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STRUCTURAL AND COMPOSITIONAL CHANGES IN AGING BONE: OSTEOPENIA IN LUMBAR VERTEBRAE OF WISTAR FEMALE RATS

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Abstract

Changes in vertebral bone mineral content and density during aging were quantified in female Wistar rats. This study represents a longitudinal follow up utilizing single photon absorptiometry for the measurement of bone mineral content (BMC), quantitative computed tomography (QCT) for the measurement of bone mineral density (BMD), and image analysis histomorphometry for the measurement of trabecular bone volume (TBV) and bone cortical area (BCA). The above measurements were accompanied by biochemical assays of calcium concentrations in the respective bones. All aging animals experienced significant decreases in BMC, BMD, TBV, BCA and in the calcium content of their bones. The above features have been further emphasized through the use of scanning electron micrographs showing the age-related structural changes in a three-dimentional fashion. New, advanced technologies will enable the quantitation of 3-dimensional images that are currently obtained from the scanning electron micrograph; thus will provide new consideration as related to trabecular bone compactness (density). Energy dispersive x-ray spectroscopy indicated that the nature of crystals in aging bones does not differ markedly from that encountered in young specimens. Data are also provided with regard to the health of the animals, and it became apparent that aging rats undergo changes in their kidneys yet do not show any significant change in renal functional parameters as measured in both the serum and the urine. Hence, new noninvasive methodologies are currently available for longitudinal studies related to the skeleton in laboratory animals enabling reliable monitoring of age-related and hormonally induced changes in bones (spine and hip) of well defined experimental models.

KEY WORDS: Aging, Rat, Vertebra, Osteopenia, Bone, Structure, Mineralization

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Introduction

Architectural and compositional changes in bone, and in particular the progressive loss of bone density with subsequent weaknesses, are well-established features associated with menopause and/or senescence. The etiology of bone loss has been linked to hormonal, and especially, estrogen insufficiency as well as to the aging of skeletal constituents i.e. bone cells and bone marrow cellular components (Parfitt, 1988). Recent studies in our laboratory concerning age-related changes in vertebral bone of female mice, indicated that the bone mass and calcium content increased with skeletal maturation and achieved their peak by 12 months of age. Thereafter, a continuous decline in bone mass took place until 24-27 months of age (Bar-Shira et al., 1989a, b). Studies with male and female Wistar rats indicated that rats also experience age-related osteopenia (Safadi et al., 1988; Kiebzak et al., 1988). Thus, the purpose of the present study was to elucidate the *in vivo* effects of aging on vertebral bone of female rats with the aim of determining both qualitatively and quantitatively the changes taking place in a specific bone throughout the lifespan of a labaoratory rat.

Materials and Methods

One-month-old female Wistar rats were obtained from Charles River Research Animals, U.K. and were maintained, in an environment strictly controlled for temperature, light cycle, and number of animals per cage. All animals were fed Purina rat chow containing 21% protein, 3.8% fat, 0.6% calcium, 0.55% phosphorus, and drinking water *ad libitum*. A total of 156 animals were used in this study. The experimental design of the present study included: (1) a longitudinal survey of the vertebral bone mineral content (BMC) and bone mineral density (BMD) via non-invasive approaches at predetermined time intervals. These included the following age groups: 5, 8, 12, 18, 24 and 30 months of age; (2) a horizontal study whereby at each of the above time intervals 25 animals were sacrificed for structural and biochemical examinations. At each time point the same rats underwent the non-invasive tests, and only those which were sacrificed were used for invasive tests. Throughout this study the number of rats in each group for every assay was 5-6. All animals were initially subjected to basic physiological tests including: heart rate, respiration rate, systolic blood pressure and the specific oxygen consumption (milliliter of O_2/gm body weight/hour) using a specially designed physiological chamber. Heart rate

and systolic blood pressure were also measured via a tail's cuff through the use of a modern system of IITC Inc. Animals also underwent serum tests using the Technicon Autoanalyzer (Technicon Instruments, Tarrytown, NY) and a renal function test. Lumbar vertebrae were processed for bone structural and biochemical studies. In addition the kidneys of the respective animals were also examined.

Measurement of bone mineral content (BMC)

Animals were anesthetized with i.p. injections of chloral hydrate (300mg/kg body weight). The BMC at the fourth lumbar vertebrae (L_4) was measured by single photon absorptiometry as previously described (Safadi et al., 1988). The Norland-Cameron Bone Mineral Analyzer, Model 178 was used to measure the total mineral content per unit length of bone. Two sites along individual vertebrae were measured, and each site was re-scanned four times. The results were expressed as BMC per unit area of bone (g/cm^2). The precision of the above technique was 3.3%. Single photon absorptiometry examines however the entire cross-section of the bone at the level being scanned, and does not discriminate between the trabecular and cortical portions of the organ. Therefore, methods capable of measuring separately trabecular and cortical bones are advantageous for the detection of small changes in the bone status.

Measurements of vertebral bone mineral density using quantitative computed tomography (QCT)

Bone mineral density was measured on a rotary fan-beam scanner (ELSCINT EXEL 2400, Israel) using 210 mAs and 140 kVp (Safadi et al., 1988). The representative images of 0.8 x 1.7 mm^2 of trabecular bone were measured within the vertebral body of L_4 and were quantified and averaged. Calibration to correct for scanner drift was provided by a special crescent-shaped normalization phantom. The latter contained dipotassium phosphate (K_2HPO_4) as a mineral equivalent for use in calibration. This equipment enabled to calculate the mineral equivalent for each spot in individual vertebrae expressed as milligrams of K_2HPO_4 per cubic millimeter of bone. The precision of the QCT data was 3%.

Histology and histomorphometry

Specimens that were designated for quantitative morphological examinations (6 animals for each age group) were initially fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2), decalcified in Tris-buffered 10% EDTA, embedded in Paraplast (Fisher Scientific, Pittsburgh, PA), and sectioned at 6 μm. Representative longitudinal sections were obtained from the midportion of the lumbar vertebrae and were stained with hematoxylin and eosin. The determination of equal sections along the horizontal plane of the vertebra at its midportion was obtained through consequential numbering of an entire series of sections starting at the most anterior region of the vertebrae back to the vertebral central canal. It is generally accepted that aldehyde fixation and decalcification may result in loss of proteoglycans and in shrinkage of tissues. Hence, measurements were carried out in order to determine the extent of such shrinkage in rodent bones. This was carried out through direct measurements with the aid of calipers, measuring scale and an optical enlarger. In addition, vertebral bone emasurements were made on both roentgenograms of living animals and via the single photon absorptiometer. Parallel measurements were made on longitudinal histological sections using an image analyzer (Cue-2), described below.

It became evident that the histological processing of the tissues led to about 7-10% shrinkage with no real difference between young and old bones. Further, we have also tried undecalcified sections (embedded in methyl methacrylate and cut with a Zeiss's CutAll microtome) but found marked differences in the quality of the sections, especially while applying the sections to the new image analysis system. For these reasons decalcified material was used in the present quantitative study.

The Cue-2 Image Analysis System of Olympus Corporation (Lake Success, NY) was used with software (C-2 Morphometry, version 1.7, 1987) developed by Galai Corp. (Migdal HaEmek, Israel). The system consisted of a Zeiss Axiomat photomicroscope fitted with a CCD video camera (Panasonic WV-CD 50). The system was connected to an IBM PC-AT computer with a hard disc, control monochrome monitor (IBM) and an optical mouse (Mouse Systems Corp., Santa Clara, CA). The accuracy of the system is limited by the number of pixels on the one hand and the background; and in our experience showed an accuracy value of 2%. For measurements of trabecular bone volume (TBV), areas in L_4 vertebrae were analyzed at a final magnification of x 160. For each test group the mean and SD value were calculated from measurements performed in 6 animals per group. A total of 192 measurements were carried out, yet the analysis system did not take into account the repeated measures but the averages of individual animals.

Biochemical determination of calcium

Vertebrae that were designated for biochemical assays were first cleaned of all adherent soft tissues; the spinal cord was removed and the vertebrae were flushed with phosphate-buffered saline (PBS) in order to remove the bone marrow. Clean individual specimens were weighed and subsequently homogenized in 3 ml of sodium bicarbonate buffer (0,15M NaCl and 3mM $NaHCO_3$, pH 7.2) using a Polytron homogenizer (Kinematica, Luzern, Switzerland) for 1-2 min at 0°C. The homogenates were centrifuged for 15 min at 20,000 xg, and the supernatants were separated from the sediments. To determine the amount of Ca^{2+} in the vertebrae, each sediment was initially hydrolyzed with 0.5M HCl (10 ml) for 18 hours at room temperature and was centrifuged at 5,000 xg for 10 min. Samples were diluted 1:4 with lanthanum oxide in plastic tubes and were measured in a Perkin-Elmer atomic absorption spectrophotometer (Model 603).

All the data are presented as mean and standard deviation. Further, the results are presented as changes between groups of animals at different age groups; while the number of animals in each group that was assigned for any specific invasive assay was between 5 to 6. The number of animals in groups undergoing the non-invasive tests was much larger and ranged between 10-15 animals. Data were analyzed by the ANOVA test using the GLM (General Linear Model) in the SAS package (SAS User's Guide: Statistics SAS Institute Inc. Cary, NC), followed by the least squares tests.

Scanning electron microscopy

Bone samples from the various age groups were also processed for scanning electron microscopy equipped with energy dispersive x-ray spectrometry (Link X-Ray Analytical System, Model AN 10000, Link Highwycombe, UK). To that purpose specimens were initially fixed in a mixture of 4% paraformaldehyde and 4% glutaraldehyde (1:1) in cacodylate buffer (0.1M, pH 7.2), immersed in sodium hypochlorite at room temperature, dehydrated, were subjected to critical point drying in carbon dioxide and were coated with either gold (for structural examinations) or with carbon for energy

dispersive x-ray analysis. The Jeol JSM 840 scanning electron microscope was used, operating at 20 kV, and the measuring areas were 70 μm^2. 24 specimens obtained from the following age groups: 1.5, 5, 8, 12, 24 and 30 months of age were examined by energy dispersive x-ray analysis. In each group 3 measurements were performed in vertebrae of animals. The system itself has been originally standardized by the producer; in addition, we have standardized each individual element via inserting the system's memory with all the elements according to the National Bureau of Standards, USA.

Results

The animals' body weight increased by 41.4% ($p < 0.001$) from the age of 5 months to the age of 30 months (Fig. 1). The results of the physiological tests are summarized in Table 1 whereas the serological findings including the renal functional test are shown in Table 2. It could be noted that except for the values of serum alkaline phosphatase the biochemical parameters checked hereto did not differ in old animals in comparison to young ones. A noticeable difference was found with regard to creatinine clearance in old animals, yet further studies are in progress in order to determine their physiological implications. Old animals also revealed a 24.9% increase ($p < 0.05$) in the respiration rate; a 12.9% increase ($p < 0.025$) in heart rate and an 18% ($p < 0.05$) decrease in the specific oxygen consumption. No significant changes were noted with regard to the systolic blood pressure. The overall length (height) and width of the 4th lumbar vertebra did not change substantially throughout life (Fig. 2). By the same time the wet weight of the respective vertebrae decreased by 14% ($p < 0.02$).

Non invasive techniques

Using single photon absorptiometry, it became evident that the bone mineral content (BMC) in vertebrae increased until the age of 12 months, when animals attained their peak BMC (Fig. 3). Thereafter, a rapid decline was encountered, a feature that lasted until the age of 24 months with no change at older age (Fig. 3). Thus, from the time of middle age (12 months) and until senescence (24 months) rats lost 30% ($p < 0.001$) of the BMC in their lumbar vertebrae.

The mean values for measurements of trabecular and cortical bone mineral density (BMD) in L_4 as measured by the QCT, indicated that there were no real changes between the age of 7-12 months, but a significant loss was measured by the age of 24 months (-24.5%; $p < 0.001$). Almost identical values were obtained with regard to L_5 in the same animals.

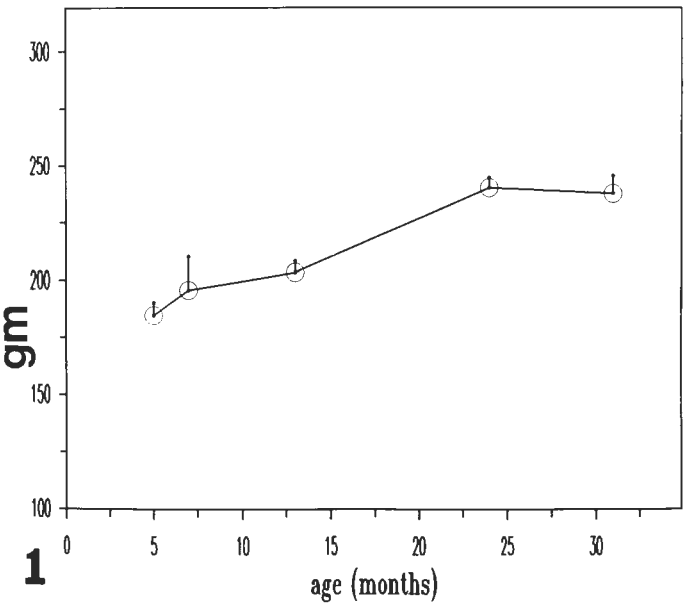


Figure 1. Mean curve indicating the age-related changes in the animals' body weight. These Wistar female rats attain their maximal weight by the age of 24 months with no further increase thereafter. The number of rats used in each group was 20.

Table 1. Physiological data of young and old female Wistar rats.

	Age of animals	
	Young (5 mths of age)	Old (30 mths of age)
Heart rate (Beats/min)	310 ± 12	348 ± 18
Respiration frequency (Breaths/min)	64 ± 8	81 ± 9
Systolic blood pressure (mm Hg)	112 ± 4	110 ± 6
Specific oxygen consumption (O_2 ml/gm BW/h)	1.06 ± 0.05	0.83 ± 0.07

All values are based upon 40 determinations obtained from 10 animals in each age group.

Table 2. Selective biochemical constituents of serum and urine in aging female Wistar rats

Age (months)	Blood Urine Nitrogen (BUN) mg/dl	Creatinine mg/dl	Calcium mg/dl	Phosphorus mg/dl	Alkaline Phosphatase IU/dl	Urine Creatinine, Creatinine clearing test mg/100 gm BW/24h
1.5	23.0 ± 1.0	0.93 ± 0.06	12.3 ± 0.9	7.8 ± 0.7	155 ± 10	
4	22.5 ± 1.2	1.0 ± 0.08	11.2 ± 0.8	7.7 ± 0.5	103 ± 8	
8	23.5 ± 1.2	0.85 ± 0.07	11.4 ± 0.7	6.9 ± 0.9	85 ± 6	0.52 ± 0.05
12	25.0 ± 3.0	1.1 ± 0.06	13.5 ± 0.5	6.4 ± 0.8	55 ± 6	
18	17.0 ± 1.5	0.95 ± 0.09	12.5 ± 0.8	7.4 ± 0.5	57 ± 6	0.42 ± 0.04
24	23.0 ± 1.5	1.3 ± 0.15	10.9 ± 0.7	9.4 ± 0.6	87 ± 7	

All values are based upon 5 determinations obtained from 5 different animals.

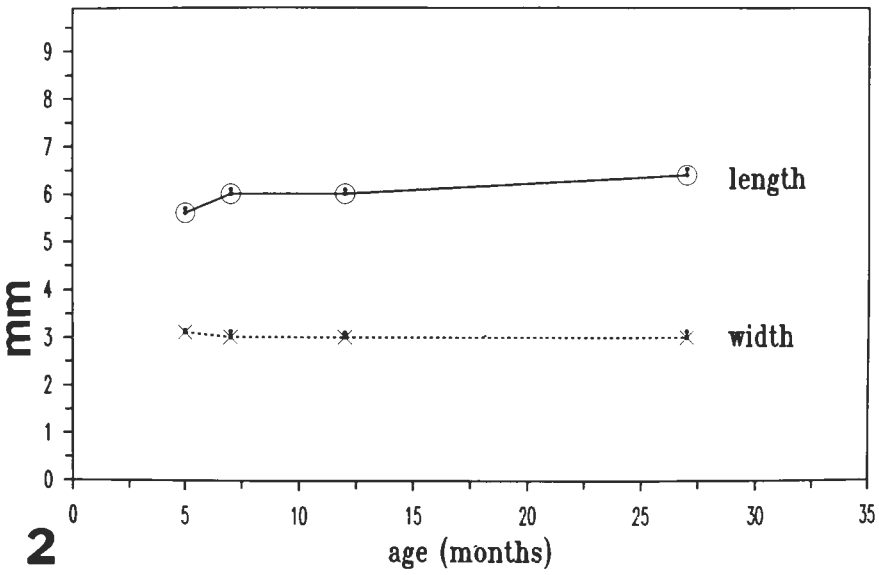


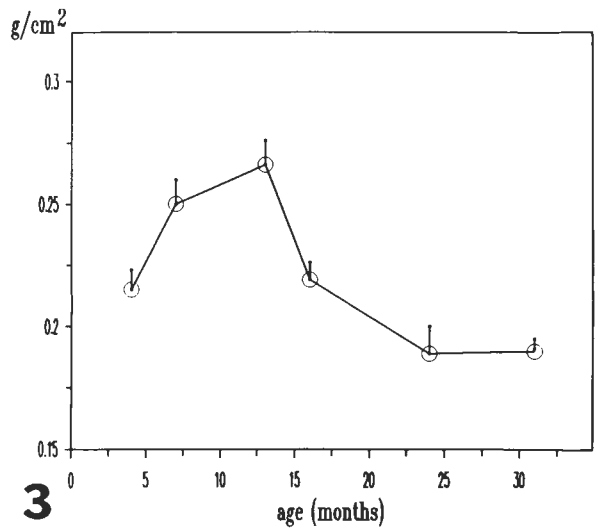
Figure 2. Mean curves referring to the length (height) and width of the L_4 vertebra in Wistar female rats. No marked changes are noted with regard the bone's length with no change at all in the latter's width. Each mean was calculated from ten measurements obtained from ten different animals.

Figure 3. Age-related changes in the values of Bone Mineral Content (BMC) in L_4 of female Wistar rats. Note the marked increase during the phase of growth and maturation. Peak values were obtained by middle age (12 months) followed by a marked decline until old age (24 months). No further changes were recorded between 24 months and 33 months. Measurements were carried out with Norland's Single Photon Absorptiometer. The number of rats used in each groups was 20.

Invasive measurements

Figure 4 exhibits the changes that take place with regard to the trabecular bone volume (TBV) in vertebrae of growing and aging animals. Again, peak values were obtained by middle age (12 months) with a very significant decline thereafter (-31.8%; $p < 0.001$). The changes were further substantiated by the histological preparations which showed that age led to a profound derangement in the internal architecture of the vertebrae. In young animals, L_4 vertebrae showed well-developed subchondral trabeculae arranged in a continuous osseous network and appeared in contact with a healthy-looking bone marrow tissue. By contrast, similar areas in old animals lacked both the trabecular continuity as well as the internal organization as noted in young specimens. The histology of rat vertebrae differs from human vertebrae in that the growth plate persists, the size of each trabecula is larger but there are fewer trabeculae.

More profound changes were encountered within the middle portion of the vertebrae. In specimens of old animals there was a noticeable decrease in the number and thickness of the trabeculae. Further, the latter revealed increased number of lacunae lacking osteocytes, and signs of fragmentations. Concomitantly, the overall number of bone cells (osteocytes and/or osteoblasts) decreased. In addition, the cortical (compact) bone surrounding the vertebral body underwent a reduction of 26.4% ($p < 0.01$) in its surface area and also showed severe structural changes: in young animals it appeared as a dense tissue containing multiple osteocytes. In comparison, the cortex of old vertebrae was found to be cavitated, filled with marrow tissue and demonstrating crack-like structures throughout.



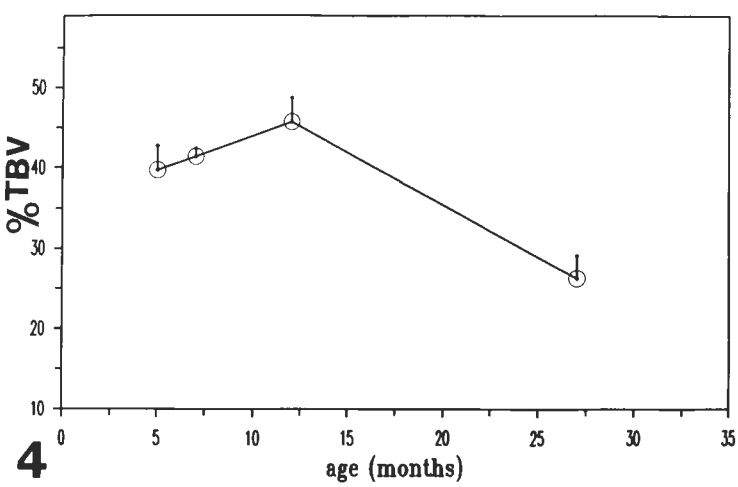


Figure 4. Effects of age on the trabecular bone volume (TBV) in L_4 of female rats as measured with the Cue-2 image analyzer. The peak of TBV is at 12 months and the decrease in old age is highly significant ($p < 0.001$). Each mean ± SD is based upon determinations obtained in 6 animals.

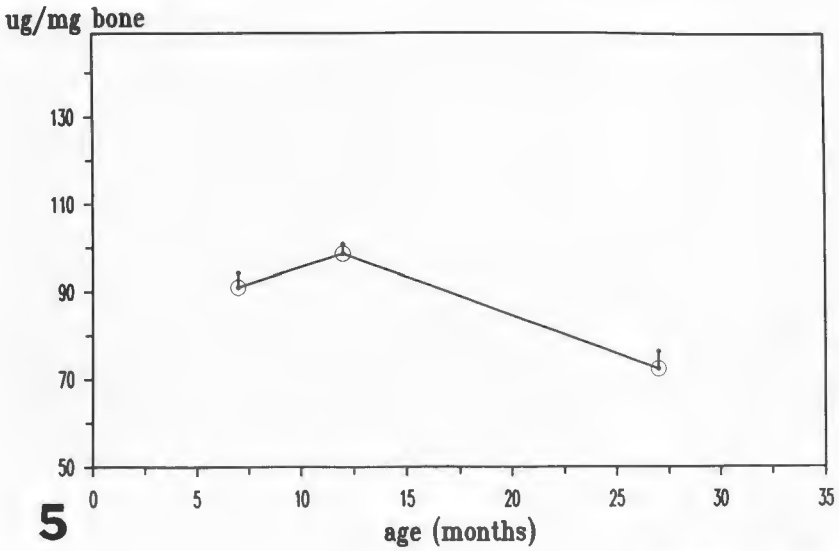
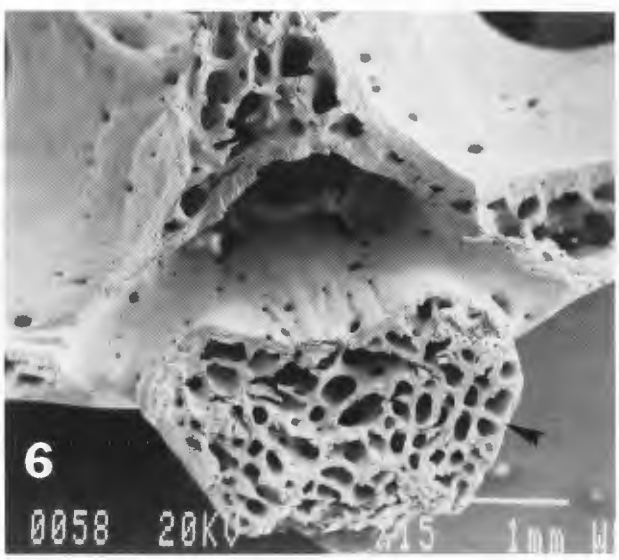


Figure 5. Effect of age on the content of calcium in lumbar vertebrae of Wistar female rats. Peak values were recorded at middle age (12 months) with a significant decline thereafter (-18%; $p < 0.05$). Measurements were carried out through the use of the Elmer-Perkin Atom Absorption Spectrophotometer. Each value is based upon measurements of 6 different vertebrae from 6 different animals at each age group.

Figure 6. General view of L_4 of 5-month-old Wistar female rat as seen with the scanning electron microscope (SEM). Note the density and well developed network of trabecular bone within the vertebral body (arrow). Bar = 1 mm.

Figure 5 illustrates the age-related changes in the content of Ca^{2+} in vertebral bones. Peak values were noted at middle age (12 months) followed by a marked decrease until old age (-25.5%; $p < 0.0001$). It should be noted that the consistency of the percentage of the recovery of mineral sample was homogenous, despite the fact that 0.5 M HCL does not hydrolyse bone of various ages uniformly. Using the multiple regression line analysis for the following parameters: BMC, BMD, TBV and Ca^{2+} a very high rate of correlative relationship was encounted with multiple R^2 of 1.0.

Through the use of the scanning electron microscope (SEM) it was possible to demonstrate in a most convincing way the involutionary changes that take place in aging bones. In a young animal, the vertebral body is occupied by a dense network of bone trabeculae which altogether yield the appearance of a sponge-like structure (Fig. 6). Following x-ray imaging it became apparent that calcium and phosphorus were evenly distributed in bones of young and old animals (not shown). The SEM reconfirmed that senescent animals experienced profound changes in their spine (Fig. 7). Instead of the well developed network of bone trabeculae the vertebra shows an advanced stage of "porosis" in all its components: body, lateral and posterior processes. Using a higher magnification, old bones revealed a marked discontinuity between their trabeculae concomitant with an increase in the intertrabecular spaces (Fig. 8). The Cue-2 Image Analyzer provides an advanced imaging device for the analysis of microscopical observations that are usually of two dimensions. In order to quantitate features making up a 3-dimentional image, as is obtained with the SEM, the image can be analyzed through the use of two algorithems of which the linear Furier transform was found to yield the better results (Wahl, 1987). We are currently trying to apply the above approach in order to provide quantitative values related to dimensional regularity of trabecular bone as a factor of age.



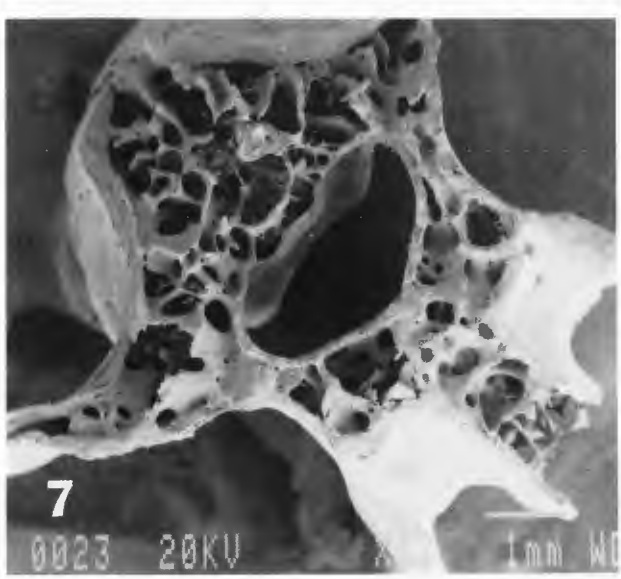


Figure 7. The appearance of L_4 vertebra as was seen in a 24-month-old rat. Note the advanced porosity throughout the vertebra (compare with Figure 6). Bar = 1 mm.

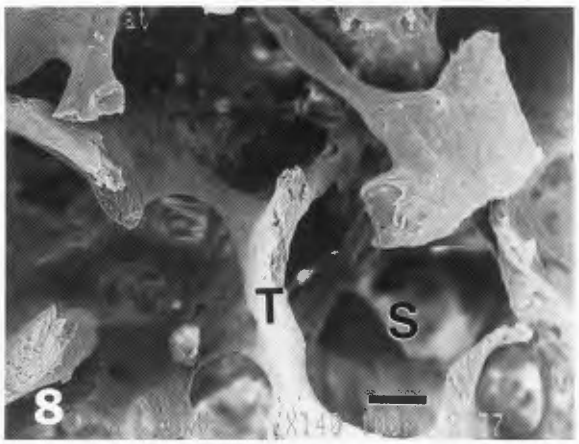


Figure 8. Higher magnification of a portion of the vertebral body of L_4 in a 33-month-old rat. Note the marked reduction in both the size and number of bone trabecules (T) along with overt discontinuity between these trabecules. The inter-trabecular spaces (S) have consequently enlarged. Bar = 100 μm.

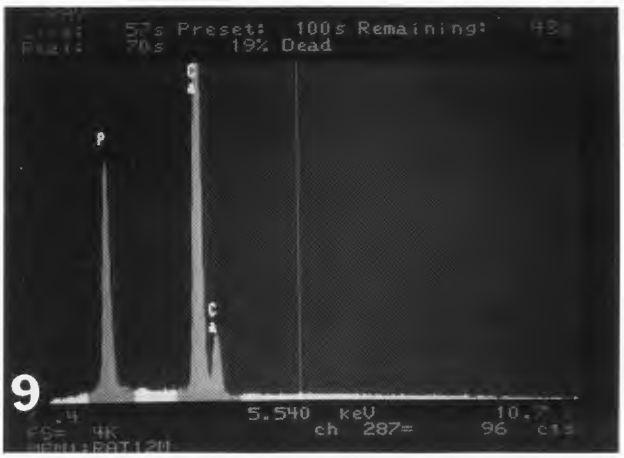


Figure 9. An energy dispersive x-ray spectrometry obtained from a trabecular bone of L_4 of a 12-month-old female rat. Note the prominent peaks of calcium (Ca) and phosphorus (P).

Figure 9 presents a spectrum obtained from a 12-month-old vertebra. In young animals the ratio of calcium to phosphorus per unit area of either trabecular or cortical bone was 2:1 (Ca^{2+} 66,9% and P^{2-} 33,1%). Bone samples that were obtained from old animals revealed a slightly different ratio: 2,2:1 (Ca^{2+} 70.1% and P^{2-} 31.2%). Hence the values in aging rats were slightly but not significantly higher in comparison to those of young bones. We have not at the moment an explanation for the higher Ca:P ratio in older rats.

Morphological changes in the Kidney

Most old kidneys revealed glomerular changes. Since inflammatory cell infiltration was not a constant feature, this apparent age related entity is referred to glomerulonephrosis. The kindeys of rats with this disease were grossly enlarged and whitish to tan in color. Multiple, bleb-like cysts were visible on the surface as well as within the parenchyma (in the cortex and upper regions of the medulla). Tubular cysts were filled with proteinaceous material. Yet, no animal had signs of renal failure (Table 2) and we have not encountered renal calcifications.

Discussion

With increasing age, osseous tissue is lost from almost every bone amenable to measurement. Rapid loss of trabecular bone occurs by both the removal of several structural elements and by profound changes in the internal architecture of the trabecular bone network. Photon absorptiometry, quantified computed tomography, quantitative histomorphometry have all confirmed that the rat's spine loses about 30% of both its trabecular bone volume as well as its mineral content.

In lumbar vertebrae of aged rats there was no diminution in the height of the vertebrae with increasing age. However, the intervertebral disks of these rats experienced a marked decline in early life before peak bone mass was reached (not shown). The significance of this observation is not as yet apparent, though it seems that the changes in the intervertebral disk precede the age-related vertebral bone loss. It is possible that the reason that aged rats do not experience vertebral compression fractures (as do postmenopausal women) is because of the different mode of posture and locomotion from that of humans.

The present QCT studies showed significant cortical and trabecular bone loss during aging. The observed bone loss was accompanied by a decrease in the number of osteocytes and osteoblasts both along the cortical and trabecular segments of the bone. This finding points toward a decreased number of functioning bone cells. A similar observation has been reported in femurs of aging female mice of the C57 BL/6 strain (Silbermann et al., 1987) and of the CW-1 strain (Silbermann et al., 1988).

We have also checked the effect of age on the activities of alkaline and acid phosphatases in lumbar vertebrae of maturing and aging Wistar rats. In as yet unpublished data, no changes were noted in the specific activity of acid phosphatase but alkaline phosphatase decreased by 43.5% ($p < 0.001$) in old rats. Hence, at the skeletal level, increases in bone loss were accompanied by an apparent osteoblast insufficiency, a fact that resulted directly or indirectly in decreased bone formation. Our scanning microscopy analysis indicated that the major structural change in the vertebral cancellous bone was the reduction in the number and size of trabecular bars, and in particular the loss of continuity among the remaining trabeculae.

A recent study of aging female rats indicated a significant rise in serum iPTH in old animals (Sacktor et al., 1987). In our animals we have encountered a decrease in alkaline phosphatase in serum of senescent rats. Also, we did not find an increase in neither serum creatinine nor BUN compared with young animals. We have found that in old animals the values for creatinine clearance test slightly decreased. In spite of the fact that most senescent rats revealed renal atrophic changes, renal function did not appear to have been seriously affected. Hence, it might be suggested that the changes in bone architecture in the vertebrae of old rats with age do not result from renal osteodystrophy.

Studies of the patterns of sex steroids and gonadotropin secretion in aging female rats revealed that the ovaries of old

rats are capable of near-normal function under appropriate gonadotropic stimulation (Huang et al., 1978; Steger and Peluso, 1987). Aging was manifested, however, by a translation defect in the hypothalamic-pituitary axis in aging rodents, suggesting that aging changes the endocrine regulating centers of the brain with defective activation of estrogen receptors (Belisle and Lehoux, 1983). As a consequence female rats, like humans, experience ovarian exhaustion due to the loss of ovarian follicles after middle age. Finch (1987) demonstrated a remarkable similarity in age-related lengthening of fertility cycles in humans and rodents, suggestive of a common mechanism. The common denominators include the decrease of blood estradiol and progesterone levels to castrate levels. Circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) generally increase to postcastrate levels because of ovarian senescence at middle age in female mice and humans. In rats the aging of the reproductive system includes the cessation of cyclicity and the entry into persistent estrus (Nelson, 1988). Previous studies already alluded to the possibility of relationships between ovarian senescence and age-related bone loss (Silberberg and Silberberg, 1962). The profound changes in estrogen levels could certainly play a role in bone loss observed with increasing age in rats. It seems, therefore, that in laboratory rodents sex hormones do appear to play a crucial role in the etiology of age-related bone loss (Wronski et al., 1988). Recently, Kiebzak et al. (1988) using Wistar male rats indicated the development of hyperparathyroidism in senescent male rats, a fact that might reflect decreased metabolic and renal clearance of PTH. Hence, it has been proposed that the aging male rat might represent a model for renal osteodystrophy. Our preliminary studies tend to point out that the aging female rat does not suffer from a significant impairment in renal function. Hence it remains to be established which factor(s) are predominantly involved in the etiology of age-related osteopenia in male versus female rats: hyperparathyroidism vs. hypogonadism respectively. In a current and as yet unpublished study in our laboratory we have screened human vertebrae from the age of 3 to 80 years. Of interest was the finding that ultrastructurally the changes noticed in aging humans resembled to a great extent those observed in the senescent rat. Still, further work is needed in order to elucidate and establish the degree of analogy between human and rodent age-related osteopenia. Also, the osteopenia that develops in aging rodents might manifest features related to growth and development. If so, this may be in contrast to the situation in human bone. Concerning the age-related mechanisms of bone loss, it has been recently suggested that changes in the activity of local growth factores (cytokines) or their interactions with systemic hormones could contribute to the onset of osteopenia (Raisz, 1988). The present results provide data associated with the development of osteopenia in a commonly used laboratory animal model. Hence, it is recommended that female rats can be used as experimental model for longitudinal studies related to age-related bone loss.

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Discussion with Reviewers

E. Bonucci: The authors suggest that changes in estrogen levels could play a role in bone loss observed in aging female rats, thus suggesting indirectly that the observed osteopenia is not simply senile osteopenia and that there may be analogy between osteopenia of old female rats and that occurring in post-menopausal women. If so, male rats could present a degree of osteopenia different from, and probably lower than that of female animals Is there any information about bone density and volume in aging male rats?
Authors: We are currently in the midst of a study following bone changes in Wistar male rats. So far we have obtained results from young age to midlife (12-months of age) and found out that the peak bone volume at middle age is higher than that recorded in Wistar female rats. Within a year we will be able to compare the pattern of bone loss in male and female vertebrae during aging. We have, however, reported that in mice vertebral bone loss was very similar in both sexes (Bar-Shira et al., 1989a). Kiebzak et al. (1988), measuring age-related changes in the femur of male Wistar rats, found significant decreases between 12 and 24 months of age. In the latter study there were, however, indications for the development of hyperparathyroidism in aging male rats. Thus, both male and female rats develop osteopenia with aging, yet it is not clear whether the etiology is common in both sexes.

Reviewer II: The changes in BMC appear to increase much more than can be accounted for by increases in TBV. This suggests an increase in mineral content during secondary mineralization of hydroxyapatite to a mature concentration, achieved at approximately 12 months. Please comment.
Authors: While comparing Figures 3 and 4 one gets the impression that the BMC increases more than the TBV between young age and middle age. However, when comparing Figures 4 and 5 a very close resemblance is noticed between the increases in TBV and calcium when measured directly with an atomic absorption. We have repeated the QCT measurements at the above time interval (7 and 12 months of age) and found slight increases in the BMD which appeared more consistent with the results obtained via the two invasive tests: histomorphology and biochemistry. It might be very possible that technical factors associated with the single photon absorptiometry measurements in small animals might yield results that are not as accurate as those obtained using direct assays. Hence, it is as yet difficult to conclude that secondary mineralization does indeed take place at middle age.

Reviewer II: The rapid drop in bone mass between 12 and 24 months, with little change thereafter suggests that the bone loss is not an age-related, slow, continual decrease in bone mass, but a rapid, short-term change related to changes in estrogen metabolism. Have you examined bone mass in male rats to determine whether a similar pattern of bone loss occurs with aging?
Authors: We fully agree with your conclusions as to the etiology of bone loss in the aging female rat. Concerning the pattern of bone loss in male rats, please note the comments in the answer to Prof. E. Bonucci's question.

Reviewer III: Creatinine clearance from a timed urine specimen is a measure of renal function in rats, although not an optimale one because of creatinine transport by tubules. Ideally, inulin clearance should be measured. Serum creatinine is not helpful because of changes in its production.
Authors: The measurement of endogenous creatinine clearance is a well established and accepted test in the clinic, although great care has to be used in measuring creatinine at low (normal) plasma concentrations. Further, the excretion rate is independent of urine flow which is an advantage. In rats creatinine clearance is a good a measure of glomerular filtration rate as inulin or mannitol (Corcoran AC, Weller JM (1968) The Kidney In: Phathologic Physiology, Sodeman WA & Sodeman WA (eds)., W.B. Saunders Co., Philadelphia and London, p. 728). The average value of serum creatinine in rodents is 1.1 mg/dl (Crispens CG (1975) In: Handbook on the Laboratory Mouse, Charles C Thomas Pub, Springfield, IL. p. 115) and the corresponding values in the present study fitted exactly with those published in the literature. Taking into account the consistent values of the blood urine nitrogen (BUN) it seems reasonable to assume that the kidneys in old Wistar rats do not undergo a significant functional impairment.

Reviewer III: The remarkable 20% drop in bone mineral content over a 4 month period, from 12 to 16 months is hard to understand on any physiologic or pathologic grounds. Were these the same animals? If so, was there some systematic error in the measurement? Please comment.
Authors: All the measurements for the BMC values were carried out on the same animals. Each measurement was routinely repeated x 4 in each individual animal. The measurements themselves were performed in a similar way to that as they are done in humans, thus it is doubtful whether we were faced with a systematic error in the measurement. The mean lifespan of female Wistar rat is 24 months and ovarian exhaustion in these animals takes place at midlife (12 months of age). Hence, during the 4 months postmenopausally the animals lost 20% of their BMC i.e. 5% per month. If one month in the rat can be considered equivalent to 3 years in humans, then it would not appear that erratic if the above value of BMC loss has been applied to female humans during 12 years postmenopausally.