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Skeletal Alterations in Ovariectomized Rats

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Summary. Female Sprague Dawley rats were subjected to either bilateral ovariectomy or sham surgery. Tetracycline derivatives were administered to each rat on two separate occasions to label sites of bone formation. All rats were sacrificed at 5 weeks postovariectomy and their proximal tibiae were processed undecalcified for quantitative bone histomorphometry. A twofold decrease in trabecular bone volume was noted in the proximal tibial metaphysis of ovariectomized rats. This bone loss was associated with elevated histomorphometric indices of bone resorption and formation. Ovariectomy increased osteoclast surface and numbers as well as osteoblast surface and numbers. Elevations in calcification rate and fractional trabecular bone surface with double tetracycline labels also suggest that bone formation was stimulated in ovariectomized rats. In addition, ovariectomized rats exhibited a greater rate of longitudinal bone growth relative to sham-operated control rats. These histomorphometric data indicate that ovariectomy induces marked bone loss and accelerated skeletal metabolism in rats.

Key words: Ovariectomy — Quantitative bone histomorphometry — Tetracycline — Osteoclasts — Osteoblasts.

Numerous studies utilizing biochemical and radiographic techniques have shown that bone loss occurs in ovariectomized rats [1–5]. However, this phenomenon is not well documented by histologic methods. Hodgkinson et al. [6] detected a reduced mass of trabecular bone in the caudal vertebrae of ovariectomized rats. These animals exhibited an increment in osteoid surface and a small, nonsignificant increase in resorption surface. Tetracycline-based analyses of skeletal alterations in ovariectomized rats are lacking. The purpose of the current study is to determine by histomorphometric methods the extent of trabecular bone loss and associated abnormalities in bone resorption and formation in ovariectomized rats.

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Materials and Methods

The experimental animals were 18 female Sprague Dawley rats that were approximately 75 days of age and weighed an average of 220 g. All rats were anesthetized with an i.p. injection of ketamine hydrochloride and xylazine at doses of 50 mg/kg body weight and 10 mg/kg body weight, respectively. Bilateral ovariectomies were performed in nine rats from a dorsal approach [7]. Nine control rats were subjected to sham surgeries in which the ovaries were exteriorized. Success of ovariectomy was confirmed at autopsy by failure to detect ovarian tissue and observation of marked atrophy of the uterine horn. After surgery, all rats were housed individually with food (Purina Rat Laboratory Chow) and water available ad libitum. Tetracycline derivatives were administered to each rat at a dose of 20 mg/kg body weight on two separate occasions preceding the day of sacrifice. Tetracycline chelates calcium and deposits primarily at sites of the initial calcification of new bone matrix [8,9]. Oxytetracycline hydrochloride (Pfizer Inc., Brooklyn, NY) was administered to each rat on the ninth day prior to sacrifice. After a 5-day period, during which no tetracycline was administered, all rats were injected i.p. with demeclocycline (Lederle Laboratories, Pearl River, NY) on the third day prior to sacrifice. This regimen results in deposition of a double tetracycline label at bone surfaces that are actively forming throughout the injection period.

At 5 weeks postovariectomy, all rats were sacrificed by cervical dislocation under ketamine anesthesia. The proximal tibiae were defleshed and placed in 10% phosphate-buffered formalin for 24 hours. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methyl methacrylate [10]. Longitudinal sections of 4 μm thickness were cut with an A0 Autocut/Jung 1150 microtome and stained according to Goldner's method [11]. Bone parameters were measured in these sections at a magnification of 400× with a Merz eyepiece reticle [12] consisting of 36 points and 6 semicircular lines within a

Table 1. Histomorphometric parameters in the proximal tibial metaphysis of ovariectomized and control rats

	Trabecular bone volume (%)	Osteoclast surface (%)	Osteoblast surface (%)	Osteoclasts/mm	Osteoblasts/mm
Ovariectomized (n = 9)	13.2[a]	12.0[b]	10.2[a]	4.1[b]	5.3[b]
	±5.1	±3.7	±5.1	±1.3	±3.5
Control (n = 9)	25.9	7.4	2.8	2.7	1.7
	±4.4	±1.8	±1.4	±0.5	±0.8

All parameters for ovariectomized animals are different from control parameters at the levels of significance listed below
[a] $P < 0.001$
[b] $P < 0.01$

square. Two sections of the proximal tibial metaphysis, equivalent to 14 mm^2 of bone tissue, were sampled in each animal. This area was standardized in relation to the growth plate–metaphyseal junction.

The number of points superimposed over mineralized tissue (calcified cartilage and bone), osteoid (unmineralized bone matrix), and bone marrow were recorded. The fractional area of mineralized tissue, commonly referred to as trabecular bone volume, was determined by dividing the number of points lying over mineralized tissue by the total number of points. The occurrence of osteoid in both control and ovariectomized rats was minimal (<1%) and, therefore, could not be reliably quantified by the manual techniques employed in this study. The intersections of the semicircular reticle lines with the bone–bone marrow interface were classified as resting, osteoblast, or osteoclast surface. Resting surface is defined as trabecular bone surface without adjacent osteoblasts or osteoclasts. Osteoblast surface is defined as trabecular surface lined with osteoblasts, whereas irregular or scalloped trabecular surface with adjacent osteoclasts is classified as osteoclast surface. Osteoblast surface (%) was determined by dividing the number of intersects with bone surfaces lined by osteoblasts by the total number of intersects. Osteoclast surface (%) was calculated in a similar manner. The numbers of osteoblasts and osteoclasts adjacent to trabecular bone surfaces of the proximal tibial metaphysis were also quantified. These data are expressed as number of bone cells per millimeter trabecular bone perimeter. This latter parameter was determined by multiplying the total number of intersects by the grid constant, d, which is equal to the distance between grid points [12].

Tetracycline-based data were collected from unstained, 10-μm thick sections of the proximal tibial metaphysis. To measure the rate of longitudinal bone growth, the distance between the fluorescent tetracycline band that parallels the growth plate and the growth plate–metaphyseal junction was quantified with a calibrated eyepiece micrometer [13] at five equally-spaced sites per section. These measurements were performed under ultraviolet illumination at a magnification of 200× in two sections per animal. The rate of longitudinal bone growth was calculated by dividing the distance between the tetracycline band and the growth plate–metaphyseal junction by the time interval between administration of the tetracycline label and sacrifice.

Discrete tetracycline labels were present in lamellar bone of the secondary spongiosa. Intersects of semicircular grid lines with trabecular bone surfaces were categorized according to the presence or absence of tetracycline labels. Trabecular bone surfaces without tetracycline labels were considered to be nonforming. The fraction of actively forming trabecular surface was determined by dividing the number of intersects with double-labeled surface by the total number of intersects [14]. In addition, the distance between the two tetracycline markers that comprise a double label was meaured with a calibrated eyepiece micrometer at three or four equally spaced sites per double label. These measurements were performed on an average of 20 double tetracycline labels per animal. Calcification rate was calculated by dividing the interlabel distance by the time interval between administration of the two tetracycline markers. These values were not corrected for the random relation between the plane of section and the plane of tetracycline markers in trabecular bone [14].

Data are expressed as the mean ± SD of the control and ovariectomized groups. Statistical differences between the two groups were evaluated with the two-tailed Student's t test. P values of less than 0.05 were considered to be significant.

Results

Ovariectomized rats weighed significantly more than control rats. The mean body weight of nine ovariectomized rats was 298.4 ± 26.3 g, while the nine control rats weighed an average of 253.3 ± 14.0 g. This difference is highly significant at the level of $P < 0.001$.

Table 1 lists values for static histomorphometric parameters in the proximal tibial metaphysis of ovariectomized and control rats. Trabecular bone mass declined by a factor of 2 in ovariectomized rats ($P < 0.001$). This marked bone loss at 5 weeks postovariectomy can be visualized in Fig. 1. Ovariectomized rats also exhibited histologic evidence for enhanced bone resorption and formation. Osteoclast surface and numbers were significantly elevated ($P < 0.01$) in ovariectomized rats relative to control rats. Ovariectomized rats were also characterized by significant increments in osteoblast surface ($P < 0.001$) and numbers ($P < 0.01$).

Tetracycline-based data from the proximal tibial metaphysis are listed in Table 2. An increased rate of longitudinal bone growth was observed in ovariectomized rats ($P < 0.001$). In addition, these animals had significant elevations in actively forming

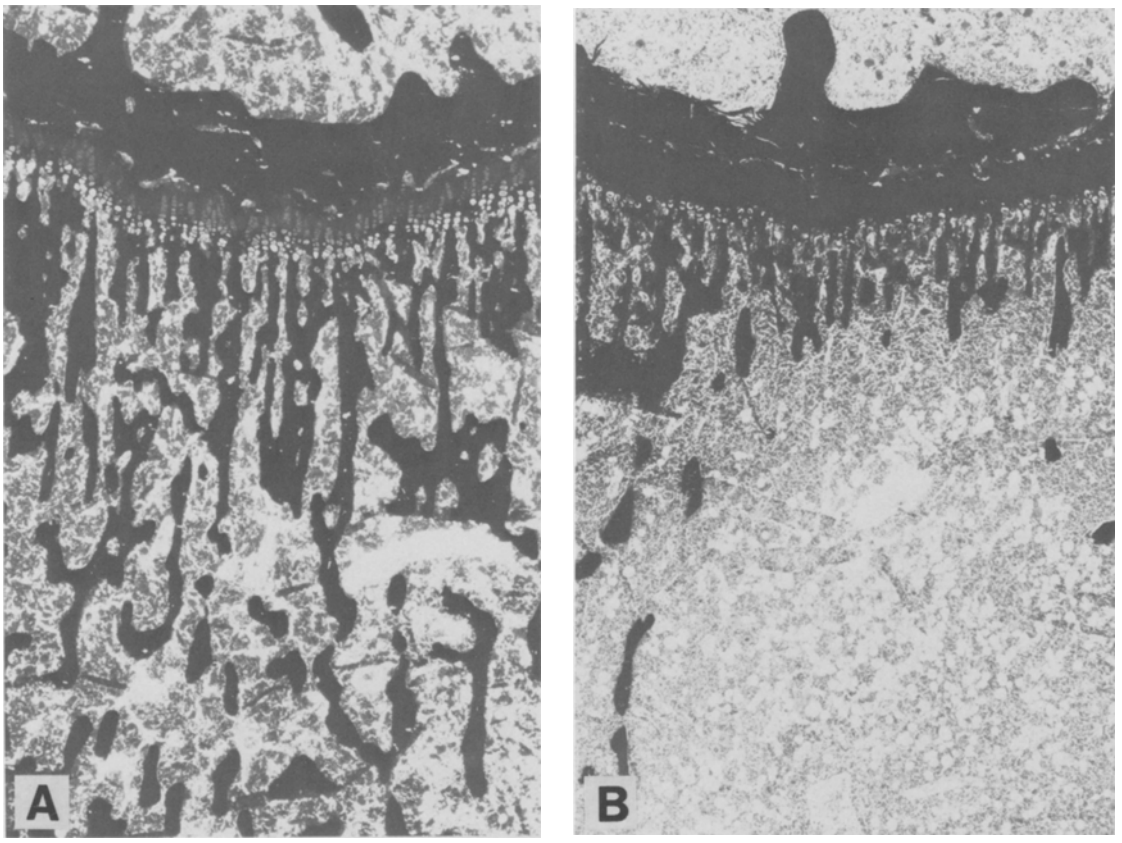

Fig. 1. Proximal tibial metaphysis of control (**A**) and ovariectomized (**B**) rats. Note the reduced mass of darkly stained trabecular bone in the ovariectomized animal. (Von Kossa stain, ×25).

Table 2. Tetracycline-based parameters in the proximal tibial metaphysis of ovariectomized and control rats

	Longitudinal bone growth (μm/day)	Forming bone surface (%)[a]	Calcification rate (μm/day)
Ovariectomized (n = 7)	36.5[b] ±6.0	12.0[d] ±10.7	1.6[c] ±0.4
Control (n = 8)	26.7 ±3.1	2.9 ±1.7	1.2 ±0.2

[a] Forming bone surface is defined as bone surface with a double tetracycline label
[b] $P < 0.001$
[c] $P < 0.025$
[d] $P < 0.05$

trabecular bone surface ($P < 0.05$) and calcification rate ($P < 0.025$).

Discussion

This study demonstrates that rapid bone loss occurs in the proximal tibial metaphysis of ovariectomized rats. A twofold decrease in trabecular bone volume was noted at 5 weeks postovariectomy. In addition, osteoclastic and osteoblastic data suggest that ovariectomy stimulates bone resorption and formation. The observed increases in actively forming trabecular bone surface and calcification rate, as determined by double tetracycline labeling, also indicate that bone formation was elevated in ovariectomized rats. Alterations in bone formation in response to ovariectomy appear to be somewhat variable, as indicated by relatively high standard deviations for osteoblastic and tetracycline-based parameters. In view of the observed increases in both bone resorption and formation, the loss of trabecular bone mass associated with ovariectomy is unexplained. The pathogenetic mechanism may involve a greater increment in bone resorption relative to the increment in bone formation so that net loss of skeletal mass occurs. Such a mechanism has been proposed for the development of postmenopausal bone loss in humans [15]. Although surface-based and cellular parameters suggest that bone for-

mation was increased to a greater extent than bone resorption in ovariectomized rats, these static measurements are not indicative of osteoclastic activity. An accelerated rate of bone resorption by individual osteoclasts as well as an increased osteoclast population may result in bone loss in ovariectomized rats even in the presence of increased osteoblast numbers and activity. It is also important to note that 5 weeks postovariectomy may not be of sufficient duration to achieve a steady state in the skeletal response to estrogen deficiency.

Ovariectomized rats exhibit an increased rate of weight gain. This phenomenon has been reported by other investigators [1–4]. In contrast to the protective effect of obesity against osteoporosis in postmenopausal women [16–18], marked bone loss occurred in obese ovariectomized rats. The effect of obesity on histologic indices of bone resorption and formation has not been documented. Nevertheless, the possibility that increased body mass affects bone turnover in ovariectomized rats cannot be ruled out.

Estrogen is thought to act as an antagonist to parathyroid hormone (PTH) [19]. The loss of this antagonism after ovariectomy may result in increased skeletal sensitivity to PTH. Our histologic data are consistent with this theory. The osteoclast and osteoblast populations in the long bone metaphysis of rats are known to increase in response to PTH [20,21]. Physiologic doses of PTH induce a coupled increase in bone resorption and formation in young adult dogs [22]. Tam et al. [23] found that rats infused with PTH had elevated calcification rates. The skeletal characteristics of ovariectomized rats appear to be similar to those of rats treated with PTH.

Estrogen is also thought to act as an antagonist to growth hormone [24]. Widening of tibial epiphyseal cartilage was inhibited by estrogen in hypophysectomized rats treated with growth hormone [25]. Whitson et al. [26] demonstrated that the most rapid phase of longitudinal bone growth in rats occurs when serum estrogen levels are minimal. Our finding of an accelerated rate of longitudinal bone growth in ovariectomized rats may be due to loss of estrogenic antagonism to the stimulatory effects of growth hormone.

Although rats have a juvenile skeleton in which modeling predominates, it is interesting to compare histomorphometric data from ovariectomized rats to that of postmenopausal patients with adult, remodeling bone. Increased resorption surfaces were detected in the iliac crest of postmenopausal women by microradiography [27] and histologic techniques [28]. On the other hand, tetracycline-based histomorphometric analyses indicated that the majority of osteoporotic patients had relatively normal indices of bone resorption and formation [29,30]. However, several subgroups were identified in which osteoporotic patients exhibited high levels of bone remodeling or a pathologically low rate of bone formation. These heterogeneous findings may reflect different phases of the disease process. Parfitt et al. [31] hypothesized that the initial rapid phase of postmenopausal bone loss is associated with an increased rate of bone remodeling and enhanced osteoclastic activity. With time, bone remodeling is thought to diminish, but loss of skeletal mass continues at a slower rate, probably due to depressed bone formation. Calcium kinetic and biochemical data support the contention that an increased rate of bone remodeling occurs in women soon after naturally occurring or surgical menopause [15,32]. Our histomorphometric data indicate that accelerated bone metabolism also occurs in rats at early times postovariectomy.

In summary, the current study demonstrates that ovariectomy induces marked loss of trabecular bone in the proximal tibial metaphysis of rats. The observed bone loss is associated with an increased rate of longitudinal bone growth and elevated histomorphometric indices of bone resorption and formation.

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