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The visualization and evaluation of bone architecture in the rat using three-dimensional X-Ray microcomputed tomography

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Abstract: Microcomputed tomography allows the true three-dimensional structure of bone to be assessed by a nondestructive analysis. This article describes how this technique has for the first time been applied to rat bone to determine the effects of aging, ovariectomy, and antiresorptive drugs on bone structure and how these results compare with those determined by histological and histomorphometric techniques. During the procedure, a micro X-ray source is directed toward the bone sample. Modifications in the X-ray beam induced by bone crystals are determined for a range of acquisitions before three-dimensional reconstruction of bone architecture is performed. Morphometric parameters determined were trabecular bone volume/tissue volume, trabecular number, and trabecular thickness. The results show that ovariectomy has a dramatic effect on rat bone structure. Following treatment with the bone resorption inhibitor tiludronate, the morphometric parameters were significantly improved. The results obtained with three-dimensional microcomputed tomography were in agreement with observations made using classical techniques. Microcomputed tomography should prove useful for evaluating the antiresorptive effects of bisphosphonates on bone architecture and in allowing between-drug comparisons.

Key words: bone, rat, 3-D X-ray microcomputed tomography, histomorphometry, bisphosphonate

Introduction

The most frequently used and best-known animal model of osteoporosis is the ovariectomized female rat [1]. Until recently, characterization of bone modifications that have been induced either by aging, ovariectomy, or by drugs were limited to changes in bone mass, evaluated by bone ash weight or dual energy X-ray absorptiometry (DEXA); bone turnover, evaluated using biochemical markers of bone formation or resorption; and bone structure, assessed by classical histology and histomorphometry. The variations of all these parameters can be used as partial indicators of modifications in bone mechanical competence, which is directly determined in some experiments.

Histological and histomorphometric techniques can detect and measure cortical or trabecular changes observed in successive bone slices. As a result, these changes are observed only in two dimensions (2-D). New techniques using innovative computer software can transform 2-D measurements into three-dimensional (3-D) examples for analysis [2–4]. However, these stereological analyses are not true 3-D images, and moreover these techniques are destructive (as are all histological analyses).

Microcomputerized tomography (μCT) is a comparatively new technique that allows assessment and measurement of the 3-D structure of bone by a nondestructive analysis. Until recently, this method has only been available for the analysis of human bone [5–7]. The aim of this study was to apply this new technology to rat bone. This kind of analysis is complicated by the fact that trabecular diameter in rats is thinner ($\leq 100\,\mu m$) than in humans ($>120\,\mu m$) [8]. We examined whether μCT can detect variations in 3-D parameters caused by age of animal, ovariectomy, or treatment by a bone resorption inhibitor. We also examined whether there are similarities between these variations in 3-D parameters and those described previously in the literature or investigated in the same animal using classical methods.

Materials and methods

Samples

Female Sprague-Dawley rats from Iffa Credo (Domaine les Oncins, France) were housed individually

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throughout the experiment in controlled conditions. They were fed a normal diet and tap water was freely available. Ovariectomy or sham operation were performed by the lateral route under isoflurane anesthesia. At the end of the experiment, euthanasia was performed by overdose of sodium pentobarbital. The first lumbar vertebra (L1) was sampled, cleaned of soft tissue, and frozen at −20°C until measurements could be taken. The protocol was approved by the Sanofi animal ethical committee.

Experimental design

Three separate experiments were performed. The first was performed on bones from 1- to 28-month-old rats (three specimens for each age; eight different ages). The second experiment was performed on bone from rats that were ovariectomized at approximately 6.5 months of age. Analysis was performed on bone from animals killed up to 19 months following ovariectomy (three animals per group; three periods). The third experiment was performed to compare sham rats with ovariectomized rats and with ovariectomized rats treated with a bone resorption inhibitor. At the beginning of the experiment, rats aged 6.5 months old were ovariectomized or sham operated. After 3 months, they were treated 2 days/week for 1 year either with distilled water or by a bone resorption inhibitor (oral tiludronate, 125 mg/kg). At the end of the experiment, the rats were approximately 21.5 months old (n = 19–23 per group).

BMC measurement

Excised vertebrae from the first and second experiments were analyzed ex vivo for their bone mineral content (BMC) using a Hologic QDR1500 (Hologic, France) bone densitometer equipped with the ultrahigh-resolution software for small animals.

Microcomputerized tomography

The μCT and software used for this experiment were from Scanco Medical (Zürich, Switzerland), as described by Ruegsegger et al. [7]. The computer system was adapted to allow a simultaneous acquisition step on one sample and an analysis step on another sample.

The details of the μCT have been previously described [7,9]. Briefly, a micro X-ray source (10 μm, 25 keV) is directed toward the sample (maximum dimensions, 36 mm length, 14 mm diameter). The quantitative modification of the X-ray beam by apatite crystals contained in the bone sample is then analyzed by a plane detector (CDD array, 1024 elements). After a data acquisition time set at 200 ms, the sample is rotated less than 1°, and a new data acquisition is performed. This is repeated until the entire plan is covered (~600 measurements). The sample is then moved upward by a fixed length of 10 μm, and a new series of data acquisitions are performed for the new plan. The whole procedure is repeated on 200 virtual successive slices (total length of the sample analyzed. ~2 mm; total time for the data acquisition, 400 min). The process is piloted by a DEC α station (Digital Equipment, Marseille, France), and an open VMS system in cluster configuration to perform the 3-D analysis. The two stations share the same external disk system (RISK disk system, 4 × 4 Gb; Digital Equipment, Marseille, France) and are linked to a band unit (10 Gb) for data archiving.

3-D image display and analysis

From 2-D pictures, a volume of interest (VOI) was determined that corresponded to the maximum possible volume of trabecular bone in the vertebral body. This was standardized so that for all specimens the same sphere (VOI) was manually chosen and centered on the middle height of the body vertebra and included only the center area part of this body. The basivertebral vein region was not included in this region.

From the data acquisition of 2-D slices and selected VOI, 3-D reconstruction was automatically performed using triangulation algorithms (divide and conquer approach). In addition to triangulation, this also allows the surface to be smoothed. Pixel size in any direction is estimated at ~10 μm and the VOI is approximately 150 × 150 × 150 voxels. Morphometric indices, which can be directly determined from the binarized VOI, were bone volume/tissue volume (BV/TV) and trabecular number (TbN); nomenclature was as described for the 2-D analysis [10]. Trabecular thickness (TbTh) and trabecular separation (TbSp) were derived from these two primary parameters.

Preliminary experiments on the rat vertebra were specifically designed to select the correct threshold value, thus allowing a correct separation between bone tissue and nonmineralized tissue. This was done for various specimens, after the acquisition step, according to the procedure used for human bone [7]: the same data were analyzed several times by changing only threshold value. Then, BV/TV values were plotted against this threshold value and the partial derivatives (∂[BV/TV]/∂[threshold value]) calculated. When this partial derivative was equal to zero, changes in the threshold did not induce changes in the bone volume and thus corresponded to the correct threshold. This fixed threshold value for rat bone was found to be 22%.

2-D histomorphometry analysis

For the third experiment only, the L3 vertebra was fixed in a 70% ethanol solution, post-fixed for 7 days in

formol–methanol mixture (1:3), and dehydrated by ethanol wash before being embedded in methylmethacrylate [11–13]. The sections were performed using a Jung K microtome (Reichert, Nuslung, Germany). The vertebra was cut in parallel to the long axis of the vertebral body and six 5-μm-thick sections located in the middle of the transverse axis of the specimen were obtained. The sections were stained with toluidine blue. Starting 100–200 μm from the growth plate, all the trabecular bone (10–12 mm^2) was analyzed. The trabecular bone volume (BV/TV, %) was measured using a software package developed specifically for bone histomorphometry (Morphométrie Osseuse; Biocom, Les Ulis, France).

Statistical analysis

In the first experiment, the effect of age was analyzed by a one-way analysis of variance. In the second experiment, considering the small number of samples, the effect of ovariectomy was analyzed using a nonparametric test only (Kruskal–Wallis). In the third experiment, the effects were analyzed by a one-way variance analysis followed by a Student–Neuman–Keuls test. The relationship between 3-D and 2-D histomorphometry was evaluated by regression analysis. All tests were performed using RS1 (statistical software for PC developed and validated by Qualilab, Orléans, France).

Results

First experiment

The results obtained in the first experiment show that values of both measured and calculated 3-D parameters presented age-related variations, but these variations depend on the parameter ($P < 0.05$, variance analysis). In the first period (from 1 to 4 months old), these parameters increased sharply (Fig. 1).

In the second period (from 4 to 25 months old), the effect of age was different, depending on the parameter. For BV/TV and the calculated parameter TbTh, there was a continuous increase, although much less pronounced than in the first period. In contrast, TbN appears as a constant value up to and including the last measured period (28 months old; Fig. 1). For rats aged between 25 and 28 months, it seemed that age induced a marked decrease in BV/TV and TbTh but not in TbN (Fig. 1), whereas modification in the shape of the vertebra body was not detected (data not shown).

Second experiment

When ovariectomy was performed in adult rats, modifications of the measured or calculated 3-D parameters at

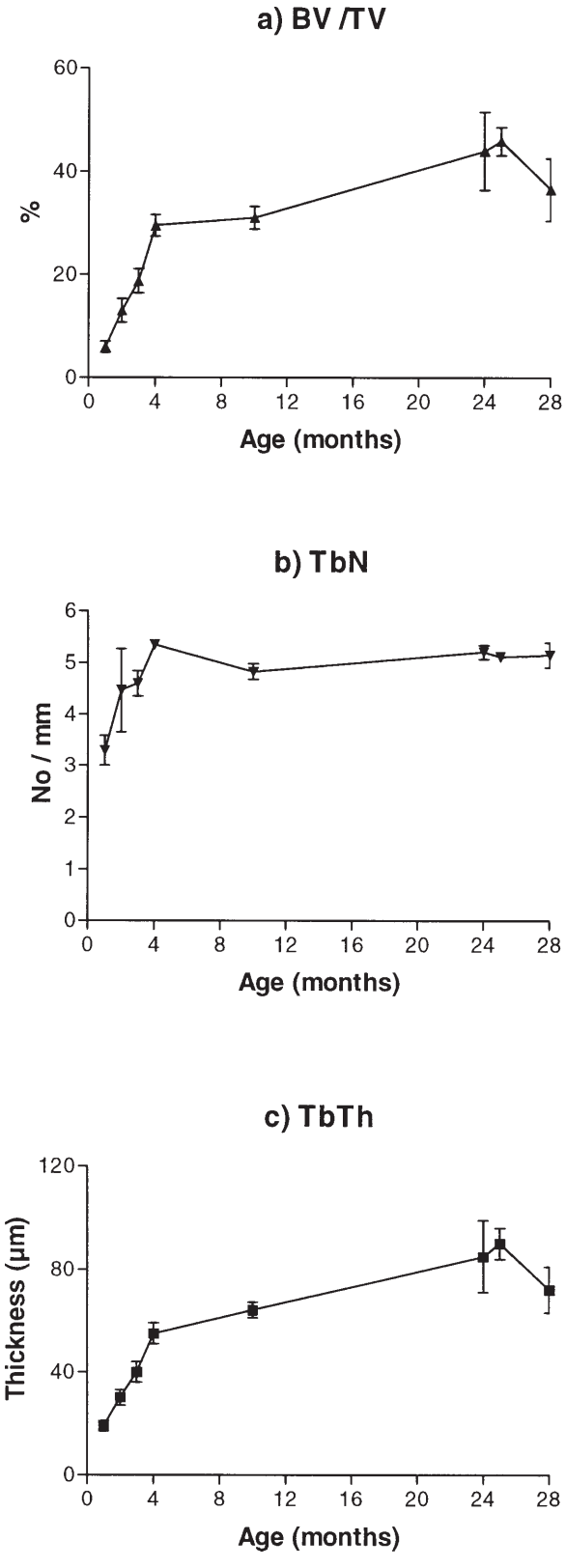

Fig. 1a–c. Influence of age of female rats on 3-D bone parameters (mean ± SEM, $n = 3$/group). For each three parameters, ANOVA analysis revealed significant intergroup differences: **a** bone volume/tissue volume (BV/TV); **b** trabecular number (TbN); **c** trabecular thickness (TbTh)

Table 1. Influence of time after ovariectomy (OVX) of female rats on measured 3-D bone parameters

Time after OVX (months)	BV/TV (%)	Δ %/normal rats, same age	TbN (no/mm)	Δ %/normal rats, same age
1	28.50 ± 4.74 (ns)	−7	5.34 ± 0.48 (ns)	+2
3	21.43 ± 1.30*	−31	4.15 ± 0.16*	−14
19	29.51 ± 1.33*	−36	4.94 ± 0.12 (ns)	−4

OVX performed at 6.5 months of age; 3 rats per group. Data are mean ± SEM
Analysis was performed by microcomputed tomography on L1 vertebra
BV/TV, bone volume/tissue volume; TbN, trabecular number
ns, nonsignificant *vs* sham-operated animals of the same age (Kruskal–Wallis test)
*, $P < 0.05$ *vs* sham-operated animals of the same age (Kruskal–Wallis test)

Table 2. Influence of the time after ovariectomy (OVX) of female rats on the calculated 3-D bone parameter

Time after OVX (months)	TbTh (μm)	Δ%/normal rats same age
1	52.6 ± 4.3 (ns)	−13
3	51.7 ± 2.8*	−19
19	59.6 ± 1.6*	−34

OVX performed at 6.5 months of age; 3 rats per group. Data are mean ± SEM
Analysis was performed by microcomputed tomography on L1 vertebra
TbTh, Trabecular thickness
ns, nonsignificant *vs* sham-operated animals of the same age (Kruskal–Wallis test)
*, $P < 0.05$ *vs* sham-operated animals of the same age (Kruskal–Wallis test)

the trabecular level were only detected after 3 months (Tables 1, 2). Values obtained 1 month after ovariectomy did not indicate significant changes (Tables 1, 2). The small decrease observed for BV/TV (−7%) was also observed for BMC, without statistical significance (data not shown).

At 3 months post ovariectomy, the decrease in BV/TV was more important than for TbTh or TbN. After 19 months, decreases in BV/TV and TbTh were equivalent, but not increased compared with BV/TV values observed at 3 months. This observation could be linked to the parallel decrease observed in nonovariectomized animals caused by age. In contrast, 19 months after ovariectomy, the decrease in TbN was not as clear.

Third experiment

In the aged rat (>21 months) 15 months after ovariectomy, the decrease in BV/TV (−47%) and in TbTh (−29%) was more marked (as illustrated by comparing Fig. 2a [sham] and Fig. 2b [ovariectomized]) than in experiment 2.

Treatment by a bone resorption inhibitor (starting after the bone loss resulting from ovariectomy was demonstrated) for 1 year led to a complete reversal of bone loss from ovariectomy (Fig. 3). Values for BV/TV, TbN, and TbTh in tiludronate-treated rats were higher than in ovariectomized rats but for two of the parameters were higher than that of sham animals (+113% and +12%, respectively, for BV/TV; +32% and −6%, respectively, for TbN; and +69% and +21%, respectively, for TbTh), as illustrated by comparing Fig. 4 (tiludronate) and Fig. 2a,b, respectively.

Two-dimensional histomorphometric analysis of the lumbar vertebra confirmed these data (Fig. 5). Ovariectomy induced a significant decrease in BV/TV (−34%), TbN (−24%), and TbTh (−14%). In these conditions, tiludronate treatment led to a normalization of all these parameters (+72% for BV/TV; +26% for TbN; +37% for TbTh) including TbSp (data not shown). Comparative analysis of 2-D histomorphometry and 3-D microtomography results show that for BV/TV (Fig. 6) and TbN (data not shown) the correlations are excellent, $r^2 = 0.750$ and $r^2 = 0.719$, respectively ($P < 0.001$). For TbTh (data not shown), the correlation between 2-D and 3-D is less ($r^2 = 0.394$) but still significant ($P < 0.001$).

Discussion

The ovariectomized rat is commonly used and considered as a good model for postmenopausal osteoporosis. This model has been widely reported in terms of bone mass changes [14], bone turnover modifications, morphometry by classical histology [15], and biomechanics [16]. The time course of vertebral osteopenia following ovariectomy has also been described using histomorphometrical parameters at the vertebral level [17]. Finally, the positive effects of bisphosphonates in this model have also been described for various parameters and compounds [18,19].

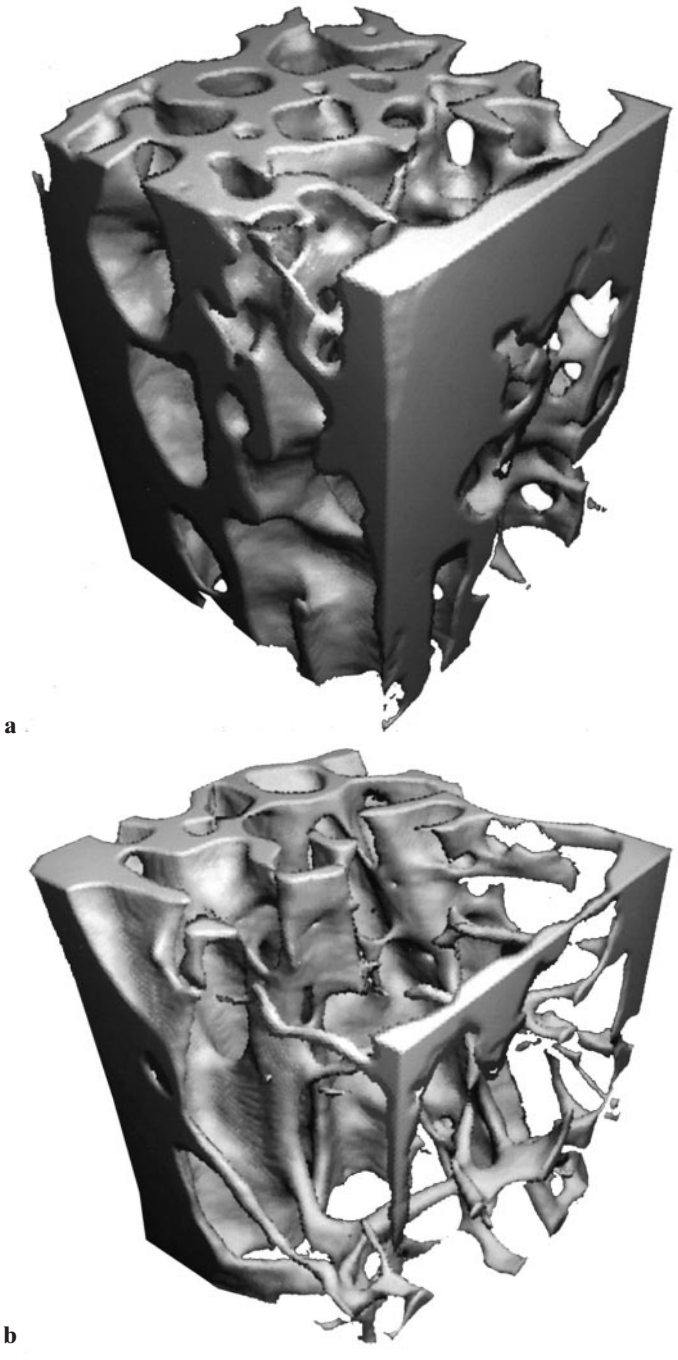

Fig. 2a,b. Three-dimensional (3-D) microcomputed tomography illustrations of L1 trabecular bone in the rat. **a** Sham-operated rat. **b** Ovariectomized rat

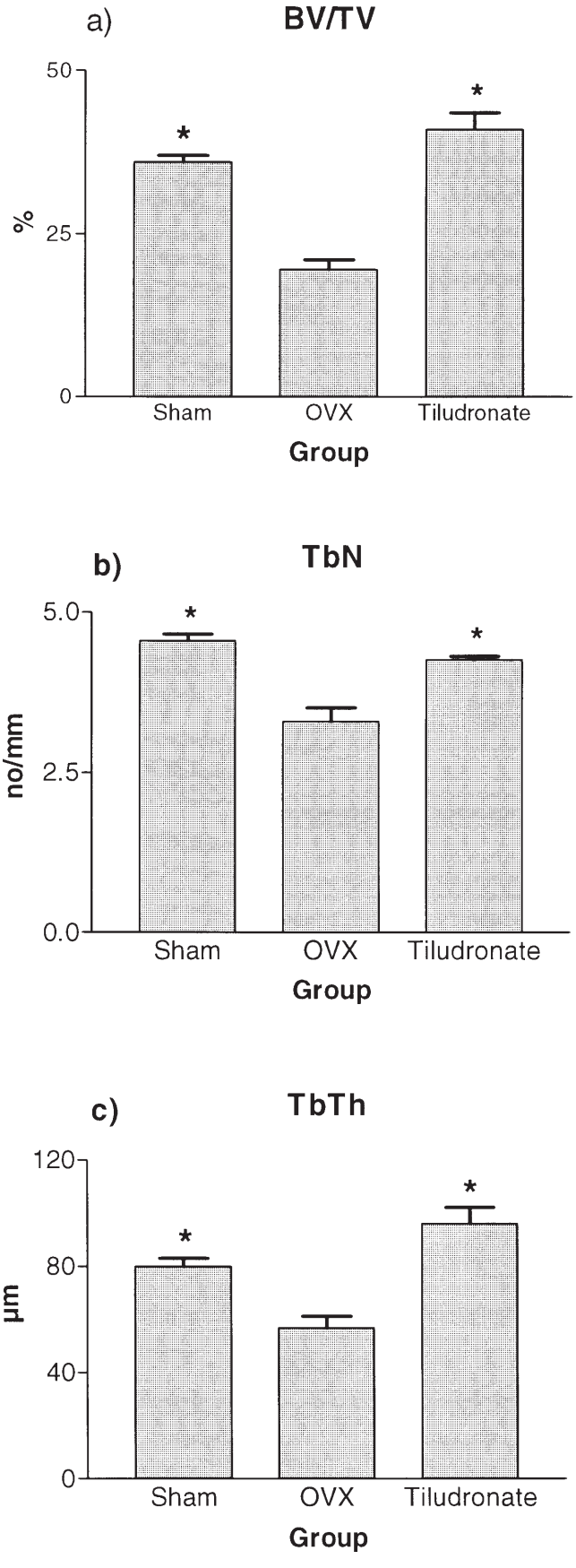

Fig. 3a–c. Influence of ovariectomy and treatment with a bone resorption inhibitor (125 mg/kg p.o., 2 days/week for 1 year) in the female rat on 3D bone parameters. *Sham*, sham-operated animals ($n = 19$); *OVX*, ovariectomized rats ($n = 23$); *Tiludronate*, OVX + tiludronate-treated rats ($n = 22$). Mean ± SEM; *, $P < 0.05$/OVX (Student–Neuman–Keuls). **a** BV/TV, bone volume/tissue volume; **b** TbN, trabecular number; **c** TbTh, trabecular thickness

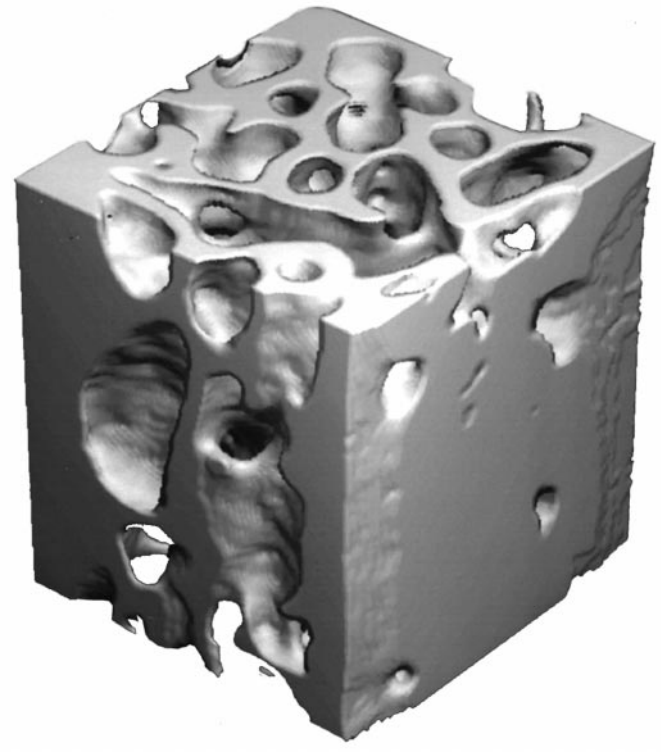

Fig. 4. 3-D microcomputed tomography illustration of ovariectomized rat trabecular bone from the L1 vertebra following treatment with the bone resorption inhibitor tiludronate (125 mg/kg p.o., 2 days/week for 1 year)

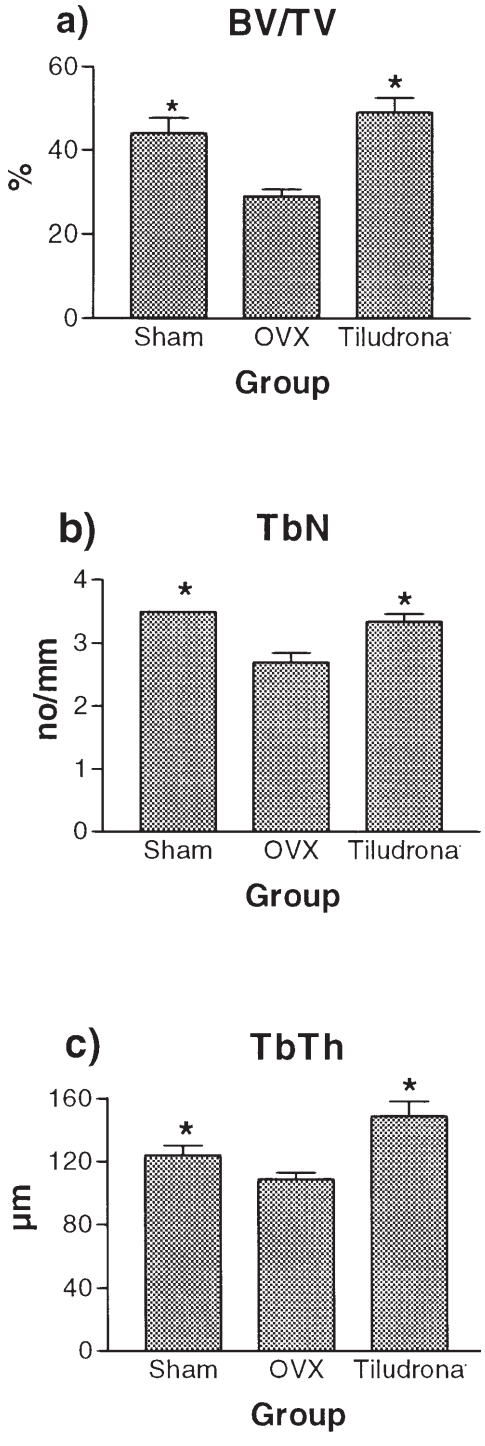

Fig. 5a–c. Two-dimensional (2-D) classical histomorphometry of rat lumbar vertebrae. *Sham*, sham-operated ($n = 19$); *OVX*, ovariectomized ($n = 23$); *Tiludrona*, OVX + tiludronate-treated rats (125 mg/kg p.o., 2 days/week for 1 year; $n = 22$). Mean ± SEM; *, $P < 0.05$/OVX (Student–Neuman–Keuls). **a** BV/TV, bone volume/tissue volume; **b** TbN, trabecular number; **c** TbTh, trabecular thickness

The development of new mathematical approaches to stereology led to an attempt to evaluate bone modifications following aging, menopause, or drug-induced effects. These techniques were primarily used for human bone [4] and then applied to rat bone [20]. These techniques are complementary to the classical histomorphometric analysis by allowing an estimation of the connectivity and anisotropy. Nevertheless, these techniques are time consuming, and the figures are extrapolated from 2-D sections. Peripheral computed tomography offers in vivo quantification of cortical and trabecular bone volume and surfaces, but it is of insufficient sensitivity to discriminate trabeculae that have a thickness (50–150 µm) which is less than the voxel dimensions of this technique (150–170 μm^3), as described by Breen et al. [21] for the rat and by Muller et al. [22] for humans.

The recent development of high-resolution computed tomography, also called microcomputed tomography (µCT), allows a nominal resolution smaller than the thickness of the trabeculae, offering true 3-D images of the trabecular network. The smallest voxel size (2 µm) was described by Bonse et al. [6] using a synchrotron source. The voxel size of the apparatus developed by Ruegsegger et al. [7], which is the basis of this system, is estimated to be 10 μm^3, taking into account the size

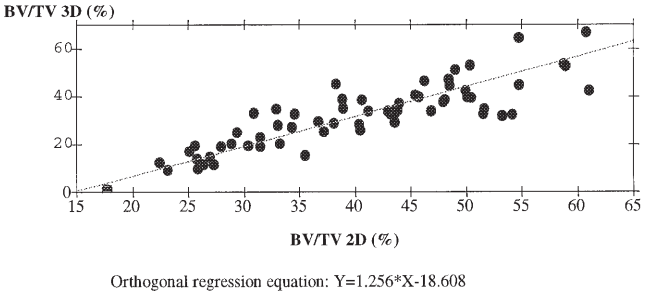

Fig. 6. Correlation for bone volume/tissue volume (BV/TV in %) between 2-D classical histomorphometry (*x*-axis) and 3-D μCT (*y*-axis). $r^2 = 0.750$, $P < 0.001$ ($n = 64$)

of our sample, and allows a spatial resolution of 20 μm, sufficient for work on the rat, even those of young age.

The results obtained with this system are the first 3-D results published to our knowledge on rat bone vertebrae. They show that this system is able to evaluate the spatial organization of ex vivo bones in a nondestructive manner and the structural variations of trabecular bone resulting from age, ovariectomy, and drug treatment. In addition to the very detailed color pictures, this system gives quantitative values about trabeculae. Interestingly, the 3-D BV/TV values are well correlated with bone mineral content as measured by DEXA on the same samples ($r = 0.831$, obtained from values of the first experiment), even though some variations can be observed between experiments, probably linked to a different number of animals per group in these experiments ($n = 3$ for experiments 1 and 2; $n = 19$–23 for experiment 3).

The results obtained in these experiments are similar to those described by several authors using conventional techniques [15,16]. However, there are some discrepancies when compared with the time course of vertebral osteopenia in ovariectomized rats described by Wronski et al. [17]. The trabecular bone volume was found to be ~40% by Wronski throughout a study using normal rats. Our values are less than 40% up to approximately 10 months of age, and then are in the same range. With ovariectomized rats, Wronski et al. [17] found a decrease in trabecular volume up to 30% at 6 months after ovariectomy, decreasing 20% by 9 months after ovariectomy. Our values are in this range, but our rats were older at the time of ovariectomy. Moreover, trabecular thickness was found to be much more important by Wronski et al. [17] (92–118 μm for controls and 75–100 μm for ovariectomized rats) than our values (65–90 μm and 50–60 μm, respectively). This discrepancy could result from an overestimation of the 2-D analysis, an underestimation of the μCT linked to the plate-model used for the calculations, or the difference in the calculation algorithms. Another explanation is that discrepancy could be linked to the 3-D chosen threshold value, whereas an insufficient 3-D resolution is unlikely. Additional experiments are needed to clarify this point. Finally, trabecular number in our study was slightly higher (~5/mm) than that found by Wronski et al. [17] (~3.5–4/mm). Nevertheless, in the two studies ovariectomy did not induce significant modification of this number (except the last time-point for Wronski), but our experiment was performed on a lower sample number. As far as modifications with age are concerned, our results are in agreement with those obtained by Sontag [23] using traditional histology: a rapid increase in trabecular bone volume in younger rats with a much reduced increase in older rats. Improvements to the μCT software are now available and will allow us to quantify anisotropy and connectivity in future similar experiments.

This new technique also provides additional information about the effects of tiludronate on aged ovariectomized rats. Studies have already shown that tiludronate increases bone mass and normalizes bone turnover in aged rats [24,25]. The 3-D examination of the rat vertebrae illustrates the improved spatial organization of trabeculae after tiludronate treatment compared with ovariectomized controls (increase in bone volume, trabecular number and thickness, decrease in trabecular separation), thus confirming the positive effects of bisphosphonate treatment on bone structure and mechanical competence [26]. Although correlations between 2-D and 3-D values are good, values obtained by microtomography are probably a more accurate appreciation of the mean thickness of the trabeculae all along the structure, whereas it is only estimated by 2-D histomorphometry.

Microcomputed tomography appears to be a very useful tool for both basic and industrial research, but is presently limited to ex vivo measurement. Advantages over conventional histomorphometry are linked to the spatial dimension of the μCT analysis and to the new informative parameters recently developed such as anisotropy evaluation and differential rod versus plate models. Linked to its nondestructive nature, another great advantage of microcomputed tomography is the possibility to run a stress test on the same sample after the 3-D analysis. Nevertheless, progress in this technology is required to improve resolution on in vivo measurements with commensurate decrease in time length of measurement. This technique should then prove a powerful tool for evaluating the effects of both antiresorptive or bone-forming agents, thus allowing between drug comparisons.

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