

Effects of Antiresorptive Drugs on Long Bone Growth, Body Weight Gain, and Serum Lipid Levels in Young Adult Ovariectomized Rats

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Abstract

Although osteoporosis is common among the female elderly, in young adults who are associated with breast cancer treatment or under ovarian abrasion are also at risk to low bone mass. The present study aimed to investigate the benefits in preventing bone deterioration due to estrogen deficiency of antiresorptive drugs, i.e. conjugated equine estrogens (CEE), alendronate and raloxifene in young adult ovariectomized (OVX) rats. Effects of the drugs on body weight gain, serum total cholesterol and serum triglyceride levels were also investigated. Nine-week old female rats were treated orally on the day of ovariectomy with 3 mg/kg/day alendronate, 0.1 mg/kg/day CEE, or 3 mg/kg/day raloxifene for 8 weeks. The results demonstrated that ovariectomy caused a decrease in the growth of long bones as evaluated by the weight, length, volume and calcium content of the femurs and tibias. Ovariectomy also caused an increase in body weight gain and serum total cholesterol while it did not cause any changes in serum triglyceride. Alendronate and CEE, but not raloxifene could effectively prevent bone deterioration due to estrogen deficiency in the OVX rats. Among the three drugs, only alendronate exhibited no significant effects on body weight gain and serum lipids of the OVX rats. Estrogen, although it could delay the body weight increment and had cholesterol-lowering effects, it caused an increase in serum triglyceride. The present results have suggested that in young adult females who are subjected to estrogen deprivation, CEE or alendronate may be effective in preventing the bone from deterioration. However, their adverse events should be taken into account in the long-term use.

Keyword: Antiresorptive drug, Ovariectomized rat, Bone growth, Body weight gain, Cholesterol, Triglyceride

INTRODUCTION

Osteoporosis is a progressive systemic disease characterized by a decrease in bone mineral density (BMD) and microarchitectural deterioration of bone tissue¹⁻³. It is the most prevalent disease among postmenopausal women, resulting from a decrease in circulating estrogens. In young adults who do not achieve their ideal peak bone mass, they may develop osteoporosis at a much earlier age^{4,5}. A recent study has shown that significant decrease in BMD is occurring in women aged 25-45 at several skeletal sites⁶. In addition, young adults who are associated with breast cancer treatment⁷ or

subjected to glucocorticoid treatment^{4,7,8} or under ovarian abrasion^{4,8} are also at risk to osteoporosis.

Antiresorptive agents (agents that block bone resorption by inhibiting the activity of osteoclasts) and anabolic agents (agents that stimulate bone formation by acting primarily on osteoblasts) including estrogens, raloxifene, alendronate, vitamin D, terparatide, and new biologics are available for the management of osteoporosis^{1,3,4,9}. Among these, estrogens, raloxifene (a selective estrogen receptor modulator or a SERM) and alendronate (a well-known bisphosphonate) which are antiresorptive drugs are still commonly used.

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Several previous studies have focused on the benefits of antiresorptive drugs in increasing BMD in osteoporotic animal models¹⁰⁻¹⁵ or in postmenopausal osteoporotic women¹⁶⁻¹⁹ but data about their effects in the prevention of estrogen deficiency-related bone deterioration in young adults are limited. Therefore, the present study aimed to investigate the benefits in preventing bone deterioration due to estrogen deficiency of antiresorptive drugs, i.e. CEE, alendronate and raloxifene in young adult OVX rats.

Although the relationship between cardiovascular disease (CVD) and osteoporosis in the elderly has remained unclear, previous studies have demonstrated in women the association between CVD and osteoporosis severity²⁰, hip fracture²¹, or low bone mass²². In addition, weight gain^{13,23} and changes in serum lipid levels^{24,25} can occur after menopause or estrogen deprivation. Benefits of antiresorptive drugs on these parameters in osteoporotic postmenopausal patients^{17,26,27} or osteoporotic animal models^{13,14,28} have been reported. Whether benefits of the antiresorptive drugs, CEE, alendronate and raloxifene in preventing changes of body weight, serum total cholesterol and serum triglyceride are also achieved in the young adults associated with estrogen deprivation were also investigated in the present study using young adult OVX rat model.

MATERIALS AND METHODS

Animals

Nine-week old young adult female Sprague-Dawley rats weighing between 190-225 g were obtained from the National Animal Center, Mahidol University at Salaya Campus, Nakorn Pathom Province, Thailand. They were housed in the air conditioning room with a 12-hour light-dark cycle. The animals were fast overnight before drug administration in the morning. All rats had free access to water. The experiment protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University.

Experimental design

Bilateral ovariectomy or sham

operation was performed under ether anesthesia. The rats were randomly divided into 5 groups of 8-9 animals each, i.e. sham, OVX control and OVX rats treated orally with raloxifene (Celvista[®] 60 mg tablets, Elli Lilly) at a dose of 3 mg/kg/day, alendronate (Fosamax[®] 70 mg tablets, Merck Sharp & Dohme) at a dose of 3 mg/kg/day, or conjugated equine estrogens (CEE; Premarin[®] 1.25 mg tablets, Wyeth-Ayerst) at a dose of 0.1 mg/kg/day. The drug doses were taken from other studies regarding anti-osteoporotic purposes²⁹⁻³². Treatment was given started on the day of ovariectomy and continued for 8 weeks. The body weight was checked weekly. Blood was collected from tail tips under ether anesthesia at a 2-week interval. At the end of treatment, the right femurs and tibias of all sacrificed rats were carefully removed for the subsequent determinations.

Determination of bone growth (weight, volume and length)

The right femur and tibia (with fibula) were dissected and cleaned off soft tissues. The bone length was measured using a Vernier caliper (Naza, China). To determine the bone volume, trapped air in bone was liberated by immersing the bone in an unstopped glass bottle filled with deionized water. The bottle was placed into a sealed plastic bag connected to a vacuum generator for 60 min so that trapped air would diffuse out of the bone. The bone volume was measured with a plethysmometer (Ugo Basile, Italy). The bone was then dried to a constant weight.

Determination of bone calcium content

Dried tibia and femur of each animal were cut into small pieces, combined and burned to ash at 600°C for 12 h in a muffle furnace (Lenton SAF11/1, UK). The calcium content was determined using a flame atomic absorption spectrophotometer (GBC 932 Plus, Australia). Briefly, about 0.0300 g of ash was accurately weighed and dissolved in 1 ml of 6 N HCl. A volume of 4 ml of 60 mg/ml lanthanum was then added and the solution was further diluted with deionized water to 25 ml in a volumetric flask. The

resulting solution was filtered through a Whatman No.1 filter paper and an adequate amount of the filtrate was submitted for calcium determination. A standard curve was conducted using different calcium solutions, 2-760 ppm.

Determination of serum total cholesterol and triglyceride levels

All rats were fasted over night before blood sampling. Blood was collected from tail tips under ether anesthesia at weeks 0, 2, 4, 6 and 8 of ovariectomy. Serum was obtained following blood centrifugation for 10 min at 1000 rpm. Serum total cholesterol and triglyceride levels were determined using commercial assay kits (CPT Diagnostics, Spain).

Data analysis

Data were analyzed by computerized programs and expressed as means \pm SEM. Differences among groups were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Statistical significance was determined using Dunnett's post hoc and the p-value of less than 0.05 indicated a significant difference between groups.

RESULTS

Effect on the body weight

Ovariectomy caused a significant increase in the mean body weight of young adult rats since week 2 till the end of the 8-week period (Table 1). CEE and raloxifene, but not alendronate, could delay (for CEE) or prevent (for raloxifene) the body weight increment.

Effect on the bone growth (weight, length, volume and calcium content)

Eight weeks of ovariectomy caused a decrease in the growth of long bones of young adult rats as evaluated by the weight, length, volume and calcium content of the femurs and tibias although significant decreases could be observed only for some parameters, i.e. bone length and calcium

content (Table 2). Treatment with CEE or alendronate, but not raloxifene, could prevent the decrease in all those parameters. Moreover, alendronate could increase all the parameters, compared with sham rats, although significant differences could be observed only for tibia weight and calcium content. Compared with CEE, alendronate seemed to be more effective in promoting bone growth and it could also produce a significantly higher increase in bone weight and calcium content.

Effects on serum total cholesterol and triglyceride

Ovariectomy caused an increase in serum total cholesterol levels in young adult rats and significant changes were found compared with sham rats since week 2 (Table 3). CEE and raloxifene, but not alendronate, could prevent the increase in serum total cholesterol due to ovariectomy. In addition, they could significantly reduce serum total cholesterol when compared with the baseline or with the levels in sham group at the corresponding time points.

Serum triglyceride levels seemed to be gradually increased from the baseline in all groups and the increases were significant at week 8 of treatment (Table 4). Ovariectomy, however, did not cause any significant changes in serum triglyceride levels of young adult rats compared with sham rats. Alendronate or raloxifene had no significant effect on changes in serum triglyceride levels while CEE significantly increased the levels, starting from week 6, either compared with OVX or sham rats.

DISCUSSION

Body weight and fat mass tend to increase with menopause²³. In the present study, as expected, there was a significant increase in the body weight of the young adult rats since week 2 post-ovariectomy. In other studies, regardless of animal age, ovariectomy also caused a significantly higher increase in body weight gain compared with non-OVX rats or sham rats^{13,30,33,34}. The results of estrogen (CEE) and raloxifene which could attenuate the body weight gain

Table 1. Effects of antiresorptive drugs on the body weight (g) of young adult ovariectomized (OVX) rats

Treatment	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	% Increase
Sham	203.25 ± 2.37	221.63 ± 2.74	236.06 ± 4.25*	245.81 ± 4.62**	251.50 ± 5.22**	262.88 ± 5.64**	266.56 ± 5.65**	272.75 ± 6.52**	271.13 ± 6.20**	33.54 ± 3.56**
OVX control	203.50 ± 2.90	228.69 ± 3.92	259.63 ± 5.77	291.25 ± 6.04	307.44 ± 6.91	317.31 ± 7.25	323.50 ± 8.61	327.50 ± 12.08	334.69 ± 11.50	64.81 ± 6.70
Alendronate, 3 mg/kg/day	201.22 ± 2.13	228.78 ± 2.90	267.28 ± 3.40**	292.83 ± 4.11**	314.22 ± 4.85**	324.44 ± 5.46**	335.28 ± 6.23**	339.78 ± 5.91**	342.33 ± 7.71**	70.22 ± 4.00**
CEE, 0.1 mg/kg/day	204.94 ± 2.28	225.28 ± 4.24	251.44 ± 4.19	270.83 ± 6.07*	288.39 ± 6.51**	299.28 ± 5.97**	311.00 ± 5.81**	315.00 ± 7.29*	315.39 ± 7.90†	53.84 ± 3.16†
Raloxifene, 3 mg/kg/day	203.83 ± 2.51	215.74 ± 3.32*	236.00 ± 3.90*	244.89 ± 5.05**	256.44 ± 5.09**	257.22 ± 3.60**	266.22 ± 5.42**	262.44 ± 8.06**	267.94 ± 6.95**	31.39 ± 2.67**

Values are means ± SEM. There were 8-9 rats per group.

CEE = conjugated equine estrogens.

^a = (body weight at week 8 - body weight at week 0) x 100 / body weight at week 0

Significant difference from OVX group at corresponding time: * p < 0.05, ** p < 0.001.

Significant difference from sham group at corresponding time: † p < 0.05, ** p < 0.001.

Table 2. Effects of antiresorptive drugs on the growth of the femur and tibia of young adult ovariectomized (OVX) rats

Treatment	Weight (g)		Volume (ml)		Length (cm)		Total Ca (mg)	
	Femur	Tibia	Femur	Tibia	Femur	Tibia	Femur + Tibia	
Sham	0.458 ± 0.009	0.360 ± 0.005	0.380 ± 0.008	0.289 ± 0.011	3.313 ± 0.017**	3.765 ± 0.020*	213.704 ± 5.493*	
OVX control	0.431 ± 0.009	0.340 ± 0.011	0.353 ± 0.008	0.275 ± 0.011	3.194 ± 0.018	3.639 ± 0.030	193.387 ± 5.049	
Alendronate, 3 mg/kg/day	0.486 ± .007**	0.405 ± 0.010***	0.389 ± 0.008*	0.314 ± 0.010*	3.316 ± 0.029**	3.806 ± 0.027**	235.159 ± 5.076**	
CEE, 0.1 mg/kg/day	0.452 ± 0.007 +	0.353 ± 0.007**	0.379 ± 0.010	0.291 ± 0.009	3.298 ± 0.021*	3.738 ± 0.027*	208.445 ± 4.355†	
Raloxifene, 3 mg/kg/day	0.427 ± 0.008	0.337 ± 0.006	0.348 ± 0.006†	0.269 ± 0.005	3.186 ± 0.015**	3.620 ± 0.020**	190.809 ± 5.222	

Values are means ± SEM. There were 8-9 rats per group.

CEE = conjugated equine estrogens.

Significant difference from OVX group: * p < 0.05, ** p < 0.001.

Significant difference from sham group: † p < 0.05, ** p < 0.001.

Significant difference from alendronate group (compared with CEE group only): + p < 0.05, ** p < 0.001.

Table 3. Effects of antiresorptive drugs on serum cholesterol levels (mg/dl) in young adult ovariectomized (OVX) rats

Treatment	Week 0	Week 2	Week 4	Week 6	Week 8
Sham	101.65 ± 2.52	105.82 ± 2.17**	104.34 ± 2.37**	107.81 ± 3.01*	108.07 ± 2.57*
OVX control	99.61 ± 3.72	125.32 ± 3.78#	126.93 ± 2.14##	128.24 ± 4.71#	131.24 ± 2.94##
Alendronate, 3 mg/kg/day	101.06 ± 2.27	111.07 ± 4.53	123.74 ± 5.61#	122.82 ± 3.07#	125.99 ± 3.72###
CEE, 0.1 mg/kg/day	104.33 ± 4.33	95.18 ± 2.15***†	89.02 ± 1.89***#	92.01 ± 2.04***†	86.69 ± 2.51***††#
Raloxifene, 3 mg/kg/day	99.44 ± 2.37	97.86 ± 2.18**	90.35 ± 2.50***†#	90.79 ± 2.12***†#	84.44 ± 2.01***††#

Values are means ± SEM. There were 8-9 rats per group.

CEE = conjugated equine estrogens.

Significant difference from OVX group at corresponding time: * p < 0.05, ** p < 0.001.

Significant difference from sham group at corresponding time: † p < 0.05, †† p < 0.001.

Significant difference from week 0: # p < 0.05, ## p < 0.001.

Table 4. Effects of antiresorptive drugs on serum triglyceride levels (mg/dl) in young adult ovariectomized (OVX) rats

Treatment	Week 0	Week 2	Week 4	Week 6	Week 8
Sham	77.67 ± 3.71	77.41 ± 2.30	81.49 ± 3.27	84.01 ± 2.01	92.00 ± 2.14#
OVX control	76.25 ± 2.89	82.46 ± 4.94	90.94 ± 3.83#	89.78 ± 2.04#	95.04 ± 1.96#
Alendronate, 3 mg/kg/day	70.40 ± 3.31	76.49 ± 4.43	83.31 ± 1.89#	82.17 ± 2.65#	84.67 ± 2.50#
CEE, 0.1 mg/kg/day	73.45 ± 4.25	78.51 ± 4.02	85.98 ± 2.35#	106.12 ± 2.08***††##	113.07 ± 3.13***††##
Raloxifene, 3 mg/kg/day	74.03 ± 3.26	75.93 ± 3.25	80.51 ± 3.26	86.59 ± 2.68#	88.27 ± 3.21#

Values are means ± SEM. There were 8-9 rats per group.

CEE = conjugated equine estrogens.

Significant difference from OVX group at corresponding time: * p < 0.05.

Significant difference from sham group at corresponding time: † p < 0.05, †† p < 0.001.

Significant difference from week 0: # p < 0.05, ## p < 0.001.

in young adult OVX rats in the present study were in agreement with previous studies which showed that treatment with either estrogen^{13,30} or raloxifene^{13,14,30} could reduce body weight increment of OVX rats to nearly equal to sham rats. On the other hand, alendronate in the present study had no effect on the increment of body weight. Recent studies also showed that alendronate could not prevent the body weight gain of OVX rats^{30,33}. As seen in many studies including the present one, the body weight of animals is gradually increased after ovariectomy and antiresorptive drugs may have different effects on the body weight gain. The results can be therefore distorted if the data are expressed in terms based on the final body weight.

Bone growth, in humans as well as in animals, includes the progressive incremental changes in length, size and mass. Female rats usually stop growth at age 85-100 days but the epiphysis, however, never completely closes³⁵. The process of bone aging in rats has begun at the age around 40-50 weeks³⁶ and to support this, in the present study weight of the femurs and tibiae of sham rats at age 17 weeks (on the day of sacrifice) were lower than those at age 25 weeks³⁷. Sex steroids are known to regulate skeletal growth and maturation in both men and women^{3,38} and also in animals³⁹. Many studies have revealed deterioration to bone (evaluated by the decrease in BMD, bone weight, or calcium content) in animals, regardless of animal age, after ovariectomy^{33,37,40} and treatment with estrogens could correct or delay bone deterioration due to estrogen deprivation^{37,40}. To support the role of sex steroids in regulating skeletal growth in young adult animals, treatment with CEE in the present study could preserve normal bone growth as evaluated by the weight, length, volume and calcium content of the femur and tibia. Since rats, unlike humans, do not undergo epiphyseal fusion^{41,42}, they are probably appropriate for studying the effect of drugs on bone elongation when the effect from epiphyseal closure is excluded. The present study has therefore demonstrated that estrogens do have the role in

enhancing longitudinal bone growth when the process of bone aging is not reached. Raloxifene, unlike estrogen, although it promotes bone antiresorptive activity in aging OVX rats^{10,11} and in osteoporotic postmenopausal women^{43,44} where ovarian estrogens are depleted, it is generally known that SERMs should be avoided in menstruating women as they can cause further bone loss⁴. In addition, previous studies showed that raloxifene inhibited longitudinal bone elongation in rats without the effect to hasten epiphyseal fusion^{31,45}. Together with the result of the present study, raloxifene may not be appropriate for preserving normal bone growth in young adults associated with estrogen deprivation.

Although many studies could confirm the preventive effect of alendronate on the bone loss induced by estrogen deficiency in adult OVX rats^{29,33}, the results of alendronate on bone growth are limited. OVX rats treated with alendronate orally at the same dose as in the present study but only for 4 weeks could not cause any significant changes in the length and diameter of the femur²⁹. Long-term treatment with other bisphosphonates, ibandronate and risedronate, for 4 months dose-dependently increased the volume of vertebral trabecular and cortical bone and also tibia cortical bone of aging rats (2 years old)⁴⁶. Alendronate treatment in children with glucocorticoid-induced osteoporosis could inhibit the loss of long bones⁴⁷. Since alendronate can bind to hydroxyapatite during the process of mineralization which becomes buried by new bone formation^{9,48} and it also promotes osteoclast apoptosis^{9,49}, therefore taken together, these could be the reasons why treatment with alendronate in the present study could overcome the decrease in bone growth after ovariectomy in young adult rats when the process of bone aging is not reached.

In postmenopausal women^{24,25,27} as well as in OVX rats^{13,14,34}, estrogen depletion consistently causes an increase in cholesterol levels. Treatment with CEE and raloxifene, but not alendronate, could effectively prevent the increase in serum cholesterol throughout

the present study which was consistent with the results from many previous studies in OVX rats regardless of animal age when treated with estrogens^{13,30,50} or raloxifene^{13,30,51} or alendronate^{28,30}. In postmenopausal women, estrogens or estrogen plus progestin⁵²⁻⁵⁴ as well as raloxifene^{27,52,55} could also reduce total cholesterol and/or low density lipoprotein cholesterol (LDL-C) while alendronate again could not cause any significant changes in serum total cholesterol in these subjects^{19,27}.

Estrogen deprivation due to ovariectomy did not cause significant changes in serum triglyceride in the present study as well as in many other studies which the investigations were performed at different periods after ovariectomy^{12,34,51}. Previous studies have suggested that oral estrogens may influence plasma triglyceride by their actions on hepatic synthesis and release of triglyceride^{56,57}. Indeed, treatment with estrogen in postmenopausal women (estrogen alone or in combination with a progestin)^{24,50,53} as well as in OVX rats (estrogen alone)^{58,59} including also in young adult rats in the present study, could increase serum triglyceride levels. Furthermore, some studies revealed that oral estrogens could increase serum triglyceride levels in a dose dependent manner^{60,61}. Raloxifene and alendronate had neutral effects on serum triglyceride as found in the present study and other studies in OVX rats^{12,51,58} as well as in postmenopausal women^{19,27,55}.

In conclusion, CEE as well as alendronate provides not only the benefit on BMD in osteoporotic subjects as revealed by many previous studies, but may also have the benefits in preventing bone deterioration due to estrogen deficiency in the young adults. Moreover, they demonstrated no undesirable effects on body weight gain and serum total cholesterol levels. Estrogen, although it has cholesterol-lowering effects, it can cause an increase in serum triglyceride levels. On the other hand, although raloxifene provides the benefit on BMD in osteoporotic subjects as revealed by many previous studies and it also demonstrated no undesirable effects on body weight gain, serum total cholesterol and triglyceride levels, it may have no benefits

in preventing bone deterioration due to estrogen deprivation in the young adults. Taken together, the present results have suggested that among the three antiresorptive drugs (CEE, alendronate and raloxifene), CEE and alendronate as a monotherapy may be appropriate for the prevention of bone deterioration in the young adult females who are associated with estrogen deprivation. However, their particular potential adverse effects, i.e. gastro-esophageal ulcers from alendronate, increased risk of endometrial and breast cancers and also some CVS adverse events from estrogen should be taken into account especially in the long-term use.

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