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Differences in Bone Turnover and Skeletal Response to Thyroid Hormone Treatment Between Estrogen-Depleted and Repleted Rats

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Abstract. This study was undertaken to compare the effect of supraphysiological doses of thyroxine (T4) on bone metabolism in SHAM and OVX young adult rats. Female Sprague Dawley rats (220 ± 2 g, approx. 5 months of age) were divided into four groups of eight animals each. The animals were intraperitoneally injected 6 days per week with vehicle (Vh): 0.001 N NaOH/0.9% NaCl (SHAM+Vh and OVX+Vh) or 250 μg of thyroxine/kg/day (SHAM+T4 and OVX+T4) during a 5-week period. Serum T4 and osteocalcin (BGP), urinary pyridinolines (Pyr), and creatinine (creat) were determined. At the beginning and at end of the experiment, skeletal bone mineral content (BMC), bone mineral density (BMD), and area (A) of the total skeleton, femur, spine, and whole tibia, as well as proximal, middle, and distal areas of the tibia were assessed by dual X-ray absorptiometry (DXA) in an ultra-high-resolution mode. T4 treatment of the SHAM rats did not induce significant changes in BGP level or Pyr/creat excretion compared with the SHAM+Vh control group. However, these two biochemical bone markers significantly increased due to T4 treatment in OVX rats compared with both OVX+Vh and SHAM+T4 groups ($P < 0.05$ and $P < 0.001$, respectively). The OVX+T4 group had a significantly lower ΔBMD than SHAM+T4 rats in all studied regions ($P < 0.05$) except for the middle tibia region. OVX+T4 groups presented a significantly lower ΔBMC and ΔA compared with SHAM+T4 animals ($P < 0.001$). OVX+T4 rats significantly impaired the ΔBMD in the femur ($P < 0.01$), spine ($P < 0.05$), whole ($P < 0.05$) and middle ($P < 0.05$) tibia whereas T4 treatment of SHAM rats only affected, significantly, the whole ($P < 0.05$) and the proximal tibia region ($P < 0.01$). T4 treatment affects bone growth in young adult rats. The effect is significantly greater in the estrogen-depleted than in the estrogen-repleted state. The bone site most adversely affected by T4 treatment depends on the estrogen status. The proximal tibia (principally trabecular bone) was the most affected area in estrogen-repleted rats. Conversely, in OVX rats, the middle tibia (principally cortical bone) presented the greatest decrease in bone density.

Key words: Bone mass — Thyroxine — Ovariectomy — Bone turnover.

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Hyperthyroidism skeletal effects were first described by von Recklinghausen in 1891 [1]. Thyroxine (T4)-related bone loss results from a significant increase in the activation frequency of bone remodeling cycles [2], with an increase in bone resorption and a less impressive increase in bone formation [3]. Bone mineral mobilization followed by changes in serum and urinary excretion of calcium (Ca), phosphorus (P), and hydroxyproline were observed [4–6]. The effect on skeletal integrity is visualized as an increased porosity of cortical bone and loss of trabecular bone [7].

Previous studies demonstrated that although trabecular and cortical bone exhibit different sensitivity to thyroid hormone, both are affected [8–11]. However, it remains debatable whether hyperthyroidism, at any individual site, has a similar detrimental effect in estrogen depletion or repletion state [12]. In view of these previous controversial reports, it is important to evaluate the interaction between reproductive and thyroid status.

In a previous report we found that thyroxine (T4) treatment to ovariectomized (OVX) rats did not cause any further increase in urinary hydroxyproline excretion (HOProl/creat) or decrease in total skeleton bone mineral density (BMD) at the end of the study compared with OVX T4-untreated rats, although bone mineral content (BMC) of the total skeleton was adversely affected [15]. In the present study we reexamined the changes in bone turnover of OVX T4-treated rats with more specific and sensitive bone turnover markers, as well as the BMD of the total skeleton and its different subareas. In addition, in view of the previous conflicting reports suggesting that the adverse effect of T4 on bone was more evident in estrogen depletion than in estrogen repletion, we compared the effect of T4 in bone turnover and in BMD between SHAM and OVX adult rats.

Materials and Methods

Details of drugs and animal care were published previously [13]. In the present report, a total of 32 female Sprague Dawley virgin rats (IBYME, Argentina), approximately 5 months of age, with an average body weight of 220 ± 2 g, were divided into two groups of 16 rats each. Under light ether anesthesia, bilateral ovariectomy was performed by a dorsal approach in one group (OVX group), and the other group was subjected to a sham operation (SHAM group). Both groups were divided into two subgroups of eight animals each. Two days after surgery, and during a 5-week period, the rats were injected 6 days per week intraperitoneally with one of the following treatments: vehicle: 0.001 N NaOH/0.9% NaCl (SHAM+Vh and OVX+Vh groups) or 250 μg of thyroxine/kg/day (SHAM+T4 and OVX+T4 groups).

Body weight (BW) was recorded once a week. All rats were

sacrificed by exsanguination under anesthesia. Blood was obtained by cardiac puncture and serum was kept frozen at −20°C for biochemical analysis. Serum Ca and P were measured as reported previously [13]. Serum T4 was measured by radioimmunoassay (RIA) (total T4 Ab-coated tube, DPC, Diagnostic Products Corp., Los Angeles, USA) using commercial kits with an intraassay coefficient of variation (CV) of 3.3%. Serum osteocalcin (BGP) was measured by EIA (Rat osteocalcin EIA kit, Biomedical Technologies Inc, Stoughton, USA). The sensitivity was 0.5 ng/ml and the CV intra- and interassay were lower than 4% and 7%, respectively. The day before sacrifice, rats were kept in metabolic cages and urine samples were collected. Urine was kept frozen at −20°C until pyridinolines (Pyr) and creatinine (creat) were determined. Urinary creatinine was measured by the colorimetric method of Jaffe and Pyr was analyzed by an ELISA method using a commercial available kit (Pyrilinks™, Metra Biosystems Inc., Palo Alto, CA). The sensitivity of the assay was 5 nM and the intra- and interassay CVs were below 6% [14]. Urinary Pyr was expressed as a ratio to urine creat level.

At the beginning and end of the experiment, BMD was measured under light anesthesia (0.1 ml/100 gr bw. ketamine hydrochloride and 0.1 mg/100 g BW acepromazine maleate) (Holliday-Scott SA., Buenos Aires, Argentina). Skeletal BMC, BMD, and area (A) were assessed by DXA using a Lunar DPX (Alfa 8034 equipment, Small Animal Software) in an ultra-high-resolution mode [13]. Femur, lumbar spine, and whole tibia BMDs were evaluated manually using an automatic ROI from the reference scan image. Taking into account the different cortical and trabecular bone content presented in the different segments of the tibia, proximal, diaphysis (middle), and distal tibia BMD were evaluated separately. CVs for five repeated *in vivo* measurements were 3.0% for total body BMC, 0.9% for total body BMD, 1.2% for A, 0.9% for femur BMD, 0.8% for tibia BMD, and 1.8% for lumbar spine BMD. The different segments of the tibia BMD presented the following CVs: 3.5% proximal, 2.7% middle, and 1.8% distal, respectively. To minimize interobserver variation, all analyses were carried out by the same technician.

All groups were studied simultaneously and the same results (serum T4, Ca, and P levels as well as the final BMD and BMC values) were published previously to evaluate the preventive effect of the bisphosphonate olpadronate on the skeletal action of T4 [11, 13]. In the present report we added the results of the SHAM+T4 group and, as mentioned above, we measured serum BGP and Pyr excretion in the four groups. Moreover, to clarify the results we expressed the bone mass as changes between the final and initial values.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (mean ± SEM). Comparison between pairs of variables was assessed by Student's *t*-test. Differences among more than two groups of variables were tested by a one-way analysis of variance (ANOVA) using the statistical package Statview™ (Macintosh). The Dunnet post hoc test was used to identify significant differences between mean values. A P value less than 0.05 was considered statistically significant.

Results

Body Weight

Changes in BW during the study are shown in Figure 1. At the onset of the experiment, all groups had the same body weight and gained weight during the study. As expected, from the third week to the end of the study, OVX rats gained significantly more BW than the SHAM-matched group ($P < 0.001$), and OVX+T4 rats gained significantly more BW than the SHAM+T4 group ($P < 0.05$). T4 treatment to both SHAM and OVX rats slows down weight gain. Although

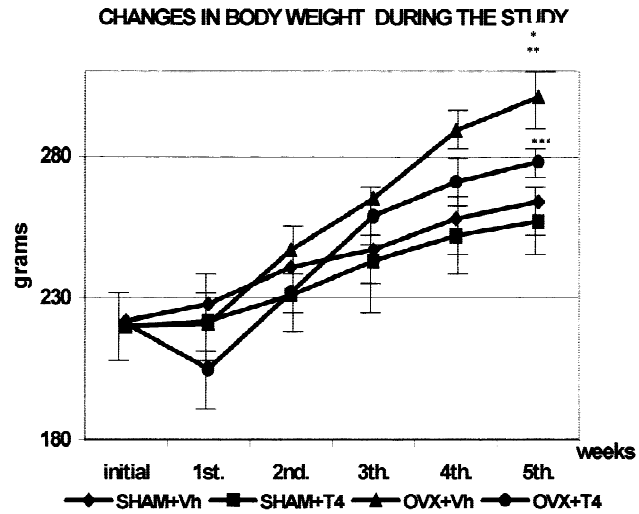

Fig. 1. Changes in body weight during the study. *$P < 0.001$ compared with SHAM+Vh; **$P < 0.05$ compared with OVX+T4; ***$P < 0.05$ compared with SHAM+T4.

the difference did not reach statistical significance in SHAM+T4 rats, the changes in BW were significantly different in the OVX+T4 group ($P < 0.05$) compared with the SHAM-matched group.

Biochemical Results

SHAM and OVX+T4-treated rats presented approximately three times higher T4 levels than the matched, untreated control rats ($P < 0.0001$). Serum T4 levels were similar in both T4-treated groups (15.1 ± 2.4 versus 15.0 ± 0.8 mg/dl) as was Ca (9.6 ± 0.1 versus 9.7 ± 0.1 mg/dl) and P (7.1 ± 0.2 versus 7.3 ± 0.1 mg/dl). Results were also similar in T4-untreated groups: T4 (5.2 ± 0.3 versus 5.2 ± 0.4 mg/dl), Ca (10.1 ± 0.1 versus 10.2 ± 0.1 mg/dl), and P (6.7 ± 0.1 versus 6.9 ± 0.2 mg/dl).

Figure 2 shows the bone marker levels at the end of the experiment. Although ovariectomy did not induce changes in serum BGP levels (19.4 ± 2.0 versus 20.6 ± 2.8 ng/dl), the urinary Pyr/creat. excretion presented a significant increment compared with their corresponding SHAM-matched group (251 ± 15 versus 357 ± 28 mmol/mmol) ($P < 0.05$). T4 treatment to SHAM rats did not induce significant changes in serum BGP level (19.4 ± 2.0 versus 20.8 ± 2.2 ng/dl) or urinary Pyr/creat excretion (251 ± 15 versus 259 ± 11 mmol/mmol). However, these two biochemical bone markers significantly increased with T4 treatment in OVX rats (20.6 ± 2.8 versus 27.4 ± 2.0 ng/dl; $P < 0.05$ and 357 ± 28 versus 608 ± 21 mmol/mmol; $P < 0.01$, respectively) (Fig. 2).

Serum BGP level (20.8 ± 2.2 versus 27.4 ± 2.0 ng/dl; $P < 0.05$) and urinary Pyr/creat excretion (259 ± 11 versus 608 ± 21 mmol/mmol; $P < 0.001$) were significantly higher in OVX+T4 rats compared with the SHAM+T4 group (Fig. 2).

Skeletal Measurements

Figure 3 shows the total skeleton, BMC, area, and BMD

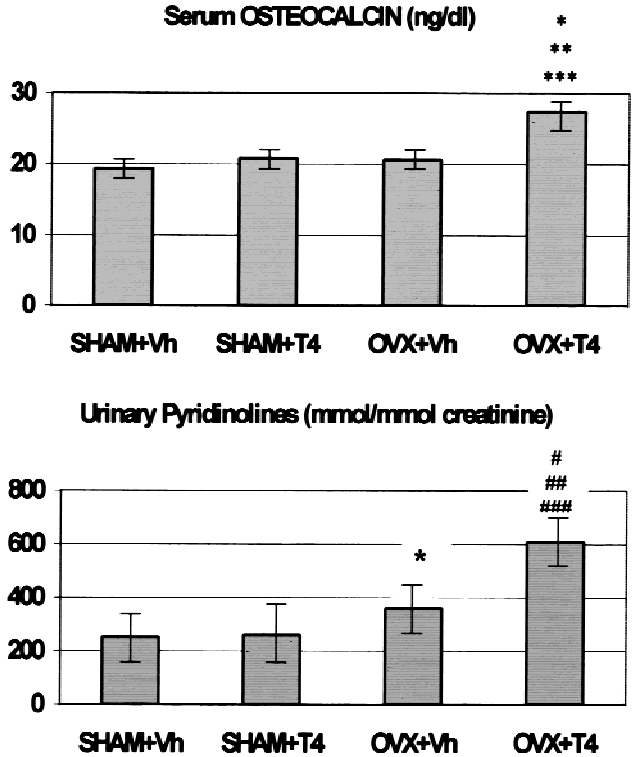

Fig. 2. Serum osteocalcin (BGP) levels and urinary pyridinolines/creatinine (Pyr) excretion at the end of the study. $*P < 0.05$ compared with SHAM+Vh; $^{\#}P < 0.001$ compared with SHAM+Vh; $**P < 0.05$ compared with OVX+Vh; $^{\#\#}P < 0.01$ compared with OVX+Vh; $***P < 0.05$ compared with SHAM+T4; $^{\#\#\#}P < 0.001$ compared with SHAM+T4.

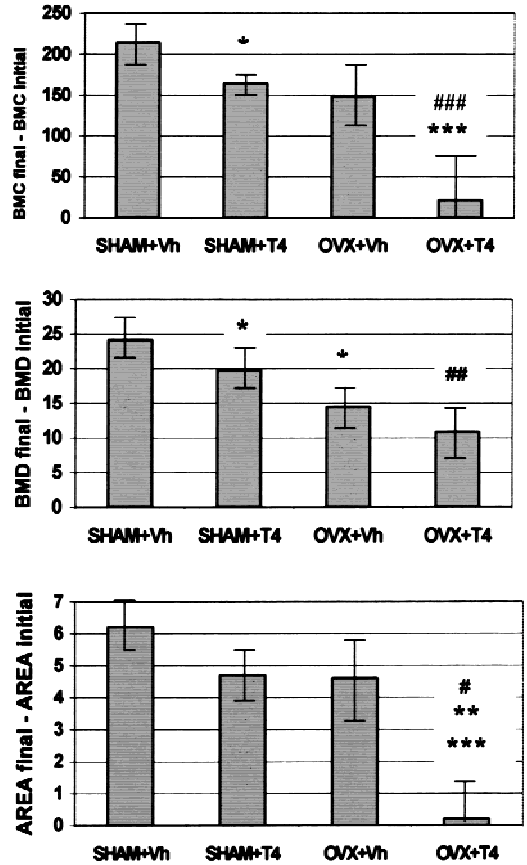

Fig. 3. Changes (final-initial) in BMC (mg), BMD (mg/cm^2), and A (cm^2) of the total skeleton. $*P < 0.05$ compared with SHAM+Vh; $^{\#}P < 0.001$ compared with SHAM+Vh; $**P < 0.01$ compared with OVX+Vh; $^{\#\#\#}P < 0.01$ compared with OVX+Vh; $***P < 0.001$ compared with SHAM+T4; $^{\#\#}P < 0.01$ compared with SHAM+T4.

changes ($\Delta BMD = BMD_{final} - BMD_{baseline}$). Table 1 summarizes the ΔBMD for the different bone regions that were analyzed.

The OVX+Vh group exhibited a significantly lower ΔBMD than SHAM+Vh rats in the total skeleton (24.1 ± 3.1 versus 14.5 ± 2.8 mg/cm^2; $P < 0.05$), whole tibia ($P < 0.01$), and proximal tibia ($P < 0.001$). The changes in BMC and A (ΔBMC and ΔA, respectively) are also shown in Figure 3. OVX groups showed lower ΔBMC and ΔA compared with SHAM animals, even though the difference did not reach statistical significance.

T4 treatment to SHAM rats significantly reduced the ΔBMD and ΔBMC of the total skeleton (19.8 ± 2.8 versus 24.1 ± 3.1 mg/cm^2 and 164 ± 26 versus 214 ± 35 mg, respectively, $P < 0.05$) (Fig. 3) as well as the ΔBMD in the whole tibia ($P < 0.05$) and its proximal segment ($P < 0.01$) (Table 1) compared with their matched T4 untreated rats. The lower ΔBMD in the other studied region (Table 1) and in the ΔA did not reach statistical significance (Fig. 3).

In contrast, T4 treatment to OVX rats significantly reduced the ΔBMC (21 ± 44 versus 148 ± 32 mg, $P < 0.01$) and completely inhibited the bone growth (ΔA) (0.0 ± 1.6 versus 4.6 ± 1.6 cm^2, $P < 0.01$) compared with the OVX+Vh group (Fig. 3). It also impaired the ΔBMD in almost all the studied regions. The differences were statistically significant in femur ($P < 0.001$), spine ($P < 0.05$), whole tibia ($P < 0.05$), and middle tibia regions ($P < 0.05$) (Table 1).

The OVX+T4 group had a significantly lower ΔBMD than the SHAM+T4 rats in the total skeleton (19.8 ± 2.8 versus 10.9 ± 2.4 mg/cm^2; $P < 0.01$) and in all other studied regions except for the middle tibia segment (Table 1). OVX+T4 rats presented lower ΔBMC and ΔA compared with SHAM+T4 rats (164 ± 26 versus 21 ± 44 mg; $P < 0.001$) and 4.7 ± 1.3 versus 0.0 ± 1.6 cm^2, $P < 0.01$) (Fig. 3).

The differences between T4-treated SHAM and OVX rats compared with their matched group is also summarized in Table 2.

Discussion

Severe hyperthyroidism is clearly detrimental to bone mass [7]. However, the skeletal effect of T4 replacement is more debatable. In this regard, our data demonstrated that T4 treatment markedly inhibited bone growth (body weight gain and skeletal area) and impaired the gain of BMC in estrogen deficiency; in the repleted state, a less marked effect was observed in BMC and BMD.

Bone turnover assessed by urinary Pyr/creat and serum BGP levels did not increase in T4-treated SHAM rats, however, bone tissue was adversely affected. In this regard, bone mass gain in estrogen-repleted T4-treated rats was comparatively lower than in the SHAM+Vh control group in all the studied regions, and the proximal tibia was the

Table 1. Effect of OVX and T4 treatment on the changes in femur, spine, total, proximal, middle, and distal tibia BMD (mg/cm^2)

	ΔSpine	ΔFemur	ΔWhole tibia	ΔProximal tibia	ΔMiddle tibia	ΔDistal tibia
SHAM+Vh	35.3 ± 6.3	44.4 ± 11.4	18.1 ± 5.6	52.0 ± 7.1	6.5 ± 7.3	27.3 ± 8.4
SHAM+T4	27.9 ± 4.3	33.5 ± 9.1	8.1 ± 3.7[a]	28.4 ± 5.5[a]*	1.4 ± 5.0	14.4 ± 3.3
OVX+Vh	23.0 ± 5.0	22.4 ± 7.0	−2.0 ± 3.4[a]*	9.6 ± 8.3[a]**	8.5 ± 12.3	12.4 ± 4.3
OVX+T4	−0.9 ± 5.3[b,c]**	6.6 ± 8.1[b]*,[c]*	−16.5 ± 4.4[b,c]*	−3.1 ± 9.1[c]**	−9.9 ± 6.9[b]	2.6 ± 5.5[c]

[a] $P < 0.05$ compared with SHAM+Vh
[a]* $P < 0.01$ compared with SHAM+Vh
[a]** $P < 0.001$ compared with SHAM+Vh
[b] $P < 0.05$ compared with OVX+Vh
[b]* $P < 0.01$ compared with OVX+Vh
[b]** $P < 0.001$ compared with OVX+Vh
[c] $P < 0.05$ compared with SHAM+T4
[c]* $P < 0.01$ compared with SHAM+T4
[c]** $P < 0.001$ compared with SHAM+T4

most affected area. This segment is mainly composed of trabecular bone, which presents a greater remodeling activity compared with cortical bone. In contrast, lumbar spine, also rich in trabecular bone, appears to be less affected by T4 treatment. This pattern could be partially explained by the high basal rate of bone turnover that takes place at the proximal tibia [15].

It is known that estrogen deficiency increases bone turnover with a predominance of bone resorption that leads to an osteopenic state that could prevent or decrease the bone mass gain, depending on the skeletal site [16]. In the present study, ovariectomy did not induce changes in serum BGP, however, urinary Pyr excretion increased. In a similar way, we previously showed an increase in HOProl excretion without changes in bone alkaline phosphatase (b-AL) [13]. These results are evidence of a disbalance between bone formation and resorption. Our hyperthyroidism also accelerates bone turnover, a synergistic effect between OVX and T4 treatment might be possible. In this regard, we reported previously that the T4 administration to OVX rats had no effect on serum b-AL levels and urinary HOProl/creat excretion [13]. However, in the present report, an approximate twofold increase in urinary Pyr/creat. levels and a significant increment of serum BGP were found. These findings could reflect the synergism between T4 treatment and estrogen withdrawal mentioned above that can only be assessed by more specific bone resorption markers. This effect could lead to an osteopenic state. The lack of an increase in the urinary HOProl/creat ratio was probably due to the lower sensitivity of this marker to assess bone turnover. In addition, we assume that the increase of bone turnover occurring after OVX may mask the effects of T4 treatment on b-AL.

Analyzing the data as changes from the initial value, the present results show that bone density was impaired in T4-treated OVX rats at the spine, femur, and whole tibia, being the middle region, rich in cortical bone and the most susceptible bone site (Fig. 4). The difference in behavior of the different sites can be partially attributed to the slower turnover rates of the lumbar vertebral body and/or differences in regional adaptation to mechanical usage [17]. Conversely, in other reports on OVX T4-treated rats, there was no decrease in femoral mass [18] or in femur and vertebral bone mass [19]. In orchidectomized male rats, the results are also controversial [4, 20–22]. The reasons for such discrepancy could be due to several factors such as differences in the experimental design, the dose of thyroid hormones used, the duration of the experiment, or the sex and age of the rats.

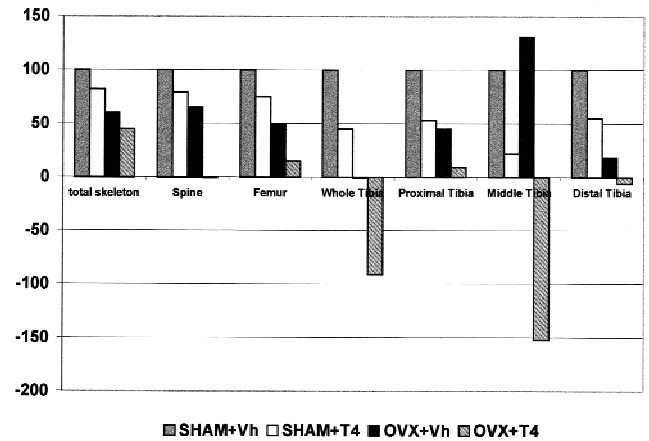

Fig. 4. Percentage of changes from the SHAM+Vh group. *$P <$ 0.05 compared with SHAM+Vh; **$P < 0.05$ compared with OVX+Vh; ***$P < 0.05$ compared with SHAM+T4.

Ovariectomy and/or thyroid hormone excess are associated with changes in body weight: OVX rats exhibit an increased weight gain and T4 administration partially inhibits this gain. Since high doses of thyroid hormones are likely to have some toxic effect, the slight influence of T4 on body growth and skeletal A (an expression of bone growth) suggests that our T4-treated rats presented a mild degree of hyperthyroidism [23]. In contrast to SHAM rats, T4 treatment of OVX rats significantly impaired body growth. We found very small changes in A and bone mass gain in the OVX+T4 group. It is known that the effects of thyroid hormones are associated with an initial increase of fatty acid formation followed by lipolysis and thermogenesis [23]. Therefore, it could be that the well-known increase in food consumption by OVX rats might have supplied the substrate source for the increased energy requirements caused by the hyperthyroid state, thus leading to a greater lipolytic action that would account for the decreased body growth found in our OVX+T4 group.

In summary, our data are consistent with the fact that T4 treatment affects bone growth in the young adult rat. The effect is significantly greater in the estrogen-depleted than in the estrogen-repleted state (Table 2). It is interesting to note that the bone site most adversely affected by T4 treatment depends on the estrogen status. The proximal tibia (principally trabecular bone) was the most affected area in

Table 2. Differences in the effect of T4 on bone markers and skeletal measurement when the rats are or are not protected by estrogen

	SHAM+Vh vs. SHAM+T4	OVX+Vh vs. OVX+T4
Body weight	n.s.	0.05
Serum BGP	n.s.	0.01
Urinary Pyr	n.s.	0.01
Total skeleton BMC	0.05	0.001
Total skeleton BMD	0.05	n.s.
Total skeleton area	n.s.	0.001
Subregions BMD		
Spine (L2–L5)	n.s.	0.05
Whole femur	n.s.	0.01
Whole tibia	0.05	0.05
Proximal tibia	0.01	n.s.
Middle tibia	n.s.	0.05
Distal tibia	n.s.	n.s.

estrogen-repleted rats. Conversely, in OVX rats the middle tibia (principally cortical bone) presented the greatest decrease in bone density.

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