Histomorphometric Changes in Iliac Trabecular Bone during Pregnancy and Lactation in Beagle Dogs

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ABSTRACT. Changes in bone metabolism throughout pregnancy and lactation in female beagle dogs were examined by histomorphometry and measurement of serum biochemical constituents related to bone. A total of eight dogs, including one dog observed repeatedly from 1 year and 8 months to 4 years and 7 months of age, were used. For double bone labeling to obtain the bone dynamic parameters, dogs were each injected twice, at an interval of 7 days, with fluorochromes such as tetracycline or calcein at the time just after mating, just after delivery, and before weaning. At the time just after weaning, the iliac bone was biopsied. Histomorphometric analysis of trabecular bone area of undecalcified iliac bone sections was performed using a semiautomatic image analyzer. Serum biochemical parameters related to bone such as parathyroid hormone, calcitonin, calcium, phosphorus and alkaline phosphatase activity were periodically measured. Bone histomorphometric analysis revealed significant increases in the mean values of osteoid volume and mineral apposition rate as compared with those of age-matched no pregnancy dogs. The mineral apposition rate measured with the passage of time was increased after delivery and was further increased before weaning compared with that at the time of mating. There were no significant changes in mean values of bone volume, mean trabecular thickness, bone formation rate and mineralizing lag time. Histological examination showed that the osteoid accumulated during the late stage of pregnancy and lactation. All histomorphometric values were significantly correlated to the age of the dam and the number of pups. Mean values of all serum biochemical parameters did not change. These results indicate that bone mineral loss occurs due to high turnover of bone metabolism during the late stage of pregnancy and lactation and is related to the age of the dam and the number of pups in dogs, in addition to the fetus mineralization and milk production.—KEY WORDS: beagle, bone histomorphometry, lactation, mineral loss, pregnancy.

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Increased maternal mineral and bone metabolism is known to occur due to skeletal mineralization of the fetus during pregnancy and milk production during lactation in mammals [2, 4, 6, 9], and often results in severe osteoporosis in humans [6, 8]. The beagle dog is the most standardized and commonly employed breed of experimental dog. In general, the same female dog is repeatedly used for reproduction. A cross-sectional study indicated that the mineral loss in female dogs occurred during lactation due to milk production [10]. The changes in mineral and bone metabolism in dams accompanying pregnancy and lactation are considered to be related to various factors such as the age, history of pregnancy and exercise of dams, numbers of fetuses and pups, nutrients including dietary calcium, in addition to skeletal formation of the fetus and milk production [1, 3, 4].

Homeostasis of serum calcium is strictly controlled within the physiological range by calcium regulating hormones such as parathyroid hormone and calcitonin. However, the clinical values of serum biochemical parameters related to bone may not always exactly reflect the changes in calcium and bone metabolism, because pregnancy and lactation are physiological events, and not diseases.

Bone histomorphometry using undecalcified bone section labeled with fluorochromes is an useful method to directly examine histological changes in bones, using not only static parameters but also dynamic parameters.

In the present study, the changes in mineral and bone metabolism in female dogs throughout pregnancy and lactation were examined by bone histomorphometry and measurement of serum biochemical parameters to determine the relations of the various factors described above.

MATERIALS AND METHODS

A total of eight female dogs, including one dog observed repeatedly, were obtained from a breeding colony in an indoor system established at our institute. Their ages, numbers of neonates and pups, and pregnancy history are shown in Table 1. They were given a diet of 250 g/day containing calcium carbonate 2.0%, phosphorus 1.6% and Vitamin D_3 200 IU/100 g. The diet was slightly increased in quantity to meet the increased demands due to the appetite of dams and number of pups. All dogs could exercise freely in a large cage from post-mating until immediately before delivery, then 1 hour/day for the first 1 month, and at least 2–3 hours/day for the next 1 month of the lactation period. Pups were weaned at about 2 months of age.

Bone double labeling was performed three times during the experimental period. For the first labeling, calcein 8 mg/kg body weight was injected subcutaneously to dogs at an interval of 7 days (1 and 8 days) after mating. For the second labeling, tetracycline hydrochloride 25 mg/kg body weight was injected intramuscularly 1 and 8 days after delivery. For the third labeling, the same dose of calcein as the first labeling was injected subcutaneously 8 and 1

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Table 1. Age, number of neonates and pups, and pregnancy history of dams

Number of dam	Age at mating	Number of neonates	Number of pups	Pregnancy history
1	1 year 8 months	3	1	First Pregnancy
2–1*	2 years 7 months	4	4	First
3	2 years 7 months	6	6	First
2–2*	3 years 3 months	4	3	Second
4	3 years 4 months	5	5	Second (First; 1 year 8 months)
5	4 years 1 month	8	8	First
6	4 years 3 months	8	8	First
7	4 years 7 months	11	9	Second (First; 3 years 1 month)

*Dog No. 2 was observed repeatedly as No. 2–1 and 2–2.

days before weaning.

Seven days after the last bone labeling, iliac bone specimens were obtained by biopsy from a site 1.5 cm below the anterior iliac spine with a trephine (8 mm internal diameter) under anesthesia with a combination of ketamine hydrochloride (25 mg/kg, Sankyo Co., Ltd.) and xylazine (10 mg/kg, Bayer Co., Ltd.).

The undecalcified biopsied specimen was stained in Villanueva's bone stain solution [11], dehydrated in ethanol and embedded in methylmethacrylate. Subsequently, the block was cut in thin slices with an inner blade cutter (MC-808D, Maruto Co., Ltd.) and sectioned at a thickness of 15 μm with grinding glasses (Asahi Glass Co., Ltd.). The histomorphometric measurements of cancellous bone area of the ilium were performed with a semiautomatic image analyzer (Kontron 64, Cal Zeiss Co.) using "Osteoplan" software [9] under fluorescence microscopy.

Histomorphometric parameters and units are as follows:

Bone volume (volume trabecular bone including osteoid/volume total bone, %), mean trabecular thickness (total trabecular surface/volume total bone, μm), osteoid volume (volume of osteoid/volume total bone, %), mineral apposition rate (mean distance of tetracycline or calcein labels/labeling intervals, μm/day), bone formation rate (mean label length × apposition rate/mean trabecular areas, %/year), and mineralization lag time (osteoid seam width/mineral apposition rate, days).

The histomorphometric values obtained by a semiautomatic image analyzer were compared with our data of age-matched no pregnancy beagles aged 1–4 years (31 dogs) in our colony [5]. In order to determine the relationships between histomorphometric values and the age of dams, and the number of pups, the correlation coefficients were calculated by a least squares method.

Except for the measurement using an image analyzer, in order to clarify the changes in mineral apposition rate with the passage of time, the distance between two adjacent labeled lines in the whole area of trabecular bone in the section was measured with an eyepiece micrometer while identifying the time of labeling by the histological features and colors of tetracycline (yellow) and calcein (light green) under fluorescence microscopy. The distances at five points per double labeling were measured and the mean value was calculated.

Blood was collected periodically from the time of mating to weaning. Blood samples were allowed to clot and were centrifuged for 15 min at 3,000 r.p.m. Serum biochemical parameters measured were as follows; total calcium (*o*-cresolphthalein complexone method), phosphorus (molybdenum blue method), alkaline phosphatase activity (Bessey-Lowry's modified method), parathyroid hormone (radioimmunoassay method), and calcitonin (radioimmunoassay method).

RESULTS

In the histomorphometric values, there were significant increases in the mean values of osteoid volume and mineral apposition rate compared with those of age-matched no pregnancy beagles (Table 2). The mineral apposition rate measured by an eyepiece micrometer was increased just after delivery and further increased at

Table 2. Comparison of bone histomorphometric values between dams in this study and age-matched no pregnancy dogs in our colony

Parameters	Examined dogs (n=8)	No-pregnancy dogs (n=31)	Significant difference
Bone volume (%)	26.6± 5.5	27.3± 4.3	
Mean trabecular thickness (μm)	275± 57	264± 56	
Osteoid (%)	0.990± 0.974	0.446± 0.330	p<0.05
Mineral apposition rate (μm/day)	1.210± 0.331	0.839± 0.167	p<0.001
Bone formation rate (%/year)	86.7± 76.4	52.8± 31.9	
Mineralization lag time	9.310± 1.785	10.545± 8.838	

Values are mean±SD.

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weaning compared with post-mating (Fig. 1). Histological examination of iliac bone sections showed marked osteoid accumulation on the trabecular bone surface (Fig. 2A and B). Figure 2A demonstrates that the osteoid accumulation commenced after delivery, whereas the first calcein line in the third double labeling was at the border between the calcified bone and osteoid. Figure 2B shows that the osteoid accumulation commenced before delivery, whereas the first line in the third bone labeling was present in the osteoid.

Fig. 1. Mineral apposition rates measured by an eyepiece micrometer just after mating, just after delivery and just before weaning in dams. Each mark and vertical bar represents mean ± SD.

The correlation coefficients between histomorphometric values and age of dams and number of pups are shown in Table 3. All histomorphometric values were significantly correlated with the age of the dam and the number of pups. High correlation coefficients were seen between bone volume, mean trabecular thickness and age of dam, and between osteoid volume and number of pups. In the dog observed repeatedly (No. 2 in Table 1), the bone volume decreased from 35.3 to 20.9% and the mean trabecular thickness from 341 to 207 μm during the first and second pregnancy and lactation, respectively.

The serum biochemical values are shown in Table 4. There were no significant differences in calcium, phosphorus, parathyroid hormone, and calcitonin throughout the period from mating to weaning, except for one dog. In one dog, the level of parathyroid hormone was elevated to 4.5 *ng*/m*l*, calcitonin to 136.0 *pg*/m*l* and the level of serum calcium was also elevated after the delivery.

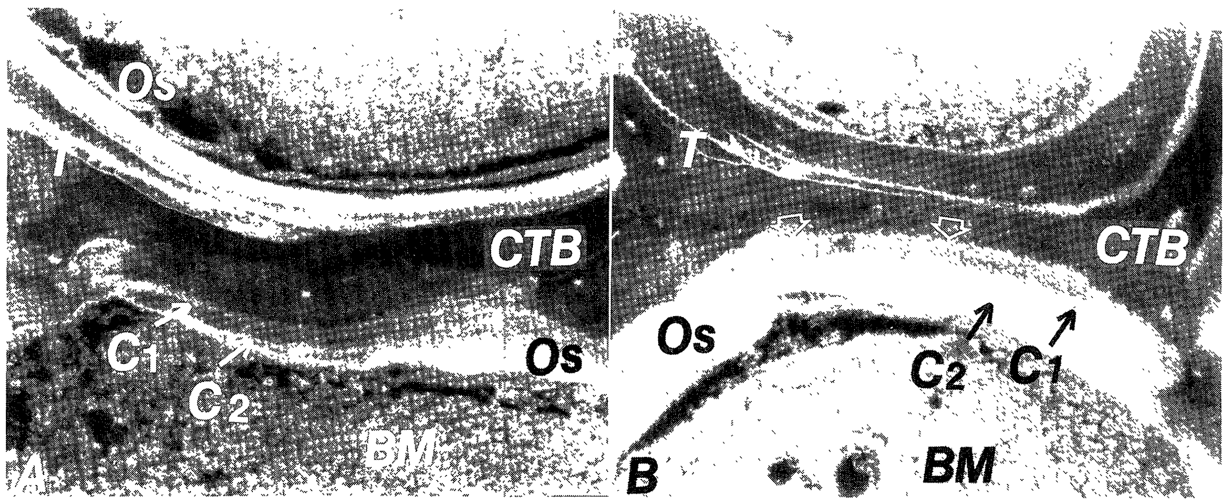

Fig. 2. Micrograph A showing the mineral loss of bone formation sites on trabecular bone surface between just after delivery and weaning. Os; accumulated osteoid, C_1; first calcein line, C_2; second calcein line labeled at just before weaning. CTB; calcified trabecular bone, BM; bone marrow. Micrograph B showing severe mineral loss starting before delivery (open arrows). C_1 and C_2; double calcein lines seen in the wide osteoid, T; tetracycline lines labeled just after delivery. Fluorescence microscopy, Villanueva's bone stain, × 200.

Table 3. Correlation coefficients between bone histomorphometric values and age of dam, and number of pups

Histomorphometric parameters	Age of dams	Number of pups
Bone volume	−0.670604 (P<0.05)	−0.340798 (P<0.05)
Mean trabecular thickness	−0.732249 (P<0.01)	−0.330815 (P<0.05)
Osteoid volume	0.209584 (P<0.05)	0.553166 (P<0.05)
Mineral apposition rate	−0.374608 (P<0.05)	−0.346158 (P<0.05)
Bone formation rate	−0.530421 (P<0.05)	−0.296386 (P<0.05)
Mineralization lag time	0.441457 (P<0.05)	0.610653 (P<0.05)

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Table 4. Serum biochemical values in dams

Items / Time	−9 weeks	−4 weeks	−1 week	1 week	4 weeks	9 weeks
Calcium (mg/g*l*)	8.8± 0.8	8.7± 0.9	8.5± 0.8	8.4± 0.9	90± 1.6	8.1± 1.3
Phosphorus (mg/d*l*)	3.9± 1.0	3.3± 0.1	2.9± 0.3	3.6± 0.6	3.3± 0.5	3.4± 0.8
Parathyroid hormone (ng/m*l*)	0.98± 0.19	1.12± 0.33	1.10± 0.19	0.95± 0.19	1.02± 0.29	1.00± 0.26
Calcitonin (*p*g/m*l*)	111.1± 23.7	106.7± 10.3	108.6± 12.3	98.9± 15.7	116.3± 13.6	103.1± 12.3

The time of delivery is expressed as 0 week. Values are mean±SD.

DISCUSSION

Both the increases of mineral apposition rate and osteoid volume in the bone histomorphometric values, and the marked accumulation of osteoid in the histological findings indicate that the bone mineral loss was due to a state of high bone turnover throughout pregnancy and lactation. The change in mineral apposition rate with time indicated that mineral loss was accelerated from the late stage of pregnancy to lactation. Osteoid volume showed a strong correlation to the number of pups. The histological findings indicated that the osteoid accumulation before and after delivery. These findings suggest that the bone mineral loss occurs due to mineralization of the fetus in the late stage of pregnancy and to milk production in dams. Our results support the results of another study in which bone mineral loss was observed during lactation in female dogs [10].

The fact that the bone volume and mean trabecular thickness did not significantly decrease as compared to those in age-matched no-pregnancy dogs indicates that bone volume is not decreased markedly in dogs by pregnancy and lactation and remains within the normal range. However, the high correlation between bone volume, mean trabecular thickness and age of dams indicates that the bone mineral loss increases with the age of the dam. Also, the bone volume and mean trabecular bone thickness in one dog were decreased by repeated pregnancy and lactation, suggesting that bone mineral loss is related to lactation history as well as age, as seen in women [8].

Although it was confirmed that all histomorphometric values were correlated with the age of dams and number of pups, the serum biochemical values hardly changed. Thus, the unchanged serum biochemical values obscured the marked changes in calcium and bone metabolism, because bone mineral loss occurs to maintain the normal calcium homeostasis. Therefore, the serum biochemical values examined in this study should generally not be utilized as indicators to detect changes in calcium metabolism. The slight elevations of serum biochemical values at the late stage of pregnancy and the early stage of lactation observed in one dog may reflect that abnormality of calcium metabolism rarely occurs in dams. In the present study, insufficient calcium intake in dams is unlikely, because the diet was not increased in quantity and calcium content throughout pregnancy and lactation. Although calcium supply is important to prevent mineral loss during pregnancy and lactation [1, 3, 8], it is necessary to examine dietary factors other than calcium [1, 3] and intestinal calcium absorption [7] in further studies.

In humans, the adverse effects on bone metabolism caused by pregnancy and lactation often result in severe osteoporosis accompanied with fractures [6]. However, it seems that such severe osteoporosis accompanied by a marked decrease of bone mass may rarely occur in dogs, because the bone volume and mean trabecular thickness remained within the range of those in non-pregnancy dogs in present study, and Miller *et al.* [10] reported that mineral loss is accelerated in a longer-lived mammals. Therefore, the magnitudes of mineral loss and bone damage depend on the period of gestation and duration of lactation in each species.

In our study, it was considered that all dams could exercise sufficiently during pregnancy and lactation, judging from the absence of significant differences in histomorphometric values between the dams observed in this study and non-pregnancy dogs in our previous study [5].

As a result, it was concluded that the bone mineral loss in dams occurs particularly between the late stage of pregnancy and the period of lactation in dogs and is related to the age of the dam and the number of pups, in addition to mineralization of the fetus and milk production, but dogs may hardly develop the osteopenia and osteoporosis with fracture and marked decrease of bone volume in pregnant women and nursing mothers.

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