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# Effects of Single and Concurrent Intermittent Administration of Human PTH (1-34) and Incadronate on Cancellous and Cortical Bone of Femoral Neck in Ovariectomized Rats

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ZHANG, L., ENDO, N., YAMAMOTO, N., TANIZAWA, T. and TAKAHASHI, H.E. *Effects of Single and Concurrent Intermittent Administration of Human PTH (1-34) and Incadronate on Cancellous and Cortical Bone of Femoral Neck in Ovariectomized Rats*. Tohoku J. Exp. Med., 1998, **186**(2), 131-141 —— The purpose of this study is to determine the efficacy of concurrent treatment with human parathyroid hormone, hPTH (1-34), and bisphosphonate (incadronate) in augmenting cortical and cancellous bone mass of femoral neck in ovariectomized (OVX) rats. Forty-eight 11-week-old female Sprague-Dawley rats were divided into eight groups (six animals in each group). The baseline control group was killed at the beginning of the experiment, at 11 weeks of age. An ovariectomy was performed in thirty rats and twelve rats were subjected to a sham surgery. OVX rats were untreated for the first four weeks of postsurgery to allow for the development of moderate osteopenia. These animals were then subjected to various treatments with either PTH, incadronate, or PTH+ incadronate for a period of 4 weeks. Right proximal femora (femoral necks) were used for bone histomorphometry. After OVX 8 weeks, there was a significant decrease in cancellous bone mass and cortical bone area of femoral neck in the OVX rats when compared to the sham control rats. In OVX rats treated with PTH alone or PTH+incadronate were completely restored lost cancellous and cortical bone mass of femoral neck by increase bone formation. The bone formation parameters (OS/BS, MS/BS) and bone turnover (BFR/BV) seen with PTH plus incadronate were similar to those seen with PTH treatment alone. This indicates that incadronate did not blunt the anabolic action of PTH when used concurrently. Our results suggest the followings: 1) the femoral neck of OVX rats is a suitable sample site for preclinical studies of the prevention of bone loss induced by estrogen depletion; 2) concurrent use of incadronate did not blunt the anabolic effect of PTH; 3) concurrent treatment showed the best results in restoring cancellous and cortical bone mass; and 4) it had additional benefits for bone strength independent of that achieved by the increase in bone mass. ———— femoral neck; bone histomor-

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phometry; concurrent treatment; parathyroid hormone; incadronate 

Osteoporotic hip fractures are most prevalent among the elderly. It is important to develop therapeutic protocols to treat osteoporosis and to decrease the incidence of hip fractures. Ovariectomized (OVX) rats are widely accepted as a model for estrogen-deficient osteoporosis in humans (Kalu 1991; Wronski and Yen 1991b; Frost and Jee 1992), and the femoral neck area appears superior to other skeletal sites routinely used for bone histomorphometry in the OVX rat model (Yamamoto et al. 1995; Bagi et al. 1997). Thus, it is important to understand the effects of new therapeutic agents for the prevention and treatment of osteopenia, especially the femoral neck of OVX rats that is a more clinically relevant skeletal site.

It has been shown that intermittent administration of parathyroid hormone has an anabolic effect in experimental animals (Hock et al. 1988; Liu and Kalu 1990; Takahashi et al. 1991; Dempster et al. 1993) and in patients with osteoporosis (Slovik et al. 1986; Lindsay et al. 1997). Bisphosphonate treatment protects OVX rats from osteopenia by the suppression of bone turnover (Wronski et al. 1989; Wronski and Yen 1991a). Thus, it is hoped that the concurrent treatment of anabolic and anti-resorptive agents may be more beneficial for increasing bone mass than the effects of either agent alone, because these agents might accelerate bone formation while suppressing bone resorption. From this point of view, concurrent treatment of parathyroid hormone (PTH [1-34]), a potent stimulator of bone formation, and bisphosphonates, a potent inhibitor of bone resorption, may be a beneficial combination. Incadronate, one of the newer bisphosphonates, is expected to be an antiresorptive agent without inhibiting bone formation when given in proper doses (Fujimoto et al. 1990; Nagao et al. 1990; Motoie et al. 1995). However, there are no histomorphometric data describing the changes of cancellous, cortical bone and their relation to bone strength during the concurrent treatment in the femoral neck of OVX rats. The purpose of this study is to evaluate the therapeutic efficacy of concurrent use of PTH plus incadronate for the restoration of lost cancellous and cortical bone mass of femoral neck in OVX rats. In addition, the following question was investigated by the present study: Does concurrent use of bisphosphonate blunt the anabolic effect of PTH ?

## Materials and Methods

### *Animal care and study protocol*

A total of forty-eight 11-week-old female Sprague-Dawley rats (Charles River Japan, Laboratories, Kanagawa) were used in the experiment. The animals weighed approximately 250 g at the beginning of the experiment. They were acclimated to local vivarium conditions (at 22°C with a 12-hour light/12-hour dark cycle) for two weeks. The rats were housed in individual cages and were fed

a pelleted commercial standard diet which contained 1.2% calcium, 0.85% phosphorus and 80 I.U. vitamin $D_3$/100 g (Oriental Yeast Co., Ltd., Tokyo). All rats were given food and water ad libitum during the experimental period. The animal experiment protocol was approved by the Animal Care and Use Committee of Niigata University School of Medicine.

Synthetic human PTH (1-34) (Asahi Chemical Industry Co., Ltd., Shizuoka) was prepared in a vehicle of acidified saline containing 0.1% bovine serum albumin. Incadronate disodium (Yamanouchi Pharmaceutical, Tokyo).

The animals were divided into eight groups of six animals each (Fig. 1). Bilateral ovariectomies (OVX) were performed on 30 rats through a dorsal approach. Twelve rats were subjected to sham surgery in which the ovaries were exposed but not removed. The baseline control rats (Group 1) were killed at the beginning of the experiment with 11 weeks of age. All the other animals were left untreated for the first 4 weeks of post-surgery. Four weeks after ovariectomy, the sham group (Group 2) and OVX (Group 3) rats were killed. The remaining rats were divided into five groups based on the following treatments. Vehicle was injected into the sham-operated rats (Group 4) or ovariectomized rats (Group 5); PTH (30 $\mu$g/kg/day, 5 days/week) was injected into the ovariectomized rats (Group 6); incadronate (1 $\mu$g/kg/day, 3 days/week) was injected into the OVX rats (Group 7); or a combined treatment with PTH+incadronate to OVX rats (Group 8). All of the treatment was initiated at 4 weeks postsurgery for a four-week period. All the animals were double-labeled with subcutaneous injections of 10 mg/kg of calcein (Wako Pure Chemical Co., Osaka) six days and two days before euthanization. All the rats were killed by exsanguination from the

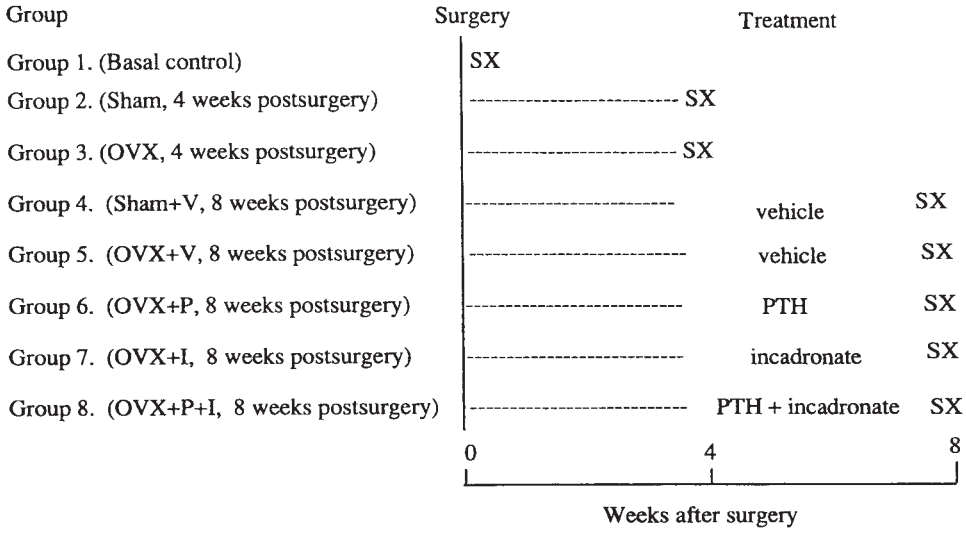


Fig. 1. Experimental protocol. Sham +V (Sham+Vehicle); OVX+V (OVX+Vehicle); OVX+P(OVX+PTH); OVX+I(OVX+incadronate); OVX+P+I(OVX+PTH+incadronate). PTH (30 $\mu$g/kg/day, 5 days/week) and incadronate (1 $\mu$g/kg/day, 3 days/week). OVX, ovariectomy; SX, rats killed.

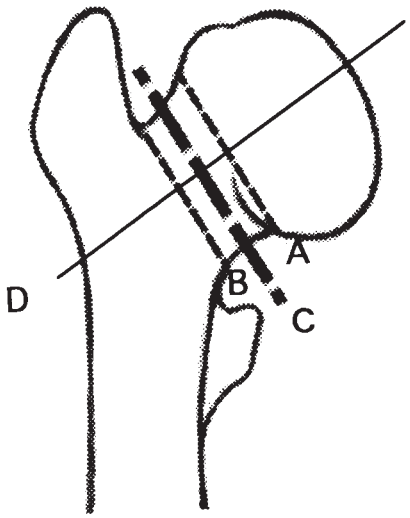


Fig. 2. The area used for bone histomorphometric analysis.
Right femur was sawed through the subcapital line (A) and the basal neck line (B) with a low-speed metallurgical saw to obtain femoral neck samples. Femoral midneck line (C) is perpendicular to the longitudinal axis of neck (D) for the section of bone histomorphometric measurement.

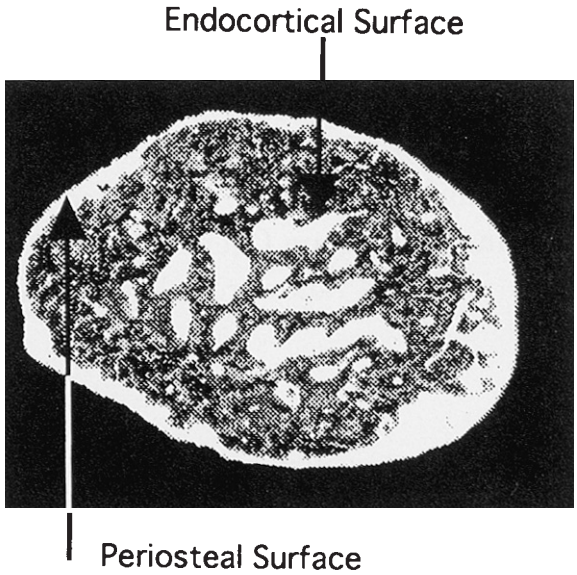


Fig. 3. Cross-sections at the femoral midneck (Fig. 2C) were chosen for histomorphometric analysis of cancellous and cortical bone, as explained in Materials and Methods.

abdominal aorta under ether ketamine hydrochloride and xylazine anesthesia.

*Bone histomorphometric measurements of cancellous and cortical bone of femoral neck*

The right femora were sawed through the subcapital line and the basal neck line (Fig. 2) with a low-speed metallurgical saw to obtain femoral neck samples. Each sample was removed and fixed with 70% ethanol, and then dehydrated in ascending grades of ethanol, defatted in an acetone/ethanol mixture (1 : 1), and

embedded in methyl-methacrylate without decalcification. Then the samples were mounted on plastic slides and ground to 50 $\mu$m using a precision lapping machine (Maruto Co., Tokyo) to examine the midneck region (Fig. 3). The midneck region was chosen for histomorphometric measurements because this site contains clear cortical envelopes and a trabecular network.

Histomorphometric measurements were performed using a semi-automatic digitizing system (System Supply, Nagano). To analysis the cortical and cancellous bone separately, we employed the methods described by Yamamoto et al. (1995) and Bagi et al. (1997). Cortical bone parameters were measured in the femoral neck included total tissue area (Ct.T.Ar), cortical bone area (Ct.B.Ar), cortical width (Ct.Wi), area within the endocortical envelope (Ec.Ar) (Frost and Jee 1992), periosteal mineralizing surface (Ps.MS/BS), endocortical mineralizing surface (Ec.MS/BS), periosteal mineral apposition rate (Ps.MAR), and endocortical mineral apposition rate (Ec.MAR), respectively. For cancellous bone measurements, the following parameters were measured: Bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th); trabecular number (Tb.N); trabecular separation (Tb.Sp), osteoid surface (OS/BS), eroded surface (ES/BS), mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate-bone volume referent (BFR/BV). We employed the histomorphometric terminology recommended by Parfitt et al. (1987).

*Statistical Analysis*

Data are expressed as the mean$\pm$standard error of the means (S.E.M) for each group. Group differences were analyzed for variance (ANOVA), with the Fisher protected least-significant-difference (PLSD) test. The $p$ values less than 0.05 were considered to be significant. The values of the basal group were provided as a reference for comparison but were not included in the analysis.

## Results

*Effects of treatment on histomorphometric indices of cancellous bone in the femoral neck*

Table 1 shows that there were significant differences in BV/TV, Tb.Th, Tb.N, Tb.Sp, OS/BS, ES/BS, MS/BS, MAR, BFR/BV between the sham and OVX control group, after 4 weeks ovariectomy. Treatment with PTH alone significantly increased BV/TV and Tb.Th accompanied by an increase in OS/BS, MS/BS, MAR, BFR/BV when compared with the OVX control group. Incadronate treatment alone significantly decreased ES/BS, and shows nonsignificant higher values of BV/TV than the OVX control group. PTH+incadronate treatment increased BV/TV, Tb.Th, and was accompanied by increased OS/BS, MS/BS, and decreased ES/BS when compared with the OVX control group.

TABLE 1. *Static and dynamic histomorphometric indices of cancellous bone in the femoral neck in sham and ovariectomized (OVX) rats during the treatment period*

| | Basal | 4 weeks | | 8 weeks | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Sham | OVX | Sham+V | OVX+V | OVX+P | OVX+I | OVX+P+I |
| BV/TV (%) | 48.9±6.8 | 50.4±3.2 | 43.4±0.6[a] | 49.1±1.1 | 41.8±1.8[a] | 51.3±2.1[b] | 44.1±2.6 | 54.1±3.3[b] |
| Tb.Th (mm) | 89.1±4.1 | 105.6±4.9 | 87.1±2.9[a] | 97.2±4.3 | 86.3±3.9[a] | 119±5.6[b] | 99.1±2.9 | 131.8±16[b] |
| Tb.N (N/mm) | 4.9±0.5 | 5.4±0.2 | 4.6±0.1[a] | 4.9±0.1 | 4.4±0.1[a] | 4.7±0.2 | 4.7±0.1 | 4.8±0.2 |
| Tb.Sp ($\mu$m) | 121±26 | 114±9 | 139±11[a] | 124±9 | 143±12[a] | 120±11[b] | 128±11 | 109±8[b] |
| OS/BS (%) | 2.4±0.3 | 5.1±0.8 | 13.5±1.9[a] | 2.9±0.4 | 12.2±1.8[a] | 18.3±3.9[a,b] | 6.2±1.9[a] | 15.1±3.2[a,b] |
| ES/BS (%) | 2.9±1.6 | 3.3±0.7 | 8.1±1.2[a] | 2.9±0.4 | 8.9±1.5[a] | 9.7±1.8[a] | 2.4±1.1[b] | 4.7±0.7[a,b] |
| MS/BS (%) | 3.9±1.4 | 3.7±0.6 | 9.7±1.7[a] | 4.9±0.6 | 10.2±1.1[a] | 19.3±2.3[a,b] | 6.1±1.6 | 18.1±4.9[a,b] |
| MAR ($\mu$m/day) | 0.4±0.1 | 0.8±0.1 | 1.4±0.2[a] | 0.7±0.2 | 1.0±0.2[a] | 1.4±0.1[a,b] | 0.6±0.1[b] | 1.1±0.1[a] |
| BFR/BV (%/year) | 11±5 | 24±6 | 115±31[a] | 51±9 | 96±24[a] | 159±23[a,b] | 34±7[b] | 98±26[a] |

Data are the mean±S.E.M. of six values per group.

[a] $p<0.05$, significantly different from the time-corresponding Sham group.

[b] $p<0.05$, significantly different from the time-corresponding OVX group.

Bone histomorphometric variables are abbreviated as follows: trabecular bone volume (bone volume/tissue volume, BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), osteoid surface (OS/BS), eroded surface (ES/BS), mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate-bone volume referent (BFR/BV).

TABLE 2. *Static and dynamic histomorphometric indices of cortical bone in the femoral neck in sham and ovariectomized (OVX) rats during the treatment period*

| | Basal | 4 weeks | | 8 weeks | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Sham | OVX | Sham+V | OVX+V | OVX+P | OVX+I | OVX+P+I |
| Ct.T.Ar ($mm^2$) | 4.27±0.13 | 4.29±0.16 | 4.36±0.41 | 4.37±0.27 | 4.51±0.18 | 4.62±0.15 | 4.51±0.17 | 4.71±0.16 |
| Ct.B.Ar ($mm^2$) | 3.15±0.18 | 3.21±0.11 | 2.79±0.07 | 3.23±0.16 | 2.52±0.07[a] | 3.08±0.13[b] | 2.78±0.12 | 2.98±0.09[b] |
| Ct.Wi (mm) | 0.53±0.03 | 0.57±0.01 | 0.49±0.01[a] | 0.58±0.03 | 0.46±0.01[a] | 0.54±0.01[b] | 0.51±0.03 | 0.53±0.01[b] |
| Ec.Ar ($mm^2$) | 0.76±0.03 | 0.83±0.04 | 0.96±0.11[a] | 0.86±0.08 | 0.99±0.07[a] | 0.83±0.03[b] | 0.79±0.04[b] | 0.81±0.03[b] |
| Ps.MS/BS (%) | 4.03±2.1 | 3.65±0.49 | 5.7±1.5[a] | 1.02±0.41 | 4.51±0.52[a] | 14.4±6.1[a,b] | 5.6±2.9[a] | 9.3±1.9[a,b] |
| Ps.MAR ($\mu$m/day) | 1.28±0.23 | 1.12±0.21 | 1.19±0.11 | 0.7±0.12 | 1.06±0.13[a] | 1.44±0.21[a,b] | 1.16±0.17[a] | 1.31±0.18[a,b] |
| Ec.MS/BS (%) | 20.4±3.4 | 10.8±3.7 | 18.2±3.1[a] | 9.3±2.8 | 16.2±4.5[a] | 28.8±4.7[a,b] | 16.3±1.1[a] | 19.9±1.4[a,b] |
| Ec.MAR ($\mu$m/day) | 1.38±0.37 | 1.49±0.18 | 1.56±0.19 | 1.11±0.32 | 1.41±0.17[a] | 1.52±0.14[a] | 1.14±0.22[b] | 1.31±0.09[a] |

Data are the mean±S.E.M. of six values per group.

[a] $p<0.05$, significantly different from the time-corresponding Sham group.

[b] $p<0.05$, significantly different from the time-corresponding OVX group.

Bone histomorphometric variables are abbreviated as follows: cortical tissue area (Ct. T. Ar), cortical bone area (Ct. B. Ar), cortical width (Ct. Wi), area within endocortical envelope (Ec. Ar), periosteal mineralizing surface (Ps. MS/BS), periosteal mineral appositional rate (Ps. MAR), endocortical mineralizing surface (Ec. MS/BS) and endocortical mineral appositional rate (Ec. MAR).

*Effects of treatment on histomorphometric indices of cortical bone in the femoral neck*

Table 2 shows that there were no significant differences in Ct.T.Ar between the sham and OVX groups, after OVX of 4 or 8 weeks. Four weeks after OVX, there were no significant differences in Ct.B.Ar, but there were significant differences in Ct.Wi, Ec.Ar, Ps.MS/BS, Ps.MAR, Ec.MS/BS between the sham and OVX controls. Eight weeks after ovariectomy, there were significant decreases in Ct.B.Ar between the sham and OVX control groups. PTH treatment alone showed a significant increase in Ct.B.Ar and was accompanied by increases in Ps.MS/BS, Ps.MAR, and Ec.MS/BS, when compared with the OVX control group. PTH + incadronate treatment showed a significant increase in Ct.B.Ar and Ct.Wi, and was accompanied by an increase in Ps.MS/BS, Ps.MAR, Ec.MS/BS and a decrease in Ec.Ar when compared with the OVX control group. Incadronate treatment alone shows no significantly high value of Ct.B.Ar, when compared with the OVX control group.

## Discussion

This study demonstrated that: 1) the femoral neck of OVX rats is a suitable sample site for preclinical studies of the prevention of bone loss induced by estrogen depletion; 2) concurrent use of incadronate did not blunt the anabolic effect of PTH; and 3) concurrent treatment shows the best results in restoring cancellous and cortical bone mass.

The femoral neck of OVX rats is an important site of the skeleton in osteoporotic bone loss since fractures occur frequently there. Losses of both cortical and trabecular bone mass are believed to contribute to decrease bone strength. Increased endocortical resorption in the OVX rats was a major contributing factor to a decrease in cortical bone of the OVX rats (Bagi et al. 1997). Our results show that ovariectomy-induced cancellous bone loss was due to a transient OVX-induced negative bone balance, and may be a contributing factor to the reduced mechanical strength in the femoral neck of OVX rats reported previously (Lauritzen et al. 1993). It suggests that the femoral neck of rats has advantages as a skeletal site for investigating bone changes induced by estrogen deficiency or for testing new therapeutic agents for the prevention and treatment of osteopenia in preclinical studies. Because the femoral neck of OVX rats is more clinically relevant to human osteoporosis than other sites such as the proximal tibia of rats, it could develop cancellous and cortical osteopenia associated with high bone turnover. This indicates that the femoral neck of OVX rats appears superior to other skeletal sites used for bone histomorphometry in the OVX rat model.

Treatment with PTH increased bone formation at both periosteal and endocortical envelopes, and increased the cortical bone area and width. In cancel-

lous bone, treatment with PTH alone significantly increases the BV/TV and Tb. Th, through an increase in bone formation related to indices such as OS/BS, MS/BS, MAR, and BFR/BV. Incadronate treatment alone could further prevent the OVX-induced cancellous bone loss by a decrease in bone resorption (ES/BS) and bone turnover (BFR/BV). Concurrent use of PTH+incadronate increased the cortical bone area and width accompanied by increased bone formation at both periosteal and endocortical envelopes. Concurrent treatment completely restored the OVX-induced cancellous bone mass, by increasing the bone formation related parameters (OS/BS, MS/BS), and significantly inhibiting the bone resorption related parameter (ES/BS). In the concurrent treatment group, trabecular number was preserved to the sham control level, and trabecular thickness increased when compared to the OVX controls. Our findings suggest that concurrent PTH+incadronate treatment creates a positive bone balance, and increases cancellous bone mass. In the OVX+incardronate treatment group, Ps.MS/BS was preserved to the OVX+V group level, whereas trabecular MS/BS and MAR were decreased. This suggests that there is a difference in the effect of incardronate on the recruitement of osteoblasts between trabecular and cortical envelope. Several studies have shown that reduced bone mass accompanies decreased bone strength of femoral neck in OVX rats (Peng et al. 1994a and b; Bagi et al. 1997). Taken together with these results, concurrent treatment could add an additional benefit on mechanical bone strength independent of that achieved by the increase in bone mass.

Our data show that PTH can increase bone formation in the presence of pretreatment with incadronate. The incadronate treatment alone clearly decreased bone resorption (ES/BS) and bone turnover (BFR/BV). When incadronate combination with PTH shows an increase in bone formation parameters (OS/BS, BFR/BV) and a decrease in bone resorption parameter (ES/BS). This indicates that bisphosphonate does not block the anabolic action of the PTH when used concurrently. This agrees with clinical human studies, Cosman et al. (1998) treated osteoporotic women with bisphosphonate (alendronate) with PTH, they found that alendronate did not block the anabolic effect of PTH. It is further suggested that a combination of PTH and bisphosphonate may be a viable treatment option for postmenopausal women with osteoporosis.

In summary, the findings of the present study indicate that the femoral neck is a suitable sample site for preclinical studies of the prevention and treatment of bone loss induced by estrogen depletion. The anabolic effect of PTH was not diminished by incadronate treatment. The increase in bone mass could add an additional benefits to long bone strength. Concurrent treatment shows the best results in restoring cancellous and cortical bone mass of femoral neck in OVX rats, and is a promising therapy for human osteoporosis.

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