Alfacalcidol prevents aromatase inhibitor (Letrozole)-induced bone mineral loss in young growing female rats

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Abstract

Long-term aromatase inhibitor use causes bone loss and increases fracture risk secondary to induced estrogen deficiency. We postulated that alfacalcidol (A; vitamin D₃ analog) could help prevent the Letrozole (L)-induced mineral bone loss. Fifty intact 1-month-old female rats were randomly divided into basal group; age-matched control group (AMC); L group: oral administration of 2 mg/kg per day; A group: oral administration of 0.1 µg/kg per day; and group L+A for a period of 8 weeks. Eight-week administration of L resulted in a significant increase in body weight, bone length, bone area, bone formation, and bone resorption activities when compared with the AMC group. However, the bone mass and bone mineral density (BMD) were significantly lower than the AMC group. Serum levels of testosterone, LH, FSH, and IGF-1 were significantly

higher and serum estrone and estradiol were lower along with a decrease in ovary+uterus horn weight, when compared with the AMC groups. None of those parameters were affected by A treatment, except suppression of bone resorption activities and increased trabecular bone mass and femoral BMD, when compared with the AMC group. Results of L+A combined intervention showed that bone length, bone area, and bone formation activities were higher than the AMC group, and the bone resorption activities were lower and BMD was significantly higher than that of the L group. This study demonstrates that the combined intervention of L and A not only enhances bone growth, but also increases bone density, and the effects of L and A are independent and additive.

Journal of Endocrinology (2009) **202**, 317–325

Introduction

Short stature associated with constitutional growth delay of puberty (CGDP), idiopathic short stature (ISS), and isosexual precocious puberty represent a pediatric endocrine challenge. Aromatase inhibitor (Letrozole, L) is an emerging therapeutic tool that can be of great hope for those children (Dunkel & Wickman 2001, Shulman *et al.* 2008). When children start their early puberty, estrogen has a major role in shaping and dictating future bone health through initiation of modeling that includes all stages of mineral acquisition, linear growth of long bone, and growth plate closure. During adulthood, remodeling is established to help keep bone mass and strength (Frost 2004), and estrogen plays a major role in the remodeling process also (Compston 2001, Vanderschueren *et al.* 2004).

Estrogen, mainly 17β-estradiol (E₂) via estrogen receptor α (ERα), is an essential regulator of bone maturation, growth plate fusion, and cessation of longitudinal growth (Nilsson *et al.* 2001).

During puberty, the maximal acquisition of bone mineral is achieved (Finkelstein *et al.* 1992, Bonjour 1998) via the effect of estrogen through the nuclear receptor ERα, and its modulator, ERβ (Bord *et al.* 2001, Nilsson *et al.* 2001,

Heldring *et al.* 2007). During this critical period of growth, children suffering from CGDP have reduced bone mineral acquisition with subsequent osteopenia in adult life (Finkelstein *et al.* 1992, Bonjour 1998); a clinical situation that will be further complicated by the use of L. During adult life, estrogen plays an important role in bone remodeling to maintain density, mineral content, and strength in both human males and females. Clinically, when there is estrogen deficiency, as in postmenopausal women, it is associated with bone mineral loss and osteoporosis (Albright *et al.* 1941, Riggs *et al.* 2002). Genetic estrogen deficiency states due to aromatase defects, and genetic ER resistance associated with severe osteopenia, and continued linear growth of the long bone with failure of growth plate closure (Carani *et al.* 1997), illustrate the major role of estrogen in bone maturation, and achieving final adult height.

L is a non-steroidal inhibitor of the aromatase enzyme system that is related to cytochrome P450. L, a potent competitive inhibitor of CYP2A6, moderately inhibits CYP2C19, and has a low affinity for CYP3A4. L causes depletion of estrogen via suppression of testosterone conversion to E₂ (Buzdar & Howell 2001, Buzdar 2003). This effect is utilized to increase the predicted adult height, in children with ISS and CGDP (MacGillivray *et al.* 1998,

Dunkel & Wickman 2001, Shulman *et al.* 2008). CGDP is reported in literature to be associated with reduced bone mineral acquisition, and reduced bone mass in adult life (Finkelstein *et al.* 1992, Bonjour 1998). The population of children with CGDP may utilize the L to improve the predicted adult height, and the induction of an estrogen deficient state after the treatment may further compromise the bone mineral acquisition (Carani *et al.* 1997, MacGillivray *et al.* 1998, Dunkel & Wickman 2001).

The emerging role of aromatase inhibitors in pediatrics and in breast cancer therapy stimulates a need to find a therapeutic intervention that can prevent or even reverse the bone mineral loss associated with the aromatase inhibitor (L).

1 α -Hydroxyvitamin D₃ (alfacalcidol, A), a pro-drug analog of calcitriol, has been used to prevent age-related bone loss in aged male rats (Li *et al.* 2004) and to treat postmenopausal women with osteoporosis (Nuti *et al.* 2006). A has been effectively used to prevent glucocorticoid-induced bone mineral loss in laboratory animals (Turnquist *et al.* 1992, Iwamoto *et al.* 2007). However, the efficacy of A in preventing bone loss induced by L is not known.

The current study was designed to evaluate the effect of A on preventing bone loss in pre-pubertal female rats treated with L.

Pre-pubertal female rats were used as opposed to male rats, as they have a high level of estrogens (E₁ and E₂) and lower pool of androgen (testosterone). This helps to evaluate the minor changes between both pools of sex steroids secondary to L therapy. Furthermore, female rats achieve their maturity and peak bone mineral deposition much faster than male rats (Jansson *et al.* 1983, Hefferan *et al.* 2003, Legato 2003).

Materials and Methods

Treatment of animals

Fifty female Sprague–Dawley rats, at 4 weeks of age, were purchased from Hilltop Lab Animals, Inc., (Scottsdale, PA, USA). They were maintained on a 12 h light:12 h darkness cycle at 22 °C with access to tap water and standard pellet diet (containing 0.93% calcium, 0.65% phosphorus, and 3 IU/g vitamin D₃ (Robert Laboratory Chow 5001; Ralston Purina, Madison, WI, USA)) ad lib. Ten rats were administered 10 mg/kg of calcein (Sigma Chemical) at 3 and 7 days after delivery, and then killed on the 8th day as the BASAL group before the experiment. We chose 4 day intervals of calcein labeling when the rats were 5 weeks of age during rapid growth stage, as we intended to avoid the first label resorption before the second calcein injection. One week after reception and adaptation to the environment, the rest of the 40 rats were randomized by stratified weight method into four experimental groups of 10 rats each; age-matched control (AMC), L, A, and L + A groups. L (Novartis International Pharmaceutical Ltd) was suspended in one-to-one mixture of distilled water and 1, 2-propanediol

solution, and was administered orally with a dosage of 2.0 mg/kg per day in 0.1 ml volume solution. A (Sigma Chemical) was dissolved in ethanol and diluted with saline-buffered solution with ethanol concentration <1% and was also orally administered with a dosage of 0.1 μ g/kg per day in 0.1 ml volume. The doses of L and A were determined based on the results of previous studies (Shiraishi *et al.* 2000, Erben *et al.* 2002, Goss *et al.* 2004, Gasser *et al.* 2006). Animals of AMC group received 0.1 ml vehicle saline solution per day. The body weight of the rats was monitored weekly for 8 weeks, the total duration of the experiment. The present study was carried out at the animal facility of the Winthrop University Hospital according to the National Institute of Health guide for Care and Use of Laboratory Animals, and the animal experiment protocols were approved by the Laboratory Animal Care Committee of Winthrop-University Hospital.

Preparation of specimens

Our previous experience suggests that 4 day intervals of calcein injection would be too short to distinguish the double labeling clearly when the animal's age is over 3 months or more, as their bone formation and bone turnover rates decrease compared with that of the 1-month-old animal. Therefore, all the rats, except the BASAL group, were labeled with 10 mg/kg of calcein (Sigma Chemical) injected i.m. 10 days and 3 days before they were killed. The animals were anesthetized with 80 mg/kg of ketamine injected i.p., together with 12 mg/kg of xylazine, and were killed by exsanguinations. The serum, right femurs, and the right tibiae were collected.

Serum biochemical analysis

Serum calcium levels were measured by an automated instrument (Dada Behring Model RXL., Bakersfield, CA, USA). Serum E₁, E₂, testosterone, LH, and FSH were measured using commercial kits (ALPCO Diagnostics, Salem, NH, USA). Serum levels of insulin-like growth factor-1 (IGF-1) were measured using rat-specific RIA kit (DSL, Inc. Webster, TX, USA). Serum levels of procollagen 1 N-terminal propeptide (PINP) as a bone formation marker (Melkko *et al.* 1996, Jensen *et al.* 1998, Shankar & Hosking 2006, Rissanen *et al.* 2008) were measured using rat-specific ELISA kit (IDS Inc. Fountain Hills, AZ, USA).

The femurs were used for the measurement of bone length, bone area, bone mineral content (BMC), and bone mineral density (BMD), as described below. The tibiae were used for static and dynamic bone histomorphometric analyses. The bones were fixed overnight in 40% cold ethanol, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphysis and tibial diaphysis with the fibular junction were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. The specimens were then dehydrated

sequentially in ascending concentrations of ethanol (70, 95, and 100%) and xylene, and then embedded in methyl methacrylate (EM Science, Gibbstown, NJ, USA) at 42 °C. Cross-sections of the tibial diaphysis, just proximal to the tibia-fibular junction, were sectioned at 8 µm thickness, and frontal sections of the proximal tibial metaphysis were sectioned at 5 µm thickness using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany) then transferred onto chromium–gelatin-coated slides, dried overnight under pressure at 62 °C, and cover slipped with Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY, USA) for static and dynamic histomorphometric analyses.

Bone histomorphometric analysis of the tibia

A digitizing morphometric system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasurement High Resolution Color Subsystem (OsteoMetrics) coupled to an IBM computer, and a morphometry program (OsteoMetrics). The measured parameters for cancellous bone included total tissue volume (TV), trabecular bone volume (BV), bone surface (BS), erosion surface (ErS), single- and double-labeled surfaces (sLS and dLS respectively), and interlabel width. These data were used to calculate percent trabecular BV/TV, trabecular number (Tb N), trabecular thickness (Tb Th), trabecular separation (Tb Sp), mineralizing surface (MS)/BS ((sLS/2 + dLS)/BS), mineral apposition rate (MAR), bone formation rate (BFR)/BS, and ErS/BS, in accordance with the standard nomenclature proposed by Parfitt *et al.* (1987). In the present study, the region of trabecular bone measured in the BASAL group rats was 1.5–4.5 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, while in the remaining four experimental groups was 1–4 mm distal to the lower margin of the growth plate. Since the animals in the BASAL group were 4 weeks old and were growing rapidly, it was necessary to skip 1.5 mm beneath the growth plate in order to avoid primary spongiosa. The area measured from 1.5 to 4.5 mm consists of secondary spongiosa and can be comparable with that of the 3-months-old experimental groups. In addition to the measurement of the above parameters, interlabel width beneath the growth plate was used to calculate longitudinal growth rate (LGR/day). The measured parameters for cortical bone were total tissue area, medullary area, and periosteal and endocortical BS, sLS, dLS, ErS, and interlabel width. These data were used to calculate cortical area, periosteal and endocortical MS/BS ((sLS/2 + dLS)/BS), MAR, BFR/BS, and endocortical ErS/BS.

Femoral bone area, BMC, and BMD

Bone area, BMC, and BMD of the whole right femur were determined by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-4500 Plus (Hologic Inc.,

Bedford, MA, USA). The instrument was adapted for an ultra-resolution mode, with a line spacing of 0.0254 cm, resolution of 0.0127 cm, and collimation of 0.9 cm diameter. The bone was placed in a petri dish, and to simulate soft-tissue density, tap water was poured around the bones to a depth of 1 cm. BMC and bone area were measured, and BMD of this area was calculated by dividing BMC by bone area. The coefficient of variation of these measurements at our laboratory was <1.0% (Iwamoto *et al.* 2007, Shen *et al.* 2008).

Statistical analysis

All the data was expressed as means and s.d. Multiple comparisons of data among the groups were performed by ANOVA with the Tukey–Kramer test. Two-way ANOVA was used to evaluate the respective effect of L and A and their interaction. All statistical analyses were performed using Prism-5.0 program on a Hewlett Packard computer. A minimal significance level of $P < 0.05$ was used for all the comparisons.

Results

Table 1 shows that there was no significant difference in the initial body weight among the experimental groups. The final body weight of the BASAL group was not different from the initial experimental groups either. In comparison with BASAL and AMC groups, animals of the BASAL group were in a rapid growth stage with relative high in LGR/day. After the 8-week experiment, LGR was decreasing with age; the bone length, size, and the density of femur were increasing. The final body weight, tibial LGR/day, femoral length, and bone area of the L group were significantly higher, and the weight of ovary + uterine horn and the femoral BMD was significantly lower than that of the AMC group. The final body weight, ovary + uterine horn weight, tibial LGR/day, and femoral length of the A group were not significantly different from that of the AMC group, but the femoral bone area, BMC, and the BMD were significantly higher than that of the AMC group. The combined intervention of L + A resulted in, not only a further increase in the body weight and femoral bone area, but also in an increase in the femoral BMC and BMD when compared with the L group. Results of two-way ANOVA showed that the effects of L on body weight, ovary + uterine horn weight, tibial LGR/day, femoral length, bone area, BMC, and BMD were significant, and the effects of A on body weight gain, femoral bone area, BMC, and BMD were significant. None of these parameters had any significant interaction between the L and A treatments. Therefore, these treatment effects are independent with a positive additive effect.

Table 2 shows that L had no significant effect on serum levels of calcium, but it significantly decreased the levels of E_1 and E_2 , and increased the testosterone, LH, FSH, IGF-1, and P1NP when compared with the AMC group. In comparison

Table 1 Body weight, ovary + uterine horn weight, femoral length, femoral bone area, bone mineral content, and the bone density of the experimental groups

Group	Femoral					
	Initial body weight (g)	Final body weight (g)	Ovary + uterine horn (g)	Longitudinal growth rate (µm/day)	Femoral length (mm)	Bone area (cm ²) BMC (mg) BMD (mg/cm ²)
BASAL	119±3.3	138±7.9	0.59±0.10	40.5±1.26	27.5±0.40	0.99±0.07 143±16.4 144.0±5.93
AMC	133±4.8 ^a	251±17.0 ^a	0.64±0.10	13.9±0.76 ^a	33.4±0.44 ^a	1.64±0.07 ^a 375±18.4 ^a 229.8±4.94 ^a
Letrozole (L)	133±6.9 ^a	342±23.8 ^{a,b}	0.33±0.07 ^{a,b}	16.2±1.07 ^{a,b}	35.5±0.55 ^{a,b}	1.80±0.09 ^{a,b} 393±17.3 ^a 217.8±4.41 ^{a,b}
Alfacalcidol (A)	134±4.9 ^a	262±17.6 ^{a,c}	0.67±0.13 ^c	14.3±0.98 ^{a,c}	34.0±0.69 ^a	1.78±0.07 ^{a,b} 444±25.5 ^{a,b,c} 249.7±5.87 ^{a,b,c}
L + A	138±7.3 ^a	375±28.7 ^{a,b,c,d}	0.28±0.06 ^{a,b,d}	16.3±0.89 ^{a,b,d}	35.7±0.77 ^{a,b,d}	1.91±0.09 ^{a,b,c,d} 456±34.7 ^{a,b,c} 236.8±8.04 ^{a,c,d}
Two-way ANOVA						
Letrozole	P<0.001	P<0.001	P<0.01	P<0.001	P<0.001	P<0.001
Alfacalcidol	P<0.01	NS	NS	NS	P<0.001	P<0.001
Interaction	NS	NS	NS	NS	NS	NS

Data are expressed as mean ± s.d. One-way ANOVA with Tukey comparison test was used to compare the data among groups. Two-way ANOVA was used to examine the effects of each factor, letrozole and alfacalcidol, and their interaction.
^aSignificant versus BASAL.
^bSignificant versus AMC.
^cSignificant versus (L) group.
^dSignificant versus (A) group.

with the AMC group, A administration resulted in a significant increase in serum levels of calcium, but had no significant effect on the serum levels of E₁, E₂, testosterone, LH, FSH, IGF-I, or P1NP. The experimental animals' daily intake of dietary vitamin D₃ was ~1.1 µg, calculated based on daily intake of chow diet 15 g (15 g×3 IU/g= 45 IU or 1.1 µg of vitamin D₃). Yet, a daily supplementation of A, 0.026–0.038 µg (0.1 µg/kg per day) in the A and A+L groups, resulted in a significant increase in serum calcium when compared with the AMC group. It showed that A is a highly potent vitamin D analog. This dose of A did not result in hypercalcemia in the current study. As an effect of A, the L + A combined intervention also resulted in an increase in the serum calcium, and as a result of L, there was a decrease in the E₁ and E₂ levels and increase in the testosterone, LH, FSH, IGF-1, and P1NP. Significant interactions of A and L on serum E₁, E₂, and LH respectively indicate that the combined intervention of A and L results in a further suppression of these serum levels. The significant interaction on serum P1NP indicates that the augmentation of P1NP by the L was suppressed by the A in combined intervention.

The static histomorphometry of trabecular bone in the proximal tibial metaphysis and cortical bone of the cross-section at proximal to the tibia–fibular junction was shown in Table 3. In comparison with BASAL and AMC groups, the trabecular BV (%) and thickness and the cortical total area and cortical bone in percentage were increased during the 8 week growth. L administration resulted in a significant decrease in the trabecular BV/TV and the Tb N and an increase in the Tb Sp when compared with the AMC group. Conversely, A administration resulted in a significant increase in the BV/TV, Tb Th, and Tb N and a decrease in the Tb Sp when compared with the AMC group. The results of combined intervention showed that trabecular bone mass, BV/TV, and the microarchitecture, Tb Th, and Tb N were higher and Tb Sp was lower than the L group. Two-way ANOVA showed that the effects of L and A on those parameters were independent.

The results of cortical bone did not show any significant difference among the experimental groups on total area, cortical area, or the cortical area in percentage (Table 3). However, the result of two-way ANOVA showed a significant effect of the increase by L on the total area, cortical area, and medullar area, and also an increased effect of A on the cortical area gain without a significant effect on the cross-sectional total area. The two significant interactions of L and A indicate a synergistic effect of the increase on bone gain in cortical area and a suppression effect of the increase on medullar area induced by L in the combined intervention.

While the experimental animals were rapidly growing during this 8-week period, the comparison of AMC with the BASAL groups shows that trabecular formation parameters, MS/BS, BFR/BS, and the resorption parameter of ErS/BS were decreasing with age (Fig. 1). L administration resulted in a significant increase in both MAR, MS/BS,

Table 2 Serum levels of calcium, estrone, estradiol (E₂), testosterone, LH, FSH, insulin-like growth factor-1 (IGF-1), and procollagen 1 N-terminal propeptide (P1NP) of the experimental groups

Group	Serum calcium (mg/dl)	Serum estrone (pg/ml)	Serum E ₂ (pg/ml)	Serum testosterone (ng/ml)	Serum LH (ng/ml)	Serum FSH (ng/ml)	Serum IGF-1 (ng/ml)	Serum P1NP (ng/ml)
BASAL	10.60±0.28	35.2±6.80	20.1±5.35	0.19±0.02	0.60±0.33	5.13±3.70	1304±133	8.07±1.04
AMC	9.80±0.44 ^a	24.9±3.45 ^a	21.8±2.93	0.17±0.04	0.59±0.23	3.60±2.81	1347±256	1.71±0.41 ^a
Letrozole (L)	9.80±0.21 ^a	15.5±7.28 ^{a,b}	13.1±5.87 ^{a,b}	0.66±0.22 ^{a,b}	1.40±0.36 ^{a,b}	14.1±2.88 ^{a,b}	1603±152 ^{a,b}	3.83±0.62 ^{a,b}
Alfacalcidol (A)	10.5±0.30 ^{b,c}	32.0±6.33 ^c	27.0±6.74 ^c	0.19±0.06 ^c	0.68±0.20 ^c	4.73±3.36 ^c	1430±188 ^c	1.49±0.21 ^{a,c}
L+A	10.2±0.19 ^{a,b,c}	14.7±6.76 ^{a,b,d}	9.6±3.75 ^{a,b,d}	0.61±0.29 ^{a,b,d}	1.05±0.28 ^{a,b,d}	15.4±3.70 ^{a,b,d}	1588±105 ^{a,b}	2.76±0.58 ^{a,b,c,d}
Two-way ANOVA	NS	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.01	P<0.001
Letrozole (L)	P<0.001	NS	NS	NS	NS	NS	NS	P<0.001
Alfacalcidol (A)	NS	P<0.05	P<0.05	NS	P<0.05	NS	NS	P<0.01
Interaction								

Data are expressed as mean ± s.d. One-way ANOVA with Tukey comparison test was used to compare the data among groups. Two-way ANOVA was used to examine the effects of each factor, Letrozole and alfacalcidol, and their interaction.

^aSignificant versus BASAL.

^bSignificant versus AMC.

^cSignificant versus (L) group.

^dSignificant versus (A) group.

BFR/BS, and the ErS/BS when compared with the AMC group. The trabecular MAR, Ms/BS, and BFR/BS of the A group were not significantly different from the AMC group, but the ErS/BS was significantly lower than the AMC group. The MAR, MS/BS, and BFR/BS of the L+A group were lower than the L group, but higher than both AMC and A groups. The ErS/BS of the L+A group was significantly lower than the L group, and not different from the AMC group.

In comparison with the AMC and BASAL groups, **Figures 2 and 3** show that periosteal and endocortical bone formation parameters such as the MS/BS, MAR and BFR/BS, and endocortical ErS/BS (%) as a resorption parameter were decreasing during this rapid-growth stage. L treatment resulted in a significant increase in the periosteal MAR, BFR/BS, endocortical MS/BS, MAR, BFR/BS, and the ErS/BS (%), while A had no significant effect on bone formation parameters of both surfaces, but a significant decrease in the endocortical ErS/BS in percentage in comparison with the AMC group. Bone formation parameters of both surfaces of the L+A group were significantly higher than that of the AMC. ErS of the L+A group was higher than the A group and not different from the AMC group.

Discussion

The emerging role of L in children with CGDP and ISS prompted our quest to investigate the efficacy of A in preventing bone mineral loss, while maintaining the therapeutic effect of L on bone elongation. Bone mineral loss associated with aromatase inhibitor use has been demonstrated in animal experiments and clinical studies (Thurlimann *et al.* 2005, Gasser *et al.* 2006). The current finding confirms that even though the experimental animals were in a rapid-growth stage, L treatment induces further bone elongation, bone area growth, and body weight gain; it also induces significant decrease in percentage of trabecular volume, Tb N, and BMD. Furthermore, the current study demonstrates that combined intervention of L and A does not only enhance bone elongation, bone area enlargement, and body weight gain, but also improves trabecular BV, microarchitecture, and bone density, with concomitant suppression of bone resorption in young rats. We also demonstrate that the effects of L and A on bone metabolism, bone density, and serum levels of estrogen and testosterone are independent and additive.

The results of our dynamic histomorphometry and serum bone turnover marker, P1NP, reveal that L treatment-induced increase in bone turnover rate and significant bone loss is associated with estrogen deficiency, since those phenomena bear a resemblance to the results observed in ovariectomized rat. When we examine the net BMC of L-treated group as measured by DXA, it may not be decreased when compared with the AMC group. Therefore, the

Table 3 Static histomorphometry of trabecular bone in the proximal tibial metaphysis and cortical bone of the tibial shaft at proximal to the tibia–fibular junction

Group	Proximal tibial metaphysis					Tibial shaft				
	Trabecular BV/TV (%)	Trabecular thickness (mcm)	Trabecular number (#/mm)	Trabecular separation (mcm)	Total area (mm ²)	Cortical area (mm ²)	Medullar area (mm ²)	Cortical percentage (%)		
BASAL	13.9 ± 1.96	42.9 ± 1.47	3.25 ± 0.42	270 ± 45	2.95 ± 0.24	2.32 ± 0.27	0.63 ± 0.05	78.4 ± 2.86		
AMC	21.3 ± 3.60 ^a	51.8 ± 3.91 ^a	4.10 ± 0.49 ^a	195 ± 29 ^a	4.14 ± 0.38 ^a	3.57 ± 0.31 ^a	0.56 ± 0.07	86.2 ± 1.85 ^a		
Letrozole (L)	13.6 ± 2.88 ^b	53.1 ± 3.24 ^a	2.55 ± 0.48 ^{a,b}	353 ± 84 ^{a,b}	4.38 ± 0.25 ^a	3.71 ± 0.25 ^a	0.67 ± 0.05 ^b	84.6 ± 1.43 ^a		
Alfacalcidol (A)	32.4 ± 3.05 ^{a,b,c}	59.1 ± 4.53 ^{a,b,c}	5.50 ± 0.66 ^{a,b,c}	125 ± 18 ^{b,c}	4.23 ± 0.33 ^a	3.65 ± 0.31 ^a	0.60 ± 0.08	86.3 ± 2.09 ^a		
L+A	21.3 ± 2.11 ^{a,c,d}	62.3 ± 4.11 ^{a,b,c}	3.43 ± 0.32 ^{b,c,d}	232 ± 27 ^{c,d}	4.51 ± 0.41 ^a	3.89 ± 0.36 ^a	0.62 ± 0.07	86.2 ± 1.92 ^a		
Two-way ANOVA	P<0.001	P=0.08	P<0.001	P<0.001	P<0.05	P<0.001	P<0.01	NS		
Letrozole	P<0.001	P<0.001	P<0.001	P<0.001	NS	P<0.01	NS	NS		
Alfacalcidol	NS	NS	NS	NS	NS	P<0.001	P<0.05	NS		
Interaction										

Data are expressed as mean ± s.d. One-way ANOVA with Tukey comparison test was used to compare the data among groups. Two-way ANOVA was used to examine the effects of each factor, letrozole and alfacalcidol, and their interaction.
^aSignificant versus BASAL.
^bSignificant versus AMC.
^cSignificant versus (L) group.
^dSignificant versus (A) group.

Figure 1 Bone dynamic histomorphometric analysis of trabecular bone of the proximal tibial metaphysis. Data are expressed as mean ± s.d. ANOVA with Tukey–Kramer test was used to compare the data among the groups ($P<0.05$). a, significant versus BASAL; b, significant versus AMC; c, significant versus L; d, significant versus A group. AMC, age-matched control; L, Letrozole; A, alfacalcidol, L+A, combined treatment of Letrozole and alfacalcidol; MAR, mineral apposition rate; MS/BS, mineralized surface/bone surface; BFR, bone formation rate.

increase in bone area and bone size without a concurrent increase in BMC could also contribute the decrease in the bone density.

Suppression of serum estrogen level is believed to be the primary mechanism through which L induces BMD loss and enhances bone elongation and bone growth. Our current finding of L treatment-enhanced serum levels of IGF-I with the increase in serum testosterone levels may provide another explanation of increased long-bone growth beside the cause of estrogen deficiency. In supporting this presumption, serum levels of GH have been reported to be increasing after 10 days of treatment with L to the peripubertal male mice (Eshet *et al.* 2004). Although the serum levels of IGF-I was not found to be increasing significantly by a relatively short time of 10 day L treatment, the IGF-I receptor in the tibial epiphyseal growth plate was reported to be increasing in comparison with the non-treated group. It is known that IGF-I is an important modulator in bone matrix formation, growth plate elongation, and improvement of bone mechanical properties (Van der Eerden *et al.* 2003). IGF-I has been utilized experimentally to induce new bone formation in an aged mouse model (Meinel *et al.* 2003), to improve bone mass in women with profound osteoporosis secondary to anorexia nervosa (Grinspoon *et al.* 1996), and to induce new bone formation in experimental segmental tibial defects (Fowlkes *et al.* 2006). On the other hand, it has been demonstrated that testosterone directly stimulates the expression of IGF-I receptor and increases chondrocyte proliferation on an *in vitro* model of mandibular condyle (Maor *et al.* 1999). Increased IGF-1 receptors are associated with testosterone-stimulated growth plate elongation (Nilsson *et al.* 1986, Abbaspour *et al.*

Figure 2 Bone dynamic histomorphometric analysis of periosteal surface of the cortical bone of the tibial shaft. Data are expressed as mean $\pm$ s.d. ANOVA with Tukey–Kramer test was used to compare the data among the groups ($P < 0.05$). a, significant versus BASAL; b, significant versus AMC; c, significant versus L; d, significant versus A group. AMC, age-matched control; L, Letrozole; A, alfacalcidol; L+A, combined treatment of Letrozole and alfacalcidol; MAR, mineral apposition rate; MS/BS, mineralized surface/bone surface; BFR, bone formation rate.

2008, Soliman *et al.* 2008), bone growth (Phillip *et al.* 2001) and body weight gain via augmentation of GH at target organs (Zung *et al.* 1999). Therefore, the increment in serum levels of IGF-I and testosterone, along with the increase in IGF-1 receptors and GH after aromatase inhibitor treatment, indicates that the GH/IGF-1 axes might be involved in mediating the effects of the aromatase inhibitor on the bone elongation and weight gain.

The effect of A on preventing osteopenia has been demonstrated using numerous animal models, such as ovariectomized rats, orchidectomized rats, aged rats, glucocorticoid-treated rats, and in human subjects (Shiraishi *et al.* 1999, Nishikawa *et al.* 2000, Li *et al.* 2004, Schacht *et al.* 2005, Iwamoto *et al.* 2006, 2007, 2008, Ringe *et al.* 2007, Majima *et al.* 2008). The anabolic and anti-resorptive properties of A have been observed and proposed as attributing factors to the increase in bone mass and improvement of bone strength. The effect of A on bone could be secondary due to an increase in intestinal calcium absorption and changes in parathyroid hormone (PTH), since D-hormone can reduce serum PTH levels (Schacht *et al.* 2005) and blocking parathyroid gland proliferation and secretion (Szabo *et al.* 1989, Brown *et al.* 2006). On the other hand, there is also evidence that A could have directly suppressed osteoclastogenesis *in vivo* by decreasing the pool of osteoclast precursors in bone marrow (Shibata *et al.* 2002). Therefore, the beneficial effect of A on bone metabolism is not only due to stimulation of intestinal absorption of calcium, thereby enhancing the positive balance of calcium, but also a significant suppression of endocortical and trabecular bone resorption while maintaining the periosteal,

endocortical, and trabecular BFRs, as we observed in the A-treated animals in comparison with the AMC group.

The advantage of combined intervention of A and L in promoting growth in young growing stage is that the enhancement of bone elongation, bone area growth, serum levels of IGF-1, LH, FSH, and testosterone by L is not suppressed by A, but is additive to the anti-resorptive effect of A. Furthermore, the suppression of estrogen by L is not affected by A either, and the side effect of L can be prevented by the combined intervention with A. These findings may imply a difference in functions of these two agents on bone metabolism in young growing rats. A may enhance calcium absorption from the gastrointestinal tract; suppress bone resorption and PTH secretion, while L enhances bone modeling and remodeling; increases bone growth and bone turnover. Interestingly, in the combined intervention of L + A, bone formation activities of the trabecular and periosteal bones remained higher than that of the AMC rat, yet the bone resorption activities of trabecular and endocortical surfaces were suppressed regardless of the serum level of estrogen that was decreased by L treatment. This indicates that the enhancement of bone gain in the combined treatment is independent from (not affected by) estrogen deficiency.

Such additive effect would be suitable to promote bone growth, bone gain in mass, and bone density and would improve the calcium balance in children with CGDP and ISS.

Third-generation aromatase inhibitors, L, have demonstrated efficacy for the treatment of postmenopausal women with hormone receptor-positive breast cancer as first- and second-line therapy for advanced metastatic disease (Buzdar *et al.* 2001, Buzdar 2003) via blocking the biosynthesis of

Figure 3 Bone dynamic histomorphometric analysis of endocortical surface of the cortical bone of the tibial shaft. Data are expressed as mean $\pm$ s.d. ANOVA with Tukey–Kramer test was used to compare the data among the groups ($P < 0.05$). a, significant versus BASAL; b, significant versus AMC; c, significant versus L; d, significant versus A group. AMC, age-matched control; L, Letrozole; A, alfacalcidol; L+A, combined treatment of Letrozole and alfacalcidol; MAR, mineral apposition rate; MS/BS, mineralized surface/bone surface; BFR, bone formation rate.

estrogen from testosterone. Thus, the theoretical advantage of this combined intervention in application to those women with hormone receptor-positive breast cancer will be preventing bone loss without affecting the suppression of estrogen biosynthesis.

In conclusion, L-treated young, female rats showed significant increase in bone elongation, bone growth with concomitant side effects of increased bone loss, bone separation, and reduced bone density. These side effects were effectively antagonized by the concomitant use of A without affecting the enhancement of long-bone elongation induced by L. This could be of clinical importance among pubertal children treated for CGDP and ISS. On the other hand, the combination therapy could be of great help for breast cancer patients treated with L, to prevent L-associated bone mineral loss in this group of patients, a theoretical advantage that needs further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Idromedical Cor under the service agreement with the Winthrop University Hospital (grant number 350-104).

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Received in final form 29 April 2009

Accepted 5 May 2009

Made available online as an Accepted Preprint
5 May 2009