More than 50% of Pregnant Japanese Women with an Intake of 150 μg Dietary Folate per 1,000 kcal Can Maintain Values above the Cut-Off

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Summary Most Japanese pregnant women do not take the estimated average requirement (EAR) of folate for pregnant women, which is 400 μg/d. Nevertheless, folate deficiencies have not been reported. We examined biomarkers for evaluating the status of folate in pregnant Japanese women. Apparently healthy pregnant Japanese women were cross-sectionally recruited from a private obstetric hospital. We measured nutritional biomarkers of folate in these women, as well as their folate intake. The numbers of subjects were 230 (49, 62, and 81, and 38 in the first (up to 15 wk), second (16–30 wk), and third (over 31 wk) trimesters of pregnancy, and 1 mo after delivery, respectively). Folate intakes (medians) in the first, second, and third trimesters, and 1 mo after delivery were 235±147 (194), 226±83 (218), and 256±85 (254), and 300±105 (305) μg/d, respectively. Folate concentrations in plasma and erythrocytes appeared to be valid indicators for assessing folate status, with cut-off values of 7 nmol/L and 300 nmol/L, respectively. Plasma folate concentrations (medians) in the first, second, and third trimesters, and 1 mo after delivery were 17.6±9.6 (16.7), 12.4±8.3 (9.4), and 12.1±8.4 (9.4), and 10.7±8.9 (7.9) nmol/L, respectively. Each of the folate values was over the cut-off value. Erythrocyte folate concentrations (medians) in the first, second, and third trimesters, and 1 mo after delivery were 358±108 (365), 389±154 (365), and 325±150 (315), and 308±158 (276) nmol/L, respectively. Each of the folate values was near or over the cut-off value. In conclusion, the data obtained demonstrated that the estimated average requirement of folate for pregnant Japanese women was =250 μg/d.

Key Words folate, requirement, pregnant women, blood, Dietary Reference Intake

Better nutrition is important for maintaining health, especially for pregnant women, as well as fetuses. Generally, the requirements of nutrients increase during pregnancy compared with the pre-pregnant stage. Therefore, additional amounts of nutrients are set for pregnant women in Dietary Reference Intakes (DRIs) for Japanese women (1).

Folate is essential for one-carbon transfer reactions required for the normal synthesis and metabolism of amino acids, purines, and pyrimidines. The estimated average requirement (EAR) of folate has been set as 400 μg/d (1). However, the EAR of folate for pregnant Japanese women is likely to be higher than the real requirement because nutrient intake surveys (2, 3) have reported that the intake of folate of pregnant women was approximately 250 μg/d, which is considerably less than the EAR of 400 μg/d. However, there are no reports on deficiency of folate in Japan.

Folate concentrations in erythrocytes have been suggested as a valid indicator for assessing the long-term state of folate (4). Plasma folate concentrations have been suggested as a valid indicator for assessing the short-term status of folate (4). In a study on folate requirements in pregnancy published in 1968 (5), the authors calculated that a dose of an additional 100 μg of pteroylmonoglutamic acid (equivalent to 200 μg dietary folate) was required during pregnancy based on serum folate levels in non-pregnant women. A limitation of this report was that only the additional amount of 100 μg of pteroylmonoglutamic acid was mentioned, with no description of the folate intake of the subjects. Therefore, a smaller addition of pteroylmonoglutamic acid could maintain normal serum levels. Willoughby and Jewell (6) reported that the minimum dose of folate needed during late pregnancy, in addition to a dietary folate intake of 50 μg per day, was approximately 300 μg/d.

There is one paper about the changes of the blood folate concentrations in Japanese women during pregnancy (7): In that report, average folate intake was less than 300 μg/d with a mean energy intake of about 1,800 kcal/d, and the mean erythrocyte folate con-
centrations were 1,317, 909, 813 nmol/L in the first, second, and third trimesters of pregnancy, respectively. The folate concentrations were lower in the second and third trimesters of pregnancy than in the first trimester; however, each of the values was much higher than the cut-off value of 300 nmol/L (4). In addition, Takimoto et al. (7) reported that the serum mean folate concentrations were 23.2, 19.3, 23.1 nmol/L in the first, second, and third trimesters of pregnancy, respectively. Each of the values was much higher than the cut-off value of 7 nmol/L (4). This report indicates that pregnant Japanese women can be maintained above the cut-off values of blood folate at the intake of ≈300 μg dietary folate per day. But, to confirm this finding, further studies were needed. Thus, we investigated blood concentrations of folate in Japanese women during pregnancy, and determined whether they can be maintained over the cut-off value without folate supplement.

MATERIALS AND METHODS

Chemicals. Pteroylmonoglutamic acid (folic acid) (C_{19}H_{19}N_{7}O_{6} \cdot 441.40) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest purity available from commercial sources.

Subjects and design. This study was reviewed and approved by the Ethical Committee of the University of Shiga Prefecture and was conducted according to the guidelines laid down in the Declaration of Helsinki.

Healthy pregnant Japanese women who were all married were cross-sectionally recruited from a private obstetric hospital in Hikone, Japan, between May 2011 and December 2012. The purpose and protocol of this study were explained to all participants.

Before joining the study, each participant gave written informed consent. We excluded participants who had taken multi-vitamin supplements at least once during the previous month. A total of 369 married Japanese women (90, 116, and 116, and 47 women in the first (up to 15 wk), second (16–30 wk), and third (over 31 wk) trimesters of pregnancy, and 1 mo after delivery, respectively) took part in the survey. To investigate folate concentrations in plasma and erythrocyte, whole blood samples were collected at a pregnancy checkup. Furthermore, to determine the habitual intake of energy and nutrients in the participants, we gave a self-administered comprehensive diet history questionnaire (DHQ) to the participants. However, 139 of the participants did not return the DHQ (41, 54, and 35, and 9 women in the first, second, and third trimesters, and 1 mo after delivery, respectively). As a result, the number of the participants with blood and DHQ data was 230 (49, 62, and 81, and 38 women in the first, second, and third trimesters, and 1 mo after delivery, respectively).

Blood samples. Non-fasting blood samples (2 mL) were taken into a Venoeject II (code no. VP-DK052K; Terumo Corporation, Tokyo, Japan) from a cubital vein at a pregnancy checkup. A pure erythrocyte fraction is hard to collect. Some investigators used the special formula for determining the erythrocyte folate concentration (8), but we used the blood cells fraction instead of erythrocytes because this fraction comprises greater than 95% erythrocytes. The collected blood samples were centrifuged for 30 min at 1500 ×g to separate plasma and precipitated materials (mainly erythrocytes) at room temperature. The resulting supernatants were retained and stored at −80°C until analysis. A volume of 1 mL of saline was gently added to the precipitated materials. The suspension was rinsed and then centrifuged at 1,500 ×g for 5 min. The precipitated materials (blood cells, mainly erythrocytes) were stored at −80°C until required.

Determination of folate concentrations. A volume of 10 μL of thawed plasma was used for measuring plasma monoglutamated folate (mainly 5-methyltetrahydrofolate) (9). Plasma concentrations of folate were determined by the microbioassay method using Lactobacillus rhamnosus, ATCC 27773 (10).

For measuring folate compounds in blood cells (pteroylmonoglutamic acid, 5-formyltetrahydropteroylmonoglutamic acid, 5,10-methylenetetrahydropteroylmonoglutamic acid), 0.1 mL of thawed blood cells was added to 0.9 mL of 0.1 mol/L KH₂PO₄-K₂HPO₄ buffer (pH 6.1) containing 0.1 mol/L L-ascorbic acid, and the suspension was gently sonicated for 2 s at room temperature. The sonicated solution was placed into a boiling water bath for 10 min. After cooling, 0.5 mL of protease MS (200 U/mL of water; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) was added. The mixture was incubated at 37°C for 3 h to digest proteins and to release polyglutamated folate compounds from the protein-bound types. The reaction was stopped by placing the mixture into a boiling water bath for 10 min. After cooling, 0.1 mL of conjugase (folyglycyl-γ-glutamate carboxypeptidase, which catalyzes the reaction of deconjugation of polyglutamate to monoglutamate) (extract from porcine kidney acetone powder, type II; Sigma-Aldrich, St. Louis, MO) was added to the mixture and incubated overnight at 37°C to convert polyglutamated folate compounds to monoglutamated folate compounds. The reaction was stopped by placing the mixture into a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant was retained, the precipitated materials extracted again with 1 mL of water, and the resulting supernatant retained. Both retained supernatants were combined, and the extract was used for measuring folate levels. The conjugase solution was made up as follows. A total of 60 mL of 50 mmol/L KH₂PO₄-K₂HPO₄ buffer (pH 7.0) containing 0.2 mol/L 2-mercaptoethanol was added to 20 g of porcine kidney acetone powder and stirred for 30 min at 4°C. The suspension was centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant was dialyzed against a large amount of 50 mmol/L KH₂PO₄-K₂HPO₄ buffer (pH 7.0) containing 0.2 mmol/L 2-mercaptoethanol to remove endogenous folate from the kidney acetone pow-
der. The dialyzed conjugase solution was directly used for measuring folate. Blood cell (mainly erythrocyte) concentrations of folate were determined by the microbioassay method using *Lactobacillus rhamnosus*, ATCC 27773 (10).

**Diet history assessment.** Dietary habits during the preceding month were assessed using a previously validated DHQ (2, 3, 11–13).

Estimates of dietary intake for 150 food and beverage items, energy, and nutrients were calculated using an ad hoc computer algorithm for the DHQ based on the Standard Tables of Food Composition in Japan (14). Detailed descriptions of the methods used to calculate dietary intake and the validity of the DHQ have been published (2, 3, 11–13).

**Statistical analyses.** Since the data were not distributed normally, the nonparametric Kruskal-Wallis test following Dunn’s post test was used to analyze statistical differences among the first, second, and third trimester, and 1 mo after delivery. A \( p < 0.05 \) was considered statistically significant. Pearson correlation coefficients were calculated to determine the association between plasma folate and blood cell folate concentrations. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

## RESULTS

**Subject characteristics and dietary history assessment**

Subject characteristics are shown in Table 1. The average age of the subjects was \( \sim 30 \) y old, and the average height was \( \sim 160 \) cm. Body weight and energy intake increased with the progress of pregnancy. Fat intake in

| Table 1. Basic characteristics of pregnant Japanese women. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 1st trimester   | 2nd trimester   | 3rd trimester   | 1 mo after delivery |
| **n**           | 49              | 62              | 81              | 38              |
| Age (y)         | 30.1±4.7        | 29.3±4.6        | 30.1±4.7        | 31.4±5.8        |
| Pregnant week   | (30.0)          | (29.0)          | (29.0)          | (32.0)          |
| **Height (cm)** |                 |                 |                 |                 |
| (10.0)          | (28.0)          | (36.5)          |                 |                 |
| Body weight (kg)| 52.3±7.6a       | 57.4±7.1bc      | 61.3±7.9a       | 55.1±7.4ac      |
| (50.6)          | (57.3)          | (59.9)          | (52.8)          |                 |
| BMI (kg/m²)     | 20.5±3.0a       | 22.9±2.7ab      | 24.0±2.5b       | 22.0±2.8a       |
| (19.9)          | (22.5)          | (23.4)          | (21.1)          |                 |
| Energy intake (kcal/d)| 1,503±566a     | 1,567±421ab     | 1,720±427bc     | 1,926±490c      |
| (1,425)         | (1,592)         | (1,655)         | (1,839)         |                 |
| Protein intake (%E)| 12.3±2.4       | 13.1±1.9        | 13.3±1.8        | 12.7±1.7        |
| (12.4)          | (13.5)          | (13.6)          | (12.6)          |                 |
| Fat intake (%E) | 26.4±6.0a       | 30.0±5.4b       | 30.5±5.2b       | 28.9±5.6ab      |
| (26.5)          | (29.9)          | (30.6)          | (28.9)          |                 |
| Carbohydrate intake (%E) | 60.3±8.0a     | 55.9±6.0b       | 55.3±5.8b       | 57.3±6.4ab      |
| (58.8)          | (55.8)          | (55.9)          | (57.6)          |                 |
| Folate intake (µg/d) | 235±147a       | 226±83a         | 256±85ab        | 300±105b        |
| (194)           | (218)           | (254)           | (305)           |                 |
| Folate intake (µg/1,000 kcal) | 153±59       | 147±43          | 155±44          | 156±42         |
| (132)           | (143)           | (152)           | (151)           |                 |

Values are means±SD (medians). The means in a row without a common superscripted letter differ, \( p < 0.05 \), determined by nonparametric Kruskal-Wallis test following Dunn’s post test.

| Table 2. Plasma and blood cell folate concentrations in pregnant Japanese women. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 1st trimester   | 2nd trimester   | 3rd trimester   | 1 mo after delivery |
| **n**           | 49              | 62              | 81              | 38              |
| Plasma folate (nmol/L) | 17.6±9.6a     | 12.4±8.3b       | 12.1±8.4b       | 10.7±8.9b       |
| (16.7)          | (9.4)           | (9.4)           | (7.9)           |                 |
| Blood cells folate (nmol/L) | 358±108     | 389±154         | 325±150         | 308±158         |
| (365)           | (365)           | (315)           | (276)           |                 |

Values are means±SD (medians). The means in a row without a common superscripted letter differ, \( p < 0.05 \), determined by nonparametric Kruskal-Wallis test following Dunn’s post test.
terms of % energy also increased throughout pregnancy.
The intake of folate is shown in Table 1. Folate intake was much lower than the EAR of folate for 30–49-y-old women during pregnancy (400 μg/d) (1).

Biomarker for evaluating the status of folate

Plasma folate concentration is used as a biomarker of folate status. Table 2 shows plasma folate concentrations in the first, second, and third trimesters, and 1 mo after delivery. Plasma folate concentrations decreased in the second and third trimesters compared with those in the first trimester. Plasma folate concentrations did not recover to the first trimester values, even 1 mo after delivery.

We analyzed the relationship between folate intake and plasma folate concentrations. Plasma folate concentrations were divided into three groups depending on folate intake: bottom tertile, middle tertile, and upper tertile (Fig. 1). No significant relationships between plasma folate concentrations and folate intake were observed in the first, second, or third trimesters of pregnancy, or 1 mo after delivery. The percentages of the subjects whose plasma folate concentrations were below the cut-off value were 18, 31, and 41, and 47% in the first, second, and third trimesters, and 1 mo after delivery, respectively (Fig. 1).

Another indicator of folate status in the mid- and long-terms is erythrocyte folate concentrations (4): The cut-off value for erythrocyte folate concentrations is 300 nmol/L (4). Folate concentration in blood cells was used as erythrocyte levels did not change throughout the period of pregnancy and delivery (Table 2).

We analyzed the relationship between folate intake and blood cells folate concentrations. Blood cells folate concentrations were divided into three groups depending on folate intake: bottom tertile, middle tertile, and upper tertile (Fig. 2). There were no significant relationships between folate concentrations in blood cells and folate intake in the first, second, or third trimesters of pregnancy, or 1 mo after delivery. The percentages of the subjects whose blood cells folate concentrations

Fig. 1. Plasma folate concentrations according to tertiles. (A) the first (up to 15 wk), (B) second (16–30 wk), and (C) third (over 31 wk) trimesters of pregnancy, and (D) 1 mo after delivery. The dotted line indicates the cut-off value of plasma folate for evaluating folate status. Each dot represents the value of one subject. Each mean ±SD is shown in the figure. The non-parametric Kruskal-Wallis test following Dunn’s post test was used to analyze differences among tertiles. None of the means differed among any of the tertiles.
Blood Folate Levels in Pregnancy

Correlation between plasma and blood cells folate concentrations

Figure 3 shows the association between plasma and blood cell folate concentrations in the first, second, and third trimesters of pregnancy, and 1 mo after delivery. A significant association between plasma and blood cells folate concentrations was observed in the first trimester of pregnancy, and also in the second and third trimesters, and 1 mo after delivery.

Dietary folate intake in the food categories

Figure 4 shows the contribution of food categories to folate intake in pregnant Japanese women. Beverages (mainly Japanese tea), green-yellow vegetables, and cereals were the main contributors to folate intake.

DISCUSSION

The cut-off value (300 nmol/L of erythrocyte) was obtained by the hemolysate method which is done as follows. Hemolysate is made by mixing 25 mL of a freshly collected blood sample with 725 mL of freshly prepared 1% ascorbic acid. Polyglutamated folates in the hemolysate are converted to monoglutamated folates using conjugase in the plasma by incubation for 20 min at 37°C. The whole blood folate is measured using the treated hemolysate. The remainder of the freshly collected blood sample is centrifuged at 3,000 rpm for 10 min to separate plasma from blood cells. The plasma folate is measured. On the other hand, the hematcrit is measured using 25 mL of whole blood. Then, the concentration of erythrocyte folate is calculated by the following equation: \( \text{concentration of erythrocyte folate} = \left( \frac{\text{whole blood folate} - \text{plasma folate}}{3} \right) \times \left( \frac{100}{\text{hematocrit}} \right) \). For example, if the concentration of whole blood folate is 150 nmol/L, that of plasma folate is 10 nmol/L, the hematocrit is 40%, and the concentration of erythrocyte folate becomes 360 nmol/L. According to their report (15), the concentration of...
erythrocyte folate is about 10% lower in the packed blood cell method compared to the hemolysate method. Therefore, the cut-off value would be 270 nmol/L in the present method. The percentages of the subjects whose blood cells folate concentrations were below the cut-off value became 30, 23, and 38, and 46% in the first, second, and third trimesters, and 1 mo after delivery, respectively.

We found that folate intake in pregnant Japanese women was \( \sim 250 \mu \text{g/d} \), which is much less than the EAR of 400 \( \mu \text{g/d} \) (1). Nevertheless, more than 50% of pregnant Japanese women could maintain blood folate values greater than the cut-off value. A similar result was already reported by Takimoto et al. (7). Therefore, the present EAR of folate (1) might have been set at too high a level.

Increased folate catabolism and urinary folate excretion during pregnancy have been reported (16–18), and
these may contribute to increased folate needs in pregnancy. In contrast, another study did not find an increase in urinary catabolites during the second trimester in women (6, 16). Furthermore, with the use of stable-isotope-labeled folate, no differences in urinary excretion of labeled folate or its catabolites between pregnant and non-pregnant women was reported (19). In our previous study, we reported that the urinary excretory level of folate was higher during pregnancy than in non-pregnant women (20). Folate concentration in our previous study (20) was measured by microbiological assay using Lactobacillus rhamnosus, ATCC 27773 (10, 21). Therefore, the urinary value did not contain catabolites of folate. We consider the amount of urinary excretion of water-soluble vitamins to reflect surplus intake of water-soluble vitamins (22). Our previous study results (20) suggest that the requirement of folate for pregnant women is lower than that of non-pregnant women, but this might not be the case because it is well known that a folate deficiency, macrocytic anemia, occurs in late pregnancy (23). A possible mechanism for the increase in urinary excretion of folate is increased absorption of folate during late pregnancy (24).

We analyzed the quality of folate but not quantity because many forms of folate compounds occur in food resources, and the availability is different depending on the form of folate. The contribution of folate intake in non-pregnant women is 39% from vegetables, 13% from cereals (cereals are not fortified with folic acid in Japan), 12% from beverages (mainly Japanese tea), 6% from eggs, and 31% from other sources (25). The form of folate compound that is used in supplements is synthetic and is called pteroerythronigrummonoglutamic acid (= folic acid). This synthetic form is different from the predominant form of naturally occurring folate compounds, the polyglutamated form of tetrahydrofolate, and its derivatives in the naturally occurring diet (26). Folic acid has a substantially higher bioavailability than does natural dietary folate, such as the polyglutamated form of 5-methyltetrahydrofolate (27, 28). The monoglutamated form of 5-methyltetrahydrofolate is more effective than folic acid supplementation for improving folate status (8). In Japan, vegetables, such as spinach, cauliflower, asparagus, and Brussels sprouts, are eaten after cooking in boiling water, which might increase the monoglutamated form of 5-methyltetrahydrofolate. As reported by Konings et al. (26), 100 g of cooked white rice contains 22 μg 5-formyltetrahydrofolate, which accounts for almost 100% of total folate. In contrast, the concentration of total folate in cooked white rice (item no. 01088 in the Standard Tables of Food Composition in Japan (2010) (14)) is 3 μg of folate/100 g of cooked white rice. Folate concentrations in the Standard Tables of Food Composition in Japan (2010) (14) are determined by the microbiological assay, Lactobacillus rhamnosus, ATCC 7469, and the method for extracting folate from food is the two-enzyme method, the protease and conjugase treatment method (14). Konings et al. (26) used the tri-enzyme method, amylase, protease, and conjugase, for the determination of total folate in cooked white rice, and the extracted samples were measured by high-performance liquid chromatography (HPLC). If the value of 22 μg of folate per 100 g of cooked white rice was to be accepted, the folate intake of pregnant women in the present study would increase to 88 μg from 12 μg supplied from cooked white rice because the subjects habitually ate 400 g of cooked white rice per day. Because pregnant Japanese women had approximately 250 μg/d of folate in the present food survey analysis, the folate intake would become 320 μg/d if the value reported by Konings et al. (26) was adopted, but this value is still lower than the EAR for pregnant women of 400 μg/d (1). Nevertheless, the folate status in pregnant Japanese women was good. Therefore, there must be another reason for pregnant Japanese women’s ability to maintain a suitable folate status.

Konings et al. (26) stated that it is impossible to compare folate intakes between different countries because of the absence of reliable data for folate in food products. Tamura (29, 30) suggested that all food folate tables should be reevaluated to obtain more reliable values, and he proposed that the extraction method used for folate should be the tri-enzyme method, not the two-enzyme method. He also reported that the bioavailability of the monoglutamated form of folate compounds might vary between 70% and 120% relative to folic acid (100%) (31). Therefore, Japanese food might contain much more folate in usually available food than that calculated by the Standard Tables of Food Composition in Japan (2010) (14), or the Japanese cooking style might convert folate into a form that has higher availability. Folate in Japanese food, in raw as well as cooked items, should be investigated using the tri-enzyme method and a newly developed analytical technique.

In conclusion, more than 50% of pregnant Japanese women with an intake of 150 μg dietary folate per 1.000 kcal, namely 250–300 μg per day, can maintain higher than cut-off values. Therefore, the present EAR of folate for pregnant women according to the DRIs for Japanese might be higher than the necessary amount for pregnant Japanese women.

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REFERENCES


