

## —Originals—

## Femoral bone density and changes therein associated with differing histories of pregnancy and lactation in aged rats

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### Abstract

**Objective:** To examine the effect of prior pregnancy and lactation on bone density in aged rats.

**Materials and Methods:** 2 month-old female Fischer-344 rats were divided into 4 groups: 1) 13 rats were allowed to get pregnant and to nurse their offspring for two or three cycles; 2) 14 rats were allowed to get pregnant two or three times and were immediately separated from their young after each delivery prior to lactation; 3) 10 rats were not allowed to become pregnant; and 4) 7 rats were sacrificed at the beginning of the experiment. All rats were fed ad libitum. After the rats had completed two or three pregnancies with or without subsequent lactation, all were fed a restricted diet until they were 25 months old (aged rats). Then they were sacrificed and both femurs were removed from each rat. One bone was used for analysis of mineral content and the other bone was used for photodensitometry of the diaphysis.

**Results:** 31 rats (83.8%) survived until they were sacrificed. The femurs of the aged rats showed increased ash weight compared to those of 2 month-old rats. Aged rats with prior pregnancies with or without subsequent lactation had higher femoral ash weight than those without prior pregnancies. There were no differences among aged rats in regard to marrow diameter, bone diameter, cortical width or bone density of the diaphysis in femur by photodensitometry.

**Conclusion:** In aged rats, past pregnancies but not lactation were related to increases in bone density of the femur. (J Nippon Med Sch 2000; 67: 18–23)

**Key words:** pregnancy, lactation, aged rat, bone density

### Introduction

There are significant alterations in the calcium metabolism during pregnancy and lactation in rats. There is evidence that the maternal skeleton accumulates calcium during early to mid-pregnancy in rats before the onset of fetal skeletal mineralization<sup>1</sup>. When dietary calcium intake is adequate, the calcium requirements for fetal development are met with no reduction in the amount of calcium in the maternal skeleton. Most authors have found increased bone mass at the end of pregnancy in rats<sup>1,2</sup>. However, decreased bone density of lumbar vertebrae in rats at the end of pregnancy has been reported<sup>3</sup>.

In contrast to the changes brought on by fetal mineralization during pregnancy, lactation results in substantial mobilization of calcium from bone irrespective of the amount of calcium in the diet<sup>1,2,4,5</sup>. Although a very significant decrease in bone mass ordinarily occurs during lactation, there is little evidence to suggest that pregnancy and lactation are harmful to the long-term health and integrity of the skeleton of rats<sup>5</sup> or human beings<sup>6</sup>. In part, this may reflect compensation by the accumulation of skeletal calcium stores during early to mid-pregnancy so as to meet the calcium demands of fetal skeletal development in late pregnancy and subsequent milk production during lactation as described above.

There is evidence that reconstitution of skeletal mineral stores occurs in human beings<sup>7</sup> and rats<sup>1,4,8</sup> following lactation and weaning. Ellinger and others<sup>9</sup> studied the effect of differing diets over three consecutive pregnancies and the associated lactation in each instance. They reported that bone mass lost during the postpartum period was restored by the end of a subsequent pregnancy.

As far as we know, there have been no reports concerning bone density in aged rats with differing histories of pregnancy and lactation. In that regard, in the present study we examined the long-term effects of physiological bone loss associated with the reproductive cycle through measurement of bone density in older rats with varying reproductive histories.

## Materials and Methods

### 1. Experimental animals and protocol

Fischer-344 female rats (Charles River, Kanagawa) were used in this study. At 2 months of age, the rats were divided into 4 groups: 1) aged rats with history of pregnancy and lactation; 2) aged rats with history of pregnancy alone; 3) aged rats with no history of pregnancy or lactation; and 4) 2-month-old rats.

For group 1, 16 rats were placed for 7 days with male rats for mating. Following delivery, they were allowed to rear their offspring for 3 weeks. Ten days following separation from their offspring, each rat was again placed for 1 week with a male rat for mating. The cycle was repeated twice, whereby 3 pregnancies with subsequent lactation were established for each rat when possible. For the group 2 rats, 18 rats were subjected to a regimen identical to that of the group 1 rats, except that the rats were separated from their offspring immediately after each pregnancy. There by, 3 pregnancies without subsequent lactation were established if possible. For group 3, 10 rats were fed ad libitum until all of the rats in groups 1 and 2 had finished three pregnancies with or without subsequent lactation. In group 4, 7 control rats were sacrificed at the start of this experiment (age 2 months plus 1 week).

All group 1, 2, and 3 rats were fed a laboratory diet containing 1.27% calcium and 0.84% phosphorus (CRF-1, Oriental Yeast Co., Tokyo) ad libitum until ap-

proximately 11 months of age, at which time all of the group 1 and 2 rats had completed 3 reproductive cycles with or without lactation. All rats were allowed free access to water. Thereafter, all rats were fed a restricted diet (70% of ad libitum feeding). Each rat was individually caged. The conditions in the animal laboratory were as follows: temperature was kept at  $21 \pm 2^\circ\text{C}$ , humidity was maintained at  $55 \pm 15\%$  and exposure to light was controlled as a 12 hr. alternating light-dark cycle. When age 2 years and 1 month old, each rat was anesthetized with ether and the femoral bones were removed. One femoral bone was kept at  $-50^\circ\text{C}$  and the other femoral bone was kept in 70% ethanol until use. Blood, hearts, brains, livers were taken for other use.

### 2. Measurement of bone density and mineral level

One femoral bone from each rat was cleaned, dried and weighed. The bone was reduced to ash using a muffled furnace at  $550\sim 600^\circ\text{C}$  for 20 hr. The ash thus obtained was then dissolved in 1N nitric acid and total bone calcium was determined by atomic absorption spectrophotometry. Phosphorus content was determined by the Fiske-Subbarow method<sup>10</sup>. The other femoral bone from each rat was placed on an aluminum plate corresponding to soft tissue together with a 10 step aluminum gradient and an x-ray photograph was taken. The x-ray photograph was scanned at the mid point of the femur and analyzed by a photodensitometer according to a reported method<sup>11,12</sup> (performed by Teijin laboratory, Tokyo). Thus, bone density of an x-ray photo was determined based on the density of a 10 step aluminum gradient. From this the bone diameter (D), marrow diameter (d), cortical width (D-d), CTI (cortical thickness index) and  $\Sigma\text{GS}/\text{D}$  (bone density) of the diaphysis were obtained (**Fig-1**).

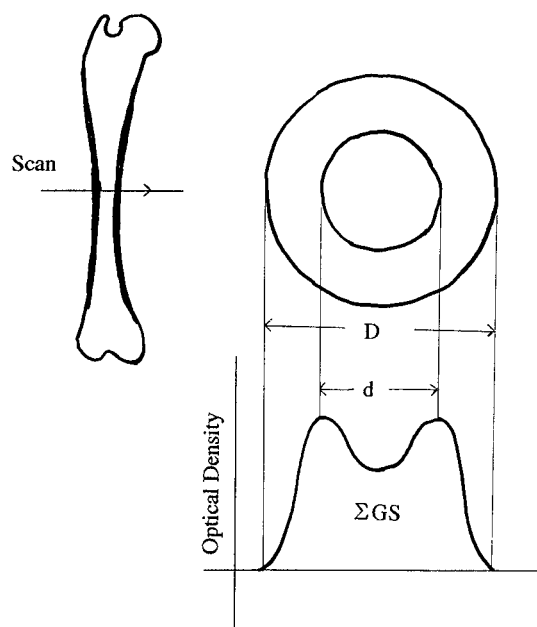
Values are means  $\pm$  SEM. Analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine significant differences when more than two means were compared. A p-value less than 0.05 was considered significant.

All procedures on rats were in compliance with the American Journal of Physiology guidelines.

## Results

In group 1, 6 rats completed 3 pregnancies with lac-

tation, 7 rats experienced 2 pregnancies with lactation. During this period, 3 rats were excluded because they did not get pregnant more than once. Ten of these 13 rats survived more than 2 years (survival rate: 76.9%). In group 2, 4 rats had 3 pregnancies, 10 rats had 2 pregnancies, and after exclusion of 4 rats



D: bone diameter  
d: marrow diameter  
D-d: cortical width  
CTI: cortical thickness index =  $(D-d)/D$   
 $\Sigma GS/D$ : bone density.

Fig. 1 Photodensitometric measurement<sup>10,11</sup>  
X-ray photo of each femur was taken together with an aluminum gradient. The bone x-ray was scanned at the mid-point of the femur and the optical density of the bone was determined using a photodensitometer.

for the same reason as above, 13 rats lived more than 2 years (survival rate: 92.9%). In group 3, 8 rats out of 10 lived more than 2 years (survival rate: 80%). There was no difference in survival rate between the 3 groups.

The results of the bone mineral level study are shown in **Table 1**. In terms of dry weight and ash weight, two month-old rats differed significantly compared with the aged rats in the three other groups ( $p < 0.001$ ). Aged rats with no history of pregnancy had lower mineral levels than aged rats with history of pregnancy ( $p < 0.05$  as dry weight,  $p < 0.01$  as ash weight). Aged rats with earlier pregnancy and lactation showed higher ash weight compared with aged rats with no history of pregnancy ( $p < 0.05$ ). Two month-old rats had lower calcium content (mg/bone) than aged rats in the three other groups ( $p < 0.001$ ). Also, 2-month-old rats showed a decreased level of phosphorus content (mg/bone) compared with the three other groups ( $p < 0.001$ ), and phosphorus content (mg/bone) was increased in rats with prior pregnancy and lactation compared with rats with history of no pregnancy ( $p < 0.05$ ). Phosphorus content, expressed as ash weight, was higher in two-month-old rats compared with aged rats with history of pregnancy ( $p < 0.05$ ). There were no differences in calcium content (dry% and ash%) or phosphorus content (dry% and ash%) among the 4 groups.

The results of quantitative assessment of bone density in x-ray photos of the diaphysis are listed in **Table 2**. Two-month-old rats differed in bone diameter

Table 1 Bone mineral content of the femur

	Two-month-old rats (n=5)*	Aged rats with no history of pregnancy (n=9)	Aged rats with history of pregnancy (n=12)	Aged rats with history of pregnancy and lactation (n=8)	ANOVA
Dry weight (g/bone)	0.392 ± 0.015 <sup>a</sup>	0.492 ± 0.011 <sup>b</sup>	0.533 ± 0.010	0.533 ± 0.012	$p < 0.001$
Ash weight (g/bone)	0.183 ± 0.014 <sup>a</sup>	0.278 ± 0.010 <sup>c,d</sup>	0.325 ± 0.009	0.324 ± 0.011	$p < 0.001$
Ca content (mg/bone)	69.620 ± 5.612 <sup>a</sup>	108.233 ± 4.183	121.033 ± 3.622	123.263 ± 4.436	$p < 0.001$
(dry %)	21.164 ± 0.627	22.002 ± 0.467	22.630 ± 0.405	23.139 ± 0.496	$p = 0.090$
(ash %)	38.160 ± 0.724	38.911 ± 0.539	37.283 ± 0.467	38.125 ± 0.572	$p = 0.176$
P content (mg/bone)	27.928 ± 2.086 <sup>a</sup>	41.950 ± 1.555 <sup>d</sup>	47.124 ± 1.347	48.150 ± 1.649	$p < 0.001$
(dry %)	8.488 ± 0.237	8.528 ± 0.177	8.814 ± 0.153	9.048 ± 0.188	$p = 0.164$
(ash %)	15.320 ± 0.216 <sup>b</sup>	15.078 ± 0.161	14.517 ± 0.139	14.925 ± 0.170	$p = 0.014$

\* The number of rats analyzed is in parenthesis

<sup>a</sup> $p < 0.001$  compared to all aged rats, <sup>b</sup> $p < 0.05$  compared to aged rats with history of pregnancy, <sup>c</sup> $p < 0.01$  compared to aged rats with history of pregnancy, <sup>d</sup> $p < 0.05$  compared to aged rats with history of pregnancy and lactation, Ca: calcium, P: phosphorus.

Table 2 Photodensitometry of the diaphysis

	Two-month-old rats (n=7) *	Aged rats with no history of pregnancy (n=7)	Aged rats with history of pregnancy (n=10)	Aged rats with history of pregnancy and lactation (n=8)	ANOVA
D (mm)	3.510 ± 0.055 <sup>ab</sup>	3.648 ± 0.055	3.838 ± 0.046	3.822 ± 0.051	p < 0.001
d (mm)	2.520 ± 0.044	2.499 ± 0.044	2.548 ± 0.036	2.579 ± 0.041	p = 0.568
D-d (mm)	0.990 ± 0.066 <sup>cd</sup>	1.149 ± 0.066	1.290 ± 0.055	1.243 ± 0.062	p = 0.011
CTI	0.282 ± 0.013	0.315 ± 0.013	0.327 ± 0.001	0.324 ± 0.013	p = 0.076
ΣGS/D	3.913 ± 0.148 <sup>e</sup>	5.207 ± 0.148	5.326 ± 0.124	5.505 ± 0.139	p < 0.001

D: bone diameter, d: marrow diameter, D-d: cortical width, CTI: cortical thickness index = (D-d)/D, ΣGS/D: bone density.

\* The number of rats analyzed is in parenthesis, <sup>a</sup>p < 0.01 compared to aged rats with history of pregnancy and lactation, <sup>b</sup>p < 0.001 compared to aged rats with history of pregnancy, <sup>c</sup>p < 0.05 compared to aged rats with history of pregnancy and lactation, <sup>d</sup>p < 0.01 compared to aged rats with history of pregnancy, <sup>e</sup>p < 0.001 compared to all aged rats.

from aged rats with history of pregnancy (p<0.001) and aged rats with prior pregnancy and lactation (p<0.01). Also two-month-old rats differed in cortical width from aged rats with history of pregnancy (p<0.01) and aged rats with prior pregnancy and lactation (p<0.05). There were no differences in the cortical thickness index or marrow diameter among the 4 groups. Two-months-old rats differed in ΣGS/D from aged rats in the three other groups. However, there was no difference in these parameters among aged rats, regardless of history of pregnancy and lactation.

### Discussion

As to longevity of Fischer-344 rats, the ages in weeks for female rats with 90% and 50% survival that were fed ad libitum were reported to be 92 and 115, respectively. For the female rats fed a restricted diet, the ages were 91 and 132, respectively<sup>13</sup>. In the present study at the 109 weeks of age, 83.8% of rats survived, which is compatible with the cited report.

Considerable bone loss during lactation in rats occurs whether dietary calcium intake is sufficient or not. Brommage<sup>14</sup> reported that lactating rats had dramatically higher intestinal absorption of calcium and phosphorus. However, by balance study, he calculated that 19% of the calcium transferred to milk was derived from the maternal skeleton with the maternal diet supplying the remaining calcium.

Bone loss during the lactation period is more severe in the cancellous bone than in the cortical bone in

rats<sup>15</sup>. Within the proximal epiphysis of the femur, cancellous bone loss was as high as 51%, whereas cortical bone loss was only 0.7%. The response of the cortical bone to lactation is not the same at different levels of the femur. Loss of diaphysis of the femur was reported to be 11%<sup>15</sup>.

In rats, various differing etiologies for bone mass loss are manifested as differing patterns of bone loss. The Metaphyseal cancellous bone of the femur serves both metabolic and mechanical functions<sup>16</sup>. The Cancellous bone in the central metaphyseal regions in the femur serves a metabolic function, while that in the lateral metaphyseal regions serves more of a mechanical function. Following ovariectomy, the loss of cancellous bone occurs mostly in the central metaphyseal region, whereas following immobilization, cancellous bone is lost throughout metaphysis<sup>16</sup>. During the lactation period, the central metaphyseal bone is absorbed<sup>1</sup>. Similar findings can be observed in the cortical bone of the femoral diaphysis. Bone loss during lactation occurs almost exclusively in the endosteal layer, which may be subject to lesser mechanical demands than the subperiosteal layer, which is not absorbed<sup>14</sup>.

In humans, various studies have suggested that lactation is associated with loss of bone, although no evidence of significant negative effects of lactation on subsequent bone mass or incidence of fracture has been reported, and recovery of bone mineral during and after weaning has been reported to occur<sup>6</sup>. Smith<sup>17</sup> reported that in humans, there is a statistical relation-

ship between parity and both cortical thickness and periosteal diameter of the femur, and that these femoral measurements for women with four or more children exceeded those of the childless by 1.7 and 1.6 mm, respectively. Restoration of the bone loss from lactation in rats has been observed. Increased trabecular bone formation, an increased proportion of double-labeled surface and increased mineral apposition of the lumbar vertebrae take place in late lactation<sup>1</sup>.

The present study shows that aged rats had a higher ash weight compared with 2-month-old rats. There was increased ash weight in aged rats with history of pregnancy with or without lactation when compared to aged rats with no history of pregnancy. Calcium content and phosphorus content remained unchanged among aged rats irrespective of history of pregnancy or lactation. However, in terms of bone diameter, marrow diameter, cortical bone width and bone density at the femoral diaphysis as determined by photodensitometry, there was no difference seen among aged rats having different histories of pregnancy and lactation. We did not determine which part of the femur increased in bone density with the reproductive cycle.

During the lactation period, a certain amount of bone is lost and restoration of bone occurs subsequently. This change may be enhanced in aged rats for which this cycle has been repeated two or three times. This observation, however, was made more than one year after the last reproductive cycle at which time they were sacrificed. Nnakwe<sup>18</sup> reported the effect of aging on bone composition of female Fischer rats ranging from 5 to 29 months of age. He concluded that steady increase in bone length, bone ash weight and calcium content occurs with increased age. In contrast, it has been reported by Sato et al.<sup>19</sup> that aging effects are manifested by a small but significant decline in volumetric bone mineral density at the proximal tibia metaphysis as determined by computed tomography over a 5 month period starting at 9 months of age in Sprague-Dawley female rats. In addition to aging, changes of gonadal steroids have an effect on bone density<sup>5</sup>. Nelson and others<sup>20</sup> reported that long-term caloric restriction retarded both neural and ovarian aging processes. In the present study, we

looked at vaginal smears of aged rats for 8 days shortly before they were sacrificed. No rats showed normal estrous cycles, although the period of 8 days was not long enough to completely evaluate the cycle (data not shown), and it was not possible to determine how much change was secondary to aging or change of gonadal steroids, if there is such change. All rats, however, were kept under the same condition after reproduction, for which reason it is likely that changes observed in the bones of aged rats reflect effects caused by pregnancies or lactation.

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