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ORIGINAL CONTRIBUTION

Relationship between dietary folate intakes, maternal plasma total homocysteine and B-vitamins during pregnancy and fetal growth in Japan

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Abstract *Background* Adequate folate status in pregnancy is important for satisfactory pregnancy outcome. *Aim of the Study* The objective of the present study was to evaluate folate status in healthy pregnant women by assessing dietary folate intakes and measuring changes in folate-related biomarkers including plasma tHcy, serum vitamin B_{12} (B_{12}), and serum and RBC folate concentrations in each trimester and to examine their relation to fetal growth. *Methods* From 94 pregnant women, 3-day-dietary records were obtained and blood was collected for plasma total homocysteine (tHcy), serum B_{12}, and serum and red-blood cell (RBC) folate measurements. Infant anthropometric measurements were made immediately after birth. *Results* Average folate intake was less than 300 μg/day with a mean energy intake of about 1800 kcal. Mean serum and RBC folate concentrations declined significantly during gestation ($p < 0.05$). Mean serum B_{12} also significantly decreased ($p < 0.01$), whereas plasma tHcy increased from 5.1 in the first trimester to 5.9 μmol/l in the third trimester ($p < 0.01$). Multiple regression analyses, after controlling for maternal age, parity and pre-pregnancy body-mass index indicated that a 1.0 μmol/l increase in plasma tHcy in the third trimester corresponded to a 151 g decrease in birth weight ($p < 0.01$). Neither B_{12} nor folate concentrations in all three trimesters showed any significant associations with birthweight. Plasma pyridoxal-5′-phosphate concentrations were markedly low, and were consistent with low intake of vitamin B_6 in our population. *Conclusion* Our data suggest that higher plasma tHcy in the third trimester is a predictor of lower birth weight. In general, the dietary intake of B-vitamins and energy may be inadequate in our population, suggesting intervention is necessary.

Key words pregnancy – folate – homocysteine – fetal growth – neonatal anthropometric measures

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Introduction

Adequate folate status is important not only in the periconceptional period but also throughout pregnancy to achieve satisfactory pregnancy outcome [1]. In 2000, the Japanese government issued a recommendation that all women planning to conceive consume daily supplementation of 400 μg of folic acid (pteroylglutamic acid) to reduce the risk of pregnancy complicated with neural-tube defects [2]. However, this recommendation appears to have little impact on obstetric care, because folic acid supplemental use among Japanese women of reproductive age is still low [3], and the birth prevalence of spina bifida seems to be on the rise [4, 5]. Another important issue in maternal and child health in Japan is the increasing trend of infants with low birth weight. Prevalence of low birth weight in term singletons has increased in the last 20 years. This increase was not due to preterm deliveries [6] and coincided with the increased rate of underweight young women [7]. Although the relationship between the increase in underweight young women and fetal-growth restriction has not been established, a significant proportion of these women are suspected to have inadequate nutritional status of both macro- and micronutrients [5].

Maternal B-vitamin status, as determined by various biomarkers, such as blood folate and total homocysteine (tHcy) concentrations, is reported to have an impact on fetal growth [8–11]. We reported that mean concentrations of serum and red-cell (RBC) folate in Japanese women not taking folic acid in the first trimester were relatively high (23.3 and 1176 nmol/l, respectively) in spite of low dietary folate intake of 289 μg/day [12]. However, our previous study was limited to pregnant women in the first trimester [12], and there have been no other studies to examine the changes in maternal micronutrient status throughout pregnancy in Japanese women. The present study was undertaken to evaluate folate status in healthy pregnant women by assessing dietary folate intake and measuring changes in folate-related biomarkers including plasma tHcy, serum vitamin B_{12} (B_{12}), and serum and RBC folate concentrations in each trimester and to examine their relation to fetal growth.

Materials and methods

Subjects

Two obstetric units in Tokyo were the sites of this study, which was carried out between 2001 and 2003. One was a public hospital in the metropolitan area which accepts referrals from other hospitals, and the other was a private hospital in downtown Tokyo. Women with no major complications such as diabetes, hypertension, etc. were invited to participate in the study. Each subject was given detailed information on the study protocol and signed an informed consent. At the initial prenatal visit, a self-administered questionnaire was given to each subject that included their parity, dietary habits, supplemental use of vitamins and minerals and drinking and smoking habits. In addition, self-reported height and prepregnancy weight were obtained, and current weight was measured at each visit. Various pregnancy outcome measures including infant gender, birth weight, length, head circumference, and the presence or absence of congenital anomalies were obtained immediately after birth. The study was approved by the Institutional Ethics Committee of the authors' affiliations.

A total of 153 women in the first or second trimester showed willingness to participate in the study. Of these, maternal anthropometric data (height or pre-pregnancy weight) were not available in 16 women, and we were unable to obtain 25 birth records and eleven dietary records. In addition, four women had a preterm infant and two had twin pregnancy. One infant had Down syndrome. Therefore, a total of 59 women were excluded from further consideration, and the data obtained from 94 women were analyzed.

Blood samples and laboratory analysis

Non-fasting blood samples were obtained at the first prenatal visit as well as the visits at the second and third trimesters for the measurements of plasma tHcy, serum vitamin B_{12}, serum and RBC folate and plasma pyridoxal-5′-phosphate (PLP). Blood samples were obtained from only 51 women who made their first visit within the first trimester, and the numbers of blood samples obtained in the second and third trimesters were 77 and 82, respectively. Of these, only 39 women provided samples at all three trimester visits, whereas 43 women provided blood samples at the first and the second trimester visits, 67 women gave at the second and the third trimester visits, and 45 women gave at the first and third trimester visits.

Plasma and serum samples were separated from whole blood collected into tubes containing EDTA-2Na and those without any anticoagulant, respectively. For the measurement of RBC folate concentrations, whole blood was collected into heparinized tubes and then mixed with 0.5% ascorbate solution. All blood samples were stored at −80°C until measurements. An HPLC with fluorescent-detection method was used for the measurement of plasma tHcy [13]. Chemiluminescent immunoassay (ADVIA Centaur Immunoassay System, Bayer HealthCare, New

York, NY, USA) was used for the measurements of serum folate and B_{12}. RBC folate concentrations were measured by microbiological assay using *Lactobacillus rhamnosus* (*L. rhamnosus*, formerly known as *L. casei*) [14]. Plasma PLP concentrations were assayed by the tyrosine-apodecarboxylase method using [^{3}H]-tyrosine as substrate (Alpco Diagnostics, Windham, NH, USA) in a limited number of subjects. The coefficients of variation using control samples for tHcy, serum folate, serum B_{12}, RBC folate and PLP were 8, 4, 6, 10 and 11%, respectively.

Dietary intake analysis

Each subject weighed and recorded all food consumed using a digital scale (Tanita, Tokyo, Japan) for three days within a week before blood sampling at each prenatal visit, where these records were checked by trained dieticians. The daily intake of individual nutrients was calculated using the Standard Food Composition Table [15], where food folate content was measured by *L. rhamnosus* microbiological assay after treatments with protease and folate conjugase. At the time of the study, there were no foods available that were enriched with folic acid.

Statistical analysis

The data were expressed in mean ±SD where appropriate. Wilcoxon's signed rank tests were used to compare continuous variables (i.e.: biomarkers and dietary intake data) between the two visits. Spearman's correlation coefficients were used to examine the correlations between biomarkers, dietary intake data, and infant anthropometry data at each visit. The p value of less than 0.05 was considered significant. All analyses were performed using the SPSS 11.5-J statistical package program (SPSS, Tokyo, Japan).

Results

Subject characteristics

Mean age of 94 mothers at delivery was 29 (±4.7) years old, and 77% was primiparous. Their mean height and prepregnancy weight were 158 (±5) cm and 51.7 (±8.0) kg, respectively. Their mean prepregnancy BMI was 20.8 (±3.2) kg/m^2, and 20 mothers (21.3%) were underweight with BMI below 18.5 kg/m^2, whereas only six mothers (6.4%) had BMI more than 25.0 kg/m^2, including two with BMI over 30 kg/m^2. Mean weight gain at 36 weeks of gestation, as calculated by the subtraction of self-reported prepregnancy weight from the actual weight at 36 weeks, was 9.2 ± 3.7 kg. The changes in maternal anthropometric measurements during pregnancy are presented in Table 1.

The mean gestational age at delivery was 39.6 (±1.0) weeks. The female/male ratio of newborns was 56/38, and their mean birth weight was 3120 (±411) g. Five newborns had low-birth weight. Mean length and head circumference were 49.3 (±2.0) and 33.0 (±1.3) cm, respectively. During the first and second trimesters, 17.7% and 13.4% of subjects, respectively smoked, whereas this information was not obtained at the third trimester.

Table 1 Mean (±SD) of maternal characteristics, biomarkers and nutrient intakes in each trimester

	First trimester (7–14 weeks, n = 51)	Second trimester (26–29 weeks, n = 77)	Third trimester (34–36 weeks, n = 82)
Characteristics			
Height (cm)	158.3 ± 4.9	157.4 ± 5.1	157.6 ± 4.8
Pre-pregnancy weight (kg)	52.5 ± 8.6	52.1 ± 8.1	51.9 ± 8.1
Current weight (kg)	53.1 ± 8.0	57.5 ± 8.2	61.1 ± 7.5
Biomarkers			
Plasma tHcy (μmol/l)	5.1 ± 1.5	5.0 ± 1.3	5.9 ± 1.4[b]
Serum vitamin B_{12} (pmol/l)	405 ± 146	301 ± 96[a]	265 ± 95[b]
Serum folate (nmol/l)	23.2 ± 9.7	19.3 ± 23.8[a]	23.1 ± 69.3[d]
Red-cell folate (nmol/l)	1317 ± 824	909 ± 551[c]	813 ± 475
Daily dietary intake			
Energy (kcal)	1709 ± 466	1816 ± 336	1800 ± 344
Protein (g)	59 ± 21	66 ± 15[c]	66 ± 15
Fat (g)	57 ± 21	61 ± 15	60 ± 16
Carbohydrate (g)	237 ± 58	245 ± 48	245 ± 48
Folate (μg)	276 ± 168	284 ± 131	271 ± 131
Vitamin B_{12} (μg)	5.5 ± 4.8	6.2 ± 7.9	7.9 ± 19
Vitamin B_6 (mg)	1.1 ± 0.8	1.5 ± 2.8	1.2 ± 1.3

[a] $p < 0.01$ between the second and first trimesters (by Wilcoxon's signed rank test), [b] $p < 0.01$ between the third and second trimesters (by Wilcoxon's signed rank test), [c] $p < 0.05$ between the second and first trimesters (by Wilcoxon's signed rank test), [d] $p < 0.05$ between the third and second trimesters (by Wilcoxon's signed rank test)

Changes and associations in biomarkers during pregnancy

As shown Table 1, biomarkers showed significant differences between the trimesters. Plasma tHcy concentrations showed little change during the first and second trimesters, whereas they were significantly higher in the third than the second trimester ($p < 0.01$ by Wilcoxon's signed rank test). Both serum B_{12} and folate concentrations decreased significantly between the first and second trimesters ($p < 0.01$). Serum B_{12} concentrations decreased significantly between the second and third trimesters ($p < 0.01$), but serum folate significantly increased ($p < 0.05$). RBC folate significantly decreased between the first and second trimesters ($p < 0.05$), whereas the values were similar between the second and third trimesters. The mean plasma PLP concentrations were 5.1 (±3.4, $n = 10$) and 4.7 (±6.0, $n = 18$) nmol/l in the second and third trimesters, respectively.

During the second and third trimesters, 24 of 77 subjects (31%) and 31 of 82 (38%) had serum folate concentrations below the cutoff of 12 nmol/l, and 7 of 77 (9%) and 18 of 82 (22%) had RBC folate concentrations below 454 nmol/l. In addition, serum B_{12} concentrations lower than 148 nmol/l were found in 6 of 77 subjects (8%) and 13 of 82 (16%) in the second and third trimesters. All subjects had plasma PLP concentrations below 30 nmol/l. These are generally accepted cutoffs of normal values [16].

In the first trimester, there was a significant negative correlation between plasma tHcy and serum folate concentrations ($r = -0.40$, $p < 0.01$). RBC folate concentrations showed significant correlations with serum B_{12} and serum folate ($r = 0.38$, $p < 0.01$ and $r = 0.43$, $p < 0.01$, respectively). In the second trimester, plasma tHcy showed negative associations with serum B_{12}, and serum and RBC folate concentrations ($r = -0.22$, $p = 0.05$, $r = -0.35$, $p < 0.01$ and $r = -0.43$, $p < 0.01$, respectively). Serum B_{12} had positive correlations with RBC and serum folate ($r = 0.32$, $p < 0.05$ and $r = 0.36$, $p < 0.01$, respectively), and serum and RBC folate correlated significantly ($r = 0.33$, $p < 0.05$). In the third trimester, there was a negative association between tHcy and serum folate ($r = -0.43$, $p < 0.01$), and serum B_{12} correlated significantly with serum folate ($r = 0.26$, $p < 0.05$).

Dietary intakes and their association with biomarkers

Dietary intakes were unchanged during pregnancy with the exception of protein intakes, which significantly increased ($p < 0.05$ by Wilcoxon's signed rank test) between the first and second trimesters (Table 1). Only two women took folic acid supplements in the first trimester and four in the second.

The Spearman's correlation coefficients between the concentrations of blood biomarkers and dietary B_{12} and folate intakes varied between trimesters. In the first trimester, dietary folate intakes had no significant correlation with serum and RBC folate or plasma tHcy concentrations. Similarly, dietary B_{12} intakes did not correlate with serum B_{12} or plasma tHcy concentrations. In the second trimester, dietary folate intakes positively correlated with both serum and RBC folate concentrations ($r = 0.41$, $p < 0.01$ and $r = 0.32$, $p < 0.05$, respectively), and negatively with plasma tHcy ($r = -0.23$, $p < 0.05$). Dietary B_{12} intake was positively correlated with serum B_{12} ($r = 0.44$, $p < 0.01$), but not with plasma tHcy. In the third trimester, dietary folate intake had no significant correlations with serum and red-cell folate or plasma tHcy concentrations. Dietary B_{12} intakes were positively correlated with serum B_{12} ($r = 0.24$, $p < 0.05$), and negatively correlated with plasma tHcy ($r = -0.35$, $p < 0.01$).

Relationship of dietary intakes and maternal biomarkers to fetal growth

The association of dietary intakes and blood biomarkers with newborn's anthropometry was examined using multiple-regression analyses controlling for parity, maternal age and pre-pregnancy BMI (Table 2). The analysis indicated that a 1.0 mol/l increase in plasma tHcy in the third trimester corresponded to a 151 g decrease in birth weight ($p < 0.01$). Although few other blood biomarkers showed significant associations with birth weight, length or head circumference, the effect sizes appears to be too small for physiological significance. Dietary B_{12} and folate intakes in all three trimesters were not related to newborns' anthropometry. Due to the small sample size of PLP values, these were not included in the regression analyses.

Discussion

Vitamins related to homocysteine metabolism, such as folate and vitamins B_{12} and B_6 are essential in adequate fetal growth [1, 9, 17]. In the present study, we found that higher plasma tHcy in the third trimester is a predictor of lower birth weight among Japanese women. Other statistically significant data from the multiple-regression analyses, such as the association between birth weight and red-cell folate in the third trimester (Table 2), had relatively small effect sizes; therefore, they may not have physiological

Table 2 Multiple-regression analyses[a] to evaluate the association of various parameters in each trimester with birth weight, length, and head circumference

Regressor	First trimester		Second trimester		Third trimester	
	Effect size	*p*	Effect size	*p*	Effect size	*p*
Birth weight						
Plasma homocysteine (μmol/l)	28.3	0.48	−32.5	0.62	−151.1	<0.01
Serum vitamin B_{12} (pmol/l)	−1.05	0.08	−5.349	0.38	0.776	0.44
Serum folate (nmol/l)	−2.60	0.80	−3.51	0.36	−0.12	0.87
RBC folate (nmol/l)	0.03	0.65	0.38	0.27	0.36	0.04
Dietary folate (μg/day)	1.07	0.09	0.48	0.31	0.008	0.99
Dietary vitamin B_{12} (μg/day)	−8.67	0.55	−5.35	0.46	−4.27	0.73
Gestational age at delivery (weeks)	168.61	<0.01	225.59	<0.01	65.47	0.28
Maternal smoking (yes = 1, no = 0)	−213.37	0.16	7.20	0.97	30.26	0.87
Newborn gender (being male = 1, female = 0)	191.76	0.10	84.61	0.56	154.96	0.17
Birth length						
Plasma homocysteine (μmol/l)	0.44	0.05	−0.27	0.27	−0.42	0.10
Serum vitamin B_{12} (pmol/l)	−0.01	0.09	−0.02	0.33	0.00	0.50
Serum folate (nmol/l)	0.02	0.69	0.004	0.77	−0.01	0.07
RBC folate (nmol/l)	0.0003	0.49	−0.0001	0.90	0.002	0.07
Dietary folate (μg/day)	0.00	0.43	0.00	0.08	0.00	0.23
Dietary vitamin B_{12} (μg/day)	−0.03	0.72	−0.02	0.33	0.09	0.12
Gestational age at delivery (weeks)	0.84	<0.01	1.22	<0.01	0.88	<0.01
Maternal smoking (yes = 1, no = 0)	0.02	0.98	−0.41	0.56	0.41	0.63
Newborn gender (being male = 1, female = 0)	0.93	0.23	0.58	0.23	1.33	0.02
Birth head circumference						
Plasma homocysteine (μmol/l)	0.02	0.92	1.54	0.03	−0.24	0.53
Serum vitamin B_{12} (pmol/l)	−0.003	0.20	0.02	0.08	0.01	0.10
Serum folate (nmol/l)	0.08	0.09	−0.03	0.07	0.004	0.57
RBC folate (nmol/l)	−0.0003	0.56	0.005	0.01	−0.0003	0.90
Dietary folate (μg/day)	0.0001	0.98	0.009	0.07	−0.006	0.16
Dietary vitamin B_{12} (μg/day)	−0.08	0.17	−0.40	0.08	0.04	0.72
Gestational age at delivery (weeks)	0.23	0.32	0.50	0.23	−0.29	0.59
Maternal smoking (yes = 1, no = 0)	−0.72	0.32	2.78	0.10	−0.22	0.93
Newborn gender (being male = 1, female = 0)	1.06	0.04	−0.54	0.44	0.31	0.77

[a] Controlled for maternal age, pre-pregnancy BMI and parity

significance. Conflicting results have been reported regarding the association between fetal growth and maternal plasma tHcy concentrations during pregnancy. There are reports suggesting that high maternal plasma tHcy is related to fetal growth restriction [8, 11, 18] but other reports do not [16, 19–22]. We do not know the biochemical as well as physiological mechanisms as to why an increase in maternal plasma tHcy concentrations in the third trimester corresponded to such a marked decrease in birth weight (151 g decrease in birth weight for 1.0 μmol/l increase in plasma tHcy) in our subjects. Plasma tHcy concentration has been well established to be low during pregnancy [23, 25]. In our study, only two subjects had tHcy values that were higher than 10 μmol/l (one each in the first and third trimesters). Our findings of the overall changes in blood biomarkers during gestation and their associations with each other are consistent with the data in the literature [23, 25–27].

It has been demonstrated that associations exist between birth weight and maternal serum folate [10, 27] as well as red cell folate concentrations [9, 28]. However, such an association was not found in our study. Although it has been well established that folate concentrations in the circulation decline as pregnancy advances [1], over 30% of our subjects had serum folate below 12 nmol/l in the second and third trimester and over 20% had red-cell folate below 454 nmol/l in the third trimester, suggesting the presence of folate inadequacy. Thus, it may be reasonable to note that folate status assessed by the determination of serum and red-cell folate concentrations was poorer than the ideal in our subjects, and their average dietary folate intake was 271–284 μg/day. This intake was calculated by including supplemental folic acid consumed by a few subjects and is far below the intake of 440 g/day recommended for pregnant women [29]. In addition, mean vitamin B_6 intake was 1.5 mg/day or less which is more than 25% less than the recommended intake of 2.0 mg/day for pregnant women [29]. This is consistent with our findings that plasma PLP concentrations were below 30 nmol/l (general cut off of normal range [16]) in all subjects, although it was measured only in a small subgroup. Furthermore, our subjects consumed diets containing 1700–1800 kcal/day which was below the intakes of 2100–2500 kcal/day recommended for pregnant women [29]. Although their mean protein and B_{12} intakes were sufficient to meet the recommended intakes [29], the overall dietary intake may be

insufficient or imbalanced in our subjects. This potential overall "undernutrition" or "inadequate nutrition" is similar to that observed in non-pregnant women in Japan [5]. The mean birth weight of 3120 g and maternal BMI of 20.8 kg/m^2 in this study further suggest that the overall nutrition is insufficient in our subjects, as it is a problem for young women along with a rising smoking rate in Japan [5].

To our knowledge, this is the first investigation to study the relationship of homocysteine and related B-vitamin status to fetal growth in a Japanese population. However, our study has certain limitations. Due to the budgetary constrains and limited time period, we were unable to conduct a larger scale investigation than the one presented here. Moreover, not all women participated longitudinally, and only 41% of women provided their blood samples in all trimesters.

Impaired fetal growth is not only a risk factor for higher mortality early in infancy [30], but also a risk for chronic diseases later in life [31–33]. Recently, a new dietary guideline for pregnant and lactating women has been published in Japan [34]. This aims to reduce growth-restricted infants through promoting a balanced diet and adequate weight gain during pregnancy. While the dissemination of this guideline may improve the nutritional status of pregnant women at the population level and improve fetal growth, another immediate approach may be necessary. Considering the increasing birth prevalence of spina bifida in Japan [5], and the recent reports on positive effect of the multivitamin supplementation on fetal growth [35, 36], it may be an urgent matter for the governmental bodies to educate young women on the importance of adequate nutrition during pregnancy and to implement the antenatal supplementation of multivitamins, including folic acid. It would not be difficult to disseminate information on healthy diet in pregnancy through this existing system, together with providing multivitamin supplements. Such practice would be cost effective, since the Japanese have a well-established pregnancy registering system where home-kept antenatal health records (the *Boshi-Kenko techo*) are handed out to all pregnant women through local municipalities.

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