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Long-Term Effects of Ovariectomy and Aging on the Rat Skeleton

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Summary. The long-term skeletal effects of ovariectomy and aging were studied in female Sprague-Dawley rats sacrificed at 270, 370, and 540 days after bilateral ovariectomy (OVX) or sham surgery at 90 days of age. The proximal tibia was processed undecalcified for quantitative bone histomorphometry. For continuity, data from these late time points were combined with previously published data from earlier time points (0–180 days). A biphasic pattern of cancellous bone loss was detected in the proximal tibial metaphysis of OVX rats. An initial, rapid phase of bone loss out to 100 days was followed by an intermediate period of relative stabilization of cancellous bone volume at the markedly osteopenic level of 5–7%. After 270 days, a slow phase of bone loss occurred during which cancellous bone volume declined to 1–2%. Both the initial, rapid phase and the late, slow phase of bone loss in OVX rats were associated with increased bone turnover. In control rats, cancellous bone volume remained constant at 25–30% out to 270 days (12 months of age), then decreased to ~10% by 540 days (21 months of age). This age-related bone loss was also associated with increased bone turnover. It is interesting to note that the proximal tibial growth plates were closed in approximately a quarter of the control rats by 15–21 months of age. Our data indicate that a slow rate of bone loss and increased bone turnover persist in OVX rats during the later stages of estrogen deficiency. Therefore, the development of osteopenia is coincident with increased bone turnover in OVX rats as well as in aged, control rats.

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Ovariectomy has been shown to induce osteopenia [1–6] and increased bone turnover [7–10] in rats. Estrogen-deficient dogs [11], baboons [12], and humans [13] also exhibit increased bone turnover. To emphasize the close temporal relationship between the development of osteopenia and increased bone turnover in ovariectomized rats, we reported that the initial rapid phase of bone loss in these animals is coincident with the maximal increase in bone turnover [14]. At later times postovariectomy, bone loss and bone turnover both subside. These findings were based on a histomorphometric analysis of osteopenic changes in ovariectomized rats as a function of time from 0–180 days postovariectomy. In the current study, we extend our observations to include histomorphometric data out to 540 days postovariectomy.

Materials and Methods

The experimental animals were female Sprague Dawley rats (Charles River Laboratory Inc., Wilmington, MA) that were 90 days of age and weighed an average of 240 g at the beginning of the study. 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 (OVX) were performed in half of the rats from a dorsal approach [15]. The remainder were subjected to sham surgeries. All rats were housed individually at 25°C with a 13 hour/11 hour light/dark cycle. Food (Purina Rat Laboratory Chow, St. Louis, MO) was available *ad libitum* to the sham-operated control rats. To minimize the increase in body weight associated with ovariectomy [16], the food consumption of OVX rats was restricted to that of the control rats (pair-feeding). Demeclocycline (Lederle

Laboratories, Pearl River, NY) and calcein (Sigma Co., St. Louis, MO) were administered to each rat by i.p. injection at a dose of 10 mg/kg body weight on the 14th and 7th days before sacrifice, respectively. This regimen resulted in deposition of a double fluorochrome label at bone surfaces that were actively mineralizing throughout the injection period.

Twelve control and 12 OVX rats were sacrificed by exsanguination under ketamine/xylazine anesthesia at 270 days and at 370 days postovariectomy. Although the same number of control and OVX rats was scheduled for sacrifice at 540 days postovariectomy, the animal population was reduced by premature death due to mammary tumors, extensive renal calculi, and severe respiratory infections. Therefore, the sample size at 540 days consisted of 7 control and 10 OVX rats. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. 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 [17]. Longitudinal sections (4 μm thick) were cut with an AO Autocut/Jung 1150 microtome and stained with a modified Masson-Goldner trichrome [17]. Bone parameters were measured in the proximal tibial metaphysis with the Bioquant Bone Morphometry Package (R&M Biometrics Corp., Nashville, TN). Cancellous bone areas and lengths were traced with a cursor and Hipad digitizing tablet adjacent to a Nikon Labophot microscope. The light within the cursor can be visualized in the microscopic field when used in conjunction with a camera lucida. Raw data were stored in an Apple IIe microcomputer interfaced to the digitizing tablet. Values for bone histomorphometric parameters were then calculated with Bioquant software. Cancellous bone volume (%) and osteoclast and osteoblast surfaces as percentages of total cancellous surface length were measured in this manner. Bone parameters were quantified in cancellous bone tissue at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. Additional details of the sample site and data calculations have been published elsewhere [7, 8]. In general, two sections of the proximal tibia with ~40 mm of cancellous bone perimeter were sampled in each control animal. The relative lack of bone spicules in OVX rats made it necessary to sample additional sections in these animals. Therefore, surface-based parameters were measured in three or more sections in each OVX rat to approximate the cancellous bone perimeter sampled in control rats.

Fluorochrome-based parameters were measured in unstained, 10 μm-thick sections of the proximal tibial metaphysis. The rate of longitudinal bone growth, percentage of cancellous bone surface with a double fluorochrome label (mineralizing surface), and mineral apposition rate were measured with the Bioquant system described above. In addition, bone formation rate (tissue level, total surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate [18]. Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone [18].

Data are expressed as the mean ± SD of the control and OVX groups at each time point. For continuity, data from the later time points (270, 370, and 540 days postovariectomy) have been added to previously published data [14] from the earlier time points (0–180 days). Statistical differences between the control and OVX groups at a given time point were evaluated with the two-tailed Student's t test. The large standard deviations of certain parameters at certain time points were suggestive of a non-normal distribution. Therefore, the nonparametric Kruskal-Wallis test was also used for statistical comparisons. As the levels of significance obtained with the two tests did not differ, only the results of Student's t test are presented below. Within each group, changes in cancellous bone volume as a function of time were evaluated by linear regression analysis [19]. In some cases, data for two different time points within a group were compared with Student's t test. P values less than 0.05 were considered to be significant.

Results

Despite pair-feeding, OVX rats weighed significantly more than control rats at all times between 35 and 370 days postovariectomy, inclusive (data not shown). At 540 days, the final body weights of OVX and control rats were 507.5 ± 47.9 g and 445.3 ± 104.0 g, respectively (NS).

Bone histomorphometric parameters in the proximal tibial metaphysis are plotted as a function of time postovariectomy in Figure 1. Cancellous bone volume (Fig. 1A) in control rats remained constant with time at ~30% out to 270 days (slope = −0.0075, r = 0.0939, NS), then declined linearly to ~10% between 270 and 540 days (slope = −0.0571, r = 0.6443, $P < 0.001$). The slopes for these two time periods are significantly different ($P < 0.001$). OVX rats exhibited an initial, rapid phase of cancellous bone loss between 0 and 100 days postovariectomy (slope = −0.1976, r = 0.7732, $P < 0.001$). The rate of bone loss during this period was 0.82%/day. Afterwards, a period of relative stabilization of cancellous bone volume at the markedly osteopenic level of 5–7% occurred between 100 and 270 days (slope = 0.0028, r = 0.0521, NS). A late, slow phase of cancellous bone loss was detected in OVX rats between 270 and 540 days (slope = −0.0204, r = 0.5601, $P < 0.001$) as cancellous bone volume declined to ~1%. The rate of bone loss during this late period was 0.08%/day. Within the OVX group, significant differences between the slopes of the lines were observed for the initial phase of bone loss vs. the intermediate period of bone stabilization ($P < 0.001$) as well as for the intermediate period vs. the late phase of bone loss ($P < 0.01$). Differences in cancellous bone mass between control and OVX rats are seen in Figure 2.

A transient increase in longitudinal bone growth (Fig. 1B) occurred in OVX rats at 14 days postovariectomy, followed by a decline to control levels out to 270 days. OVX rats then exhibited a slight, but statistically significant, increased rate of longitudinal bone growth relative to control rats at 370 and 540 days. This difference is due primarily to closure of the proximal tibial growth plate in some of the control animals at these later times (Fig. 3). At 370 days, closed growth plates were observed in

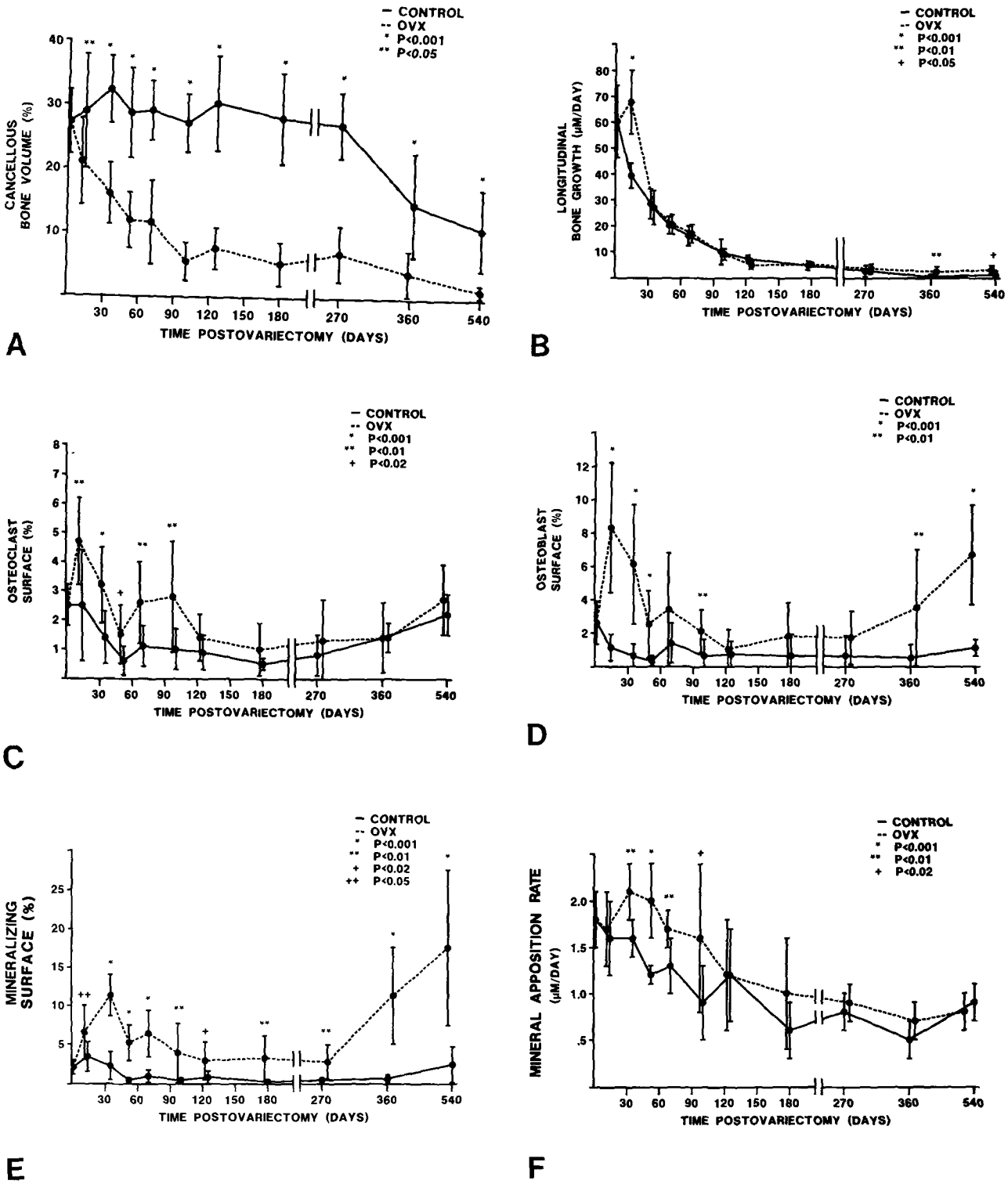

Fig. 1. Cancellous bone volume (**A**), longitudinal bone growth (**B**), osteoclast surface (**C**), osteoblast surface (**D**), mineralizing surface (**E**), and mineral apposition rate (**F**) in the proximal tibial metaphysis are plotted as a function of time postovariectomy. Each data point for the control (solid lines) and OVX (broken lines) groups is the mean ± SD of 10–12 animals, with the exception of the control group at 540 days (N = 7). The data point for the baseline control group (day 0) is the mean ± SD of 8 animals. Levels of significance were determined with the two-tailed Student's *t* test.

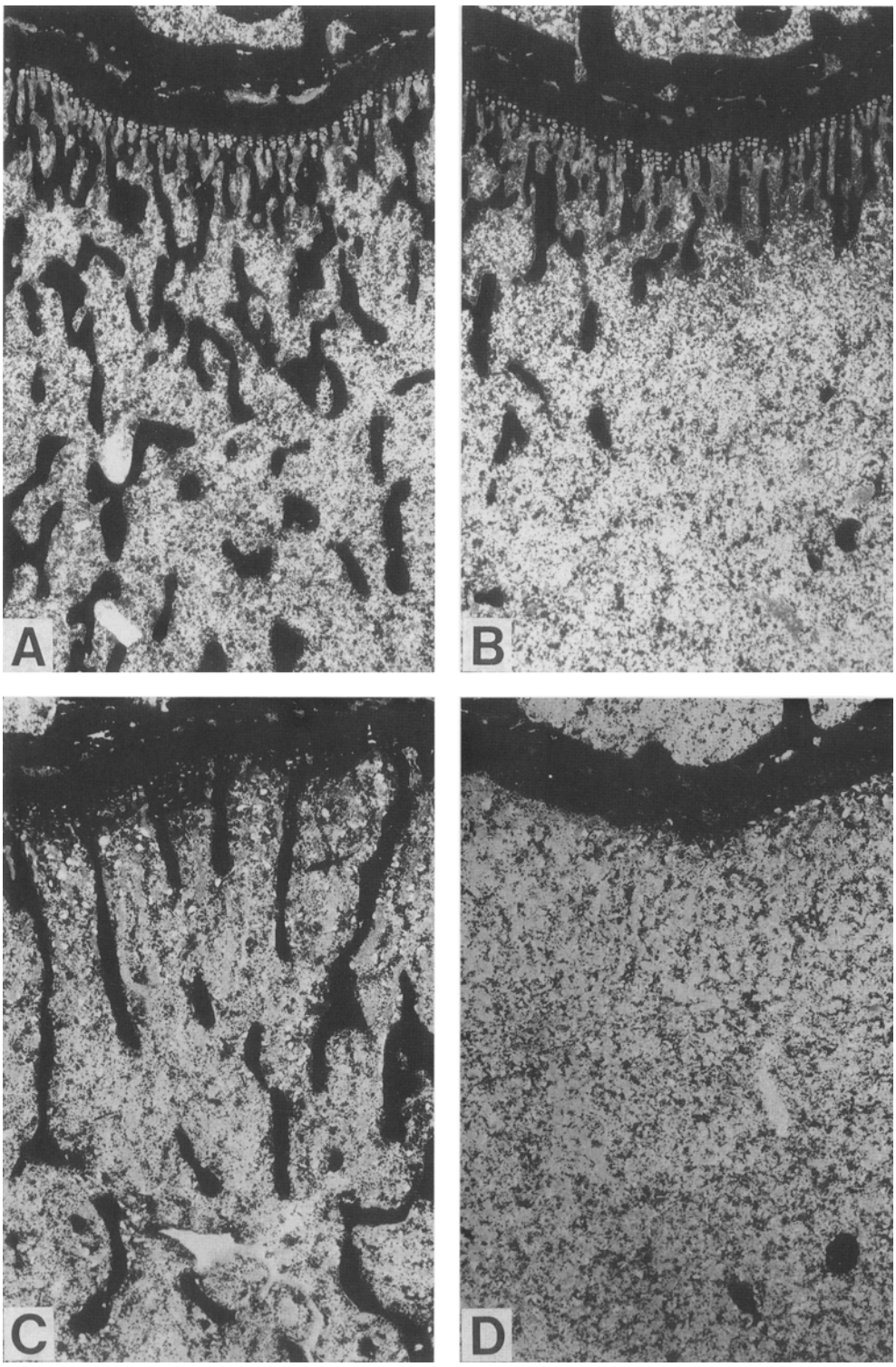

Fig. 2. Proximal tibial metaphyses from a control rat at 70 days after sham surgery (5 months of age) **(A)**, an OVX rat at 70 days **(B)**, a control rat at 540 days (21 months of age) **(C)**, and an OVX rat at 540 days **(D)**. Note the reduced mass of darkly stained cancellous bone in the OVX rats relative to the control rats. Note also the age-related loss of cancellous bone in the older control rat. Von Kossa stain, ×25.

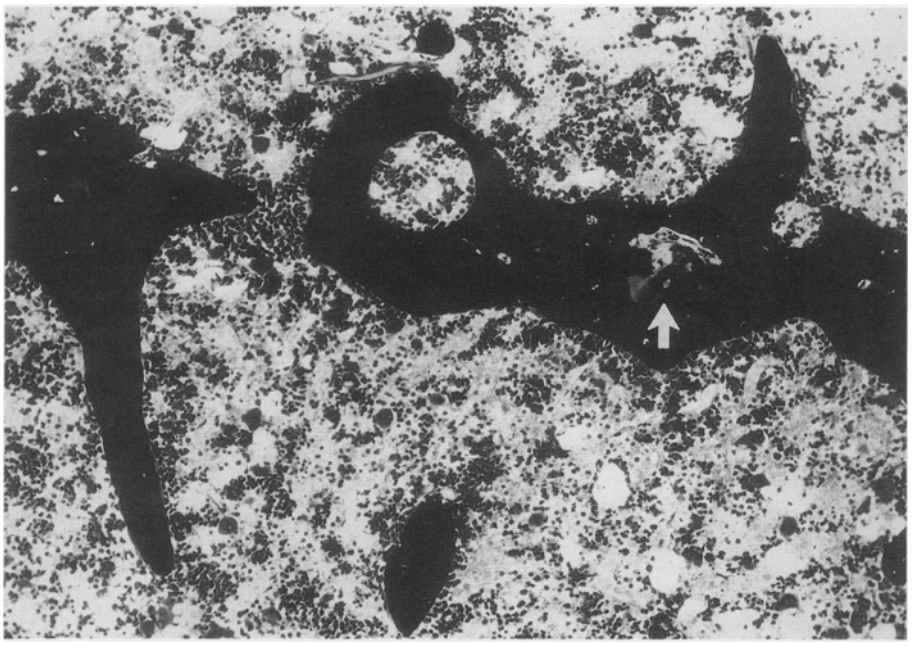

Fig. 3. Cancellous bone tissue at the epiphyseal-metaphyseal junction of the proximal tibia in a control rat at 540 days after sham surgery (21 months of age). Note the absence of a growth plate in this animal. Only a few remnants of hyaline cartilage persist (arrow). Approximately a quarter of the control rats from 15 to 21 months of age had closed growth plates. Von Kossa stain, ×100.

3 of 12 control rats whereas at 540 days, 2 of 7 control rats had closed growth plates. In contrast, the proximal tibial growth plates of all OVX rats remained open at these late times.

Values for osteoclast and osteoblast surface are depicted in Figures 1C and 1D, respectively. Both parameters were significantly increased in OVX rats from 14 to 100 days. Between 125 and 270 days, OVX rats exhibited a trend for increased osteoclast and osteoblast surface, but statistical significance was not observed. Osteoclast surface then increased at later times in both control and OVX rats. This parameter was significantly greater at 540 days than at 270 days in the control ($P < 0.001$) and OVX ($P < 0.025$) groups. Osteoblast surface also increased at 370 and 540 days in OVX rats, but remained nearly constant in control rats.

Figures 1E and 1F illustrate fluorochrome-based parameters as a function of time. Mineralizing surface was significantly increased in OVX rats relative to control rats throughout the duration of the experiment (Fig. 1E). However, the maximal increase in the mineralizing surface of OVX rats occurred during the early and late times postovariectomy. Within the control group, the mean mineralizing surface at 540 days was significantly greater than the mean value at 270 days ($P < 0.02$). OVX rats were characterized by an increased mineral apposition rate between 35 and 100 days, inclusive (Fig. 1F). This parameter was nearly identical in control and OVX rats at all later times.

OVX rats exhibited an increased bone formation rate relative to control rats at all time points (Fig. 4). This parameter was maximally increased during the first several months postovariectomy, then declined toward control levels out to 270 days. A second phase of maximally increased bone formation rate occurred in OVX rats at later times. Within the control group, bone formation rate was significantly greater at 540 days than at 270 days ($P < 0.02$).

Discussion

A biphasic pattern of cancellous bone loss was detected in the proximal tibial metaphysis of OVX rats. The great majority of the bone loss occurred during the first several months postovariectomy. This initial, rapid phase of bone loss was followed by an intermediate period between 100 to 270 days during which cancellous bone mass appeared to stabilize at the markedly osteopenic level of 5–7%. Finally, a late phase of slow bone loss occurred between 270 and 540 days postovariectomy as cancellous bone volume declined to 1–2%. These phases of bone loss in OVX rats correlate well with temporal patterns in bone turnover. We previously reported that the initial, rapid phase of bone loss is coincident with the maximal increase in bone turnover [14]. During the intermediate period, bone loss and bone turnover both subside. A similar sequence of events has been recently described by Stepan et al. [20] in women subjected to surgical menopause. In the current study, we determined that the late,

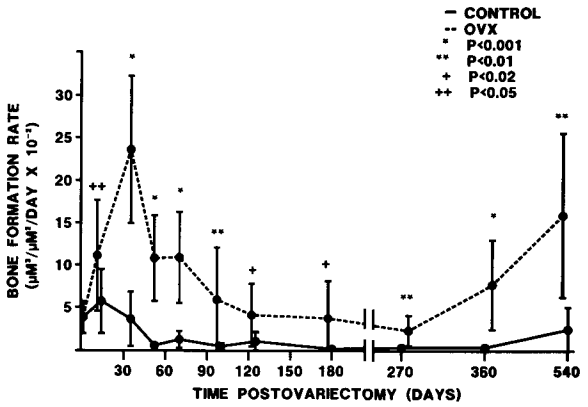

Fig. 4. Bone formation rate (tissue level, surface referent) in the proximal tibial metaphysis of control (solid line) and OVX (broken line) groups is plotted as a function of time postovariectomy. See Figure 1 legend for details.

slow phase of bone loss in OVX rats is also associated with increased bone turnover. Bone loss would be minimal if increased bone turnover was the only skeletal abnormality in OVX rats. Because marked bone loss occurs, it follows that ovariectomy also induces an imbalance between bone resorption and formation. The increment in bone resorption apparently exceeds the increment in bone formation so that net bone loss occurs, as reported in early postmenopausal women [13]. Because of this imbalance, it seems logical to assume that an increased rate of bone turnover would result in an increased rate of bone loss. This appears to be the case in OVX rats.

Women also exhibit a transient, rapid phase of bone loss during the early stages of estrogen deficiency [21]. Numerous biochemical studies indicate that increased bone turnover occurs at this time [22–25]. The mechanism for the rapid bone loss is thought to involve a predominance of bone resorption with excessive osteoclastic activity [26]. Conversely, the subsequent slow phase of bone loss in postmenopausal women is thought to be due primarily to diminished bone formation [26]. Our histomorphometric findings in OVX rats are consistent with skeletal events in women during the early stages of estrogen deficiency. However, we find no evidence for decreased bone formation in OVX rats during the late stages of estrogen deficiency. This discrepancy may be due to the relatively short life span of rodents, providing insufficient time for the long-term development of decreased bone formation after ovariectomy.

Cancellous bone volume remained constant at 25–30% in control rats out to 270 days after sham surgery (1 year of age), then declined to ~10% at 21 months of age. This age-related bone loss is associated with increased bone turnover, as indicated by significant increases in parameters such as osteoclast surface and bone formation rate at the later time points. Biochemical data indicate that bone turnover also increases with age in normal women [27, 28]. It is interesting to note that serum parathyroid hormone (PTH) is elevated in aged rats [29–31]. As serum creatinine also increases with age in rats [31], this hyperparathyroidism may be secondary to early renal failure, a period during which bone turnover is known to be accelerated [32]. Therefore, PTH may be involved in the development of the age-related bone changes. In OVX rats, the late phase of bone loss and increased bone turnover may also be age-related, but perhaps exacerbated by estrogen deficiency. The relative increase in bone turnover during the later stages of life is more pronounced in OVX rats than in control rats. This finding is consistent with the theory that bone is more responsive to PTH in the estrogen-deficient state [33].

Closed growth plates were detected in the proximal tibiae of control rats at 15 months of age, which is earlier than previously reported [34, 35]. However, it should be emphasized that growth plate closure was not a consistent event but occurred only in roughly a quarter of control animals. Growth plates remained open in all OVX rats. As estrogen is thought to be an antagonist to growth hormone [36], cartilage function may persist for a longer period of time in the estrogen-deficient state due to increased sensitivity to growth hormone.

In summary, OVX rats exhibited a biphasic pattern of cancellous bone loss in the proximal tibial metaphysis. An initial, rapid phase of bone loss was followed by an intermediate period during which cancellous bone volume stabilized at a markedly osteopenic level. The subsequent late phase was characterized by a slow rate of bone loss. Both the initial, rapid phase and the late, slow phase of bone loss were associated with increased bone turnover. Age-related bone loss in control rats was also associated with increased bone turnover. The mechanism for the bone loss in these high turnover states presumably involves an imbalance between bone resorption and formation with an emphasis on the former skeletal process. Our findings are indicative of a close temporal relationship between the development of osteopenia and increased bone turnover in OVX rats as well as in aged, control rats.

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