Red blood cell folate concentrations increase more after supplementation with [6*S*]-5-methyltetrahydrofolate than with folic acid in women of childbearing age[1–4]

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ABSTRACT

Background: For the primary prevention of neural tube defects (NTDs), public health authorities recommend women of childbearing age to take 400 μg folic acid/d 4 wk before conception and during the first trimester. The biologically active derivate [6*S*]-5-methyltetrahydrofolate ([6*S*]-5-MTHF) could be an alternative to folic acid.

Objective: We investigated the effect of supplementation with [6*S*]-5-MTHF compared with that of folic acid on red blood cell folate concentration, an indicator of folate status.

Design: The study was designed as a double-blind, randomized, placebo-controlled intervention trial. Healthy women (n = 144) aged 19–33 y received 400 μg folic acid, the equimolar amount of [6*S*]-5-MTHF (416 μg), 208 μg [6*S*]-5-MTHF, or placebo as a daily supplement for 24 wk. Red blood cell and plasma folate concentrations were measured at baseline and at 4-wk intervals.

Results: The increase in red blood cell folate over time was significantly higher in the group receiving 416 μg [6*S*]-5-MTHF/d than in the groups receiving 400 μg folic acid/d or 208 μg [6*S*]-5-MTHF/d ($P < 0.001$). No plateau was reached in red blood cell folate concentration in the 3 treatment groups during 24 wk of intervention; however, plasma folate plateaued after 12 wk.

Conclusions: We showed that administration of [6*S*]-5-MTHF is more effective than is folic acid supplementation at improving folate status. In addition, the study indicates that the recommended period for preconceptional folic acid supplementation should be extended to >4 wk for maximal prevention of NTDs based on folate concentrations. [6*S*]-5-MTHF might be an efficient and safe alternative to folic acid. *Am J Clin Nutr* 2006;84:156–61.

KEY WORDS Red blood cell folate, 5-methyltetrahydrofolate, folic acid, preconception supplementation, neural tube defect

INTRODUCTION

Periconceptional folic acid supplementation was shown to reduce the incidence of neural tube defects (NTDs) by 72–100% (1, 2). For primary prevention, health authorities recommend that women take a supplement of 400 μg folic acid/d ≥4 wk before conception and during the first trimester of pregnancy (3, 4). However, because most pregnancies are unplanned and only 18–45% of women take periconceptional supplements (5–9), some countries have implemented mandatory food fortification with folic acid (10–12). After folic acid was added to grain products, a decrease in the occurrence of NTDs was observed in the United States, Canada, and Chile (13–15). Countries that have not implemented mandatory folic acid fortification are concerned about the possible harm of a high intake of folic acid (eg, delaying the diagnosis of vitamin B-12 deficiency) (16).

A possible substitute for folic acid under consideration is the naturally occurring folate form [6*S*]-5-methyltetrahydrofolate ([6*S*]-5-MTHF) that is less likely to mask a vitamin B-12 deficiency (17). The condition for the usage of [6*S*]-5-MTHF instead of or in addition to folic acid would be to have at least equal efficacy with respect to the prevention of NTDs. A placebo-controlled trial to assess the efficacy of [6*S*]-5-MTHF on the occurrence of NTDs as primary endpoint would be unethical. However, because a relation between folate status and the risk of NTDs has been assessed, a surrogate endpoint is given which is the red blood cell folate concentration. In a case-control study conducted in Ireland, a threshold for the lowest risk of having a child born with an NTD was estimated to be a red blood cell folate concentration >906 nmol/L (18).

The objective of this double-blind, randomized, placebo-controlled intervention trial was to investigate the efficacy of daily supplementation with [6*S*]-5-MTHF compared with folic acid in increasing red blood cell folate, an indicator of folate status and a risk marker for NTD, in healthy women of childbearing age. The dosage of folic acid and the equimolar amount of [6*S*]-5-MTHF given correspond to the recommendations of periconceptional folic acid supplementation for primary NTD prevention. Further interest was on the kinetics of red blood cell and plasma folate over this long-term trial to investigate for a possible plateau effect in folate concentrations. By including a second group that received [6*S*]-5-MTHF in lower amounts, we also investigated the dose-response of [6*S*]-5-MTHF.

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SUBJECTS AND METHODS

Subjects and study design

Eligible participants for the study were healthy, young women (aged between 18 and 35 y) with normal results on routine laboratory tests (hematologic pattern, blood chemistry, and thyroid markers) and an adequate vitamin B-12 status (plasma vitamin B-12 ≥ 110 pmol/L). Women were not included if they were pregnant, lactating, or planning a pregnancy within the next months. Further exclusion criteria were regular consumption of vitamin supplements that contained folic acid or food fortified with folic acid (>100 μg folic acid/d during the past 4 mo), medical treatment interfering with folate metabolism, and abuse of alcohol or drugs.

Women were recruited through advertisement at the University of Bonn, Germany. After screening, 144 women aged 19–33 y were included in the study. The intervention was a 24-wk double-blind, placebo-controlled trial with parallel group design. Participants were randomly assigned to receive either 400 μg folic acid/d, 416 μg [6*S*]-5-MTHF/d, 208 μg [6*S*]-5-MTHF/d, or placebo. Before random assignment, participants were stratified according to their genotype for the 677C→T polymorphism of the gene encoding for 5,10-methylenetetrahydrofolate reductase *(MTHFR)* because homozygosity for the 677C→T *MTHFR* polymorphism is a risk factor for an NTD-affected pregnancy (19, 20) and affects the red blood cell folate concentration (21). During the intervention period, 1 participant withdrew because of personal reasons. After exclusion of 7 subjects with missing values (absence on blood sampling days because of vacation or illness), 136 participants were entered in the statistical analyses. The study was approved by the Ethics Committee of the Medical Association Hamburg, Germany, and all participants gave written informed consent. The study has been described previously when presenting the homocysteine-lowering potential of the different folate supplements (22).

Supplements

Supplements were taken as a capsule, one every morning before breakfast except on the blood sampling days when the capsule was taken after venipuncture. Both subjects and investigators were blinded to the treatment. The supplements were manufactured by PCI Services (Schorndorf, Germany) as hard gelatin capsules, each containing a blend of magnesium stearate and microcrystalline cellulose as a filler (placebo), and either 400 μg (906 nmol) folic acid (Caesar&Loretz GmbH, Hilden, Germany), 416 μg (906 nmol) [6*S*]-5-MTHF calcium salt, or 208 μg (453 nmol) [6*S*]-5-MTHF calcium salt (Metafolin; Merck Eprova AG, Schaffhausen, Switzerland). Folate contents of the capsules were measured by HPLC at the beginning and at the end of the study. The actual amounts in the capsules aimed to provide 400 μg folic acid, 416 μg [6*S*]-5-MTHF, and 208 μg [6*S*]-5-MTHF were 393 μg folic acid, 408 μg [6*S*]-5-MTHF, and 208 μg [6*S*]-5-MTHF, respectively, at the beginning of the study and 382 μg folic acid, 412 μg [6*S*]-5-MTHF, and 206 μg [6*S*]-5-MTHF, respectively, at the end of the study. Compliance with respect to the supplement intake was assessed by pill counting at weeks 8, 16, and 24.

Assessments

Fasting blood samples were collected by venipuncture at baseline and at weeks 4, 8, 12, 16, 20, and 24 of the study. For measurement of red blood cell and plasma folate concentrations, fasting blood samples were collected into heparinized tubes. After measurement of the hematocrit, whole blood samples for red blood cell folate analysis were diluted 1:10 with 1% ascorbic acid and incubated 30 min in the dark before storage at −80 °C. The remaining whole blood of the same sample was centrifuged (2000 × *g* for 10 min at 4 °C) and stored as plasma aliquots at −80 °C. Folate concentrations were measured by using the microbiological assay (23). The intraassay and interassay CVs were 2.6% and 7.2% for whole blood folate and 1.3% and 5.3% for plasma folate, respectively. For external validation, a whole blood folate standard (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom) was measured at each run. To avoid between-run variation, samples from each participant were measured in one run. Red blood cell folate concentrations were calculated according to the formula:

$$\text{Red blood cell folate} = \{(\text{whole blood folate} \times 100) - [\text{plasma folate} \times (100 - \text{hematocrit})]\}/(\text{hematocrit}) \quad (1)$$

Blood samples used to determine the health status were taken at baseline and at week 24 and were immediately analyzed by the central laboratory of the University Hospital, Bonn. Identification of the 677C→T *MTHFR* genotype was conducted by using the polymerase chain reaction according to the method of Frosst et al (24).

Dietary intakes were assessed by 3-d diet records administered at baseline and at weeks 8, 16, and 24. The diet records were analyzed by using EBISpro for WINDOWS (version 4; J Erhardt, University of Hohenheim, Germany).

Statistical analysis

Because the red blood cell and plasma folate concentrations were positively skewed, data were log-transformed to normalize distribution and back-transformed to geometric means with 95% CIs. For further analyses the natural logarithms of red blood cell and plasma folate were used in all statistical tests as continuous variables.

One-factor analysis of variance (ANOVA) was used to test for between-group differences with respect to baseline characteristics, dietary folate intake, and compliance. Within-group changes in dietary folate intake were determined in each intervention group by using paired *t* test adjusted to multiple comparisons ($P < 0.05/3$). Repeated-measures ANOVA was used to examine the interaction between time and intervention and to test for changes within time and intervention with respect to red blood cell and plasma folate concentrations. Tukey's honestly significant difference test was carried out as post hoc analysis. In case of significant interaction, the within-group comparison was carried out by using Bonferroni post hoc test adjusted for multiple comparisons ($P < 0.05/6$). The dose-response relation between administration of [6*S*]-5-MTHF and changes in red blood cell and plasma folate concentrations was tested with linear regression, including the groups that received placebo, 208 μg [6*S*]-5-MTHF, or 416 μg [6*S*]-5-MTHF. Results of all statistical calculations were considered statistically significant at $P < 0.05$. All analyses were done by using SPSS for WINDOWS (version 12; SPSS Inc, Chicago, IL).

TABLE 1
Baseline characteristics of the study population[1]

	400 μg folic acid/d ($n = 34$)	416 μg [6*S*]-5-MTHF/d ($n = 35$)	208 μg [6*S*]-5-MTHF/d ($n = 33$)	Placebo ($n = 34$)
Age (y)	23.6 ± 3.2[2]	24.2 ± 4.0	23.1 ± 2.7	22.6 ± 2.4
BMI (kg/m^2)	20.7 ± 2.4	21.6 ± 3.0	21.0 ± 2.3	21.3 ± 1.8
Red blood cell folate (nmol/L)	668 (593, 752)[3]	603 (525, 692)	656 (594, 726)	682 (612, 761)
Plasma folate (nmol/L)	19.3 (16.3, 22.9)	18.3 (15.9, 21.1)	19.6 (16.8, 22.8)	19.7 (17.5, 22.2)
Dietary folate intake (μg/d)	244 (212, 281)	252 (215, 295)	225 (199, 254)	232 (204, 263)

[1] [6*S*]-5-MTHF, [6*S*]-5-methyltetrahydrofolate. No significant differences were observed between the 4 groups (one-factor ANOVA).
[2] Arithmetic $\bar{x}$ ± SD (all such values).
[3] Geometric $\bar{x}$; 95% CI in parentheses (all such values).

RESULTS

Baseline characteristics of the 136 subjects included in statistical analyses are presented in **Table 1**. At baseline, the 4 intervention groups did not differ with respect to age, body mass index, red blood cell and plasma folate concentrations, and dietary folate intake. With respect to dietary folate intake, no change was observed throughout the study period; thus, it did not differ within the groups or between the groups at baseline and at week 8, 16, or 24. The compliance with respect to supplement intake was high and did not differ significantly between the groups ($P > 0.05$). Ninety percent of the subjects consumed ≥95% of the supplements, and the other 10% of subjects consumed 86–94% of the supplements.

The mean red blood cell and plasma folate concentrations of the 3 treatment groups and the placebo group are shown in **Figure 1** and **Figure 2**. A significant interaction was observed between time and intervention in both red blood cell and plasma folate ($P < 0.001$ for both). In red blood cell folate, the dimension of increase over time was significantly greater in the group receiving 416 μg [6*S*]-5-MTHF/d than in the groups receiving 400 μg folic acid/d or 208 μg [6*S*]-5-MTHF/d ($P < 0.001$ for both) and in the group receiving 400 μg folic acid/d than in the group receiving 208 μg [6*S*]-5-MTHF/d ($P < 0.05$). Similarly, in plasma folate, the dimension of increase over time was significantly greater in the group receiving 416 μg [6*S*]-5-MTHF/d than in the groups receiving 400 μg folic acid/d or 208 μg [6*S*]-5-MTHF/d ($P < 0.05$ and $P < 0.001$, respectively) and in the group receiving 400 μg folic acid/d group than in the group receiving 208 μg [6*S*]-5-MTHF/d ($P < 0.05$). Within-group analysis showed a continuous, significant increase in red blood cell folate concentration over 24 wk of intervention, in all 3 folate groups. A plateau, defined as no further significant increase between consecutive points in time, was not observed in red blood cell folate concentration during the study period. However, in plasma folate, a plateau was reached independent of the folate form after 12 wk of supplementation.

A significant dose-dependent effect was observed on the increase of red blood cell and plasma folate over the 24-wk administration of [6*S*]-5-MTHF. Pearson's correlation coefficients were 0.873 and 0.769 for changes in red blood cell and plasma folate, respectively ($P < 0.001$ for both). Linear regression, including the groups receiving placebo, 208 μg [6*S*]-5-MTHF or

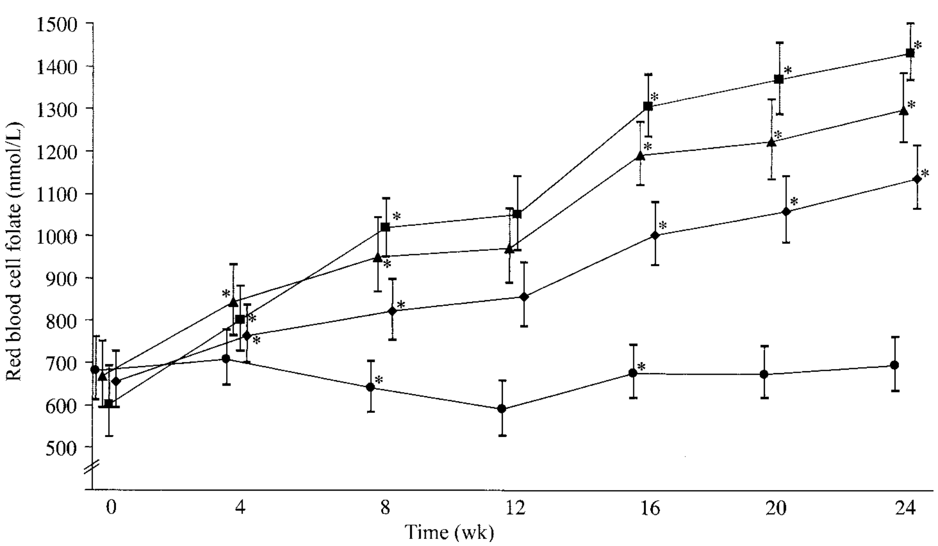

FIGURE 1. Geometric mean red blood cell folate concentrations over time after 24 wk of supplementation with 400 μg folic acid/d (▲, $n = 34$), 416 μg [6*S*]-5-methyltetrahydrofolate ([6*S*]-5-MTHF)/d (■, $n = 35$), 208 μg [6*S*]-5-MTHF/d (◆, $n = 33$), or placebo (●, $n = 34$). Bars represent 95% CIs. A significant interaction was observed between time and intervention ($P < 0.001$, repeated-measures ANOVA). The dimension of increase over time (ie, slope) was significantly greater in the group receiving 416 μg [6*S*]-5-MTHF/d than in the groups receiving 400 μg folic acid/d or 208 μg [6*S*]-5-MTHF/d ($P < 0.001$ for both, Tukey's honestly significant difference test) and in the group receiving 400 μg folic acid/d than in the group receiving 208 μg [6*S*]-5-MTHF/d ($P < 0.05$, Tukey's honestly significant difference test). *Significantly different from the previous time point within groups, $P < 0.05/6$ (Bonferroni post hoc test adjusted for multiple comparisons).

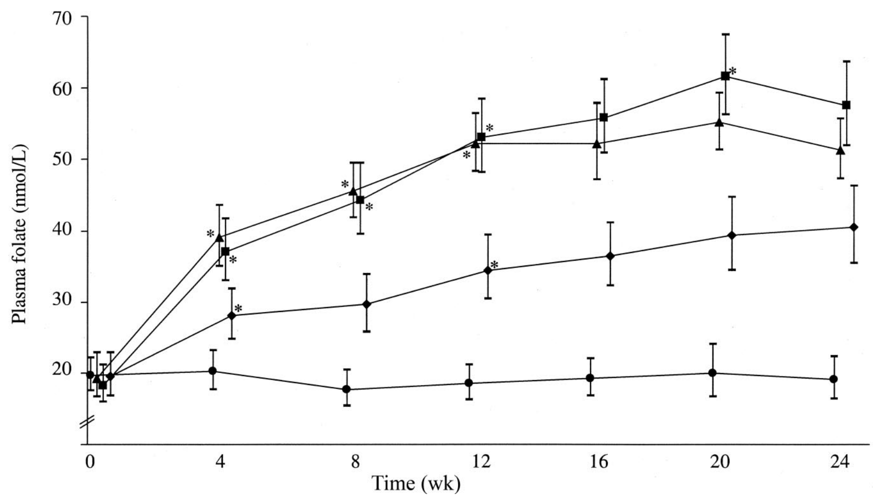

FIGURE 2. Geometric mean plasma folate concentrations over time after 24 wk of supplementation with 400 μg folic acid/d (▲, n = 34), 416 μg [6*S*]-5-methyltetrahydrofolate ([6*S*]-5-MTHF)/d (■, n = 35), 208 μg [6*S*]-5-MTHF/d (◆, n = 33), or placebo (●, n = 34). Bars represent 95% CIs. A significant interaction was observed between time and intervention ($P < 0.001$, repeated-measures ANOVA). The dimension of increase over time (ie, slope) was significantly greater in the group receiving 416 μg [6*S*]-5-MTHF/d than in the groups receiving 400 μg folic acid/d or 208 μg [6*S*]-5-MTHF/d ($P < 0.05$ and $P < 0.001$, respectively, Tukey's honestly significant difference test) and in the group receiving 400 μg folic acid/d than in the group receiving 208 μg [6*S*]-5-MTHF/d ($P < 0.05$, Tukey's honestly significant difference test). *Significantly different from the previous time point within groups, $P < 0.05/6$ (Bonferroni post hoc test adjusted for multiple comparisons).

416 μg [6*S*]-5-MTHF, showed that, per 100 μg [6*S*]-5-MTHF supplementation over 24 wk, red blood cell and plasma folate concentrations increased by 190 and 9.6 nmol/L, respectively.

DISCUSSION

This long-term intervention trial with healthy, nonpregnant women showed a higher efficacy of the biologically active folate form [6*S*]-5-MTHF than the equimolar amount of folic acid with respect to the increase in folate status. As an indicator of folate status which is related to the risk of NTDs (18, 25), red blood cell folate concentration was used. Half of the amount of [6*S*]-5-MTHF, 208 μg/d, was not as efficient as 416 μg [6*S*]-5-MTHF/d or 400 μg folic acid/d with respect to change over time. After 24 wk of each of the folate administrations, the mean red blood cell folate concentration exceeded 906 nmol/L, which is the concentration above which women were shown to have the lowest risk of an NTD-affected pregnancy than were case subjects with red blood cell folate concentrations ≤ 905nmol/L (18). Daly et al (26) showed that median red blood cell folate concentration exceeded 906 nmol/L after 24 wk of supplementation with either 400 μg or 200 μg folic acid/d in a study of Irish women. In their study, no blood samples were drawn between baseline and week 24. In the present study, mean red blood cell folate concentrations exceeded 906 nmol/L after 8 wk of intervention in the groups that received 400 μg folic acid/d and 416 μg [6*S*]-5-MTHF/d and also after 16 wk in the group that received 208 μg [6*S*]-5-MTHF/d. Venn et al (27) did not observe significant differences in the increase of red blood cell or plasma folate concentration after 24 wk of supplementation with 100 μg folic acid/d or the equimolar amount of [6*S*]-5-MTHF in a subgroup of healthy women. In their study, the subjects' red blood cell folate concentrations were already near 906 nmol/L at baseline. The analytic method used for folate determination was the microbiological assay both in the present study and in the studies from Daly et al (18), Daly et al (26), and Venn et al (27).

Red blood cell folate concentrations were used to estimate NTD risk by Daly et al (18) because the folate status was retrospectively measured at a median of 15 wk of gestation, whereas the time of interest was the folate status at neural tube closure (ie, at 4 wk of gestation). With respect to fetal development, the milieu providing folate to the embryo is the maternal plasma. Low maternal plasma folate was also observed to be related to NTDs (28, 29) and other adverse pregnancy outcomes, eg, early spontaneous abortion (30). In the present study, plasma folate concentrations reached a plateau after 12 wk of folate supplementation independent of the form and dosage of folate supplementation. At this point, mean red blood cell folate concentrations had exceeded 906 nmol/L only in the groups that received 400 μg folic acid/d and 416 μg [6*S*]-5-MTHF/d. Current recommendations are for women to use periconceptional supplementation with 400 μg folic acid/d ≥4 wk before conception and during the first trimester of pregnancy (3, 4). Because plasma folate plateaued after 12 wk of supplementation while mean red blood cell folate concentrations had reached the "safe range," the results of the present study show a possible additional preventive effect if women would start earlier with periconceptional folic acid and folate supplementation at amounts ≥400 μg/d. The amount of 208 μg [6*S*]-5-MTHF/d would be too low to reach red blood cell folate concentrations >906 nmol/L before plasma folate plateaus.

In contrast to plasma folate, red blood cell folate concentration did not reach a plateau in any of the 3 treatment groups during 24 wk of intervention. The importance of red blood cell folate in the embryonic development is that red blood cells serve as a folate storage tissue and provide folate to the maternal plasma in case of decreasing folate intake. A plateau in red blood cell folate concentration was expected after 16 wk (≈120 d) because red blood cells incorporate folate only during erythropoieses, lose it during their degeneration, and have a mean life span of ≈120 d (31–33). After 120 d of folate supplementation, the new generation of red blood cells would have incorporated high amounts of supplemented folate. However, no plateau was reached in a period of 24 wk (ie, 168 d) of folate supplementation in our study. We hypothesize that folate released during the degeneration of red blood cells is again available for incorporation in newly formed red blood cells. Therefore, a plateau would not be achieved after the red blood cells all were replaced once (ie, 120 d); however, during the second period of 120 d, these red blood cells then would benefit from the high supply of folate through the supplements and from the folate available of the first generation of red blood cells.

Folic acid supplementation was shown to decrease the mother's risk of having an NTD-affected pregnancy (1, 2), especially when supplements are taken both before and after conception (34). Although promotion campaigns and educational programs were undertaken in the United Kingdom, Ireland, and Australia to increase the awareness and usage of periconceptional folic acid supplementation, only 18–45% of women were taking supplements during the recommended time span (5–9). Prevalent characteristics of women not using folic acid supplements were unplanned pregnancy, low socioeconomic status, and late information or no knowledge about folic acid (6–8). With respect to the low usage of periconceptional folic acid supplementation and the high percentage of unplanned pregnancies, food fortification seems to be a more efficient strategy to increase folate status in young women and to lower the incidence of NTDs than education programs or campaigns for folic acid supplementation (6, 35–37). Food fortification has shown to decrease the occurrence of NTDs by ≤50% (13–15) and to increase red blood cell folate status in different populations (12, 38, 39). Countries not implementing mandatory or voluntary food fortification with folic acid generally are concerned about the possible harm of chronic exposure to high amounts of folic acid. The possible negative side effect of the consumption of foods fortified with folic acid is, mainly in combination with the usage of vitamin supplements, that some people may exceed the tolerable upper intake level (UL) of folic acid (40, 41). Folic acid above the UL of 1 mg/d potentially can delay the appearance of the hematologic symptoms of vitamin B-12 deficiency (10, 42). Vitamin B-12 deficiency mainly occurs in the elderly with a prevalence of 8–16% (42), but in this age group only a small percentage exceeded the UL of folic acid (41). So far no increase in masking of vitamin B-12 deficiency was found after food fortification in the United States (43). The possible risk for elderly people seems lower than their benefit of food fortification with respect to a decrease in plasma total homocysteine concentrations (44), an independent risk factor for vascular diseases (45). It is more of a concern that children showed a high folic acid intake with 26% exceeding the UL (41); however, the effect of high folic acid exposure is still unknown.

Unlike folic acid, [6*S*]-5-MTHF was proposed not to mask a vitamin B-12 deficiency according to 2 hypotheses. First, high amounts of [6*S*]-5-MTHF cannot be formed into folate derivatives needed for DNA and cell synthesis if vitamin B-12 is lacking for regeneration of 5-MTHF to tetrahydrofolate (THF) (46). Second, in addition to the vitamin B-12 requirement for intracellular use of 5-MTHF, the vitamin B-12–dependent production of THF is needed for cellular retention of folate, because the preferred substrate for the folylpolyglutamate synthase is THF and it only has low affinity to 5-MTHF (47, 48). Folic acid conversion to THF is independent of vitamin B-12; thus, it is available for intracellular use and storage. In the supplement forms used, [6*S*]-5-MTHF and folic acid showed equal stability in our long-term study (98.8% and 95.6%, respectively, after 6 mo). Folic acid in fortified food is stable and has a high bioavailability (49, 50). Thus, the usage of folic acid for food fortification and [6*S*]-5-MTHF in vitamin supplements would be an approach to avoid the excess of the UL of folic acid.

In conclusion, our study shows that administration of [6*S*]-5-MTHF is more effective than is folic acid supplementation at increasing red blood cell folate concentrations in women of childbearing age. Supplementation with [6*S*]-5-MTHF might be an adequate alternative to folic acid for increasing folate status and, thus, for reducing the risk of having an NTD-affected pregnancy. On the basis of red blood cell and plasma folate concentrations before conception, the recommended period of preconceptional folic acid supplementation of 4 wk should be extended to ≥12 wk to achieve maximal risk reduction. [6*S*]-5-MTHF might be an efficient and safe alternative to folic acid in vitamin supplements.

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YL and KP had the original idea for the study and recruited the subjects. YL, RP-L, and KP were responsible for designing and planning the study. YL was responsible for sample collection, laboratory analysis, and statistical analysis. YL, RP-L, SB, and KP contributed to the writing of the paper. None of the authors had a conflict of interest.

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