eroded surface are necessary to measure the relative movement of each surface during bone development.

The hypothalamic suppression of GnRH resulted in decreased serum estradiol levels and greater IGF-1 levels. Decreased estradiol levels are associated with increased bone resorption (38–40). Studies of post-menopausal bone loss have identified correlations between medullary diameter and periosteal diameter and hypothesize that decreased estrogen levels resulted in increased endocortical resorption with a concomitant periosteal expansion. Periosteal expansion is a strategy that is theorized to maintain bone strength during the rapid loss of bone following the menopause (41–43). Our results suggest a similar adaptation response, a positive correlation exists between void area and total area ($R^2=.66$, $p < 0.01$) (Figure 4). However, the potential signaling mechanism between endocortical osteoclasts and the periosteal osteoblasts remains unclear but may result from local and systemic signals including changes in diaphyseal strain levels or greater levels of serum IGF-1.

During puberty, both somatic growth (Growth Hormone/ Insulin-like Growth Factor 1 (GH / IGF-1) axis) and reproductive maturation (hypothalamic-pituitary-gonadal (HPG) axis) affect bone development. Initially, the mechanism of low bone mass resulting from amenorrhea was attributed to increased bone resorption from suppressed estradiol levels. However, recent studies have hypothesized an “estrogen-independent” mechanism of bone loss with decreased bone formation that may be associated with serum IGF-1 levels (17,44). The growth hormone receptor knockout mouse which results in decreased serum IGF-1 levels does not have any adverse effects on trabecular bone but a significantly lower periosteal bone formation rate, decreased cortical area and thickness (45). We found significant increases in serum IGF-1 levels from a delayed puberty using GnRH-a injections. An increase in IGF-1 was also found in castrated rats (46) and ovariectomized (OVX) rats (47) as well, GnRH-antagonists increased follicular fluid IGF-1 in humans (48). Supplemental IGF-1 has been found to increase the percent of mineralizing perimeter on the periosteal surface in rats (49) which was similar to the current study in which increased serum IGF-1 levels were accompanied by increased periosteal labeled surface. However, this response did not rescue the diminished bone strength in the Low and High dose GnRH-a animals. Along with the increase in serum IGF-1 levels was a significant increase in body weight in the experimental animals. IGF-1 levels were positively correlated with body weight ($R^2 = .48$, $p < 0.05$). Higher body weights have been

reported to provide a protective effect against bone loss during OVX (50). Increased IGF-1 levels, an index of stimulation of the GH/IGF-1 axis, have been hypothesized to provide a protective effect independent of body weight on bone as measured by bone mineral content (BMC) (47). However, the increased bone formation and greater body weights in the current study did not result in structural and bone quality changes sufficient to maintain bone strength. An increase in serum IGF-1 levels may not always be indicative of positive changes in bone strength.

The increase in serum IGF-1 levels and body weight in models of suppressed estradiol (OVX) may occur through increased food intake (51) or an increased energy availability due to a decline in metabolic rate and decreased cage activity (47). IGF-1 is typically synthesized in the liver; however bone can also be a source of IGF-1. It is suspected that IGF-1 is released from bone during resorption (52,53). Therefore, an increase in endocortical resorption may increase serum IGF-1 levels. Further investigation is needed to determine the source of increased IGF-1 levels in this model of delayed pubertal onset.

Multiple variables will affect skeletal development during a delay in puberty including the age at which the delay occurs, the duration of delay and the severity of the delay. To understand fully the mechanisms that affect mechanical properties due to a delay in pubertal development and the long term consequences of such a delay necessitates an animal model that reflects closely the clinical characteristics of delayed puberty. In this study, two doses of GnRH-antagonist were used to determine the best dosage to replicate the physiologic conditions associated with delayed puberty. The dose response between GnRH-a and mechanical strength was significant. However, this model did replicate key factors reported clinically in patients with delayed puberty. The estradiol levels were suppressed in these animals similar to human athletes with primary and secondary amenorrhea (5,54,55). Furthermore, the significant deficits in bone strength and stiffness parallel the increase incidence of stress fracture seen clinically. However, there are limitations to this model. The increased body weights and increased serum IGF-1 levels are not consistent with clinical populations however the data do illustrate that bone strength deficits can occur even with higher serum IGF-1 levels suggesting that using hormonal assays to assess bone status should be done with caution. The GnRH-antagonist model of delayed pubertal development only affects the hypothalamic-pituitary-gonadal axis and clinically the condition of delayed pubertal onset may be a more complex interaction of somatic and reproductive maturation. Studies that treated amenorrheic dancers for 2 years with hormone replacement therapy found no difference in BMD between treated and placebo groups (17,56) suggesting that estradiol is not the only factor in the dancer's bone loss.

In summary, delayed pubertal onset modeled using GnRH-antagonist injections resulted in significant bone strength deficits. The suboptimal bone strength was due to decreases in cortical bone area and cortical width with no significant change in total subperiosteal area. A periosteal formation response, possibly stimulated by the decreased estradiol levels or increased endocortical resorption, did not result in an increase in periosteal perimeter and polar moment of inertia. The decrease in peak stress and Young's modulus may suggest that the bone formed was not of sufficient quality to increase the stiffness of the bone. However direct measures of bone quality such as collagen content, fiber orientation and micro structural measurements and nano indentation are needed.

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Figure 1.

Time of vaginal opening (VO) resulting from increased dose of GnRH-antagonist injections in a delayed puberty model. Control animals had 100% VO by day 35. In Low Dose, the lowest dose (1.25mg/kg/dose), 50% of the animals never reached puberty by sacrifice. However, the High Dose (5.0mg/kg/dose) group had only 1 animal reach puberty prior to sacrifice.

Figure 2.

Estradiol (pg/mL) and IGF-1 (ng/mL) levels after a delayed puberty model using a low and high dose of GnRH-a. Estradiol levels were significantly suppressed in all groups. IGF-1 levels were 32% higher than control in all groups. G1 and G2 are growth control groups; IGF-1 levels increased between day 23 and 49 and G2 levels are similar to the control group. * $p < 0.05$ versus C, # $p < 0.05$ versus G1.

Figure 3.

Femoral cross-sections from control and high dose animals illustrating the drift pattern from anterior-medial to posterior-lateral. Both endocortical and periosteal surfaces are forming bone and the endocortical surface has both formation and resorption surfaces.

Figure 4.
There is a positive relationship between void area (Vd.Ar) and total subperiosteal area (T.Ar).
 $R^2=.66$

Table 1

Summary of group differences in uterine and ovarian weights, femur length and body weight at sacrifice. Mean (SD).

Parameters	Groups		
	C (n=15)	Low Dose (N=15)	HighDose (N=15)
Uterine Weight (g)	.347 ± .110	.079 ± .004 [*]	.085 ± .013 [*]
Ovary Weight (g)	.080 ± .013	.030 ± .005 [*]	.017 ± .005 [*]
Femur Length (mm)	28.12 ± .90	25.28 ± .40 [*]	24.62 ± .83 ^{*,‡}
Body Weight at Sacrifice (g)	182.96 ± 15.85	215.75 ± 20.98 [*]	214.53 ± 15.33 [*]

^{*}
p<0.05 versus C

[‡]
p<0.05 versus Low Dose

Table 2
Effect of delayed puberty on femoral mechanics, geometry, structure and composition. Mean (SD).

	C (n=15)	Low Dose (n=15)	High Dose (n=15)	ANOVA p-value	r ² Dose [#]
Mechanical Properties					
Peak Moment (Nmm)	290.6 ± 26.2	239.1 ± 34.8 [*]	218.5 ± 21.4 ^{*,‡}	0.001	0.425
Yield Moment (Nmm)	268.5 ± 28.9	229.2 ± 39.7 [*]	189.2 ± 31.8 ^{*,‡}	0.001	0.464
Stiffness (Nmm ²)	8536.5 ± 1459.2	6549.6 ± 1343.5 [*]	5603.2 ± 682.3 [*]	0.001	0.416
Post Yield Displacement (mm/mm ²)	.0123 ± .0159	.0136 ± .0121	.0278 ± .0150 ^{*,‡}	0.009	0.205
Work to Failure (Nmm-mm/mm ²)	9.58 ± 2.61	7.38 ± 2.17 [*]	9.88 ± 2.9 ^{*,‡}	0.025	0.027
Stress (N/mm ²)	169.5 ± 33.6	136.5 ± 18.3 [*]	118.9 ± 15.9 ^{*,‡}	0.001	0.364
Young's Modulus (GPa)	3.34 ± .91	2.62 ± .60 [*]	2.03 ± .42 ^{*,‡}	0.001	0.361
Geometry					
I _{tot} (mm ⁴)	2.65 ± .41	2.52 ± .23	2.82 ± .39 ^{*,‡}	0.093	0.070
J (mm ⁴)	7.09 ± .97	6.84 ± .64	7.13 ± .94 [*]	0.614	
Ct.Ar/T.Ar %	50.1 ± 3.8	48.7 ± 3.6	46.2 ± 2.8 [*]	0.002	0.245
Structure					
Ct.Ar (mm ²)	4.05 ± .21	3.72 ± .27 [*]	3.62 ± .26 [*]	0.001	0.268
Ma.Ar (mm ²)	3.84 ± .56	3.79 ± .42	4.12 ± .40	0.127	
T. Ar (mm ²)	7.98 ± .59	7.66 ± .32	7.84 ± .54	0.241	
Ps.Pm (mm)	10.50 ± .42	10.26 ± .23	10.54 ± .51	0.152	
Vd.Pm (mm)	8.98 ± 1.18	9.37 ± 1.42	9.58 ± 1.86	0.568	
Ec.Pm (mm)	6.85 ± .68	6.93 ± .85	7.43 ± .57	0.066	
% Po/Ct.Ar	1.6 ± 1.3	2.6 ± 2.2	3.0 ± 3.2	0.282	
Med Ct.Wi (mm)	.39 ± .04	.35 ± .05 [*]	.32 ± .04 [*]	0.001	0.277
Lat Ct.Wi (mm)	.60 ± .15	.67 ± .21	.59 ± .19	0.544	
Avg Ct.Wi (mm)	.44 ± .05	.46 ± .06	.43 ± .04	0.388	
Composition					
Ash Fraction %	69.7 ± 1.1	69.8 ± 0.8	70.0 ± 0.9	0.646	

* p<0.05 versus C

[‡] p<0.05 versus Low Dose

[#] Linear regression of the variables versus GnRH-a dosage.

Table 3

Effect of delayed puberty on kinetic histomorphometry measurements on the femoral mid-diaphysis. Mean (SD)

	C (n=15)	Low Dose (n=15)	High Dose (n=15)	ANOVA p-value
Periosteal Surface				
L.Pm/Ps.Pm (%)	6.46 ± 5.97	12.48 ± 8.74 *	15.78 ± 7.54 *	0.007
MAR (µm/day)		N/A No Double Labels		
BFR/Ps.Pm (µm/day * 100)		N/A No Double Labels		
Endocortical Surface				
L.Pm/Ec.Pm (%)	53.21 ± 13.35	45.00 ± 25.83	39.89 ± 17.40 ‡	0.232
MAR (µm/day)	6.31 ± .91	5.77 ± 1.26	5.66 ± .80	0.245
BFR/Ec.Pm (µm/day * 100)	336.6 ± 83.9	312.8 ± 121.5	253.1 ± 75.3 *	0.116

*
p<0.05 versus C‡
p<0.090 versus C