Change in Calcium Balance and Bone Mineral Density during Pregnancy in Female Rats

Naomi OMI and Ikuko EZAWA*

Department of Food and Nutrition, School of Home-Economics, Japan Women's University, 2–8–1 Mejirodai, Bunkyo-ku, Tokyo 112–8681, Japan

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Summary This study investigated changes in Ca balance and BMD during pregnancy in female rats. During pregnancy, the intestinal Ca absorption increased significantly, and Ca accumulation was also markedly elevated. However, BMD values for the lumbar spine decreased significantly during pregnancy. Twenty female SD rats, 10 weeks of age (Japan SLC Co., Shizuoka, Japan) were acclimated for 2 weeks. Then, the rats were divided into two groups; the control (no pregnancy) group (n=10) and the pregnant group (n=10). The rats in the pregnant group were kept in a cage with a male rat for 5 days at 12 weeks of age, and all 10 were successfully impregnated. During the pregnancy period, the values for intestinal Ca absorption and the rate of the intestinal Ca absorption in the pregnant group were significantly greater than those of the control group. In addition, in spite of the significant increase in urinary Ca excretion in the pregnant group, the Ca accumulation was markedly elevated during the latter half of pregnancy. On the other hand, the BMD value in the lumbar spine for the pregnant group significantly decreased during pregnancy. These findings suggest that pregnancy accelerated intestinal Ca absorption and Ca accumulation in female rats, while the lumbar spine BMD decreased during pregnancy.

Key Words pregnancy, bone mineral density (BMD), intestinal Ca absorption, urinary Ca excretion

Changes in calcium (Ca) metabolism, which can occur during pregnancy, can considerably affect bone metabolism. There have been some reports on the effects of pregnancy on bone, such as post-pregnancy osteoporosis (1), decreasing bone mass (2–4), no change in the bone mass (5, 6), or increasing bone density (2) during pregnancy; however, bone metabolism during pregnancy is still unclear. Elucidating the changes in Ca balance during pregnancy may also be important to help prevent bone loss during pregnancy. However, these changes have not been clarified, and human Ca balance is difficult to determine. In addition, at present, it is also impossible to measure the bone mineral density (BMD) in pregnant woman using X-rays, due to the amount of radiation involved, and axial bone BMD can not be determined by quantitative ultrasound measurement. Heel ultrasound measurement is effective for osteoporosis screening (7), but the heel is not useful for analyzing BMD changes during pregnancy, since it is a weight-bearing bone and body weight might increase during pregnancy.

Therefore, the purpose of this study was to elucidate changes in the Ca balance and BMD during pregnancy in female rats. Additionally, to clarify the change in Ca balance after delivery, the baby rats were removed at birth to prevent lactation.

MATERIALS AND METHODS

Experimental design. Twenty female Sprague-Dawley (SD) rats, 10 weeks of age (Japan SLC Co., Shizuoka, Japan) were acclimated for 2 weeks, and divided into two groups; the control (no pregnancy) group (n=10) and the pregnant group (n=10). All the rats were kept in separate balance-study cages during the experiment. Each rat in the pregnant group was kept in a separate cage with a male rat for 5 days at 12 weeks of age, and all 10 were successfully impregnated. All the rats in the control and pregnant groups were allowed free access to diet (F-2 diet, Funabashi Co., Japan, containing 1.2% calcium (Ca) and 0.96% phosphorus) and ion-exchanged-distilled water. The duration of the experiments after propagation was 8 weeks. In this study, in order to determine the Ca balance in the dams, the litters were separated from the dams at birth. Each dam had 7 to 14 offspring (mean ± SE = 11.1 ± 0.6). The temperature was kept at 23 ± 1°C, and humidity was maintained at 50 ± 5%. A 12 hour light cycle was maintained for all groups, with lights on from 7:00 a.m. to 7:00 p.m.

Balance study. Ca balance was evaluated at eleven intervals during this study to determine the intestinal Ca absorption and Ca accumulation. For each evaluation, feces and urine were collected over a 24-hour period. Urine was collected under acidic conditions using 1 mL of 6 N-hydrochloric acid, thus preventing Ca precipitation and putrefaction. The first evaluation was

*To whom correspondence should be addressed.
carried out on the last two days before the start of the experimental period (before pregnancy: 0 time balance). After matching, the Ca balance was evaluated once every 3 days during the pregnancy period (1st to 6th balance studies), and once a week after delivery (7th to 10th balance studies). All the collected urine was centrifuged at 2,500 rpm for 15 minutes, immediately after collection, to extract the supernatant. All daily feces were burnt to ash at 550–600°C for approximately eighteen hours, and the resulting ash was dissolved in 1 N nitric acid. Fecal and urinary Ca excretions were measured by atomic absorption spectrophotometry (Shimadzu AA-640-12, Kyoto, Japan). Intestinal Ca absorption and Ca accumulation were calculated using the amount of Ca intake, the fecal Ca excretion and the urinary Ca excretion (explained below).

Intestinal Ca absorption (mg/d)  
= Ca intake (mg/d) − Fecal Ca excretion (mg/d)

Rate of the intestinal Ca absorption (mg/d)  
= Intestinal Ca absorption (mg/d)/Ca intake (mg/d)

Ca accumulation (mg/d)  
= Intestinal Ca absorption (mg/d) − Urinary Ca excretion (mg/d)

Rate of the Ca accumulation (mg/d)  
= Ca accumulation (mg/d)/Ca intake (mg/d)

Change in bone mineral density (BMD). BMD values for the L4-L5 lumbar spine and the right tibia were determined by dual energy X-ray absorptiometry (DXA: Hologic’s QDR-1500, USA) three times in this experiment. For each rat, BMD measurements were performed under general anesthesia at the start (before pregnancy), just after delivery and 1 month after birth. The radiation beams were directed antero-posteriorly for the lumbar spine and laterally for the tibia. An ultra high-resolution scan mode (rat mode, version 4.59 software) was used. BMD values were obtained for the lumbar spine, the proximal one-third of the tibia including the epimetaphyseal region (8), and the distal two-thirds of the tibia representing the cortical diaphyseal region. The coefficient of variation of the BMD measurement was 0.88%.

Measurement of mechanical strength of bone. At the end of the experiment, the rats were weighed. After overnight deprivation of food, the rats were dissected. Femur samples were isolated after killing by exsanguination. After the adhering connective tissues were trimmed, the femora were tested for bone strength. The mechanical strength, the breaking force and energy, of the femurs was tested by the breaking property test using an instrument (Iio Co., Japan, type DYN-1255) as previously reported (9). The force and energy necessary to produce a break at the center of the femur were measured. The measurement conditions were as follows: the sample space was 1.0 cm, the plunger speed was 100.0 mm/min, the load range was 50.0 kg, and the chart speed was 120.0 cm/min.

Serum calcium, phosphorus, and total protein levels. After overnight deprivation of diet, the rats were anesthetized with ether, and blood samples were taken from the abdominal aorta. All the serum samples were stored at −90°C. Serum Ca levels were determined by the methods described previously. Phosphorous (P) was measured by the Fisk-SubbaRow method (10) and the total protein levels were determined using the biuret method (11).

Statistics. All data are expressed as the means±SE. Student’s t-tests were used to analyze the differences between the groups after F-test. A p-value less than 0.05 was considered statistically significant.

RESULTS

Body weight gain and food intake
Changes in body weight and food intake during the experiment are shown in Fig. 1 and 2. The body weight of the pregnant group significantly increased during the pregnancy period. After delivery, body weight in the pregnant group rapidly decreased, and then increased gradually until the end of the experiment. Compared with the pregnant group, the body weight of the control group increased slightly during the experiment. At the end of the experiment, there was a significant difference between body weight in the control and the pregnant groups (p<0.01).

There was no difference in food intake between the control and pregnant groups during the acclimation period (before pregnancy). There was no significant change in the food intake of the control group during the experiment. However, the food intake of the pregnant group during the pregnancy period significantly increased compared with that before the start of the experiment (p<0.001), and was significantly higher than that of the control group (p<0.001). After giving birth, the food intake in the pregnant group was still significantly higher than that of the control group (p<0.05), but it gradually decreased and finally there was no difference in food intake compared with the control group.

Serum calcium, phosphorus, and total protein levels
At the end of the experiment (1 month after delivery), the serum Ca, P and total protein values were within normal range in both the control and the pregnant groups, and there was no significant difference the parameters, between two groups (data not shown).

Ca balance study
During the pregnancy period, the Ca intake in phases I to VII (including the period just after delivery) in the pregnant group were significantly greater than those of the control group (p<0.001, p<0.01).

The fecal Ca excretions for the pregnant group during the last part of the pregnancy period (phases V and VI) decreased significantly compared with the control group. In phase VII (just after delivery), fecal Ca excretions for the pregnant group were significantly higher than those of the control group (Fig. 3).

Intestinal Ca absorption and the rate of Ca absorption are shown in Fig. 4. There was no difference in the Ca absorption before pregnancy (0 time period) between the control group and the pregnant group. During the pregnancy period (phases I to VI), however, the values for both the amount of intestinal Ca absorption and the rate of the intestinal Ca absorption in the pregnant
Fig. 1. The change in body weight during the experiment. ●—● = the control group (n=10). ○—○ = the pregnant group (n=10). Female SD rats, 10 weeks of age were acclimated for 2 weeks, and were divided into two groups: the control (no pregnancy) group (n=10) and the pregnant group (n=10). Experimental period lasted 8 weeks. The body weight of the pregnant group significantly increased during the pregnancy period. Compared with the pregnant group, the body weight of the control group increased slightly during the experiment. At the end of the experiment, there was a significant difference between the 2 groups (p<0.01).

Fig. 3. The fecal Ca excretion during the experiment. ●—● = the control group (n=10). ○—○ = the pregnant group (n=10). *p<0.05, **p<0.01 vs the control group. During this experiment, Ca balance study was carried out at eleven intervals. The fecal Ca excretions for the pregnant group during the last part of the pregnancy period (phases V and VI) decreased significantly.

Fig. 2. The food intake during the experiment. The upper figure shows a change in food intake during pregnancy. ●—● = the control group (n=10). ○—○ = the pregnant group (n=10). The bottom figure demonstrates the mean daily food intake in each period. Cont.= the control group (n=10) Preg.= the pregnant group (n=10) before= the acclimation period (before starting the experimental period) pregnant= the period during pregnancy, after= the period after delivery. *p<0.05, ***p<0.001 vs the control group. During the experiment, all the rats were allowed free access to diet (F-2, Funabashi Co., 1.2% Ca, 0.96% P). The food intake of the pregnant group during the pregnancy period increased significantly (p<0.001). After delivery, it gradually decreased and finally there was no difference.

Fig. 4. The intestinal Ca absorption and the rate of the intestinal Ca absorption during the experiment. ●—● = the control group (n=10). ○—○ = the pregnant group (n=10). *p<0.05, **p<0.01, ***p<0.001 vs the control group. During this experiment, Ca balance study was carried out at eleven intervals. Intestinal Ca absorption and rate of the Ca absorption were calculated using the amount of Ca intake and the fecal Ca excretion. During the pregnancy period (phases I to VI), the intestinal Ca absorption in the pregnant group was significantly higher.
group were significantly greater than those of the control group. In particular, in phases V and VI (the latter part in the pregnancy period) and phase VII (after delivery), the intestinal Ca absorption for the pregnant group was remarkably high. There was no marked difference in the intestinal Ca absorption of the control group during the experiment (0 time to phase X). As shown in the lower part of Fig. 4, the rate of Ca absorption was significantly higher in the pregnant group during the pregnancy period than the control group (phases I, III, IV, V, and VI).

The findings for the Ca accumulation and the rate of the Ca accumulation in the pregnancy period were also significantly higher in the pregnant group, similar to the intestinal Ca absorption and the rate of the Ca absorption (data not shown). Although the urinary Ca excretion for the pregnant group increased significantly (phases I, IV, V, and VI; Fig. 5), Ca accumulation was markedly elevated during the latter half of pregnancy.

**BMD values at the lumbar spine, tibial proximal metaphysis and the tibial diaphysis**

The BMD values for the lumbar spine in the control group slightly increased during the experiment (0 time, post-delivery, and after 1 month) (Fig. 6). The BMD value in the lumbar spine in the pregnant group at the 0 time measurement did not differ from that in the control group, but decreased during pregnancy. The lumbar spine BMD of each dam in the pregnant group decreased during pregnancy. Furthermore, the BMD value in the lumbar spine in the pregnant group just after delivery (at the point for post-delivery) was significantly lower than that in the control group. After delivery, the BMD in the lumbar spine recovered to a value similar to that at the 0 time measurement, but it was slightly lower than that in the control group (there was no significant difference between the two groups).

The BMD values in the tibial proximal metaphysis and the tibial diaphysis are shown in Fig. 6. Those BMD values did not differ significantly between the control and the pregnant groups. The BMD value in the proximal metaphysis of the pregnant group did not increase during the pregnancy, and was similar to the BMD value in the 0 time period. After 1 month of measurement, the BMD values in the metaphysis and the diaphysis tended to be higher than those of the control group.

**Bone strength of the femur**

The values for the center of the femur strength at 1 month after delivery did not differ significantly between the control and the pregnant groups (data not shown).

**DISCUSSION**

The findings in this study showed the Ca balance and BMD change during pregnancy in female rats: during pregnancy, the intestinal Ca absorption increased significantly, and Ca accumulation was also dramatically elevated, while the BMD values for the lumbar spine de-
creased significantly.

Although the values of intestinal Ca absorption during pregnancy increased significantly, it was not caused by an elevation in the Ca intake in this study. A high Ca intake was related to an increase in the food intake and usually the rate of intestinal Ca absorption decreases due to the elevation of Ca intake. However, the rate of intestinal Ca absorption during pregnancy also increased significantly in this study. In particular, the rate of intestinal Ca absorption in the latter half of the pregnancy period markedly increased. This finding was also reported by Heaney et al. (12).

Kent et al. (13) and Gertner et al. (14) reported that 24 h urinary Ca excretion was elevated during pregnancy, which Gertner et al. (14) suggested could be due to the acceleration of Ca absorption. Our findings showed a similar elevation in 24 h urinary Ca excretion in the latter half of the pregnancy period, probably due to the increase in intestinal Ca absorption. Data of the present Ca balance study clarify the acceleration of intestinal Ca absorption during pregnancy.

Both the Ca accumulation and the rate of Ca accumulation for the pregnancy group increased significantly during the pregnancy period, suggesting that the requirement for Ca increases during pregnancy. In humans, Ca transfer to the fetus transplacentally was reported to be active in the third trimester during pregnancy (15), suggesting an increased requirement for Ca in the later stages of pregnancy.

In the present study, just after delivery intestinal Ca absorption and Ca accumulation were still significantly higher in the pregnant group, but they decreased gradually according to the reduction of food intake. These findings indicate that, immediately post-delivery the requirement for Ca is probably still high, but that when lactation does not occur, Ca demand returns to within normal levels within several weeks of delivery.

The lumbar spine BMD in the pregnant group decreased significantly during pregnancy, despite the elevation in intestinal Ca absorption and Ca accumulation. This suggests that the demand for Ca in the pregnant rats might be higher than the intestinal Ca absorption increase. Although there have been some reports on the effects of pregnancy on bone mass (1–6), since the measurement sites and techniques of bone mass measurement were different in each study, it is still controversial whether bone mass decreases during pregnancy. However, it has been suggested in both the human and the rat studies that the axial bone mass might decrease during pregnancy (2–4), and that the appendicular bone mass does not decrease (2, 5, 6). In the present study, the BMD of the axial bone, which was lumbar spine, decreased during pregnancy. The bone structure of the lumbar spine is mainly trabecular bone. The trabecular bone metabolizes rapidly compared with cortical structures, such as a diaphysis in long bones. The change of Ca metabolism during pregnancy could rapidly affect trabecular bone, because the bone turnover of the trabecular bone is higher than that of the cortical bone. Moreover, since in rats the lumbar spine is not a weight-bearing bone, the increase in body weight during pregnancy would not affect lumbar spine BMD. Thus, in the present study the bone mass of the lumbar spine decreased during pregnancy.

In order to clarify the change in Ca balance during pregnancy and after delivery, the rats did not lactate in this study, and it was noted that the lumbar spine BMD increased immediately after delivery. In humans, it has been reported that post-pregnancy osteoporosis was improved after lactation was stopped (16), suggesting that the hormonal balance might influence the recovery of BMD.

Tibial BMD, the proximal metaphysis and the diaphysis BMDs, did not decrease during pregnancy, and at 1 month after delivery values tended to be higher than those of the control. Tibiae are weight-bearing bones, so the BMD probably does not decrease during pregnancy, because body weight markedly increases. In pregnant women, the measurement of BMD is carried out in the radius or calcaneus (7). The radius BMD is determined by the BMD of the cortical bone, which metabolizes more slowly than the trabecular bone and the change in BMD of the radius during pregnancy can be unclear. The calcaneus BMD is determined mainly by the trabecular BMD; however, since the heel is a weight-bearing bone and body weight increases during pregnancy affect the increase in the calcaneus BMD, the extent of change in BMD of the calcaneus during pregnancy is also unclear. In this study, the tibial BMD did not decrease, because it is a weight-bearing bone, but the lumbar spine BMD, which is not a weight-bearing bone in rats, decreased significantly during pregnancy. These findings suggest that the results of changes in bone mass during pregnancy would vary according to the site of the bone mass measurement.

In conclusion, this study confirmed that pregnancy accelerates intestinal Ca absorption and Ca accumulation in female rats, and that the lumbar spine BMD decreases during pregnancy. There have been few reports investigating both the Ca balance and BMD change during pregnancy in the same study (6), and in the present study we determined both the Ca balance and the BMD change. While the exact mechanisms are not known, the reduction in BMD during pregnancy might be due to a higher Ca requirement during pregnancy. BMD recovered 1 month after delivery in non-lactating rats, but just after delivery, the intestinal Ca absorption and Ca accumulation were still higher, which may have affected the recovery of the lumbar spine BMD.

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