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HISTOMORPHOMETRIC ANALYSIS OF THE RAT PROXIMAL TIBIAL METAPHYSIS BY "LINEAR SCANNING"

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Abstract

Twenty-four female Sprague-Dawley rats (10 weeks old, 200g BW) were either sham-operated (n = 6) or ovariectomized (ovx). Ovx rats were divided into 3 groups (n = 6 each): ovx; ovx + 1,25-$(OH)_2D_3$; ovx + 1,25$(OH)_2D_3$ + 1,24,25-$(OH)_3D_3$. The vitamin D metabolites were fed orally starting the day after surgery. After 7 weeks all rats were sacrificed and the proximal tibiae were processed undecalcified for quantitative histomorphometry. Conventional histomorphometric analysis of the distal zone (> 1 mm from the growth cartilage) of the tibial metaphysis revealed a dramatic loss of cancellous bone mass in ovx rats. Both 1,25$(OH)_2D_3$ and the combination of 1,25$(OH)_2D_3$ with 1,24,25$(OH)_3D_3$ prevented the bone loss in the distal zone in ovx animals. Measurements in the proximal zone (< 1 mm from the growth cartilage) of the tibial metaphysis were performed with a newly developed technique that utilizes the advantages of automatic image analysis, and that we propose to name "linear scanning". This method revealed a significantly decreased hard tissue mass at about 100 µm and within 800 to 950 µm distance from the growth plate in ovx rats. However, ovx rats reached normal amounts of hard tissue within 250 to 450 µm from the growth plate. The results obtained by linear scanning suggest that the obvious loss of cancellous bone mass in the distal zone of the tibial metaphysis in growing ovx rats is not a consequence of structural changes in the proximal zone.

KEY WORDS: Rat - Ovariectomy - Bone - Metaphysis - Bone growth - Histomorphometry - Osteopenia - 1,25-Dihydroxycholecalciferol - 1,24,25-Trihydroxycholecalciferol

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Introduction

For a correct histomorphometric analysis of the growing long bone metaphysis it is necessary to collect data from homogeneous tissue bands equidistant to the growth cartilage-metaphyseal junction (GCMJ), i.e. from temporally equivalent zones [11]. The manual method introduced by Kimmel and Jee [11], and the method given by Schenk et al. [26] using automatic image analysis, provide excellent means for the quantitative histomorphometric analysis of the growing long bone metaphysis. A major disadvantage of both methods, however, is their limited spatial resolution due to the use of optical measuring *fields* of considerable size (usually about 0.04 mm^2). In order to overcome this disadvantage, and to quantitatively evaluate the distribution and the architecture of cancellous bone mass in the rat proximal tibial metaphysis, we developed an automatic image analysis method that uses a measuring *line* to scan the hard tissue of the tibial metaphysis in parallel to the GCMJ. We propose to name this technique "linear scanning". The high spatial resolution of this method is particularly useful for analyses of the most proximal parts of the metaphysis close to the GCMJ, where rapid changes in hard tissue mass and composition occur within a very small area [11].

It is well established that ovariectomy induces osteopenia in the distal zone (> 1 mm from the growth cartilage) of the rat proximal tibial metaphysis [28, 29, 31-34]. On the other hand, very little is known about the effects of ovariectomy on the proximal zone (< 1 mm from the growth cartilage) of the rat long bone metaphysis. It is still unclear whether preformed changes in the proximal zone, i.e. diminished formation of bone trabeculae, contribute to the loss of cancellous bone mass in the distal zone of growing ovariectomized (ovx) rats. The main purpose of this study was to elucidate the effects of ovariectomy on the proximal zone of the tibial metaphysis in

the growing rat, using the linear scanning technique.

Several studies have shown that vitamin D metabolites can be effective in prevention and therapy of the osteopenia induced by ovariectomy in the rat [5, 8, 13-15]. Recent work from our laboratory indicated that a combination of vitamin D metabolites might be superior to the application of one vitamin D metabolite alone [20-23]. Therefore, the other aspect of this study was to evaluate the ability of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and of a combination of $1,25(OH)_2D_3$ with 1,24R,25-trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$] to prevent the development of osteopenia in the long bone metaphysis of ovx rats.

Material and Methods

Twenty-four female 10-week-old Sprague-Dawley rats (Mus-Rattus, FRG) weighing 190 - 210 g were used for this experiment. Eighteen rats were bilaterally ovariectomized by a ventral approach under xylazine/ketamine anesthesia and the remaining 6 rats were sham-operated. The animals were kept in individual cages and fed a standard laboratory diet (Altromin, FRG) containing 0.9% calcium, 0.75% phosphorus and 600 IU/kg vitamin D_3. Food and tap water were available *ad libitum*. The rats were allocated by weight in the following groups:

Group 1: sham-operated (n = 6)
Group 2: ovx (n = 6)
Group 3: ovx + 50 ng $1,25(OH)_2D_3$/rat/d (n = 6)
Group 4: ovx + 50 ng $1,25(OH)_2D_3$/rat/d + 50 ng $1,24,25(OH)_3D_3$/rat/d (n = 6)

The vitamin D metabolites were dissolved in ethanol/1,2 propandiol (1 : 10) and added to the diet, starting the day after surgery. Similar dose levels of $1,25(OH)_2D_3$ have been shown to increase bone mass in ovx rats in previous studies [5, 8, 14]. Seven weeks postovariectomy all rats were sacrificed by an ether overdose. Success of ovariectomy was confirmed by failure to detect ovarian tissue and observation of marked atrophy of the uterine horns.

According to the formula:

$$n = 2(Z_\alpha + Z_\beta)^2\sigma^2/\delta^2, \qquad (1)$$

and with a standard deviation $\sigma \leq 25\%$ (which is a reasonable value for most of the histomorphometric parameters in the rat tibial metaphysis), a sample size of n = 6 is sufficient to detect an about 50% difference δ between means at a two-tailed 5% level of significance (α = 0.05) and a power P' = 0.80 (β = 0.20) [27].

Histology

At autopsy, the right tibia was carefully defleshed and with a fine saw the proximal end of the bone (about 10 mm long) was separated from the shaft and fixed immediately in 40% ethanol at 4°C for 48 hours on a magnetic stirrer [2]. After fixation, the bones were dehydrated and embedded in methylmethacrylate (MMA) according to the method given by Baron et al. [2], with the following modifications: After dehydration, the specimens were cleared in two steps of xylene (2 x 2 days) [25], and the bones were infiltrated in each MMA solution for 7 days on a magnetic stirrer. Polymerization was carried out in a waterbath at 41°C.

Midsagittal, longitudinal, 5 µm thick undecalcified sections were prepared with a Jung Polycut sledge microtome (Reichert-Jung, FRG). The plane in which the sections were sampled was determined by the following three structures: tuberositas tibiae, eminentia intercondylaris and widest lumen of the diaphysis. This regimen ascertained that every bone was sampled in the same region. Sections for automatic image analysis were stained with von Kossa [2], and counterstained with

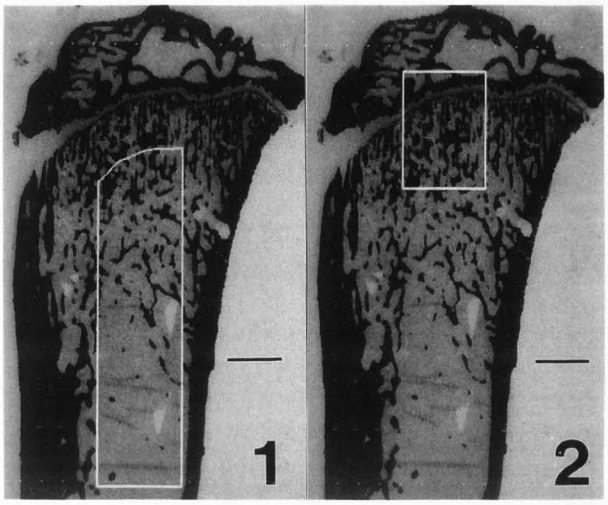


Fig. 1. Undecalcified, midsagittal, 5 µm thick section of the proximal tibia of a sham-operated rat. White overlay represents the measuring field (~10 mm²) for histomorphometry in the distal zone of the tibial metaphysis. Von Kossa/toluidine blue stain mounted in von Apáthy's gum syrup. Bar = 1 mm.

Fig. 2. Undecalcified, midsagittal, 5 µm thick section of the proximal tibia of a sham-operated rat. White overlay represents the measuring field for histomorphometric analysis with the linear scanning technique in the proximal zone of the tibial metaphysis. Von Kossa/toluidine blue stain mounted in von Apáthy's gum syrup. Bar = 1 mm.

toluidine blue at acid pH [2]. After fixation of toluidine blue with 5% ammonium molybdate (Merck No. 1182) [24], the sections were directly mounted (without dehydration) in von Apáthy's gum syrup.

Preparation of von Apáthy's gum syrup [24]: Diligently dissolve 50 g gum arabic (Merck No. 4282) and 50 g cane sugar in 50 ml dH_2O on a waterbath. Add 0.5 g thymol (Merck No. 8167) as a preservative and dH_2O to the desired consistency. The syrup can be kept indefinitely. In contact with air it becomes very hard and the von Kossa/toluidine blue stain is preserved in it for years.

The sections for the assessment of the osteoclast distribution in the rat tibial metaphysis using a semiautomatic technique were stained with toluidine blue at pH 3.7 and mounted in Eukitt (Riedel de Haën, FRG) [2].

Histomorphometry

All measurements were performed on the cancellous bone of the proximal tibial metaphysis. Structural parameters in the proximal and distal zone were determined using an automatic image analysis system (IBAS, C. Zeiss, FRG) connected to a Zeiss Universal microscope (C. Zeiss, FRG) via a TV-camera (Bosch, FRG). All measurements with the automatic image analysis system were performed on sections stained with von Kossa/toluidine blue and mounted in von Apáthy's gum syrup. The osteoclast distribution in the proximal zone of the tibial metaphysis was determined using a semiautomatic system (Videoplan, C. Zeiss, FRG).

Distal zone. For this measurement a x 1.25 plan objective was used. The measuring field is shown in Figure 1. It encompasses the whole non-trajectorial cancellous bone in the distal zone of the tibial metaphysis. The term "non-trajectorial" refers to the cancellous bone in the central part of the metaphysis that is not directly connected with the cortical bone, and that, as a consequence, does not have a direct weight-bearing function. The area within 1 mm from the growth plate was excluded from the measurements [11]. The average measuring area was about 10 mm^2 in each section. The image analysis system automatically determined the measuring area (= tissue area, T.Ar), total bone area (Tt.B.Ar), total bone perimeter (Tt.B.Pm) and the number of trabeculae within the measuring area (N.Tb). From these data the following parameters were calculated [19]:

Bone area (B.Ar/T.Ar) = Tt.B.Ar/T.Ar * 100 [%] (2)

Bone perimeter (B.Pm/T.Ar) = Tt.B.Pm/T.Ar [mm/mm^2] (3)

Trabecular width (Tb.Wi) = Tt.B.Ar/Tt.B.Pm * 2000 [µm] (4)

Trabecular number (N.Tb/T.Ar) = N.Tb/T.Ar [$\#/mm^2$] (5)

Trabecular area (B.Ar/N.Tb) = Tt.B.Ar/N.Tb [mm^2] (6)

Since the structural elements in the cancellous bone of the rat proximal tibial metaphysis show a highly anisotropic distribution, and since the three-dimensional architecture of the rat tibial metaphysis is unknown, we used only two-dimensional terms.

Proximal zone. For this measurement a x 6.3 plan objective was used. The overall magnification of the system was 3.2 µm/pixel (picture element of the monitor screen), which resulted in a measuring area of about 1.6 x 2.4 mm. Figure 2 shows the position of the measuring field within the bone section. Figure 3 depicts a typical measuring sequence. The principle of the measurement that we propose to name "linear scanning" is as follows:

1) A line right at the beginning of the first calcified structures in the hypertrophic zone of the growth cartilage was drawn interactively with the cursor (Fig. 3c). For technical reasons this line had to be 3 pixels in width (about 10 µm with the above mentioned scaling factor). The length of the line was measured automatically (Line length, Ln.Le).

2) In parallel with this first line a measuring line (also 3 pixels wide) was then shifted automatically in steps of 5 pixels (about 16 µm) through the proximal 1 mm of the tibial metaphysis (about 60 steps). On every step the following parameters were determined automatically (Fig. 3e): a) the number of trabeculae hit by the measuring line (N.Tb), and b) the total length of the measuring line that lay upon calcified structures (Total mineralized length, Tt.Md.Le). From these data the following parameters were calculated [19]:

Trabecular number-linear referent (N.Tb/Ln.Le) = N.Tb/Ln.Le [#/mm] (7)

Total trabecular width-linear referent (Md.Le/Ln.Le) = Tt.Md.Le/Ln.Le [µm/mm] (8)

Trabecular width-linear referent (Md.Le/N.Tb) = Tt.Md.Le/N.Tb [µm] (9)

These parameters were stored as a function of distance from the first calcified structures in the hypertrophic zone of the growth cartilage. For brevity, this distance will be termed "distance from the growth plate" in the

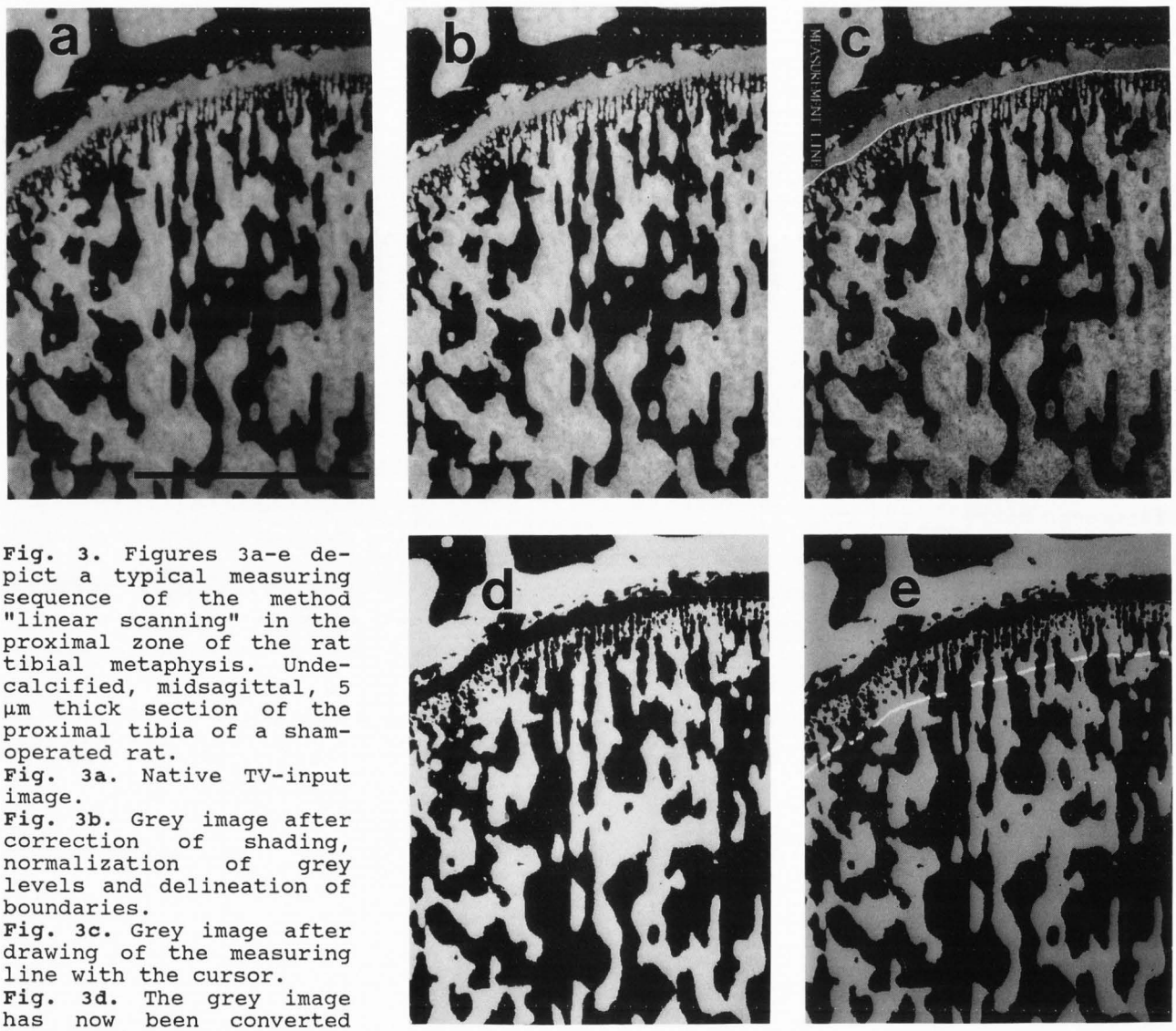


Fig. 3. Figures 3a-e depict a typical measuring sequence of the method "linear scanning" in the proximal zone of the rat tibial metaphysis. Undecalcified, midsagittal, 5 µm thick section of the proximal tibia of a sham-operated rat.
Fig. 3a. Native TV-input image.
Fig. 3b. Grey image after correction of shading, normalization of grey levels and delineation of boundaries.
Fig. 3c. Grey image after drawing of the measuring line with the cursor.
Fig. 3d. The grey image has now been converted into a binary image. Note that the white structures in the binary image are identical with the black calcified structures in Figures 3a-c.
Fig. 3e. In this binary image (to make the measuring line better visible the image was artificially darkened and the measuring line itself dilated) the measuring line is depicted at a distance from the starting point of the measurement of about 200 µm. Only the parts of the line that lie upon calcified structures (white in the binary image) are visible.
See text for detailed information. Von Kossa/toluidine blue stain mounted in von Apáthy's gum syrup. Bar = 1 mm.

following, and exclusively used in this sense. Note that "distance from the growth plate" in this context is not identical with the distance from the GCMJ, which is defined as a line connecting the last intact transverse septa of the chondrocyte lacunae. The distance between the first calcified structures in the hypertrophic zone of the growth cartilage and the GCMJ is about 30 - 50 µm in Sprague-Dawley rats of the weight and age used in this experiment.

One section was analyzed per animal. In all groups of animals, this sampling scheme resulted in coefficients of variation (CV = $SD/\bar{x} * 100$) of less than 25% for all three calculated parameters (total trabecular width-linear referent, trabecular width-linear referent, and trabecular number-linear referent) in the area within 400 µm from the starting point of the measurement. At greater distances than 400 µm, the coefficients of variation were generally higher (between

10 and 40%) due to the decreased number of structural elements (below 10/mm line length). However, since the differences between the group means also increased at distances greater than approximately 700 µm from the growth plate, the statistical probability of showing a significant difference between the groups remained almost the same as within the first 400 µm.

The time requirement for the complete measurement of one bone section with this method is about 15 minutes.

Osteoclast distribution in the proximal zone. This measurement was made with a semiautomatic system at a magnification of 250x on sections from sham and ovx animals stained with toluidine blue. In a 1.2 mm wide measuring field in the central part of the tibia, the first 500 µm of the proximal zone of the tibial metaphysis were evaluated. The location of this measuring field was similar to that shown in Figure 2. One section was analyzed per animal. The total number of osteoclasts found within this region was 420 (201 in sham-operated rats, and 219 in ovx rats, n = 6 animals each). Osteoclasts were defined as large, irregularly shaped cells with 1 or more nuclei and a foamy, slightly metachromatic cytoplasma. In parallel with the long axis of the bone, the distance of the osteoclasts from the growth plate was determined by a two-point measurement. For reasons of comparability with the above described linear scanning we chose the first calcified structures in the hypertrophic zone of the growth cartilage (and not the GCMJ) as reference structures for the distance measurement.

Statistical analysis

The data were analyzed using the Kruskal-Wallis H-test. When the Kruskal-Wallis H-test performed over all groups indicated a significant ($p<0.05$) difference among the groups, statistical differences between two groups were evaluated with the two-tailed Wilcoxon-Mann-Whitney U-test. P values of less than 0.05 were considered significant. Data are presented as the mean $\bar{x}$ ± SD.

Results

The final body weights at the end of the experiment were: 246 ± 16 g (sham), 304 ± 9 g (ovx, $p<0.01$ vs. sham), 304 ± 19 g (ovx + $1,25(OH)_2D_3$, $p<0.01$ vs. sham), and 306 ± 6 g (ovx + $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$, $p<0.01$ vs. sham).

Thus, the application of vitamin D metabolites had no influence on the significantly higher weight gain in ovx rats.

Distal zone of the tibial metaphysis

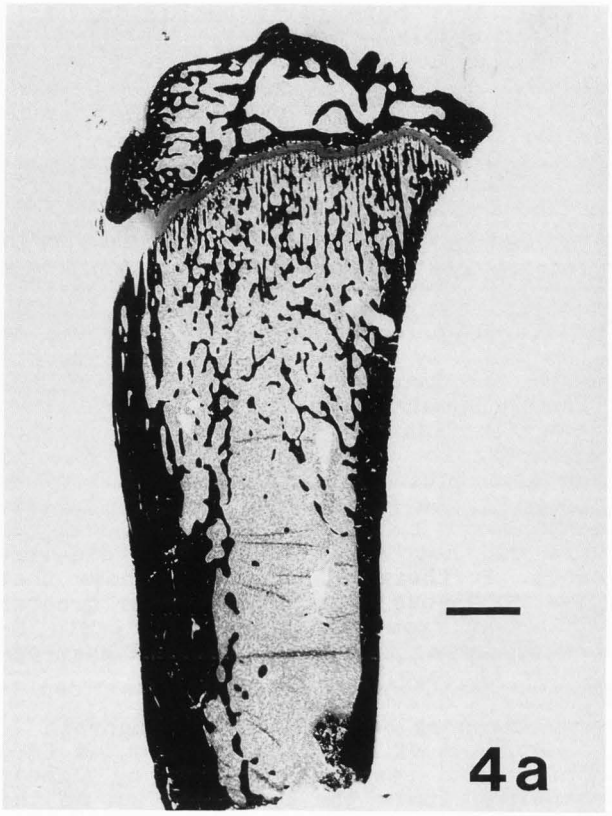
Effects of ovariectomy. Ovariectomy caused a dramatic fall in bone area (B.Ar/T.Ar), bone perimeter (B.Pm/T.Ar), and trabecular number (N.Tb/T.Ar) ($p<0.005$; Fig. 4a,b; Fig. 5a,b,d). Ovx animals also showed a reduction in trabecular width (Tb.Wi) and trabecular area (B.Ar/N.Tb) ($p<0.05$; Fig. 5c,e), when compared with the sham-operated group.

Effects of treatment with vitamin D metabolites. Both the treatment of ovx rats with $1,25(OH)_2D_3$ and the combination of $1,25(OH)_2D_3$ with $1,24,25(OH)_3D_3$ proved highly effective in preserving the cancellous bone of the proximal tibial metaphysis (Fig. 4c,d; Fig. 5). There was no statistically significant difference between the two ovx groups receiving the vitamin D metabolites and the sham group in any parameter. In most of the histomorphometric parameters treatment with the combination of vitamin D metabolites seemed to be less effective than treatment with $1,25(OH)_2D_3$ alone, although this did not reach statistical significance. Furthermore, Figure 4 shows that the cancellous bone extended to greater distances from the GCMJ in vitamin D-treated rats, when compared with sham-operated animals.

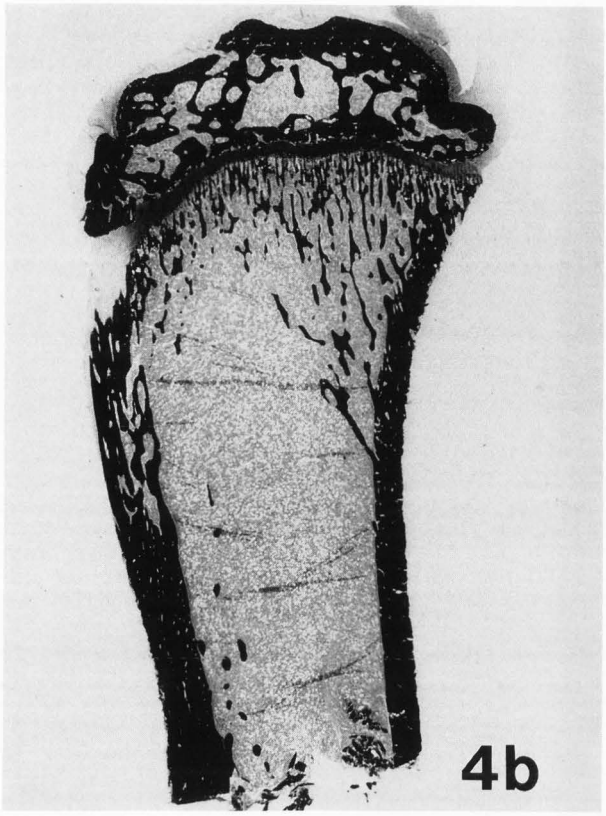
Proximal zone of the tibial metaphysis

Effects of ovariectomy. Figures 6a-c depict the results obtained by linear scanning within the first 600 µm of the proximal zone of the tibial metaphysis. The parameter total trabecular width-linear referent (Md.Le/Ln.Le) (Fig. 6a), which is an index of the total amount of hard tissue, was significantly lower in ovx animals within 96 to 127 µm ($p<0.05$) distance from the growth plate. Also, ovx rats demonstrated a significant decrease in trabecular width-linear referent (Md.Le/N.Tb) (Fig. 6b) within 80 to 111 µm ($p<0.01$) from the growth plate. The parameter trabecular number-linear referent (N.Tb/Ln.Le) did not show significant differences between the sham and the ovx group (Fig. 6c). Within approximately 250 to 450 µm distance from the growth plate, ovx rats reached normal values for total trabecular width-linear referent (Md.Le/Ln.Le), trabecular width-linear referent (Md.Le/N.Tb), and trabecular number-linear referent (N.Tb/Ln.Le).

The osteoclast distribution within the first 500 µm of the proximal zone of the tibial metaphysis is depicted in Figure 7. The number and distribution of osteoclasts was almost identical in sham and ovx animals. Figure 7 demonstrates that the total number of osteoclasts was high in the area within 100 µm from the growth plate where initial resorption of calcified cartilage trabeculae takes place, and low within about 100 to 200 µm distance from the growth plate where intense osteoblastic bone formation is found [10-12]. Thus, the osteoclast distribution correlated well with the dis-



4a



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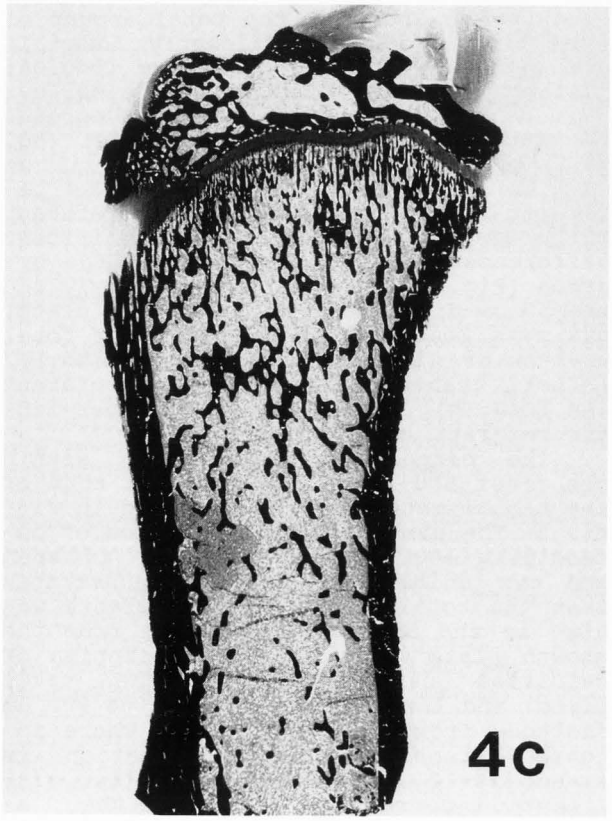
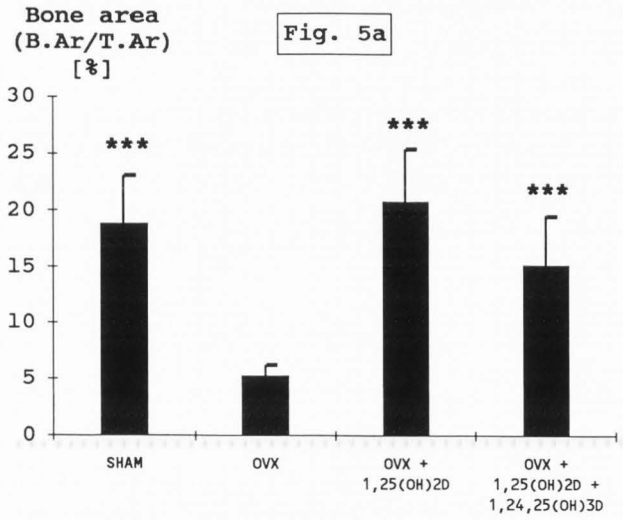
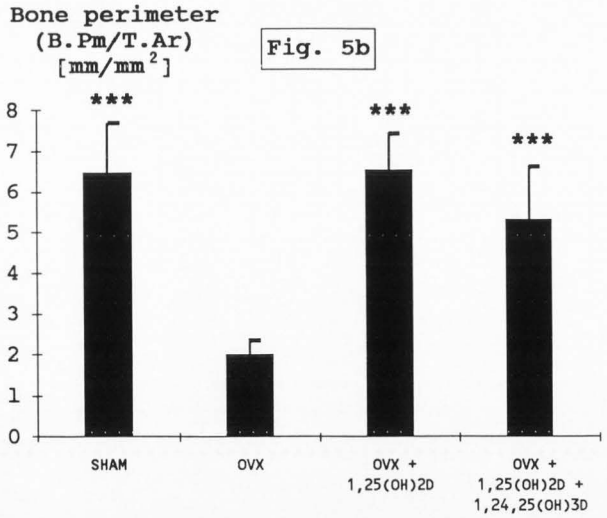


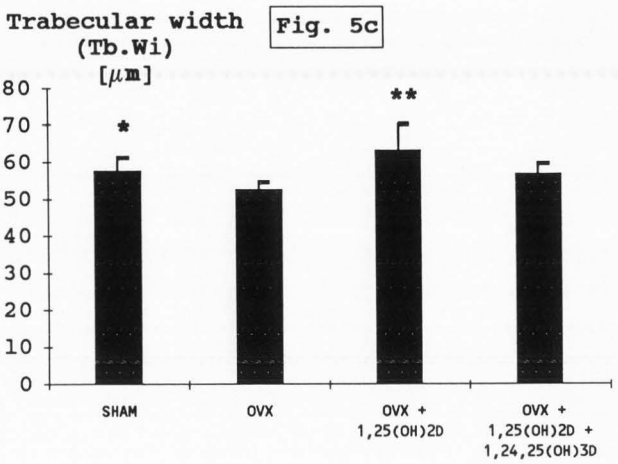
4c

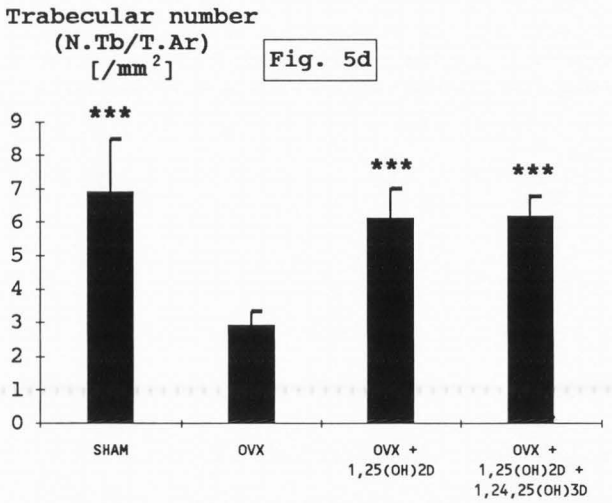
4d

Fig. 4. Representative undecalcified, midsagittal sections (5 μm thick) of the proximal tibiae of sham-operated rats (**Fig. 4a**), ovx rats (**Fig. 4b**), ovx rats treated with $1,25(OH)_2D_3$ (**Fig. 4c**), and ovx rats treated with $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$ (**Fig. 4d**). Note that vitamin D metabolites prevented the cancellous bone loss in the proximal tibial metaphysis of ovx rats and that, compared with sham-operated animals, the cancellous bone in vitamin D-treated rats extended to greater distances from the growth cartilage-metaphyseal junction. Von Kossa/toluidine blue stain mounted in von Apáthy's gum syrup. Bar = 1 mm.









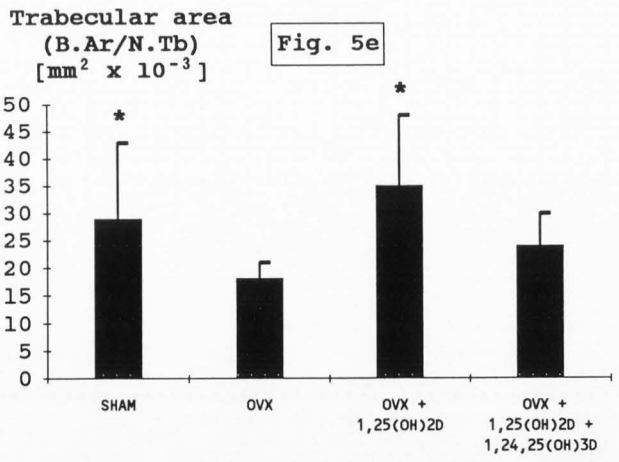
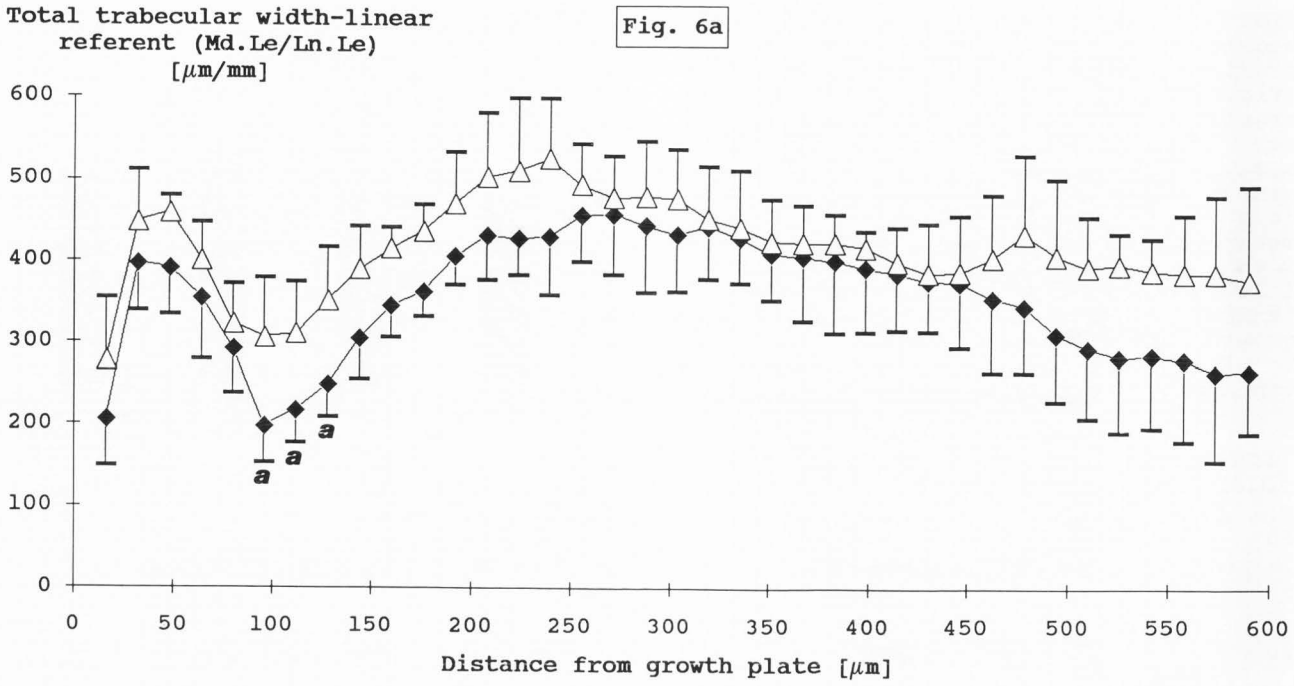


Fig. 5. Bone area (B.Ar/T.Ar) (**Fig.5a**), bone perimeter (B.Pm/T.Ar) (**Fig. 5b**), trabecular width (Tb.Wi) (**Fig. 5c**), trabecular number (N.Tb/T.Ar) (**Fig.5d**), and trabecular area (B.Ar/N.Tb) (**Fig. 5e**) in the distal zone of the tibial metaphysis in sham-operated rats, ovx rats, ovx rats treated with $1,25(OH)_2D_3$, and ovx rats treated with $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$. n = 6 for each group. All values are means ± SD. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.005$ vs. ovx.



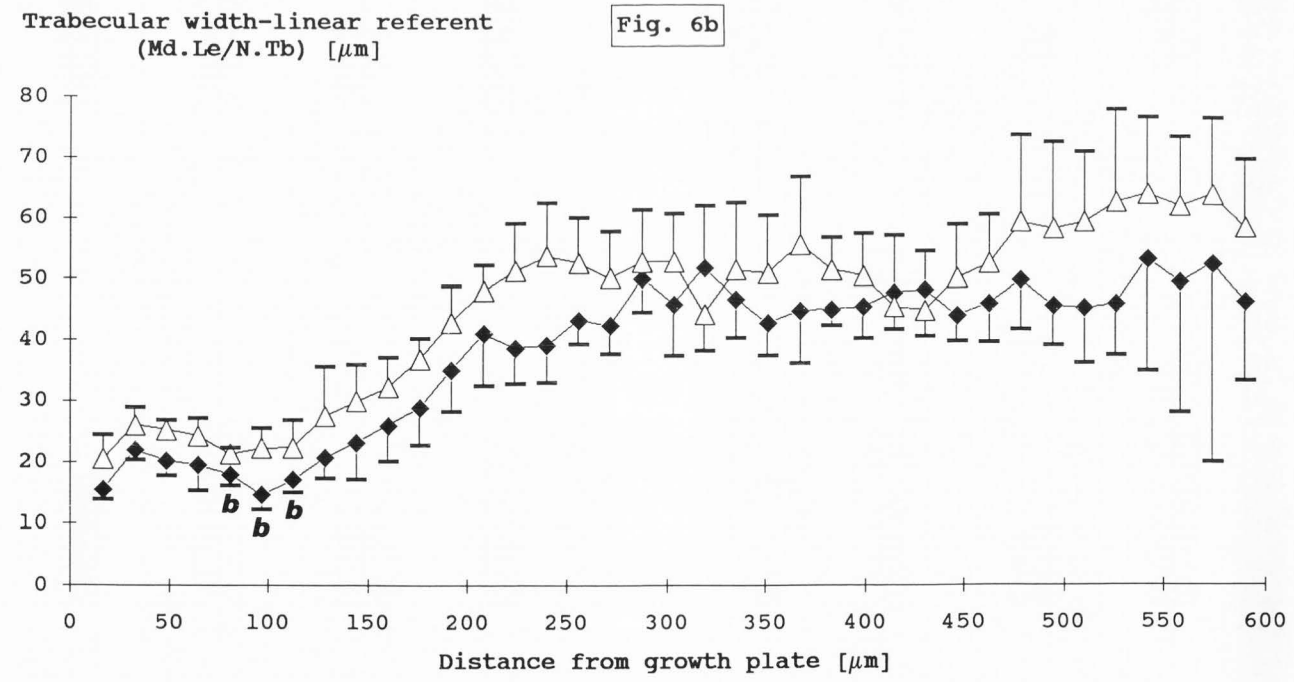
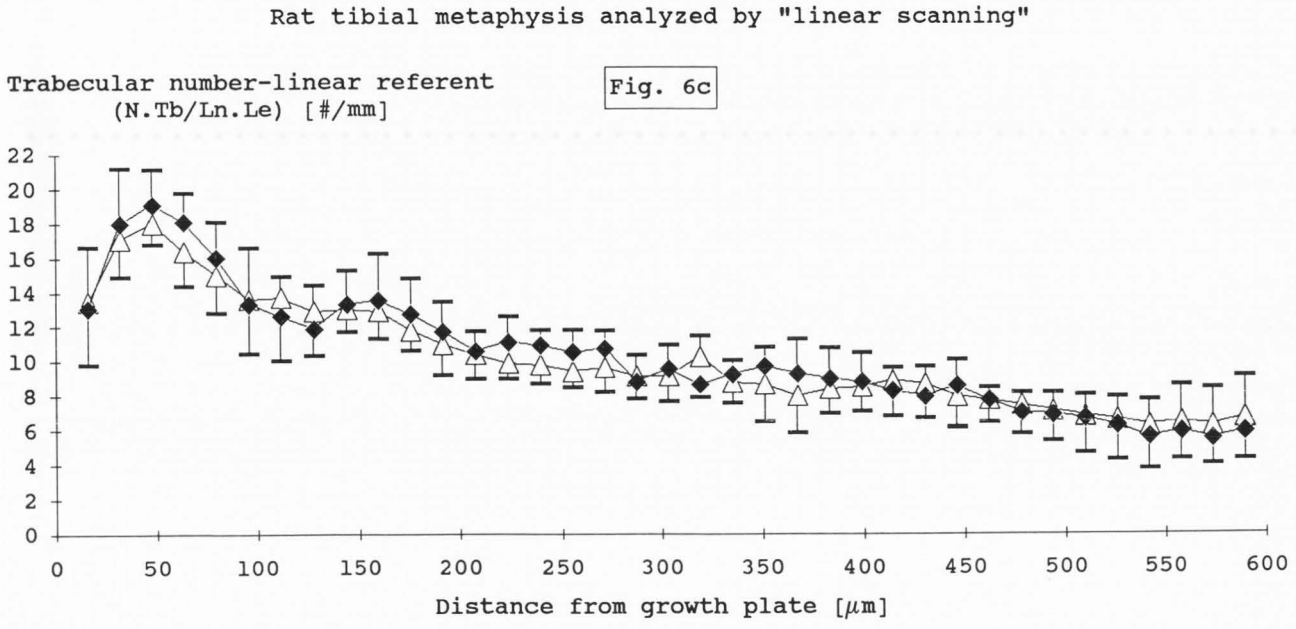


Fig. 6. Histomorphometric analysis of the proximal zone of the tibial metaphysis by linear scanning in sham-operated (△) and ovx rats (◆). The parameters total trabecular width-linear referent (Md.Le/Ln.Le) (**Fig. 6a**), trabecular width-linear referent (Md.Le /N.Tb) (**Fig. 6b**), and trabecular number-linear referent (N.Tb/Ln.Le) (**Fig. 6c**) are plotted as a function of distance from the first calcified structures in the hypertrophic zone of the growth cartilage (termed "distance from growth plate"). Each data point is the mean ± SD of 6 animals. (**a**) $p<0.05$; (**b**) $p<0.01$ vs. sham.



tribution of hard tissue in the most proximal parts of the tibial metaphysis.

Between 600 and 955 μm distance from the growth plate, ovx rats exhibited significantly decreased values for total trabecular width-linear referent (Md.Le/Ln.Le) within 796 to 955 μm ($p<0.01$; Fig. 8), and for trabecular width-linear referent (Md.Le/N.Tb) within 876 to 924 μm from the growth plate ($p<0.01$; data not shown).

Effects of treatment with vitamin D metabolites. Both $1,25(OH)_2D_3$ alone (Gp 3) and the combination of $1,25(OH)_2D_3$ with $1,24,25(OH)_3D_3$ (Gp 4) prevented the ovariectomy-induced decrease in trabecular width-linear referent (Md.Le/N.Tb) (data not shown) and total trabecular width-linear referent (Md.Le/Ln.Le) (Fig. 8) within the first 130 μm of the proximal zone in ovx animals (trabecular width-linear referent: $p<0.05$ Gp 3 vs. ovx, $p<0.01$ Gp 4 vs. ovx; total trabecular width-linear referent: $p<0.01$ Gps 3 and 4 vs. ovx; 96 μm distance from the growth plate). Within approximately 850 to 955 μm distance from the growth plate, ovx rats treated with vitamin D metabolites showed higher values for total trabecular width-linear referent (Md.Le/Ln.Le) than untreated ovx rats (Fig. 8), but control levels were not reached and statistical significance was observed only for the group receiving $1,25(OH)_2D_3$ ($p<0.05$ within 892 to 955 μm, Gp 3 vs. ovx). Compared with untreated ovx rats, the parameter trabecular number-linear referent (N.Tb/Ln.Le) remained almost uninfluenced by treatment with the metabolite combination (Gp 4, data not shown). In ovx rats treated with $1,25(OH)_2D_3$ alone (Gp 3), however, there was a (non-significant) tendency towards increased numbers of trabeculae in the whole proximal zone (data not shown).

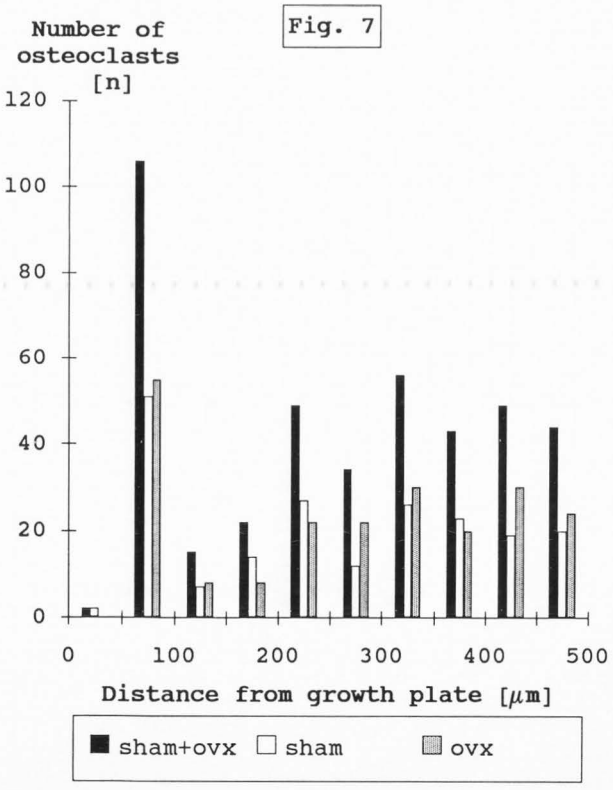


Fig. 7. Figure 7 shows a histogram of the osteoclast distribution within the first 500 μm of the proximal zone of the tibial metaphysis, evaluated in ovx and sham-operated rats (n = 6 each). The distance of 420 osteoclasts (total number; 201 and 219 osteoclasts in sham and ovx animals, respectively) from the first calcified structures in the hypertrophic zone of the growth cartilage (termed "distance from growth plate") was measured.

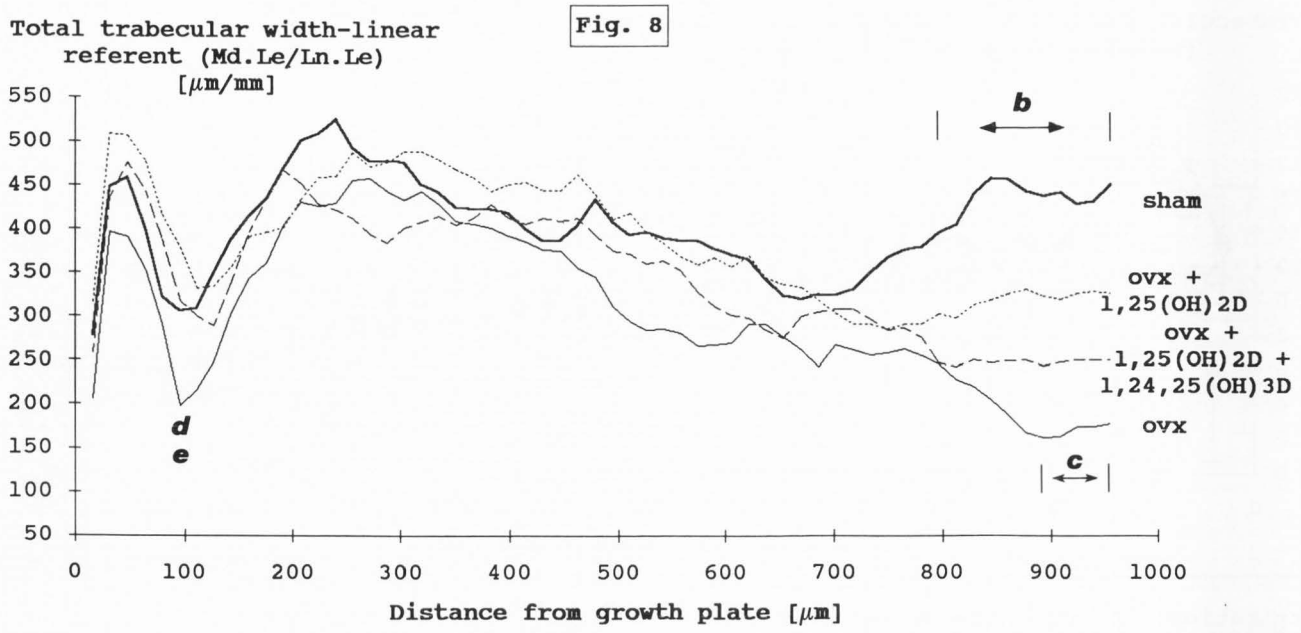


Fig. 8. Histomorphometric analysis of the proximal zone of the tibial metaphysis by linear scanning in sham-operated rats, ovx rats, ovx rats treated with 1,25-$(OH)_2D_3$ (Gp 3), and ovx rats treated with $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$ (Gp 4). The parameter total trabecular width-linear referent (Md.Le/Ln.Le) is plotted as a function of distance from the first calcified structures in the hypertrophic zone of the growth cartilage (termed "distance from growth plate"). For reasons of clearness, the standard deviations were not included in this graph. Each data point is the mean of 6 animals. (**b**) $p<0.01$ sham vs. ovx; (**c**) $p<0.05$ Gp 3 vs. ovx; (**d**) $p<0.01$ Gp 3 vs. ovx; (**e**) $p<0.01$ Gp 4 vs. ovx.

Discussion

The advantages of the above described method "linear scanning" can be summarized as follows: 1) high spatial resolution, 2) minimal time requirement, 3) the spatial and temporal structure of the proximal zone of the rat tibial metaphysis can be allowed for by the use of a curved measuring line, and 4) with the help of special staining techniques it is possible to measure all structures that can be selectively stained (e.g. calcified cartilage).

Since linear scanning is based upon automatic image analysis, the use of artefact-free sections is a sine qua non [9]. The preparation of nearly artefact-free undecalcified sections was accomplished with the help of a modified von Kossa stain together with a hydrophilic mounting medium.

Figures 9a and 9b demonstrate that even the smallest structures in the vicinity of the GCMJ were distinctly longer than the width (~10 μm) and the distance (~16 μm) of the measuring lines. Thus, the applied measuring technique also allowed an adequate sampling of the smallest hard tissue trabeculae near the GCMJ.

Ovariectomy-induced bone loss in the distal zone of the tibial metaphysis [28, 29, 31-34] accompanied by increased bone turnover [7, 16, 28, 29, 31-34] is a well-documented phenomenon. The current study also demonstrates a highly significant loss of cancellous bone mass in the distal zone of the tibial metaphysis in ovx rats 7 weeks postovariectomy.

The application of vitamin D metabolites prevented the development of osteopenia in the distal zone of the tibial metaphysis in ovx rats. However, when compared to sham-operated controls, ovx rats treated with vitamin D metabolites exhibited an altered morphology of the cancellous bone in the distal zone (Fig. 4). The mechanism of action of vitamin D metabolites probably consists in inhibition of bone resorption. When compared with sham-operated animals, the cancellous bone in vitamin D-treated ovx rats extended to greater distances from the GCMJ (Fig. 4), which is indicative of reduced bone resorption. In a very similar fashion this was observed in the study by Wronski et al. [30]. Several studies have shown that chronic administration of vitamin D metabolites inhibits bone resorption *in vivo* in the rat [3, 4, 18, 30], and also in humans [1, 6, 17]. The combination of $1,25(OH)_2D_3$ with $1,24,25(OH)_3D_3$ in the present study did

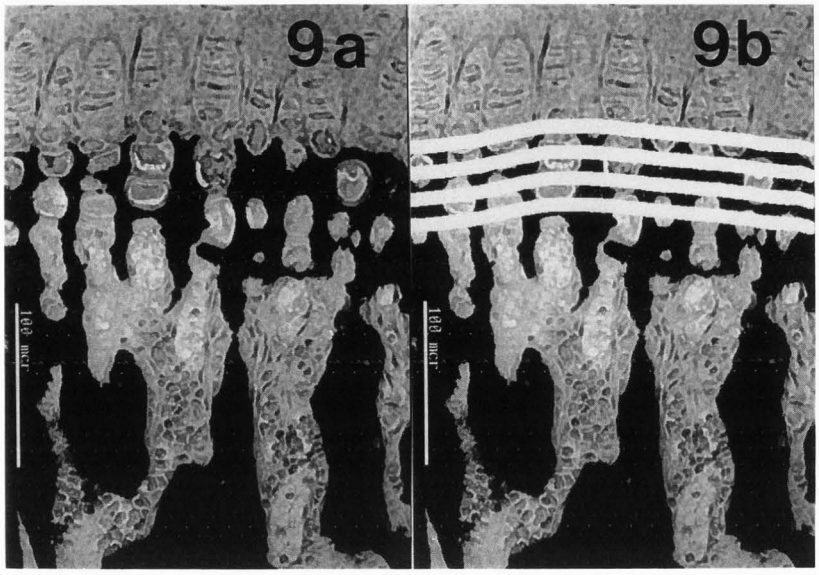


Fig. 9. Undecalcified, mid-sagittal section (5 µm thick) of the proximal tibia of a sham-operated rat, depicting the growth cartilage and the proximal zone of the metaphysis (**Fig. 9a**). In Figure 9b (**Fig. 9b**) 4 measuring lines of the method "linear scanning" were drawn (to scale) into the TV-image of Figure 9a. The width (~10 µm) and the distance (~16 µm) of the measuring lines correspond to the true dimensions in linear scanning (according to the applied measuring technique: scaling factor 3.2 µm/pixel; measuring line 3 pixels in width; 5 pixels distance between the measuring lines). Von Kossa/toluidine blue stain mounted in von Apáthy's gum syrup. Scaling factor 0.485 µm/pixel; x 40 objective; Bar = 100 µm.

not result in additive or over-additive, synergistic effects. On the contrary, the combination of the two vitamin D metabolites seemed to be less effective than the application of $1,25(OH)_2D_3$ alone.

The present study with 10-week-old Sprague-Dawley rats extended over a period of 7 weeks. Using data from other experiments with Sprague-Dawley rats of comparable age [29, 33], longitudinal bone growth in the proximal tibial growth plate of both ovx and sham-operated rats can be expected to amount to approximately 2 - 3 mm over the whole experimental period. Thus, at the end of the experimental period, the proximal parts of the cancellous bone in the distal zone of the tibial metaphysis consisted of bone newly formed under the influence of ovariectomy. Therefore, ovariectomy-induced structural changes in the proximal zone could contribute to the bone loss observed in the distal zone. The serial histomorphometric study by Wronski et al. [33] showed that alterations in longitudinal bone growth in ovx rats, which could affect the interpretation of histomorphometric data obtained in the proximal zone, are restricted to the first several weeks after ovariectomy. A significant increase in longitudinal bone growth in ovx rats was observed only at 2 weeks postovariectomy. At all times later, this parameter was not different in ovx and sham-operated rats.

By linear scanning we could demonstrate that ovx rats reached normal values for total trabecular width-linear referent (Md.Le/Ln.Le), trabecular width-linear referent (Md.Le/N.Tb), and trabecular number-linear referent (N.Tb/Ln.Le) in the area within 250 to 450 µm distance from the growth plate. Thus, the osteoblastic formation of new bone trabeculae in the proximal zone of the rat tibial metaphysis was not disturbed by ovariectomy. It is concluded that the osteopenic changes in the distal zone of the tibial metaphysis in growing ovx rats are caused by a process leading to destruction of a normal bone architecture built up within the first 300 - 400 µm of the tibial metaphysis. Ovariectomy-induced structural changes in the first 500 µm of the proximal zone do not seem to play a causative role in the development of osteopenia in the distal zone of the tibial metaphysis in growing ovx rats. This concept is consistent with the finding that a site of major cancellous bone loss in ovx rats is located beyond approximately 800 µm distance from the growth plate (Fig. 8), i.e. in the transitional area between the proximal and distal zone.

The significantly decreased values for total trabecular width-linear referent (Md.Le/Ln.Le) and trabecular width-linear referent (Md.Le/N.Tb) at about 100 µm distance from the growth plate in ovx rats are indicative of elevated osteoclast activity in the zone immediately below the GCMJ. However, with the applied measuring technique the possibility cannot be excluded that diminished formation of calcified cartilage tissue in ovx rats contributed to this phenomenon. In the same area, ovx rats treated with vitamin D metabolites demonstrated normal values for total trabecular width-linear referent (Md.Le/Ln.Le) and trabecular width-linear referent (Md.Le/N.Tb). The finding that the number of osteoclasts located within the first 100 µm of the proximal

zone was not different in sham and ovx animals does not argue against increased osteoclast activity in ovx rats at this skeletal site. The osteoclasts close to the GCMJ usually present as very large cells containing multiple nuclei, and appropriate estimation of osteoclast activity in this zone requires quantification of the number of nuclei per osteoclast, and of osteoclast cellular size.

Within 100 µm distance from the growth plate, osteoclastic (chondroclastic) resorption of calcified cartilage trabeculae is the dominating process (Fig. 10). Although osteoblasts forming osteoid at calcified cartilage surfaces are frequently found within this region also, mineralization of osteoid does not start before approximately 100 µm distance from the growth plate (Fig. 10). Since osteoid is stained only lightly (light blue to yellow) by the von Kossa/toluidine blue stain used for automatic image analysis in this study, osteoid was not measured by the linear scanning technique. Thus, the changes in histomorphometric parameters documented by linear scanning within the first 100 µm from the growth plate are predominantly due to osteoclastic resorption of calcified cartilage. In the area within 100 to 200 µm distance from the growth plate, where osteoclasts were only infrequently found, osteoblastic woven bone formation and mineralization of osteoid are the dominating processes (Fig. 10). This phenomenon is documented by rapidly increasing values for total trabecular width-linear referent (Md.Le/Ln.Le) and trabecular width-linear referent (Md.Le/N.Tb) in this region (Fig. 6a and 6b), reflecting osteoblastic bone formation capacity. By linear scanning it is therefore possible to spatially separate calcified cartilage resorption from bone formation and to measure the "functional output" of the cells involved in these processes. The proximal zone of the rat tibial metaphysis is a site of very high bone cell activity [11]. Therefore, linear scanning might prove to be a valuable tool in the investigation of hormonal or drug effects on bone metabolism.

Acknowledgements

The vitamin D metabolites were kindly supplied by Dr. H. Weiser, Hoffmann-La Roche Ltd, Basel. The authors gratefully thank Mr. W.E. Köditz, Kontron Electronics, Eching, FRG and Mr. J. Famula, C. Zeiss, München, FRG for providing the IBAS automatic image analysis system and the Videoplan semiautomatic system, and for their expert technical assistance. We are grateful to Mr. J. Stanglmeier for the statistical analyses. This research was supported in part by Deutsche Forschungsgemeinschaft.

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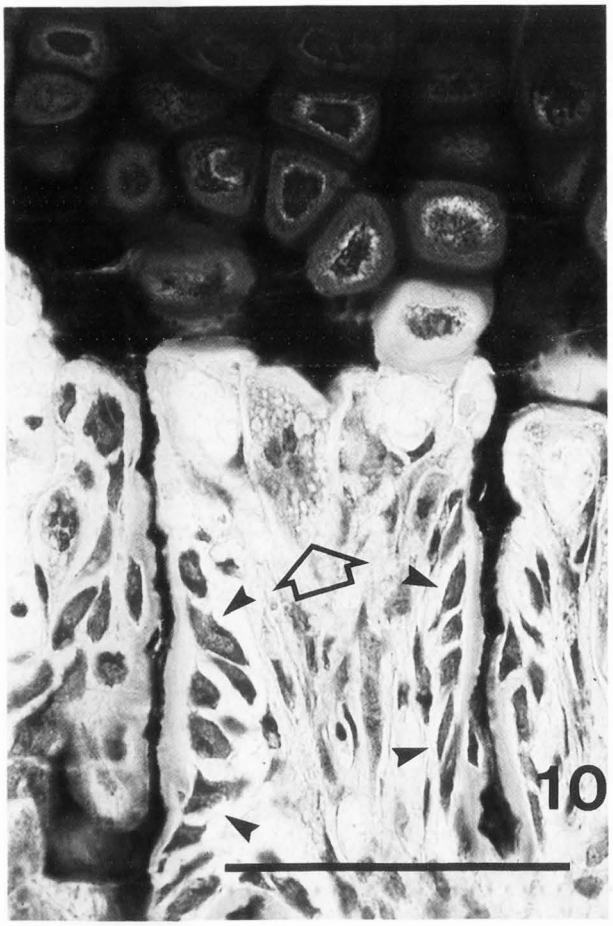

Fig. 10. Undecalcified, 5 μm thick section of the proximal tibia of an ovx rat, depicting the GCMJ and the most proximal parts of the metaphysis. Note osteoclast (chondroclast) resorbing calcified cartilage (→) and osteoblasts synthesizing unmineralized bone matrix (▸) on calcified cartilage trabeculae. Osteoid light blue; calcified cartilage dark purple; mineralized bone light purple. Toluidine blue stain mounted in Eukitt. Bar = 100 μm.

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Discussion with Reviewers

H.H. Malluche: Appropriate design of this experiment should include a group of rats receiving $1,24,25(OH)_3D_3$ only and a group receiving $1,25(OH)_2D_3$ at a dose of 100 ng/day, i.e. equivalent to the total dose of vitamin D metabolites given in the group receiving both metabolites.

Authors: We generally agree with this comment. However, we have conducted a series of experiments in the past few years that have characterized the effects of $1,24,25(OH)_3D_3$ in the ovx rat model at different dose levels (unpublished data). A group of rats receiving $1,25(OH)_2D_3$ together with $1,24,25(OH)_3D_3$ was included in this experiment to specifically test whether there would be pronounced synergistic effects on calcium and bone metabolism at the pharmacological dose of $1,25(OH)_2D_3$ used in this study (50 ng/rat/d). The activity of $1,24,25(OH)_3D_3$ is lower than that of $1,25(OH)_2D_3$ by a factor of about 2 to 5 [35]. Thus, a dose of 100 ng $1,25(OH)_2D_3$/rat/d cannot be considered to be equivalent to a dose of 50 ng $1,25(OH)_2D_3$/rat/d combined with 50 ng $1,24,25(OH)_3D_3$/rat/d.

H.H. Malluche: The animals were fed *ad libitum* and the metabolites were mixed with the rat diet. How was the amount of metabolites absorbed by the animals controlled and what is the activity of the metabolite(s) when added to the diet and over time under those conditions? Since one of the limitations of the use of vitamin D metabolites is the occurrence of hypercalcemia and hypercalciuria, measurements of serum and urinary calcium are needed.

Authors: In order to keep the daily dose of vitamin D metabolites constant in each group, the concentration of the vitamin D metabolites in the diet was adjusted to the food intake of the rats at regular intervals. Several studies in rats have shown that orally administered hydroxylated vitamin D metabolites are readily and almost completely absorbed in the upper small intestine [38, 39]. When mixed with the diet, the activity of vitamin D metabolites in various bio-assays is equal to or even slightly higher than the activity of the same vitamin D metabolites given by stomach tube (personal communication, Dr. H. Weiser, Hoffmann-La Roche Ltd). Over the short time intervals used in this experiment, the applied vitamin D metabolites can be regarded as sufficiently stable in the diet (personal communication, Dr. H. Weiser, Hoffmann-La Roche Ltd).

The activity of the orally administered vitamin D metabolites can also be demonstrated by their pronounced hypercalcemic and hypercalciuric effects in the current study: 50 ng $1,25(OH)_2D_3$/rat/d caused a 13% rise in serum calcium, and a 6-fold increase in urinary calcium in ovx rats [37].

H.H. Malluche: Accuracy and precision of linear scanning are not provided as well as the optimal number of measurements needed to ascertain representative results.

Authors: The precision of linear scanning almost solely depends on the optomanual skills of the investigator, provided that artefact-free sections are used. The most critical step in the whole measuring procedure is the drawing of the measuring line. If this is done carefully, the reproducibility of the results obtained with linear scanning in one section is good (intraobserver variation < 5%). When employed at an appropriate magnification, automatic image analyzers are very accurate measuring instruments with a measuring error of generally less than 1%. Since the measuring line is curved and forms an angle of about 10 - 20° with the perpendicular on the long axis of the tibia, the results obtained for the (primary) parameter Tt.Md.Le are subject to an error of about 5 - 10% (compared with a "true" value measured perpendicularly to the long axis of the bone). Thus, the results for the parameters total trabecular width-linear referent (Md.Le/Ln.Le) and trabecular width-linear referent (Md.Le/N.Tb) are biased and cannot be considered to be very accurate. However, when analyzing the proximal zone of the tibial metaphysis, it is a must to sample temporally equivalent zones [11], which necessitates the use of a curved measuring line in parallel to the growth plate in linear scanning.

In order to ascertain representative results for each group of rats, we considered the optimal number of measurements to be 1 section per animal, since this sampling regimen resulted in acceptable coefficients of variation (< 25%) for all parameters within the first 400 μm of the tibial metaphysis [36].

H.H. Malluche: The "linear scanning method" has been only applied to the proximal zone. Comparisons of results obtained with linear scanning and semiautomatic method should have been done in proximal and distal zone. This would validate the technique and allow an accurate comparison between proximal and distal zone.

M.W. Lundy: It would be helpful if the authors state why conventional histomorphometric techniques have not been applied to the proximal zone.

Authors: As stated in the Introduction, the main advantage of linear scanning over conventional histomorphometric techniques is its high spatial resolution. With a reasonable expenditure of time, it is not possible to obtain the same kind of information on the spatial distribution of the metaphyseal hard tissue (and with the same spatial resolution as in linear scanning) with semiautomatic or manual techniques. Moreover, the results obtained with conventional semiautomatic or manual techniques using measuring *fields* can only partially be compared with the results obtained with the linear scanning technique using a measuring *line*, i.e. a completely different measuring geometry. The changes in cancellous bone architecture in the distal zone of the tibial metaphysis in ovx rats are obvious (Fig. 4) and well characterized, and it was not the aim of this study to compare the proximal and distal zone of the metaphysis, using the linear scanning technique.

R.W. Boyce: Would the authors like to comment how the changes in the coefficient of variation of the parameters affect the sensitivity of the measurement? Does this limit the practical utility of this method to the most proximal region of the metaphysis?

Authors: It is clear that the increase in the coefficient of variation, observed for all three linear scanning parameters at distances greater than about 400 μm from the growth plate, reduces the sensitivity of the measurement to detect a given difference between group means in a statistically significant fashion. However, this does not limit the practical utility of the method, since the coefficients of variation in the more distal parts of the metaphysis could be decreased either by increasing the number of sections analyzed per animal and/or by increasing the number of animals per group [36].

T.J. Wronski: The authors are probably correct in their contention that the protective effect of vitamin D metabolites against osteopenia in ovx rats involves inhibition of bone resorption. However, they should support their contention with histomorphometric indices of bone resorption and also determine the effect of vitamin D metabolites on bone formation.

M.W. Lundy: A disadvantage of this method (linear scanning) is that it cannot distinguish osteoid, as one of the effects of excess vitamin D metabolites may be hyperosteoidosis. It would be interesting to know whether the inclusion of $1,24,25(OH)_3D_3$ with $1,25(OH)_2D_3$ alters osteoid formation and mineralization.

Authors: Histomorphometric analysis of the cancellous bone of the first lumbar vertebra revealed a highly significant reduction ($p<0.005$) in osteoclast number (N.Oc/Md.Pm) in ovx rats treated with vitamin D metabolites in the present study [37]. We did not perform tetracycline labeling in this experiment. As judged by histological examination, there was no obvious hyperosteoidosis in $1,25(OH)_2D_3$-treated ovx rats in the tibial metaphysis. However, static histomorphometric data from the vertebral cancellous bone indicated that the treatment with $1,25(OH)_2D_3$ alone resulted in an impairment of osteoid mineralization, as indicated by a reduction in the parameter osteoblast-osteoid ratio (Ob.Pm/O.Ar = osteoblast perimeter/osteoid area) [mm/mm^2], which from our experience is a very sensitive static index of mineralization in the cancellous bone of the rat:

144 ± 57	sham
136 ± 15	ovx
112 ± 22	ovx + $1,25(OH)_2D_3$
142 ± 23	ovx + $1,25(OH)_2D_3$ + $1,24,25(OH)_3D_3$ (unpublished data)

The changes in this parameter were not significant, though. The inclusion of $1,24,25(OH)_3D_3$ with $1,25(OH)_2D_3$ antagonized the reduction in osteoblast-osteoid ratio (Ob.Pm/O.Ar), and (non-significantly) decreased osteoid area (O.Ar/B.Ar), osteoid perimeter (O.Pm/B.Pm), osteoblast perimeter (Ob.Pm/B.Pm), and osteoid width (O.Wi) in vertebral cancellous bone, when compared with animals receiving $1,25(OH)_2D_3$ alone (unpublished data). Interestingly, the impaired mineralization in $1,25(OH)_2D_3$-treated ovx rats was also demonstrable with the linear scanning technique: The normally rapid increase in total trabecular width-linear referent (Md.Le/Ln.Le) within 100 to 200 μm distance from the growth plate (Figures 6a and 8) was either delayed or otherwise distorted in rats receiving $1,25(OH)_2D_3$ alone (except for 1 animal). In the same area, rats treated with the

metabolite combination showed a normal rate of increase in total trabecular width-linear referent (Md.Le/Ln.Le). Thus, it can be inferred that 1,24,25-$(OH)_3D_3$ improves osteoid mineralization in combination with hyperosteoidosis-inducing doses of $1,25(OH)_2D_3$. This effect of $1,24,25(OH)_3D_3$ is more pronounced, though, when the molar ratio of 1,24,25-$(OH)_3D_3$:$1,25(OH)_2D_3$ is about 2:1 (unpublished data).

H.H. Malluche: Inferences are made about osteoblastic formation, direct measurements of osteoblastic number and activity obtained by tetracycline labelling would have been more appropriate.
Authors: The main aim of this study was to address the question whether ovariectomy results in structural changes in the proximal zone of the metaphysis that could partially explain the well-documented cancellous bone loss in the distal zone of the metaphysis in the growing rat. There is ample evidence that ovariectomy stimulates osteoblast activity and enhances osteoblast and osteoclast number in the rat [7, 16, 28, 29, 31-34]. It cannot be deduced from these well-known data, however, whether the formation of new bone trabeculae in the proximal metaphysis would be normal or abnormal. It was our intent to show in this study that linear scanning could provide a means to measure the *result* of cellular activity and number, i.e. the amount and the structure of the hard tissue in the proximal zone of the metaphysis.

T.J. Wronski: Since there are major differences in trabecular structure between untreated and treated ovx rats in the distal zone, did the authors perform linear scanning at this skeletal site? The results would certainly be more dramatic than in the proximal zone.
R.W. Boyce: What happens to the scan data beyond 1 mm?
Authors: We did not perform linear scanning in the distal zone of the tibial metaphysis in this experiment. Nevertheless, this method could be used to quantify the abnormalities in cancellous bone distribution in the distal zone induced by ovariectomy or treatment of ovx rats with vitamin D metabolites. In the present study, however, these structural changes were relatively gross and could easily be demonstrated with conventional histomorphometric methods.

T.J. Wronski: In future studies, pair-feeding of sham-operated and ovx rats is recommended to minimize body weight differences between groups as an experimental variable.
Authors: We agree with this comment. Despite increased body weight, however, ovx rats fed *ad libitum* develop pronounced cancellous bone osteopenia [32]. Thus, augmented body fat mass does not seem to be a major protective factor against ovariectomy-induced osteopenia in the rat.

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