



Original Article

Bone Density and Morphology of Mammary Glands of Ovariectomized Rats Treated with Combined Raloxifene and Alendronate

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Abstract Osteoporosis is the most prevalent disease among postmenopausal women, resulting from a decrease in circulating estrogens. Estrogens, raloxifene and alendronate are among the commonly used drugs for the management of osteoporosis. The relationship between estrogens and the risk of breast cancer is well documented; however, few data are available for other drugs. Recently, many studies have demonstrated the benefits of combined antiresorptive drugs in preventing bone loss. This study aimed to investigate the beneficial effect of raloxifene in combination with alendronate on bone density and also to investigate the effect of the drug combination on morphology of the mammary glands. Female Sprague-Dawley rats weighing 200-240 g were divided into 5 groups of 8-9 animals each, *i.e.* sham, ovariectomized control rats and ovariectomized rats treated with raloxifene at a dose of 3 mg/kg/day, alendronate at a dose of 3 mg/kg/day, and the drug combination. Treatment was given orally for 8 weeks. At the end of treatment, the right femur and tibia as well as mammary glands were carefully removed for determination of bone density and morphological changes, respectively. The results demonstrated that raloxifene either alone or in combination with alendronate could significantly increase the relative femoral and tibial bone density compared to ovariectomized rats and restore bone length to the sham levels. The drugs, alone or as a combination, exhibited neutral effects on morphology of the mammary glands. The present study provides evidence to support the benefit of combined antiresorptive drugs, raloxifene and alendronate, on the bone without increasing risk to develop disease of the mammary glands. ©All right reserved.

Keywords: alendronate, antiresorptive drugs, raloxifene

INTRODUCTION

Osteoporosis is a progressive systemic disease characterized by a decrease in bone mineral density (BMD) and microarchitectural deterioration of bone tissue, resulting in bone fragility accompanied by increased susceptibility to fractures. It is the most prevalent disease among postmenopausal women, resulting from a decrease in circulating estrogens. Several pharmacologic options are available for the management of osteoporosis including estrogens, raloxifene and alendronate, the commonly used drugs.¹⁻³

Estrogen has an impact on many body tissues and organs: bone, brain, blood vessels, heart, and reproductive organs. The relationship between estrogens and the risk of breast cancer is well-documented⁴⁻⁶ while few data are available for other antiosteoporotic drugs. Recently, several studies have found that greater skeletal benefits are obtained when two antiresorptive therapies are administered in combination, compared with either agent given alone.⁷⁻⁹ Alendronate added to ongoing hormone replacement therapy, in postmenopausal women with osteoporosis, produced increases in bone mass at the lumbar spine and

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hip trochanter that were greater than those seen with continued hormone replacement therapy alone.⁷ Due to several side effects of estrogen, other therapeutic options including drug combinations are convincing. The aims of the present study were to investigate the beneficial effect of raloxifene in combination with alendronate on bone density and also to investigate the effect of the drug combination on morphology of the mammary glands.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats weighing between 200-240 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. All rats had free access to a standard commercial pellet diet (C.P. Thailand) and tap water. The experiment protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University.

Experimental Design

The rats were divided into 5 groups of 8-9 animals each, *i.e.* sham, ovariectomized control rats and ovariectomized rats treated with raloxifene (Celvista® 60 mg tablets, Eli 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, and the drug combination. Treatment was given orally for 8 weeks. At the end of treatment, the right femur and tibia as well as mammary glands were carefully removed for determination of bone density and morphological changes, respectively.

Determination of Bone Density and Length

The right femur and tibia were removed and cleaned of soft tissues. The bone density was determined using a plethysmometer (Ugo Basile, Italy), calculated by Archimedes' principle and expressed as a density on the basis of body weight. The bone length was measured using a Vernier caliper (Naza, China).

Morphology Detection of Mammary Glands

The mammary glands were fixed in 10% neutral buffered formalin and the sections of 3- μ m were prepared by a rotary microtome (Shandon, England) and stained with hematoxylin-eosin. For each animal a uniform area was used for data collection. Terminal ducts (terminal mammary structures with diameters between 50 and 100 μ m), terminal end buds (structures of diameter greater than 100 μ m), and lobule types 1, 2 and 3 were visualized by a digital microscope (Nikon Eclipse E600, Japan) and quantified to determine the degree of differentiation. The number of terminal ducts, terminal end buds, and gradation of lobule type 1 (containing up to 11 alveolar buds), lobule type 2 (12-47 alveolar buds), and lobule type 3 (more than 48 alveolar buds) in 3 to 4 areas were counted per section.¹⁰

Data Analysis

Data were analyzed by computerized programs and expressed as means \pm S.D. One way analysis of variance (ANOVA) was used to compare data from each group. Statistical significance was determined according to the multiple-range test of Scheffe or Dunnett's T3 Post Hoc multiple comparisons and *p*-value of less than 0.05 indicated a significant difference between groups.

RESULTS

Bone Density and Length

Ovariectomy caused a significant decrease ($p < 0.05$ to $p < 0.001$) in the relative bone density (per 100 g body weight) as well as bone length of the femur and the tibia when evaluated after 8 weeks of operation. Treatment with alendronate at 3 mg/kg/day demonstrated no effects on bone density and bone length of ovariectomized rats. Raloxifene at 3 mg/kg/day either alone or in combination with alendronate could significantly increase ($p < 0.05$ to $p < 0.001$) the relative femoral and tibial bone density compared with ovariectomized rats and could restore bone length to the sham levels. A trend of synergistic effect of the two drugs on bone density was also demonstrated (Table 1).

Morphology Detection of Mammary Glands

A trend of decreasing the number of terminal end buds, terminal ducts, lobules - types 1 and 2 and total ducts was found in ovariectomized control rats compared with sham rats but no significant differences were

reached. The treatment groups (each drug alone or the combination) also showed no significant differences in the number of mammary ducts either compared with sham or ovariectomized control rats (Table 2 and Figure 1).

Table 1. Effects of combined raloxifene with alendronate on bone density and bone length of ovariectomized (OVX) rats

Group	N	Bone density (g/ml) per 100 g body weight		Bone length (cm) per 100 g body weight	
		Femur	Tibia	Femur	Tibia
Sham	8	0.432 ± 0.034 **	0.458 ± 0.036 *	1.27 ± 0.07 **	1.35 ± 0.07 **
OVX control	8	0.345 ± 0.030	0.379 ± 0.057	1.07 ± 0.08	1.13 ± 0.08
Raloxifene	9	0.438 ± 0.038 **	0.470 ± 0.036 *	1.28 ± 0.08 **	1.36 ± 0.09 **
Alendronate	9	0.341 ± 0.027	0.356 ± 0.032	1.05 ± 0.06	1.12 ± 0.07
Raloxifene + Alendronate	9	0.474 ± 0.028 **	0.509 ± 0.027 **	1.28 ± 0.05 **	1.35 ± 0.07 **

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

Table 2. Effects of combined raloxifene with alendronate on mammary glands of ovariectomized (OVX) rats, evaluated based on the number of terminal end buds, terminal ducts, lobule types and total ducts

Group	N	Number detected per visual field (x100) (mean ± S.D.)			
		Terminal end buds	Terminal ducts	Lobules - types 1, 2	Total ducts
Sham	8	0.10 ± 0.28	10.20 ± 3.32	12.35 ± 10.55	22.65 ± 9.74
OVX control	8	0.04 ± 0.11	7.49 ± 3.19	9.68 ± 6.03	17.18 ± 9.04
Raloxifene	9	0	9.63 ± 2.86	12.99 ± 4.16	22.61 ± 6.19
Alendronate	9	0.08 ± 0.23	6.18 ± 2.02	10.58 ± 3.68	16.82 ± 5.41
Raloxifene + Alendronate	9	0.02 ± 0.07	9.61 ± 6.38	12.33 ± 6.92	21.96 ± 12.59

Lobules - type 3 were not detected.

There were no significant differences in all treatment groups compared with the OVX control rats.

DISCUSSION

Bone Density and Bone Length

It is well known that in rodents after ovariectomy, bone loss occurs in the secondary spongiosa.¹¹ The increased bone turnover and cancellous bone loss that follow ovariectomy in rats have proven especially useful as an experimental model for postmenopausal bone loss.¹² The study by Murthy *et al.*¹³ showed that, 30 days post-ovariectomy in rats, the BMD of the neck region of femur and the proximal tibia had decreased by 15% and 17%, respectively. Ovariectomized rats of the present study also

exhibited a reduction in the bone density and length of the femur and the tibia.

In the present study, treatment with alendronate alone at a dose of 3 mg/kg/day for 8 weeks demonstrated no beneficial effects either on bone density or bone length while treatment with raloxifene could increase bone density as well as bone length. The advantage of raloxifene on bone density was supported by the following findings. Martel *et al.*¹⁴ showed that ovariectomized rats given raloxifene at doses of 0.01-1 mg/kg had 88-91% of the femoral BMD. Turner *et al.*¹⁵ also showed that, raloxifene treatment resulted in greater bone strength in the lumbar vertebrae

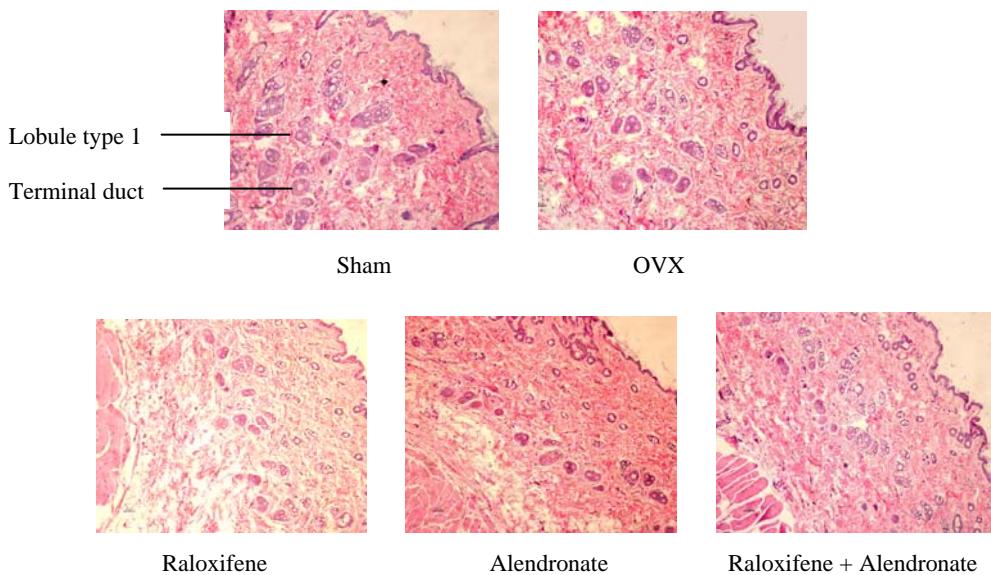


Figure 1. Microscopic morphological features of mammary glands in ovariectomized (OVX) rats treated with raloxifene, alendronate and the combination.

and femoral neck and greater BMD at the proximal tibia and lumbar vertebrae when compared with ovariectomized rats. The positive effects on bone biomechanical properties from raloxifene treatment were not different from those associated with ethinyl estradiol treatment. Sato *et al.*¹⁶ showed that raloxifene had effects similar to but not identical with ethinyl estradiol in preventing bone loss in vertebrae and tibiae.

Alendronate, an antiresorptive drug, could have effects in decreasing bone resorption and preventing bone loss in ovariectomized rats.¹⁷⁻¹⁹ Treatment with alendronate alone in the present study did not increase the density of the femur and the tibia. The difference may be at least partly due to differences in drug dosing.^{18,20,21}

Several studies found that greater skeletal benefits were obtained when two anti-resorptive therapies were administered in combination.⁷⁻⁹ Johnell *et al.*²² reported that combined raloxifene and alendronate reduced bone turnover more than either drug alone, resulting in greater BMD increment. Those findings were supported by the present results which demonstrated the synergistic effect on the bone density of the femur and the tibia in

drug combination-treated group, *i.e.* raloxifene and alendronate.

Effect of ovariectomy on bone length is inconclusive.^{12,23-25} In this study, longitudinal growth of the femur and the tibia was significantly inhibited by ovariectomy after 8 weeks of operation. Raloxifene but not alendronate could restore the length. Sliwinski *et al.*²⁵ also showed that the length and diameter of the femur were not different among the alendronate treated (3 mg/kg orally), ovariectomized and sham groups. Up to now, there were no studies about the effects of the drug combination and raloxifene on bone length.

Morphology of Mammary Glands

Estrogen and estrogen-like substances produced a rapid acceleration in the formation of progressively more differentiated mammary epithelial structures (such as lobules type 3 and 4).^{10,26,27} In the present study, a trend of decreasing the number of terminal end buds, terminal ducts, lobules - types 1 and 2 and total ducts was found in ovariectomized control rats compared to sham rats. The treatment groups did not show any significant differences in the number of mammary ducts.

These could be due to lack of estrogen-like effects on the mammary glands of raloxifene, alendronate, and the combination. In contrast, previous studies revealed that raloxifene could antagonize estradiol stimulation of mammary gland development in ovariectomized rats and caused regression of the mammary glands in intact female rats, and some studies suggested the role for raloxifene in the prevention of breast cancer.^{28,29} Up to now, there were no informative studies about the effects of alendronate and alendronate in combination with raloxifene on morphology of mammary glands.

In conclusion, we demonstrated that raloxifene either alone or in combination with alendronate could significantly increase the relative femoral and tibial bone density in ovariectomized rats and restore bone length to the sham levels. The drugs, alone or as a combination, exhibited neutral effects on morphology of the mammary glands. The present study provides evidence to support the benefit of combined antiresorptive drugs, raloxifene and alendronate, on the bone without increasing risk to develop disease of the mammary glands.

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