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Differences in bone metabolism between singleton pregnancy and twin pregnancy

Soichiro Nakayama [a], Toshiyuki Yasui [b,*], Masanori Suto [a], Miki Sato [a], Takashi Kaji [a], Hirokazu Uemura [c], Kazuhisa Maeda [a], Minoru Irahara [a]

[a] Department of Obstetrics and Gynecology, Institute of Health Biosciences, The University of Tokushima Graduate School, Japan
[b] Department of Reproductive Technology, Institute of Health Biosciences, The University of Tokushima Graduate School, Japan
[c] Department of Preventive Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, Japan

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ABSTRACT

Objective: The objective of this study was to examine the influence of twin pregnancy on calcium metabolism, including bone turnover markers and calcium-regulating factors, by comparison between singleton pregnancy and twin pregnancy in women during pregnancy and puerperium in cross-sectional and longitudinal studies.
Methods: Women with singleton and twin pregnancies were recruited from the outpatient clinic of Tokushima University Hospital. In both cross-sectional and longitudinal studies, bone formation and resorption markers, mineral metabolism and calcium-regulating factors were measured at 10, 25, 30 and 36 weeks of pregnancy and at 4 days and 1 month postpartum in women with singleton and twin pregnancies.
Results: Urinary levels of cross-linked type I collagen N-telopeptides and C-terminal telopeptides of type I collagen in women with twin pregnancy were significantly higher than those in women with singleton pregnancy and those high levels were observed earlier than those in women with singleton pregnancy. In the cross-sectional study, serum levels of bone-specific alkaline phosphatase, calcium and phosphate in women with twin pregnancy were higher and the levels of 1,25-$(OH)_2$ vitamin D and 25-(OH) vitamin D in women with twin pregnancy were lower than those in women with singleton pregnancy.
Conclusion: Changes in bone metabolism in women with twin pregnancy are different from those in women with singleton pregnancy. Early and large increases in bone turnover markers allow women with twin pregnancy to meet high fetal demand for calcium during pregnancy.

Introduction

Calcium and bone metabolism in pregnant women is different from that in non-pregnant women from the point of view of calcium homeostasis. Calcium homeostasis in non-pregnant women is maintained by absorption from the intestine, re-absorption in the kidney and bone metabolism. In women during pregnancy, placental calcium transport increases from 50 mg/day at 20 weeks of pregnancy up to 350 mg/day at term to maintain bone calcium accretion in the fetus [1–3]. Bone turnover during pregnancy has been demonstrated to be highly activated due to an increase in bone resorption preceding an increase in bone formation [4]. We have also shown in a longitudinal study that bone resorption markers increased during late pregnancy and decreased at postpartum, while a bone formation marker was increased at 1 month postpartum in women with singleton pregnancy [5].

Calcium and bone metabolism may be greatly influenced by twin pregnancy because bone changes occur in anticipation of demand of the growing twin fetuses. Although it has been reported that estimated requirement was 2000 mg in women with singleton pregnancy [6], the most recent review has shown that recommended dietary allowance (1000 mg) for calcium intake in pregnant women is similar to that in non-pregnant women of the same age [7]. Bone turnover in women with singleton pregnancy and that in women with multiple pregnancy have been compared at the third trimester [8]. However, to the best of our knowledge, the influence of twin pregnancy on calcium metabolism and bone turnover throughout pregnancy and postpartum has not been fully elucidated.

The objective of this study was to examine the influence of twin pregnancy on calcium metabolism, including bone turnover markers, mineral metabolism and calcium-regulating factors, by comparison between singleton pregnancy and twin pregnancy in women during pregnancy and puerperium in both cross-sectional and longitudinal studies.

Subjects and methods

Subjects

We performed a cross-sectional study for comparison of bone turnover markers, mineral metabolism and calcium-regulating hormones in women with singleton pregnancy and women with twin

* Corresponding author at: Department of Reproductive Technology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Fax: +81 88 631 2630.
E-mail address: yasui@medsci.tokushima-u.ac.jp (T. Yasui).

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Table 1
Baseline characteristics in women with singleton pregnancy and women with twin pregnancy in the longitudinal study.

	Singleton	Twin	P value
Number	15	11	
Age (years)	30.7 ± 4.1	30.8 ± 4.8	0.93
Nulliparity (%)	7 (47)	8 (73)	0.18
Body height(cm)	159.9 ± 6.2	158.0 ± 4.3	0.40
Body weight before pregnancy (kg)	55.4 ± 9.9	50.3 ± 5.2	0.14
BMI before pregnancy	21.6 ± 3.7	20.2 ± 2.5	0.29
Body weight gain(kg)	9.2 ± 2.7	13.8 ± 4.5	0.003
Weeks of pregnancy at delivery (weeks)	39.6 ± 1.1	37.8 ± 1.1	0.0005
Rate of breast-feeding (%)	13 (87)	10 (91)	0.62
Rate of smoking (%)	1 (7)	1 (9)	0.68
Intake of calcium (mg)	673 ± 158	795 ± 287	0.47
Intake of vitamin D (μg)	7.1 ± 2.7	7.4 ± 3.4	0.87
Intake of phosphate (mg)	1013 ± 166	1022 ± 204	0.94

BMI: body mass index, mean ± SD.

pregnancy during pregnancy and puerperium (Study 1) and a prospective comparison study of bone turnover markers, mineral metabolism and calcium-regulating hormones in women with singleton pregnancy and women with twin pregnancy during pregnancy and puerperium (Study 2).

Study 1

The study population comprised 191 women with singleton pregnancy, 131 women with twin pregnancy, 139 postpartum women who had delivered a single baby and 53 postpartum women who had delivered twin babies. All women were recruited for the cross-sectional study from the outpatient clinic of the Department of Obstetrics and Gynecology, Tokushima University Hospital, and informed consent was obtained from all women. The Ethics Committee of Tokushima University Hospital approved the study. For women with singleton pregnancy, 36, 46, 51 and 58 women were studied at 10, 25, 30 and 36 weeks of pregnancy, respectively, and 70 and 69 women were studied at 4 days and 1 month postpartum, respectively. For women with twin pregnancy, 45, 35, 26 and 25 women were studied at 10, 25, 30 and 36 weeks of pregnancy, respectively, and 25 and 28 women were studied at 4 days and 1 month postpartum, respectively. None of the women had any disorders that affected their metabolism of calcium or bone, any history of endocrine, renal or liver illness, pregnancy-induced hypertension or gestational diabetes, and none of the women were prescribed bed rest for any reason. All of the women intended to breastfeed for at least 6 months postpartum. Serum bone-specific alkaline phosphatase (BAP), serum cross-linked type I collagen N-telopeptides (NTx), urinary NTx, urinary C-terminal telopeptide of type I collagen (CTx), serum calcium and phosphate, urinary calcium and phosphate, intact parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25-$(OH)_2$ vitamin D) and 25-(OH) vitamin D were measured at 10, 25, 30 and 36 weeks of pregnancy and at 4 days and 1 month postpartum.

Study 2

Forty women (20 women with singleton pregnancy and 20 women with twin pregnancy) who attended the Tokushima University Hospital from an early stage of pregnancy were recruited during pregnancy and puerperium. All of the women with twin pregnancy were diagnosed by using ultrasonography at 9 weeks of pregnancy. Subjects with any disorders that affected their metabolism of calcium or bone, any history of endocrine, renal or liver illness, hypertension or diabetes and subjects receiving glucocorticoid treatment at an early stage of pregnancy were excluded from the study. Serum BAP, serum NTx, urinary NTx, urinary CTx, serum calcium and phosphate, urinary calcium and phosphate, intact PTH, 1,25-$(OH)_2$ vitamin D and 25-(OH) vitamin D were measured at 10, 25, 30 and 36 weeks of pregnancy and at 4 days and 1 month postpartum. We asked about body weights and heights before pregnancy at the first visit to our hospital. None of the women during pregnancy and puerperium received any nutrient supplements. Levels of calcium, phosphate and vitamin D intake were estimated using a questionnaire in which subjects were asked to estimate their daily intake of food for three consecutive days. The contents of calcium, phosphate and vitamin D in each food were calculated and then expressed as an average daily intake.

Collection of blood and urine samples

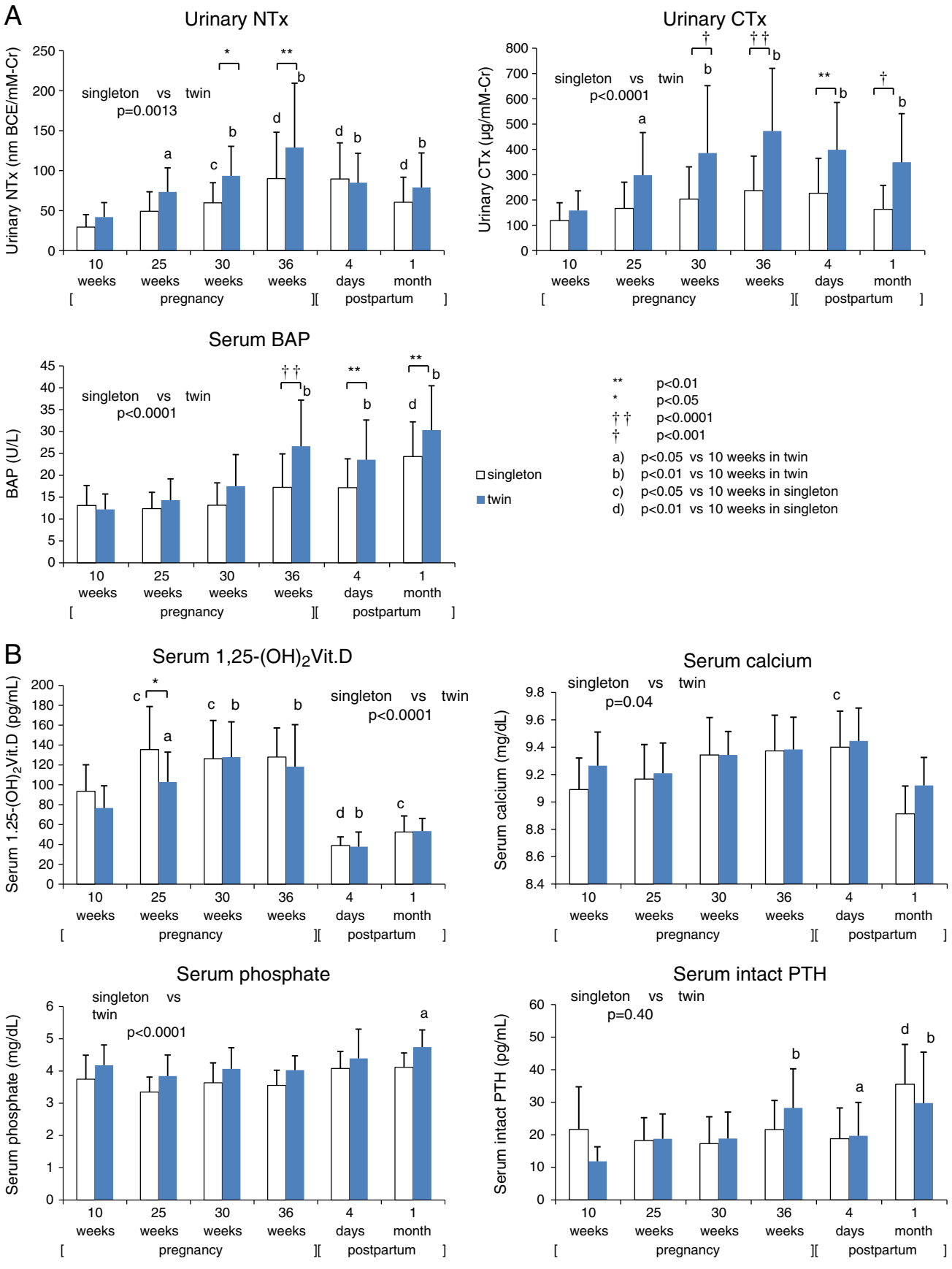
Fasting blood and urine samples were collected during pregnancy and puerperium. Serum was immediately separated after blood collection and promptly frozen at −40 °C until assay. Urine samples were collected from a second voiding at the same time as blood collection and they were stored at −40 °C until assay. Measurements were carried out simultaneously for all samples at the end of the study.

Measurements of mineral metabolism, calcium-regulating hormones and bone turnover markers

We measured serum BAP as a bone formation marker and serum NTx, urinary NTx and urinary CTx as bone resorption markers. The serum BAP concentration was measured by an enzyme-linked immunosorbent assay (ELISA) using a kit from Quidel Co. (Santa Clara, CA, USA). The intra- and inter-assay coefficients of variation (CVs) were 4.8% and 5.8%, respectively, and the sensitivity of the assay was 0.7 U/l. Urinary NTx concentration was measured by ELISA (Osteomark NTX, Ostex International, Inc., Seattle, WA, USA). Inter- and intra-assay CVs were 4% and 8%, respectively. Urinary CTx concentration was measured by ELISA (Urnine BETA CrossLaps® ELISA, Nordic Bioscience Diagnostics, Herplev, Denmark). Inter- and intra-assay CVs were 5.0–6.5% and 8.8–9.9%, respectively. Urinary concentrations of NTx and CTx were corrected for dilution by urinary creatinine analysis, and the results are expressed in nM BCE per millimolar creatinine (nM BCE/mM creatinine). Serum NTx concentration was also measured by ELISA (Osteomark NTX, Ostex International, Inc., Seattle, WA, USA). Inter- and intra-assay CVs were 6.9% and 4.6%, respectively.

Concentrations of calcium and phosphate in serum and urine were determined by an automatic analyzer (Olympus AU 2000, Tokyo, Japan). Serum calcium was corrected by serum albumin. Urinary calcium and urinary phosphate were adjusted for creatinine excretion. Serum intact PTH concentration was measured by an electrochemiluminesence immunoassay (Rosh Diagnostic Co., Basel, Switzerland), with a normal range of 10–65 pg/ml and an assay sensitivity of 3 pg/ml. Inter- and intra-assay CVs of PTH were 3.5% and 3.2%, respectively. Serum 1,25-$(OH)_2$ vitamin D concentration was determined with a radioimmunoassay kit (Immunodiagnostic Systems Ltd, Boldon, UK). The sensitivity was 2.0 pg/ml, and inter- and intra-assay CVs were 8.7% and 7.5%, respectively. Serum 25-(OH) vitamin D concentration was determined with a radioimmunoassay kit (DiaSorin Ltd., Boldon, UK). The sensitivity was 5.0 ng/ml, and inter- and intra-assay CVs were 4.9% and 5.5%, respectively. Serum vitamin D-binding protein concentration was measured by a commercial enzyme-linked immunosorbent assay kit (R&D Systems

Fig. 1. A. Comparison of levels of urinary NTx, urinary CTx and serum BAP in women with singleton pregnancy and women with twin pregnancy in the cross-sectional study. Open bar: women with singleton pregnancy. Closed bar: women with twin pregnancy. *$p<0.05$, **$p<0.01$, †$p<0.001$, ††$p<0.0001$. a) $p<0.05$ vs 10 weeks in twin, b) $p<0.01$ vs 10 weeks in twin, c) $p<0.05$ vs 10 weeks in singleton, d) $p<0.01$ vs 10 weeks in singleton. B. Comparison of serum levels of 1,25-$(OH)_2$ vitamin D, calcium, phosphate and intact PTH in women with singleton pregnancy and women with twin pregnancy in the cross-sectional study. Open bar: women with singleton pregnancy. Closed bar: women with twin pregnancy. *$p<0.05$. a) $p<0.05$ vs 10 weeks in twin, b) $p<0.01$ vs 10 weeks in twin, c) $p<0.05$ vs 10 weeks in singleton, d) $p<0.01$ vs 10 weeks in singleton.


A
Urinary NTx
Urinary NTx (nm BCE/mM-Cr)
singleton vs twin
p=0.0013
Urinary CTx
Urinary CTx (μg/mM-Cr)
singleton vs twin
p<0.0001
Serum BAP
BAP (U/L)
singleton vs twin
p<0.0001
singleton
twin
** p<0.01
* p<0.05
†† p<0.0001
† p<0.001
a) p<0.05 vs 10 weeks in twin
b) p<0.01 vs 10 weeks in twin
c) p<0.05 vs 10 weeks in singleton
d) p<0.01 vs 10 weeks in singleton
B
Serum 1,25-(OH)2Vit.D
Serum 1.25-(OH)2Vit.D (pg/mL)
singleton vs twin
p<0.0001
Serum calcium
Serum calcium (mg/dL)
singleton vs twin
p=0.04
Serum phosphate
Serum phosphate (mg/dL)
singleton vs twin
p<0.0001
Serum intact PTH
Serum intact PTH (pg/mL)
singleton vs twin
p=0.40
10 weeks
25 weeks
30 weeks
36 weeks
4 days
1 month
[pregnancy][postpartum]

Inc., Boldon, UK). The sensitivity was 1.3 μg/ml, and inter- and intra-assay CVs were 6.2% and 7.4%, respectively.

Statistical analysis

Data are expressed as means ± SD. Fisher's exact test was used for statistical analysis of baseline characteristics. Differences between groups were tested by two-way ANOVA (in the cross-sectional study) or repeated measures ANOVA (in the longitudinal study), and multiple comparisons in the cross-sectional and longitudinal studies were made by the Tukey–Kramer test. We analyzed areas under the curve (AUC) in bone turnover markers, mineral metabolism and calcium-regulating hormones for the time-related data throughout pregnancy in order to clarify the difference between singleton pregnancy and twin pregnancy. In addition, we compared the values of slope between 10 weeks and 25 weeks of pregnancy of bone turnover markers, mineral metabolism and calcium-regulating hormones in women with singleton pregnancy and women with twin pregnancy with respect to the time course. All analyses were performed with the Statistical Analysis System software package (version 8.2; SAS Institute, Inc., Cary NC). All P values are two-tailed, and α was set at a significant level of 0.05.

Results

Baseline characteristics of women with singleton pregnancy and women with twin pregnancy

Mean values of age, body height, body weight and body mass index (BMI) before pregnancy in the groups at 10, 25, 30 and 36 weeks of pregnancy and at 4 days and 1 month postpartum in the cross-sectional study were not different. In the longitudinal study, 26 of the 40 women completed this study during pregnancy and puerperium. Nine women with twin pregnancy and 5 women with singleton pregnancy dropped out of the study during pregnancy because of premature delivery and/or pregnancy-induced hypertension. Baseline characteristics of the 26 women with singleton pregnancy and twin pregnancy in the longitudinal study are shown in Table 1. The duration of pregnancy in women with twin pregnancy was significantly shorter than that in women with singleton pregnancy. There were no significant differences between the two groups in age, rate of nulliparity, BMI before pregnancy, and daily intake of calcium, phosphate and vitamin D. In addition, rates of breast-feeding and smoking in the two groups were not different.

Comparisons of bone turnover markers between women with twin pregnancy and women with singleton pregnancy

As shown in Fig. 1A, two-way ANOVA in the cross-sectional study showed significantly different transitions in the levels of NTx and CTx in urine and BAP in serum between women with twin pregnancy and women with singleton pregnancy. Urinary levels of NTx and CTx rose progressively to a maximum at 36 weeks and fell during the postpartum period to levels just above or the same as those at 25 weeks of pregnancy in both groups. Serum BAP levels at 36 weeks and during the postpartum period were significantly higher than that at 10 weeks of pregnancy in women with twin pregnancies. Although the pattern in women with singleton pregnancy was similar, serum BAP levels were only significantly elevated in the postpartum period. In the longitudinal study, patterns of changes in urinary NTx and CTx and change in serum BAP were similar to those found in the cross-sectional study, but the differences were not significant at most time points probably because of the small sample size (Fig. 2A). In addition, areas under the curve of urinary NTx, urinary CTx and BAP throughout pregnancy and puerperium in women with twin pregnancy (mean ± SD: 16,070.8 ± 4998.8, 68,668.1 ± 27,118.6 and 4442.4 ± 1258.9) were significantly larger than those in women with singleton pregnancy (12,199.8 ± 3103.9, 30,699.3 ± 14,390.9 and 3195.2 ± 804.6) ($p = 0.033$, $p = 0.0003$ and $p = 0.01$, respectively). The value of the slope between 10 weeks of pregnancy and 25 weeks of pregnancy of urinary CTx in women with twin pregnancy (107.4 ± 26.3 nM BCE/mM creatinine/15 weeks) was significantly larger than that in women with singleton pregnancy (37.7 ± 14.1 nM BCE/mM creatinine/15 weeks) ($p = 0.023$). As shown in Tables 2 and 3, there was no significant difference in serum NTx level between women with twin pregnancy and women with singleton pregnancy.

Comparisons of mineral metabolism and calcium-regulating hormones between women with twin pregnancy and women with singleton pregnancy

In the cross-sectional study, serum levels of calcium and phosphate were higher and levels of 1,25-$(OH)_2$ vitamin D and 25-(OH) vitamin D in women with twin pregnancy were lower than those in women with singleton pregnancy (Fig. 1B, Table 2). There were no significant associations of serum 25-(OH) vitamin D with 1,25-$(OH)_2$ vitamin D and calcium at 10 weeks of pregnancy in women with twin pregnancy. Intact PTH level at 36 weeks of pregnancy was significantly higher than that at 10 weeks of pregnancy in women with twin pregnancy. In the longitudinal study, there were no significant differences in mineral metabolism and calcium-regulating hormones between women with singleton pregnancy and women with twin pregnancy by repeated measures ANOVA (Fig. 2B, Table 3). The 25-(OH) vitamin D levels at 25 and 30 weeks of pregnancy in women with twin pregnancy were significantly lower than those in women with singleton pregnancy.

To further examine the differences in 1,25-$(OH)_2$ vitamin D levels from 25 weeks to 30 weeks of pregnancy between women with singleton pregnancy and women with twin pregnancy, we compared vitamin D-binding protein (DBP) levels in the two groups of women at 25 and 30 weeks of pregnancy. There were no significant differences in DBP levels between women with twin pregnancy and women with singleton pregnancy at 25 weeks (344.2 ± 295.6 and 306.7 ± 131.5 μg/ml) and at 30 weeks of pregnancy (309.2 ± 130.8 and 306.7 ± 168.8 μg/ml).

Discussion

To the best of our knowledge, this is the first study in which the influence of singleton pregnancy and the influence of twin pregnancy on calcium and bone metabolism were compared throughout pregnancy and puerperium. In both cross-sectional and longitudinal studies, we found that levels of bone resorption markers, such as NTx and CTx in urine, in women with twin pregnancy were significantly higher and that those high levels were observed earlier than those in women with singleton pregnancy.

Okah et al. reported that carboxyterminal telopeptide of type 1 collagen, which is a bone resorption marker, in multiple pregnancy was higher than that in singleton pregnancy at the third trimester [8]. Bone turnover in women with twin pregnancy may be highly activated due to an increase in bone resorption preceding an increase in bone formation since bone changes occur in anticipation of demands of growing twin fetuses. The mechanism for the anticipatory response has not been clarified, although involvement of hormones such as estrogen, progesterone and prolactin has been suggested [2,3,9].

It has been shown in previous studies that alterations in calcium and bone metabolism during pregnancy were accompanied by an increase in 1,25-$(OH)_2$ vitamin D [10–12]. Okah et al. reported that 1,25-$(OH)_2$ vitamin D level in multiple pregnancy was significantly lower and 25-(OH) vitamin D level was significantly higher than those in singleton pregnancy at the third trimester [8]. In our cross-sectional study, both levels of 1,25-$(OH)_2$ vitamin D and 25-(OH) vitamin D in women with twin pregnancy were lower than those in women with singleton pregnancy. It has been reported that bed rest during

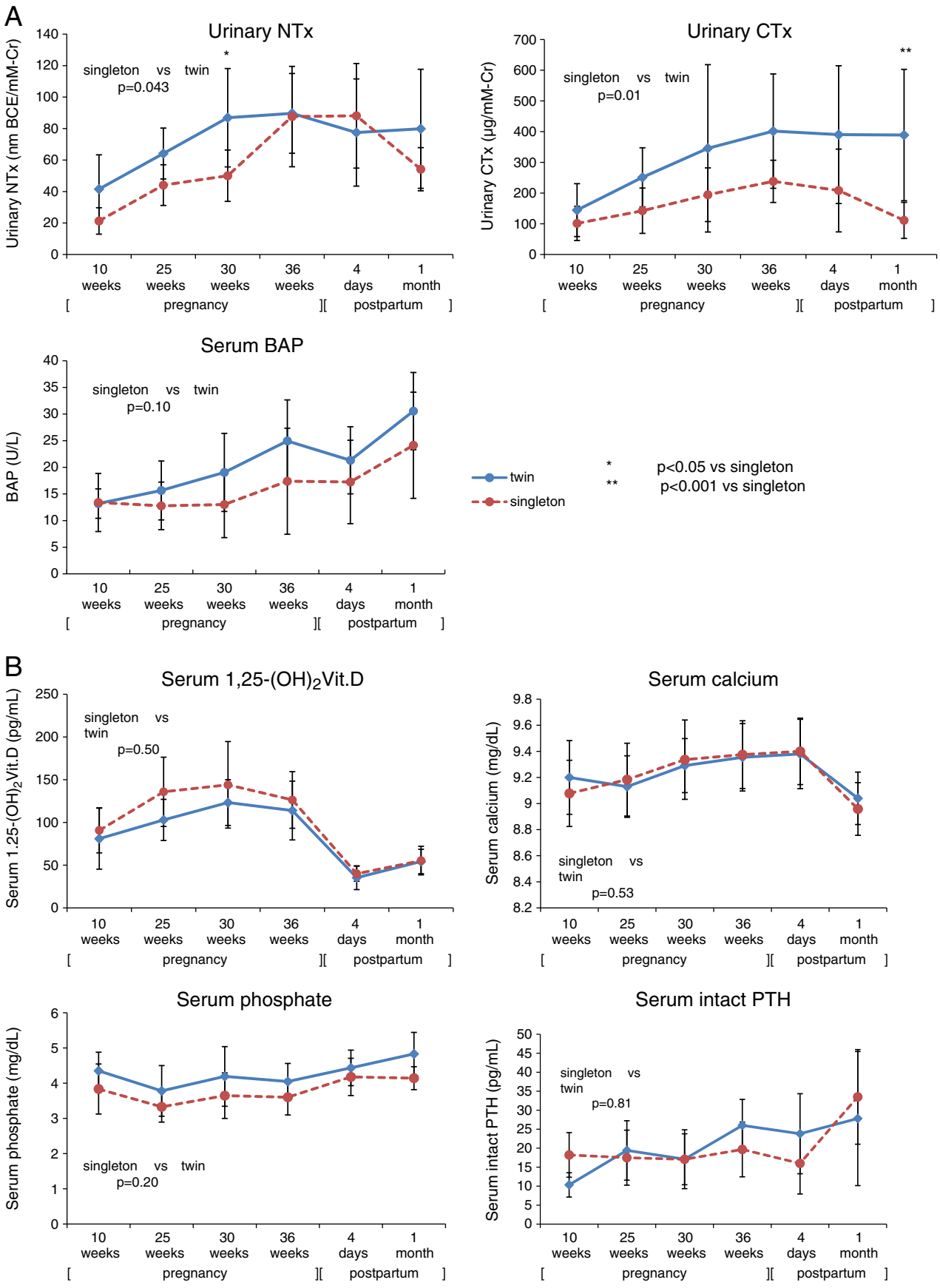


Fig. 2. A. Comparison of the changes in urinary NTx, urinary CTx and serum BAP in women with singleton pregnancy and women with twin pregnancy in the longitudinal study. Dotted line: women with singleton pregnancy. Solid line: women with twin pregnancy. *$p<0.05$, **$p<0.001$. B. Comparison of the changes in serum levels of 1,25-$(OH)_2$ vitamin D, calcium, phosphate and intact PTH in women with singleton pregnancy and women with twin pregnancy in the longitudinal study. Dotted line: women with singleton pregnancy. Solid line: women with twin pregnancy.

Table 2
Comparison of concentrations of serum NTx, serum 25-(OH) vitamin D, urinary calcium and urinary phosphate in women with singleton pregnancy and women with twin pregnancy in the cross-sectional study.

		10 weeks	25 weeks	30 weeks	36 weeks	4 days	1 month	P value singleton vs twin
Serum NTx (nmol BCE/L)	Singleton	12.5 ± 3.4	11.6 ± 3.4	14.9 ± 3.2	16.3 ± 3.9[c)]	16.7 ± 4.2[c)]	15.6 ± 3.8[c)]	0.67
	Twin	10.5 ± 4.4	13.9 ± 8.8	15.0 ± 3.9[b)]	9.1 ± 5.9[b)]	17.1 ± 4.0[b)]	17.6 ± 5.6[b)]	
Serum 25-(OH) Vit.D (ng/ml)	Singleton	16.6 ± 6.1	21.6 ± 8.6	22.8 ± 10.3	25.3 ± 8.9	19.9 ± 7.9	19.3 ± 8.0[c)]	<0.0001
	Twin	12.8 ± 5.1	12.5 ± 4.3	15.1 ± 5.5	15.0 ± 6.6	12.1 ± 5.5	15.9 ± 6.3[§)]	
Urinary calcium (mg/g·CREA)	Singleton	170.0 ± 87.1	114.0 ± 46.2	142.1 ± 88.7	122.4 ± 91.6	100.8 ± 98.4	65.0 ± 60.3	0.58
	Twin	169.5 ± 147.5	111.5 ± 80.2	131.1 ± 101.8	68.8 ± 84.8[b)]	83.1 ± 96.3[a)]	57.5 ± 47.6[b)]	
Urinary phosphate (mg/g·CREA)	Singleton	382.1 ± 156.3	466.0 ± 210.0	444.2 ± 220.4	520.1 ± 225.3	726.9 ± 142.8[c)]	520.3 ± 225.8	0.30
	Twin	441.4 ± 240.5	469.0 ± 172.3	452.9 ± 196.3	523.4 ± 189.2	719.5 ± 322.2[b)]	780.2 ± 323.2[b) #)]	

Mean ± SD, a) $p<0.05$ vs 10 weeks in twin, b) $p<0.01$ vs 10 weeks in twin, c) $p<0.01$ vs 10 weeks in singleton, #) $p<0.05$ vs singleton, §) $p<0.01$ vs singleton.
NTx: cross-linked type I collagen N-telopeptides, Vit.D: vitamin D.

pregnancy was strongly associated with significant trabecular bone loss [13] and that 1,25-$(OH)_2$ vitamin D level in pregnant women with bed rest for threatened premature delivery was lower than that in pregnant women without bed rest [5]. In the present study, levels of vitamin D intake in women with singleton pregnancy and women with twin pregnancy were not different. The low level in women with twin pregnancy may be related to decrease in sunlight irradiation by low activity for lower abdominal tension. We showed that 1,25-$(OH)_2$ vitamin D level in women with twin pregnancy was lower than that in women with singleton pregnancy at 25 weeks of pregnancy, although there was no significant difference between DBP levels in women in singleton pregnancy and women with twin pregnancy. It has been reported that the increase in 1,25-$(OH)_2$ vitamin D was secondary to the increase in DBP level at the end of pregnancy [14]. Further study on the contribution of the feto-placental unit to changes in 1,25-$(OH)_2$ vitamin D and DBP in twin pregnancy may be needed.

The results for change in albumin-corrected serum calcium level during pregnancy are in line with results of previous studies [9,15], but the level in women with twin pregnancy was higher than that in women with singleton pregnancy in the cross-sectional study. It has been reported that serum calcium levels were not different between multiple pregnancy and singleton pregnancy at the third trimester [8]. Reddy et al. also reported that serum calcium level was reduced due to low serum albumin level in twin pregnancy at delivery [16]. The reason for the discrepancy in serum calcium level in twin pregnancy is not clear. Fudge et al. reported that increase in intestinal calcium absorption was independent of the vitamin D receptor in vitamin D receptor null mice [17]. Lower 1,25-$(OH)_2$ vitamin D levels at 25 weeks of pregnancy in women with twin pregnancy may not influence circulating calcium levels.

On the other hand, serum phosphate level in women with twin pregnancy was relatively high throughout pregnancy and postpartum. The high phosphate levels in serum may be due to change in renal function involved in phosphate metabolism in twin pregnancy. Prentice et al. reported that women with breast-feeding have elevated serum phosphate concentrations, indicative of renal phosphorus conservation [18]. Further study on phosphate metabolism in twin pregnancy may be needed.

We showed that changes in serum NTx concentrations were not different between twin pregnancy and singleton pregnancy, while urinary NTx concentration in twin pregnancy was higher than that in singleton pregnancy. It has been reported that NTx concentrations in paired serum and urine samples in non-pregnant women were strongly correlated when urinary concentrations were normalized to creatinine concentrations [19,20]. The differences in changes of NTx concentrations between serum and urine may be due to the changes in clearance during pregnancy. The increment of fetal load in women with twin pregnancy is greater than that in women with singleton pregnancy. Hence, the increases in urinary excretion of NTx and CTx may be associated with fetal load in women with twin pregnancy. Although we measured NTx concentrations in spot urine, further study using 24-hour collections may be needed.

During puerperium, women with twin pregnancy need to adapt to increased calcium loss caused by the increase in volume of breast milk for two babies [21]. It has been reported that bone resorption and formation markers in breast-feeding women were higher than those in formula-feeding women [22]. We did not measure volume of breast milk, although there was no significant difference in the rates of breast-feeding in the postpartum period between women with singleton pregnancy and women with twin pregnancy. The results obtained in only lactating women were also similar to those in both lactating and non-lactating women because of the high rate of breast-feeding in both women with singleton pregnancy and women with twin pregnancy (data not shown). High levels of urinary CTx and serum BAP during puerperium in women with twin pregnancy in the present study might be due to calcium mobilization from the maternal skeleton through estrogen and PTHrP because it has been demonstrated that estrogen levels were lower and PTHrP levels were higher in lactating women than in non-lactating women and that changes in these hormones induced bone loss during lactation [23]. In women nursing twins, it has been reported that levels of 1,25-$(OH)_2$ vitamin D and calcium were increased by an increase in PTH in order to balance

Table 3
Comparison of concentrations of serum NTx, serum 25-(OH) vitamin D, urinary calcium and urinary phosphate in women with singleton pregnancy and women with twin pregnancy in the longitudinal study.

		10 weeks	25 weeks	30 weeks	36 weeks	4 days	1 month	P value singleton vs twin
Serum NTx (nmol BCE/L)	Singleton	11.3 ± 3.3	12.2 ± 2.8	15.1 ± 3.8	16.9 ± 4.1[c)]	16.6 ± 3.7[c)]	13.6 ± 2.6	0.99
	Twin	10.0 ± 4.4	11.1 ± 2.1	14.0 ± 3.9	17.3 ± 5.0[b)]	17.0 ± 4.0[b)]	17.0 ± 3.7[b)]	
Serum 25-(OH) Vit.D (ng/ml)	Singleton	17.6 ± 3.3	26.3 ± 7.3	27.4 ± 10.7	24.1 ± 9.0	18.0 ± 7.1	18.2 ± 7.4	0.18
	Twin	14.6 ± 8.7	12.1 ± 4.5[§)]	13.8 ± 5.0[#)]	14.2 ± 6.4	13.3 ± 8.1	18.4 ± 6.4	
Urinary calcium (mg/g·CREA)	Singleton	168.1 ± 90.4	112.4 ± 48.5	149.9 ± 90.7	133.0 ± 94.0	127.1 ± 103.1	75.0 ± 63.5	0.32
	Twin	157.3 ± 86.6	110.4 ± 106.0	185.7 ± 103.3	61.0 ± 52.6	113.0 ± 128.7	56.0 ± 33.6	
Urinary phosphate (mg/g·CREA)	Singleton	383.9 ± 149.7	518.6 ± 205.7	483.4 ± 221.4	565.4 ± 224.3	738.4 ± 134.7[c)]	570.7 ± 223.7	0.66
	Twin	545.5 ± 276.3	485.1 ± 146.4	508.6 ± 174.4	585.1 ± 123.7	791.8 ± 365.1	908.9 ± 320.9[a) #)]	

Mean ± SD, a) $p<0.05$ vs 10 weeks in twin, b) $p<0.01$ vs 10 weeks in twin, c) $p<0.01$ vs 10 weeks in singleton, #) $p<0.05$ vs singleton, §) $p<0.01$ vs singleton.
NTx: cross-linked type I collagen N-telopeptides, Vit.D: vitamin D.

the large calcium loss [24], but the changes in calcium, 1,25-$(OH)_2$ vitamin D and PTH in the present study are not consistent with this report.

There are some limitations to this study. First, differences in urinary NTx and CTx between women with twin pregnancy and those with singleton pregnancy were observed in both cross-sectional and longitudinal studies. The patterns of changes in BAP and calcium metabolism in the longitudinal study were not inconsistent but rather did not show statistically significant changes because of the small numbers. It is difficult to recruit women with twin pregnancy since the incidence of dropout is high due to complications, such as premature delivery and pregnancy-induced hypertension in women with twin pregnancy. Second, in the present study, body weight gain in women with twin pregnancy was significantly higher than that in women with singleton pregnancy. The changes in bone turnover markers in women with twin pregnancy must be interpreted with caution since the change in body weight during pregnancy may influence the changes in bone turnover markers. Finally, women with twin pregnancy tended to feel low abdominal tension for threatened premature delivery, and their activities were restricted. Muscle atrophy due to limited activity may influence the changes in bone turnover markers. In addition, we prescribed ritodorine hydrochloride, which is a β-stimulant, for the treatment of threatened premature delivery. It has been reported that β-adrenergic agonists stimulated bone-resorbing activity in human osteoclast-like multinucleated cells [25]. Further study is needed to clarify the accurate activity during pregnancy and the effect of ritodorine hydrochloride on bone turnover markers.

In conclusion, changes in bone metabolism in women with twin pregnancy are different from those in women with singleton pregnancy. Early and large increases in bone turnover markers allow women with twin pregnancy to meet high fetal demand throughout pregnancy. Therefore, care of calcium metabolism should be considered in women with twin pregnancy.

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