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THE EFFECTS OF DELAYED PUBERTY ON THE GROWTH PLATE

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Abstract

Background—Many athletes are beginning intense training before puberty, a time of increased bone accrual when up to 25% of total bone mineral accrual occurs. Female athletes experiencing late or delayed pubertal onset may have open epiphyseal plates open that are vulnerable to injury. This investigation's purpose was to determine whether a delay in puberty (primary amenorrhea) affects the growth plate immediately post-puberty and at maturity.

Methods—Forty-eight female Sprague–Dawley rats (23days-of-age) were randomly assigned to four groups (n=12); short-term control (C-ST), long-term control (C-LT), short-term GnRH antagonist (G-ST) and long-term GnRH antagonist (G-LT). At 25days-of-age, daily gonadotropin-releasing hormone antagonist (GnRH-a; Cetrotide™, Serono, Inc.) injections were administered delaying pubertal onset. Left tibias were analyzed. Stained frontal slices of proximal tibia (5 μm thick) were analyzed in hypertrophic, proliferative and reserve zones for total height, zone height, and cell/ column counts. All procedures were approved by (IACUC) at Brooklyn College.

Results—Growth plate height was 19.7% wider in delayed puberty (G-ST) group and at maturity was 27.9% greater in G-LT group compared to control (C-LT) ($p<0.05$). No significant differences were found in short or long-term growth plate zone heights or cell/column counts between groups ($p>0.05$). Growth plate zone height normalized to total height resulted in 28.7 % larger reserve zone in the short term GnRH-a group (G-ST) but the proliferative zone was 8.5 % larger in the long-term group compared to the control group ($p<0.05$). Normalized to growth plate height a significant decrease was found in column counts in proliferative zones of the short and long-term GnRH-a groups.

Conclusions—Current data illustrates delayed puberty using GnRH-a injections results in significant growth plate height and decreases proliferative column counts and zone height \potentially contributing to decreases in bone mass at maturity.

INTRODUCTION

Female athletes are at an increased risk for acute and long-term skeletal injury such as stress fracture and osteoporosis [1-4]. Stress fracture prevalence is upwards of 31% in military recruits and 21% in athletes [5] and is disproportionately found in women [6]. In addition, many athletes are beginning intense training prior to puberty, a time of increased bone accrual [7-10].

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Delayed menarche (primary amenorrhea) and infrequent menstrual cycles (secondary amenorrhea) are two conditions that decrease estrogen levels during adolescence [11,12]. Warren et al. [13] reported strong correlation between increased age at menarche (delayed puberty) and a fracture incidence in ballet dancers of 61 % (46 of 75 dancers); 69% of these fractures were stress fractures [13]. Reduced bone accrual has also been linked to estrogen deficiency [14,15]. Investigators have identified bone densities in young (17–35 yr) athletic women with decreased estrogen levels similar to the bone densities of 51-year-old women [11,12].

While increased incidence of stress fracture and low bone mass may increase risk of osteoporotic fractures later in life in young women, evidence exists for “catch-up” growth [16,17]. That is, bone inhibiting and enhancing strategies early in life may not have long-term effects on bone strength due to continued growth occurring in juvenile bone. In elite female athletes, compensation for a delay in pubertal growth by late acceleration of linear growth was found [18], however other studies involving athletes suggest bone mass cannot be regained after [19] delayed puberty and amenorrhea during young adulthood [13-19]. Therefore, “catch-up” growth may depend on age of onset and severity of the condition affecting normal growth [16].

Estrogen in low doses stimulates growth hormone and results in the chondroblast progenitor cells in the reserve zone beginning clonal expansion [21,22]. Suppressed estrogen levels during delayed puberty and secondary amenorrhea may have a direct negative effect on the growth plate and thus bone development. Yingling and Xiang [23,24] reported delayed puberty and suppressed estradiol in young female rats decreased trabecular bone volume, trabecular number, and increased trabecular separation[23,24]. Therefore, an understanding of the effect of delayed puberty specifically on the growth plate and the potential effects on bone development in the short term and maturity are crucial and the purpose of this study.

METHODS

Research Design

A randomized control comparison group design. Independent variables were age at sacrifice (short-term, 42days, and long-term, six months of age) and group, (delayed puberty vs. normal puberty). Dependent variables were growth plate height, zone height, and growth plate zone cell and column number in the three zones of the growth plate.

Subjects

Forty-eight female Sprague–Dawley rats (25 days-of-age) were housed and randomly assigned to one of four groups; 1) short-term control group (C-ST) (n=12), 2) long-term control (C-LT) (n=12), 3) short-term GnRH antagonist group (G-ST) (n=12) and 4) long-term GnRH antagonist group (G-LT) (n=12). Gonadotropin-releasing hormone antagonists (GnRH-a) have successfully delayed the onset of puberty in female rats and have the advantage that normal hypothalamic-pituitary function is restored after cessation of injections [25]. Eighteen days of injections (0.2 ml) of saline (C-ST, C-LT) or GnRH antagonist (G-ST, G-LT) (100 μg/day) (Cetrotide™, Serono, Inc) were given intraperitoneally. Short and long-term groups received the GnRH-a for 18 days (day 25-42). However, short-term groups were sacrificed after last injection (day 42) and long-term groups were sacrificed at 6 months of age. Power =.88333291 analysis was calculated with the use of G*Power 3 [26].

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Brooklyn College (City University of New York). After sacrifice at either day

42 or six months of age, left tibia were removed, cleaned of soft-tissue and processed for histomorphometric analysis of the growth plate.

Specimen Preparation

Left tibiae were immersion-fixed in 10% neutral buffered formalin for 48 h, dehydrated with ethylene glycol monoethyl ether (Fisher, Fair Lawn, NJ), cleared in methyl salicylate (J.T. Baker, Phillipsburg, NJ) and embedded using methyl methacrylate with 15% dibutyl phthalate (Fisher Scientific, Fair Lawn, NJ) [27]. Undecalcified thin (5μm) frontal sections of the proximal tibia were cut using a Polycut microtome (Leica, Nusseloch, Germany). Thin frontal sections (5μm) were stained (Von Kossa and Gomori trichrome) to clarify cells in each zone of the growth plate. Slides were mounted and cover slipped using the mounting medium EuKitt (Hawthorne, New York). Von Kossa stained sections were used to measure growth plate height from reserve zone to hypertrophic zone. Gomori trichrome stain was used to identify cell nuclei (blue) and cytoplasm matrix (red/ violet) [28] for growth plate cell counting using a bioquantification system (Bioquant Image Analysis Corporation: Osteo II, Nashville, TN). Growth plate zone heights and cell/column counts were taken at the reserve, proliferative and hypertrophic zones. Cell/ column counts were taken in three areas on each growth plate: mid-medial, middle and mid-lateral (Figure 1). Zones were measured and delineated by the cells in each group. The very top of the proliferative zone columns served as the bottom of the reserve zone as well as the bottom of the proliferative columns served as the top of the hypertrophic zone. Protein and gene expression markers were not utilized. Intra-tester and inter-tester reliability was determined ($r = .995$; $r = .996$ respectively). Numbers of cells were quantified in the reserve and hypertrophic zones and the number of columns in the proliferative zone. In long-term groups the septae, bone cross-bridges spanning from the proximal and distal epiphyseal plates were quantified. Growth plate analyses were not blinded to the treatment group of the specimens being examined.

Data Analysis

Data are presented as mean ± SD. Growth plate heights, zone heights, number of septae and growth plate cell/column number at mid-medial, middle and mid-lateral sections were analyzed using independent t – tests at both time points. Cell count in the reserve and hypertrophic zones and cell/column count in the proliferative zone were also normalized to growth plate height (average cell count across all three sections/ growth plate height) and statistically analyzed using an independent t – test.

RESULTS

Body Weight

Body weight was 5.8% greater in the short-term GnRH-a group compared to control (183.80g ± 16.27g vs 195.10g ± 16.95g) ($p<0.05$). Body weight at the long-term time point (6 months of age) remained 8.5% heavier than the control long-term group (337.60g ± 27.86g vs 368.90g ± 59.68g) ($p<0.05$).

Growth Plate Height

Growth plate height was 19.7% greater in the short-term GnRH-a group compared to control ($p<0.05$). Growth plate height at the long-term time point (6 months of age) showed a shortening of the height compared to short-term but the experimental growth plates remained 27.9% larger than control long-term group ($p<0.05$) (Figure 2) .

Growth Plate Zone Height

There were no significant differences in both short and long-term growth plate zone height ($p<0.05$). Growth plate zone height normalized to growth plate height resulted in a 28.7 % larger reserve zone in the short-term GnRH-a group but the proliferative zone was 8.5 % larger in the long-term group than the control group ($p<0.05$) (Table 1).

Zone Cell/Column Counts

There were no significant differences in the cell number between the short-term control and GnRH-a groups of the resting zone in the mid-medial, middle and mid-lateral areas (Figure 3). Column number in the proliferative zone was not different between groups (Figure 3). Also, cell number in the hypertrophic zone was not significantly different (Figure 3). At six months of age (long-term groups), there were no differences in cell count number in the reserve (Figure 4), proliferative column count (Figure 4) or the hypertrophic (Figure 4) zone count between groups. Normalized proliferative column count was found to be statistically significant. Specifically, in the proliferative zone there was a significant decrease (21.7%) in column count in the GnRH-a short-term group compared to control and decreased (26.6%) in GnRH-a long-term group compared to the control group (Figure 5, Table 2).

Septae Cross Bridges

No significant differences found in septae cross bridge data in the long-term between groups (Figure 6).

DISCUSSION

The GnRH-a model significantly delays the onset of puberty [25,29] resulting in suppressed estradiol levels during growth [29] which results in significant trabecular bone volume[23] and cortical bone strength deficits [29,30] yet body weight was unchanged. Significant increases in body weight at sacrifice were reported after the same GnRH-a injection model as the current study both in the short-term (5.8 %) which have translated into a heavier animal in the long-term GnRH-a group (8.5 %) [23]. The current study showed an increase in body weight, increases in growth plate height but not in zone height except when normalized to growth plate height. When individual zone height is normalized to growth plate height increases are found exclusively in the proliferative zone.

No significant differences were found between control and experimental groups in the cell and cell column counts or zone heights in all 3 zones in both short and long-term groups. However when cell counts were normalized to growth plate height there was a decrease in cell column number per unit of height in the proliferative zone in both the short and long-term groups which may translate into fewer cells forming trabecular bone. Yingling et al. 2007 reported delayed pubertal onset using GnRH-a injections resulted in significant decreases in trabecular bone volume, number and increases in the separation of trabecular architecture in the short-term [23]. Yacker [31] reported that the proximal growth plates of the tibia of IGF – 1 deficient and acid labile subunit knockout mice (LID+ALSKO) were smaller in total height as well as in the height of the proliferative and hypertrophic zones of chondrocytes compared to controls translating to a 10% decrease in bone mineral density and a 35% decrease in periosteal circumference and cortical thickness were found[31]. Increased zone height would indicate potential for more growth but decreases in zone cell number would translate into less bone formation.

A delay in growth plate fusion may represent a mechanism to counter a decrease in trabecular bone volume. In the current study there was a significantly larger growth plate at both the short-term and long-term time points which may indicate a delay in the fusion

essentially increasing the length of time to form trabecular bone. However, there were no differences in the number of septae, bone cross bridging, in both long-term groups indicating a similar rate of fusion in both the GnRH-a animals and control at 6 months of age. Fusion would minimize the duration of bone volume accumulation, therefore the bone deficit in the GnRH-a animals at 42 days of age would be difficult to overcome. Normal fusion may have occurred since the estrogen suppression was only transient. Estrogen has been shown to decrease the resting zone cell proliferation and accelerates growth plate senescence [31] . Data from this study also showed delayed pubertal onset in the GnRH-a groups resulted in increased height but bone bridging would indicate that even though statistically growth plates are at different heights senescence was near with growth plate fusion at a time point similar to control animals. Delayed growth plate senescence following the dexamethasone injections was indicated by the lack of growth plate fusion in the experimental animals. After the 16 week period, the control group had 74% more fused growth plates in comparison to the dexamethasone group. Minimization of the duration of potential catch-up growth for future maximization of long-term bone mass architecture may be the real consequence of delayed pubertal onset.

The small difference in body weight and the similar septae cross-bridging suggests that there was no catch- up growth. Systemic and local mechanisms may affect growth plate cell proliferation and senescence independent of body size. Two hypothesized mechanisms have been proposed to indicate the potential of “catch up growth”. The neuroendocrine hypothesis suggests that a mismatch between the size of the organism and the target size results in growth that is sped up or slowed down to achieve this target normal size. The lack of difference in body weight in this study suggests that there was no catch up growth through this hypothesized mechanism and deficits in trabecular bone may be long term. The growth plate hypothesis suggests that the catch up growth happens in response to the delay in senescence following the period of suppressed growth. Resting zone chondroblast progenitor cells prepare and begin clonal expansion by stimulating growth hormone [21,22]. Proliferative cells then shift to the hypertrophic zone and chondrocytes transition into bone. Growth hormone and estrogen control the rate at which cells proliferate and move to the hypertrophic zone [7]. The increased growth plate height indicates that there was preparation for continued growth, however, septae bone cross-bridging were the same between groups in the long-term indicative of an equal timeline for growth plate senescence between the delayed puberty and the control. Communication between reserve and proliferative zones may allow the growth plate to catch up due to a local hormonal response and data suggests that there is a critical exchange between the reserve and proliferative zones.

Clinically, prevention is the key recognizing delays in menarche early and intervening could be the difference between healthy adolescent bones transitioning into healthy bones at maturity. Delaying puberty delays bone accrual. However, pubertal delay does not mean growth plate senescence is also delayed. Delay in pubertal onset using GnRH- a injections, results in suppression of estrodial levels and decreased trabecular bone volume, and increased growth plate height. In this study increased reserve zone height in the short-term and greater proliferative zone height in the long-term with long-term growth plates having fewer proliferative cells per micrometer of height were found. Increases in growth plate height in response to GnRH-a injections at 6 months of age would indicate an increase in catch-up growth is possible. However, a larger growth plate and a decreased number of proliferating cells create inadequate opportunity to recover bone mass leading to increased risk of fracture such as stress fracture during youth and potentially osteoporotic fracture in maturity.

Acknowledgments

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Clinical Relevance

Growth plate height increases indicate increased potential for growth and bone accrual. However, previous models report decreased bone volume following delayed puberty via GnRH-a injections which may have detrimental effects in the long-term.

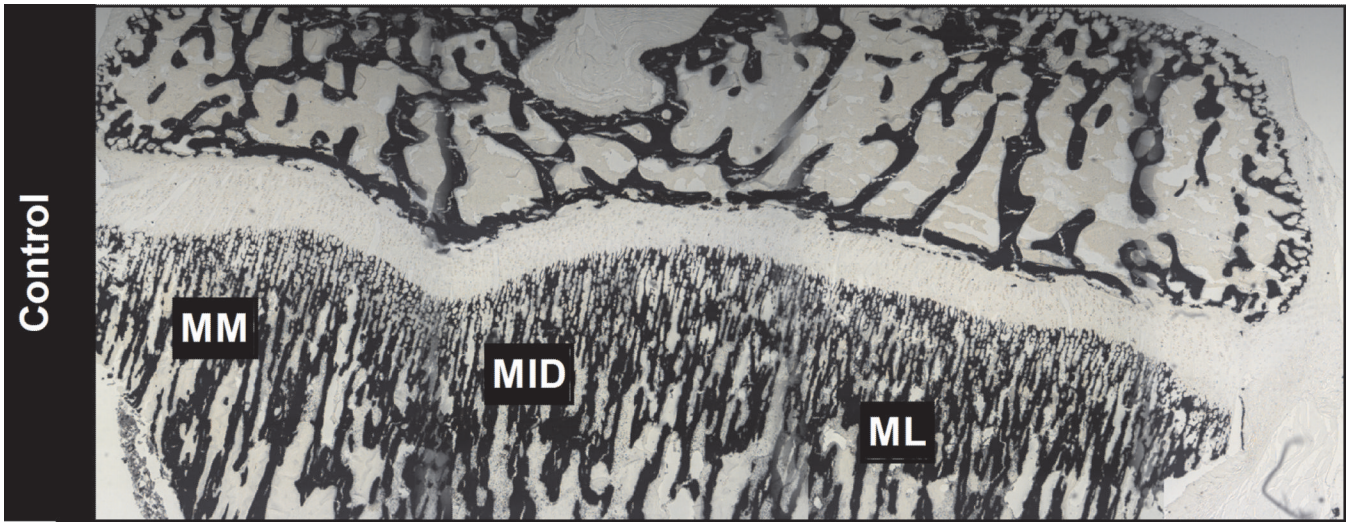

Figure 1.
Zone Cell/ Column sites for cell quantification; mid medial (MM), middle (MID) and mid lateral (ML). Magnification .04x

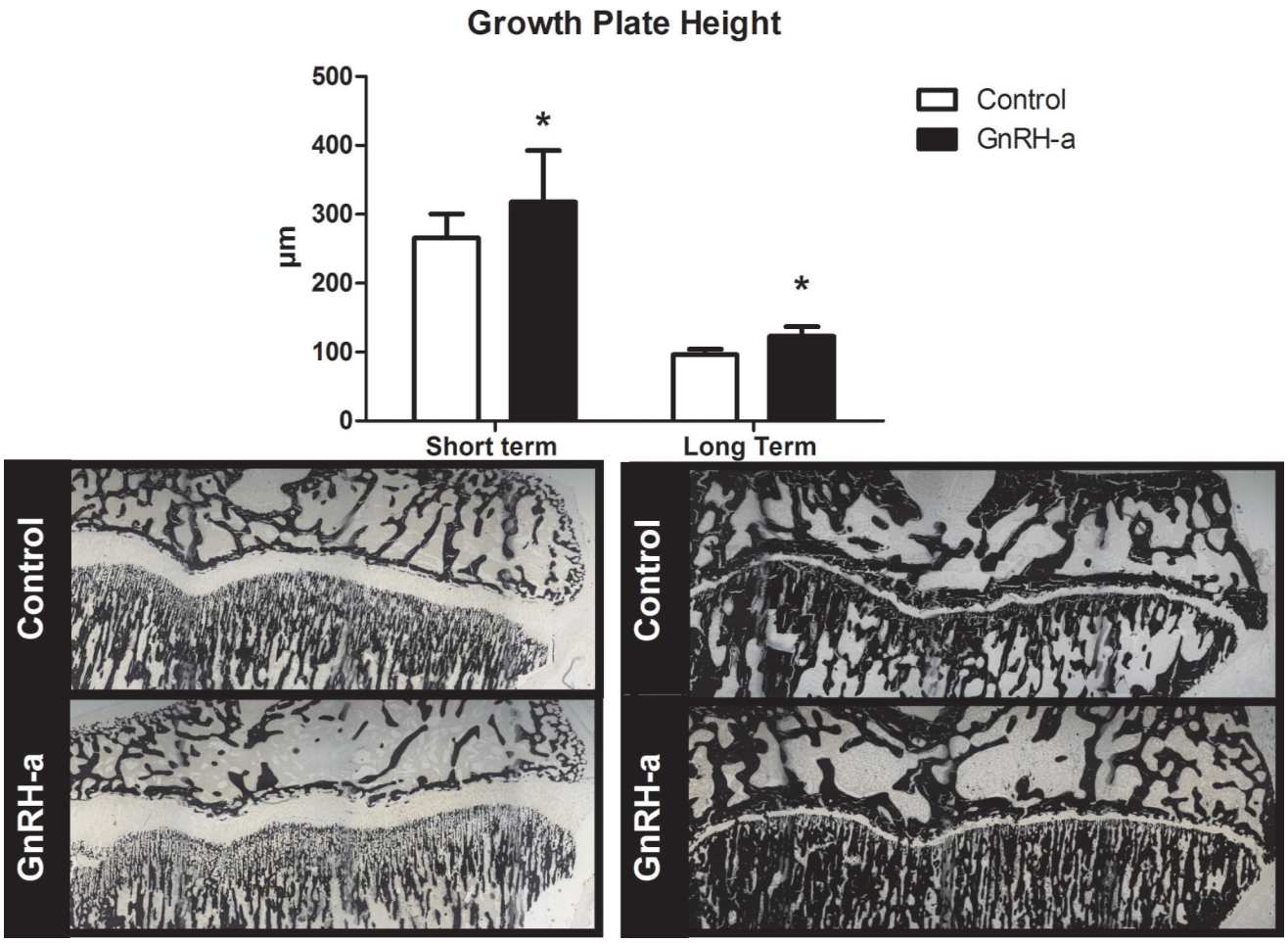

Figure 2.

Comparison of Growth Plate heights of short-term (ST) and long-term (LT) groups. (Top) The ST GnRH-a and LT GnRH-a animals had significantly larger growth plates compared to control. Height was measured in micrometers. * $p<0.05$ versus control. (Bottom) Representative images of the short-term and long-term growth plates for both the GnRH-a and control groups. Magnification 04x

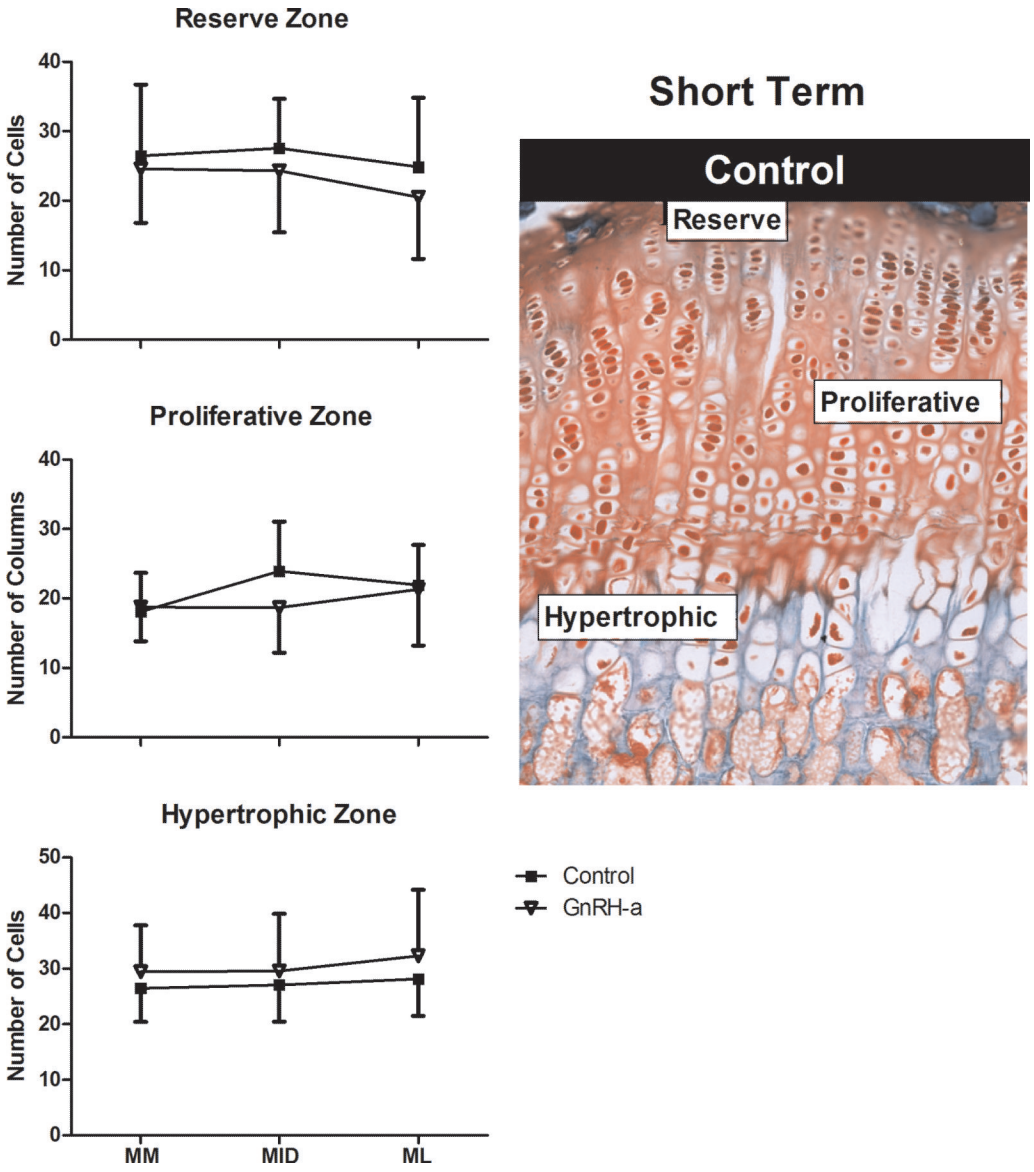

Figure 3.

Comparison of Growth Plate Zone cell/ column counts in the short-term. Cell and column counts were taken at mid medial (MM), middle (MID) and mid lateral (ML) growth plate for the resting, proliferative and hypertrophic zones. Magnification 40X

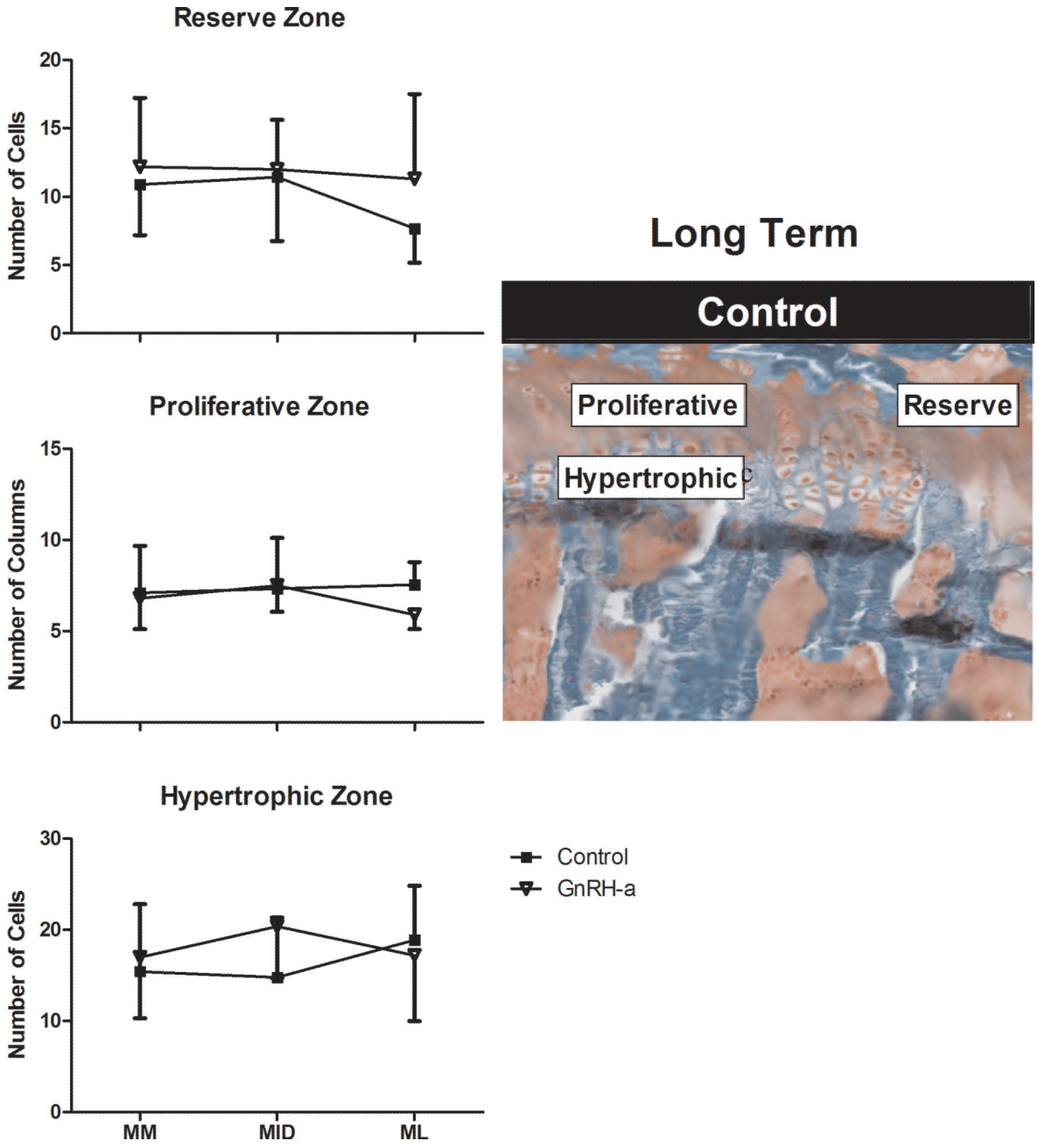

Figure 4.

Comparison of Growth Plate Zone cell/ column counts in the long-term. Cell and column counts were taken at mid medial (MM), middle (MID) and mid lateral (ML) growth plate for the resting, proliferative and hypertrophic zones. Magnification 40X

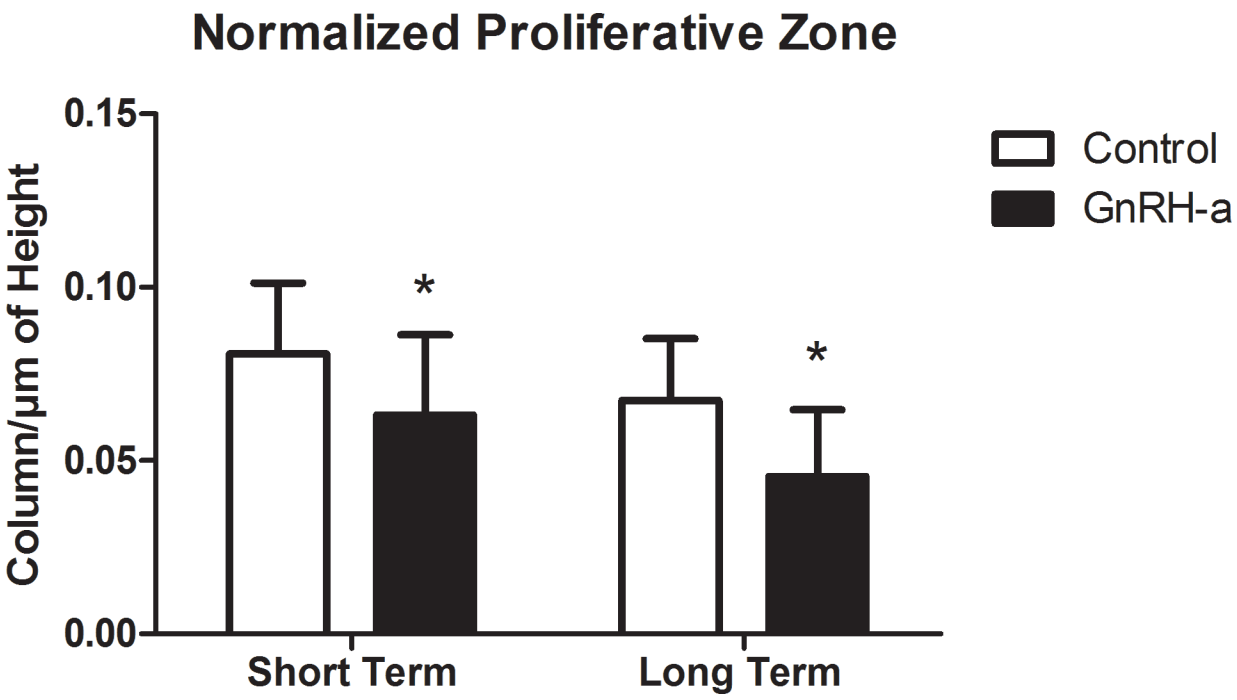

Figure 5.
Comparison of normalized growth plate zone cell column counts in the short-term and the long-term proliferative zone. Cell column count in the short-term and the long-term were normalized to growth plate height giving the amount of cell columns in the proliferative zone per micrometer of height. * $p<0.05$ versus control.

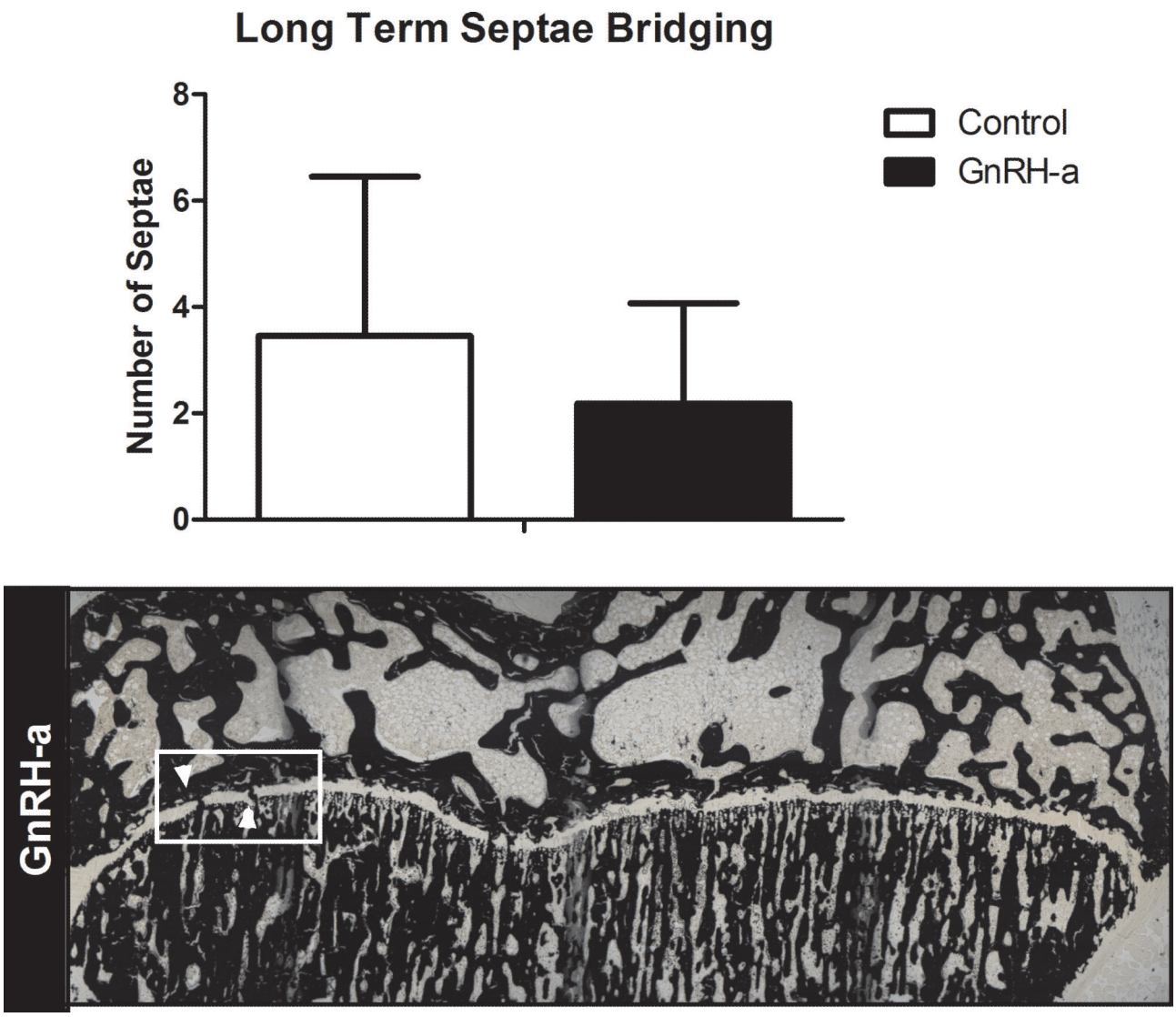

Figure 6.
Septae Bridging or Bone Bridging in long-term growth plates indicating signs of maturity. Septae in the long-term GnRH-a group were not different from control. (Bottom) Von Kossa stained image of the growth plate indicating septae. Magnification 04x.

Table 1
Short and Long Term Growth Plate Zone Data and Normalized Data

Short- Term and Long -Term Zone Width Normalized by Growth PlateWidth. Zone Widths were taken at the growth plate for the Reserve, Proliferative and Hypertrophic zones. Width was measured in micrometers.

	Short Term		Long Term	
Zone	**Control**	**Experimental**	**Control**	**Experimental**
Reserve (μm)	47.84 ±6.88	42.29 ±9.67	23.11±2.56	26.48 ±4.87
Proliferative (μm)	214.91 ± 42.46	196.88 ±59.57	65.89 ±9.56	63.35 ±10.03
Hypertrophic (μm)	107.18 ±13.84	123.12±37.47	48.37±5.07	48.63 ±10.51
N. Reserve (μm/ μm width)	5.55 ±.77	7.79 ±2.89 *	4.33 ±.25	4.99 ±4.84
N. Proliferative (μm/ μm width)	1.25 ± .20	2.00 ±1.78	1.53±.13	2.07±.29 *
N. Hypertrophic (μm/ μm width)	2.49 ±.44	3.15 ±2.70	2.10 ±.37	2.78 ±.73

Note: Values are normalized to growth plate width on day of sacrifice

* P<.05 compared to control.

Table 2
Short-term and Long-term Cell Count Data: Normalized

Short- Term and Long -Term Zone Cell Counts Normalized by Width. Cell and column counts were taken at mid medial (MM), middle (MID) and mid lateral (ML) growth plate for the resting (A), proliferative (B) and hypertrophic (C) zones. Width was measured in micrometers.

	Normalized Cell Counts			
Zone	**Short-term**		**Long-term**	
	Control	**Exp**	**Control**	**Exp**
Resting	.101 ± .026	.077 ± .026	.103 ± .022	.087 ± .016
Proliferative	.242 ± .061	.189 ± .052	.225 ± .047	.164 ± .037
Hypertrophic	.313± .067	.291± .078	.502 ± .147	.457± .187

Note: MM = Mid Medial Slice; MID = Middle Slice; ML = Mid Lateral Slice

* $p<0.05$ versus control.