Unmetabolized folic acid and total folate concentrations in breast milk are unaffected by low-dose folate supplements\(^1\)\(^–\)\(^3\)

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ABSTRACT

Background: Many lactating women in North America are exposed to high synthetic folic acid intakes because of food fortification and vitamin supplement use. Few data exist on the potential long-term effect of high folic acid intakes on milk folate concentrations, whereas no data are available on the effect of supplemental [6\(^S\)]-5-methyltetrahydrofolate ([6\(^S\)]-5-methylTHF).

Objective: The aim of the present study was to investigate the effect of 3 treatments (placebo, folic acid, and [6\(^S\)]-5-methylTHF) on milk folate and folate-binding protein (FBP) concentrations and to determine whether unmetabolized folic acid is present in milk.

Design: In this 16-wk randomized, placebo-controlled intervention, 69 lactating women were randomly assigned to receive [6\(^S\)]-5-methylTHF (416 µg/d, 906 nmol/d) or a placebo, or were assigned to receive folic acid (400 µg/d, 906 nmol/d) within 1 wk postpartum. Total milk folate, FBP, and unmetabolized folic acid concentrations were measured at 16 wk.

Results: Unmetabolized folic acid was detected in 96% of milk samples tested representing ~8% of total milk folate concentrations. Total milk folate, FBP, and the proportion of unmetabolized milk folic acid did not differ between treatments; however, FBP concentrations were significantly lower than those published before mandatory folic acid fortification of the food supply.


INTRODUCTION

In an effort to reduce the incidence of neural tube defects (NTDs), the addition of folic acid to enriched grain products became mandatory in North America in 1998 (1, 2). Before the fortification program, unsupplemented pregnant and lactating women in North America generally had intakes that were well below dietary recommendations to support optimal folate status (3–6). Today, consumption of folic acid–fortified foods (~100–200 µg/d) together with recommendations by healthcare providers to consume a prenatal nutritional supplement (1000 µg folic acid per tablet) during pregnancy and lactation, has resulted in intakes well above the recommended daily allowance for pregnancy [600 µg dietary folate equivalents (DFE)/d] and lactation (500 µg DFE/d) (7). Thus, it is not surprising that work in our laboratory showed red blood cell (RBC) folate concentrations to be, on average, well above 2000 nmol/L in pregnant women consuming prenatal supplements in the fortification era (8). A cutoff value of 305 nmol/L is indicative of folate deficiency (7).

Despite the unequivocal success of the folic acid fortification program in reducing NTD rates (9–12), the potential health effects of very high blood folate concentrations are unclear. Controversy concerning the safety of high folic acid intakes has heightened, partly because of the recent observations that they can facilitate cancer development and progression (13). Furthermore, ingestion of synthetic folic acid above a certain threshold leads to the presence of this unmodified form of folate in the blood of infants and adults (14–17). It has been postulated that the presence of unmetabolized folic acid could affect the normal homeostatic regulation of folate as folic acid is metabolized differently from the reduced folates (18). For example, it has been shown in vitro that folic acid competes with the reduced forms for folate binding sites on enzymes and carrier proteins. More specifically, the transport of folate into the cytoplasm of human milk is carried out by the mammary epithelial folate receptor, which in vitro exhibits a higher affinity for folic acid than for 5-methyltetrahydrofolate such that transport via the folate receptor appears to be blocked by physiologic concentrations of folic acid (19).

Thus, the present study was undertaken to investigate the effect of daily supplementation with 400 µg folic acid compared with that of an equimolar dose of [6\(^S\)]-5-methyltetrahydrofolate ([6\(^S\)]-5-methylTHF) on total milk folate and unmetabolized folic acid concentrations in well-nourished lactating women consuming folic acid–fortified foods. Furthermore, we examined the impact of folic acid and [6\(^S\)]-5-methylTHF supplementation on the concentration of milk folate-binding proteins (FBPs)—cleavage products of the membrane-associated mammary folate receptor (20).

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\(^2\) Supported by Merck Eprova AG (Schaffhausen, Switzerland) and the Natural Sciences & Engineering Research Council of Canada. LAH received a student stipend to carry out this work from the Canadian Institute of Health Research Training Grant in Clinical Nutrition (STP-53889) and from the Ontario Student Opportunity Trust Fund, The Hospital for Sick Children Foundation Scholarship Program.

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Received June 20, 2008. Accepted for publication October 6, 2008.


SUBJECTS AND METHODS

The breast-milk folate data presented herein for the first time were collected as part of a randomized controlled trial designed to assess the efficacy of [6S]-5-methylTHF compared with folic acid as a dietary supplement during lactation. A detailed description of the study design and blood folate results can be found elsewhere (8).

Study population and design

Briefly, healthy pregnant women (n = 72; 16–40 y; <36 wk gestation) who intended to exclusively breastfeed for >4 mo postpartum were recruited by word-of-mouth and through the Motherisk Program—a telephone counseling service at The Hospital for Sick Children, Toronto, Canada.

The intervention was a 16-wk double-blinded, randomized, placebo-controlled trial. Within 1 wk after the birth of their infants, participants were randomly assigned to receive placebo or [6S]-5-methylTHF (416 µg/d, 906 nmol/d) (Metafolin; Eprova, Schaffhausen, Switzerland). As planned a priori, a reference group of lactating women who followed all aspects of the study protocol were provided with a daily commercial folic acid supplement containing 400 µg (906 nmol/d) (Jamieson Laboratories, Montreal, Canada). The folate content of supplements was verified analytically, and both forms of folate were found to be stable over the period of the study. In addition, all participants received a folate-free daily multivitamin and mineral supplement, which contained 1 mg vitamin B-6, 3 µg vitamin B-12, and 4 mg ferrous fumarate (Exact; Pharmetrics, Quebec, Canada). Participants were instructed to consume supplements until 16 wk postpartum and to avoid any other folate-containing vitamin and/or mineral supplement during the study period. Supplemental folate intakes were determined by assessing the difference between the number of capsules dispensed at randomization (within 1 wk postpartum) and the number of capsules remaining at study completion (16 wk postpartum). Of the 72 women recruited, 69 were assigned to a treatment group within 1 wk after the birth of their infant. A qualified lactation consultant was hired to ensure breastfeeding success. Five subjects withdrew (n = 1 in the [6S]-5-methylTHF group, n = 1 in the placebo group, and n = 3 in the folic acid group) during the course of the study because they discontinued breastfeeding or found the study too difficult because of the extra demands of a sick infant. Of the 64 subjects remaining, participants who did not provide a milk sample were excluded from the present analyses. Hence, our final sample sizes at 4, 8, and 16 wk postpartum were 55, 53, and 57, respectively.

Blood samples were collected at 16 wk postpartum (±1 wk); milk samples were collected at 4, 8, and 16 wk postpartum (±1 wk); and total milk folate concentrations were measured at each time point. In addition, unmetabolized milk folic acid and soluble milk FBP concentrations were determined at 16 wk. The Human Ethics Committee at The Hospital for Sick Children approved the study, and participants gave written informed consent.

Biological sample collection and total folate analysis

Blood samples were collected in EDTA-treated tubes, transported on ice, and processed within 2 h of collection. Plasma and RBC folate concentrations were measured by microbiological assay as described by Molloy and Scott (21) using the test organism Lactobacillus rhamnoses (ATCC 7649; American Type Tissue Collection, Manassas, VA).

Complete breast expression (manually or by electric breast pump) was used to collect milk samples. Because of the wide variation in milk folate concentrations over a 24-h period, milk samples were collected between 1300 and 1450. The folate content in milk during this time period appears to be representative of the mean folate concentration in samples obtained from all feedings during a 24-h period (22). To protect labile milk folate, collection tubes contained sodium ascorbate (1%, wt:vol) and were mixed well before freezing. Each milk sample was divided into several aliquots and stored at −80°C. Total milk folate concentrations were measured by microbiological assay as described by Molloy and Scott (21) by using the test organism L. rhamnoses after trienzymatic treatment with x-amylase (EC 3.2.1.1), protease (EC 3.4.24.21) (Sigma Chemical, St Louis, MO), and rat serum folate conjugase (Harlan Bioproducts for Science, Indianapolis, IN) (23). The interassay CV for the assay was 7.7% on the basis of repeated measurements (n = 10) using an infant formula standard with a certified value (mean ± SD: 129 ± 28 µg folic acid/100 g) (Standard Reference Material 1846 Infant Formula; National Institute of Standards and Technology, Gaithersburg, MD). Analysis of the formula standard in our laboratory yielded a mean (±SD) folate concentration of 143 ± 1 µg/100 g.

Analysis of folic acid in milk

Folates were purified from enzyme-treated milk samples by affinity chromatography using immobilized bovine milk FBP isolated from dried whey milk powder. A detailed description of the methods and materials used to prepare and store affinity columns is published elsewhere (24). The recovery from the affinity column, as measured with tritiated folic acid (Amersham Pharmacia Biotech, Piscataway, NJ), was 85.5 ± 0.7% (mean ± SD; n = 5). The total folate binding capacity exceeded 500 µmol/L, solid phase. The folic acid from purified milk samples was identified by using ion-pair HPLC with electrochemical detection as described in detail by Bagley and Sellhub (25, 26) and Belz and Nau (27). The HPLC system consisted of a P580 pump with ASI-100 autosampler, a 250 × 4.6 mm Betasil Phenyl analytic column, and an ED50 electrochemical detector with set-up shift and Ag/AgCl reference potential, managed by Chromeleon version 6.2 software. All parts were purchased from Dionex Corporation (Oakville, Canada), except for the phenyl analytic column (Keystone Scientific, Thermo Electron Corporation, Waltham, MA). The mobile phase was delivered at a flow rate of 0.75 mL/min and maintained at 25% A, 7% B, and 68% water for the first 10 min. From 10 to 40 min, the concentration of B was raised linearly to 20%, providing the gradient. The folic acid derivative was identified on the basis of retention time (22.3 min) as compared with the electrochemical response of the peak of the folic acid standard (Sigma, Oakville, Canada). The between-run precision (1.4%) was determined by analyzing aliquots of a pooled human milk control.

Milk folate-binding protein analysis by competitive binding radioassay

Milk FBP concentrations were determined by using a competitive binding radioassay procedure as described by Selhub et al (28).
To dissociate endogenously bound folate, freshly thawed breast milk was acid-washed by dilution with 19 volumes (vol:vol) 0.1 mol/L acetic acid and left to stand at room temperature for 15 min. A 0.05-mL aliquot was then diluted with 17 volumes (vol:vol) 0.1 potassium phosphate buffer and then incubated with 11 ng [3H]folic acid (Amersham, Buckinghamshire, United Kingdom) for 30 min. The mixture was then passed through a cellulose-nitrate filter (0.45 μm; Pall Corporation, Ann Arbor, MI) under vacuum, and the filter was washed twice with 0.1 mol/L potassium phosphate buffer to remove residual unbound radioactivity. The filter was allowed to dry overnight, and radioactivity was determined by liquid scintillation spectrometry (LS6500 Beckman Scintillation Counter; Beckman Coulter, Fullerton, CA). The between-run CV in FBP content of a pooled milk sample was 3.6%.

**Statistical analyses**

All data were checked for normal distribution (Proc Univariate procedure), and skewed data were transformed to normalize their distribution for analyses. Square root transformation was used to normalize total milk folate and unmetabolized milk folic acid concentrations. Baseline characteristics such as age and prenatal folic acid consumption between treatment groups were compared by using a one-factor analysis of variance (ANOVA). Group differences in milk folate concentrations at 4, 8, and 16 wk postpartum were analyzed by using repeated-measures ANOVA (Proc Mixed) followed by Tukey’s post hoc test. Furthermore, one-factor ANOVA was used to compare unmetabolized milk folic acid and FBP concentrations at 16 wk postpartum between treatment groups. Possible confounding variables in the aforementioned relation, such as total milk folate concentration, plasma folate concentration, and folic acid intake (supplemental + fortified) were examined but were not prognostic of unmetabolized milk folic acid concentrations. At 16-wk postpartum, correlation analyses were used to investigate associations between total milk folate concentration and maternal blood folate indexes. All statistical analyses were performed by using SAS for Window version 9.1 (SAS Institute Inc, Cary, NC). Differences with \( p \) values <0.05 were considered significant.

**RESULTS**

The mean (±SD) age of the participants included in the present analyses was 32 ± 4 y. All participants reported that they regularly consumed a folic acid–containing prenatal supplement during pregnancy with an average daily folic acid consumption of 911 ± 251 μg. As reported in greater detail elsewhere (8), no significant differences existed between the 3 treatment groups with respect to age, prenatal folic acid supplement intake during pregnancy, family income, maternal education, and compliance with taking the study supplement. Participants were well educated and were predominantly of middle-to-upper socioeconomic status. After 16 wk of supplementation, mean (±SD) concentrations of plasma folate in the supplemented participants (folic acid: 97.0 ± 27.0 nmol/L; [6S]-5-methylTHF: 104 ± 55 nmol/L) were significantly greater than those in the placebo group (47.6 ± 24.4 nmol/L) \( (P < 0.0001) \). Likewise, mean (±SD) RBC folate concentrations in the supplemented participants (folic acid: 2257 ± 1168 nmol/L; [6S]-5-methylTHF: 2304 ± 804 nmol/L) significantly exceeded those of the unsupplemented participants (placebo group: 1462 ± 532 nmol/L) \( (P < 0.01) \).

Milk folate concentrations, as presented in Table 1, were similar between the folic acid– and [6S]-5-methylTHF–supplemented groups and did not differ from those participants not supplemented with folate. Furthermore, milk folate concentrations between all groups remained stable from 4 to 16 wk postpartum, and there was no significant correlation between maternal blood folate concentrations and total milk folate concentrations. The average pooled milk folate concentration across all treatment groups and study visits was 181 ± 70 nmol/L \( (79.7 ± 30.9 \mu g/L) \). FBP concentrations measured at 16 wk postpartum did not differ between treatment groups (Table 1). Similarly, no group differences were noted for unmetabolized milk folic acid concentrations at 16 wk postpartum (pooled mean ± SD: 14.4 ± 9.7 nmol/L, or 6.4 ± 4.3 μg/L) (Figure 1). Unmetabolized milk folic acid was detected in 55 of 57 (96%) milk samples tested and represented ≈8% of the total milk folate concentration.

**DISCUSSION**

In the present study, we found no differences in the total milk folate concentration of women provided with either a low dose of folic acid, a [6S]-5-methylTHF supplement, or a placebo during lactation. Unmetabolized folic acid was detected in most of the milk samples; however, no differences in concentrations were observed between treatment groups, despite the higher exposure to folic acid in the folic acid–supplemented participants. To our knowledge, this was the first study to compare the effect of modest supplemental amounts of [6S]-5-methylTHF (416 μg/d) with that of an equimolar dose of folic acid on milk folate concentrations and to investigate the presence of unmetabolized folic acid in milk samples of free-living, well-nourished lactating women.

The breast milk folate concentration is known to be tightly regulated, and minimally influenced by maternal folate status, except when the mother is severely folate deficient (29); however, less is known about the impact of supplementation on milk folate concentration. Concentrations of milk folate in the present study were similar between the study groups, despite the significantly lower maternal folate concentrations in the supplemented participants.

<table>
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<tr>
<th>TABLE 1</th>
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<td>Total folate and folate-binding protein (FBP) concentrations in milk collected from women supplemented with synthetic [6S]-5-methyltetrahydrofolate ([6S]-5-methylTHF) or folic acid or provided a placebo during lactation$^4$</td>
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<td>Treatment</td>
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<td>Milk folate (nmol/L)$^2$</td>
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<td>[6S]-5-methylTHF</td>
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<td>FBP (nmol folate binding/ L milk)$^3$</td>
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$^4$All values are means ± SDs.

$^2$No significant differences between groups over time, \( P > 0.05 \) (repeated-measures ANOVA).

$^3$FBPs were not measured at week 4 and week 8. No significant differences between treatment groups at week 16, \( P > 0.05 \) (one-factor ANOVA).
significantly higher blood folate concentration of the supplemented participants. Furthermore, we found no correlation between milk folate concentration and maternal blood folate indexes. These observations are consistent with findings from earlier studies (5, 30, 31), suggesting that milk folate secretion reaches a maximum threshold in women with adequate folate status. Some studies have also noted a gradual increase in milk folate concentrations with the progression of lactation, whereas others have reported no change or a decrease in milk folate concentrations (5, 6, 31–33). In the present study, we observed a relatively constant concentration of milk folate in the first 4 mo of lactation.

Circulating folic acid concentrations in our sample of lactating women were not measured; thus, the relation, if any, between plasma folic acid and milk folate acid concentration is unknown. However, the very high blood folate concentrations of our participants may offer one possible explanation for the lack of difference in human milk folic acid concentrations between treatment groups. Pfeiffer et al (34) reported a higher percentage of folic acid as total folate in the circulation of those with total serum folate concentrations >50 nmol/L than in those with serum folate concentrations <50 nmol/L. The average plasma folate concentration of our unsupplemented mothers was 48 nmol/L, and it was proposed that the high folate status of our participants precluded us from detecting a significant effect on milk folic acid concentrations.

Interestingly, although the mean milk folate concentration (181 nmol/L) in our study was highly comparable with those reported before the folic acid fortification program (range: 181–224 nmol/L) (6, 23), the mean milk FBP concentration (43.1 nmol/L) reported herein was substantially lower than those reported before fortification. With the same analytic method, the mean (±SD) milk FBP concentration of complete breast milk from 4 healthy women analyzed by our laboratory was 48 nmol/L (35). Likewise, earlier work by Selhub et al (28) reported human milk FBP concentrations ranging from 186 to 271 nmol/L. Milk folate is bound to FBPs, a cleavage product of the membrane-bound folate receptor, which is proposed to be involved in regulating folate secretion. We hypothesize that the observed decrease in the mean milk FBP concentration in our study may relate to a down-regulation of folate receptor synthesis secondary to high extracellular folate concentrations in our study participants. The mean RBC folate concentrations of our placebo and folate-supplemented mothers at 16 wk postpartum were >1462 nmol/L in comparison with a range in the mean RBC folate concentration of 667–770 nmol/L reported for lactating women consuming ≤300 µg supplemental folic acid each day before folic acid fortification of the food supply in North America (5, 6, 31). In support of this hypothesis, Ashokkumar et al (36) recently showed a decrease in the mRNA concentration for folate receptor in cultured renal epithelial cells exposed to high levels of folic acid.

In contrast with previous literature whereby human milk folate binding capacity exceeded folate concentrations by ~68 nmol/L (37), the concentration of milk folate in the present study far exceeded FBPs (~137 nmol/L). The implications of these findings may raise concern because it has been suggested that milk FBPs enhance the bioavailability of folate (31, 38). Furthermore, on the basis of the concentration of unmetabolized folic acid found in human milk reported herein (6.35 µg/L), the theoretical exposure of the exclusively breastfed infant to synthetic folic acid would be ~5 µg/d. This estimate assumes a daily milk intake of 0.78 L/d (7). Although it seems unlikely that this amount of unmetabolized folic acid would have a negative affect on the folate status of an infant per se, recent evidence suggests that the somewhat higher affinity of FBP for folic acid than for reduced forms may also decrease the bioavailability of milk folate (39).

In conclusion, our data represent the first information on human milk folate concentrations after the introduction of folic acid fortification of the food supply. Milk folate concentrations were similar to those reported before fortification, and low-dose supplementation in the form of either folic acid or [5S]-5-methylTHF did not appear to increase milk folate concentrations. The markedly lower concentration of milk FBPs compared with pretodification reports along with the detectable appearance of unmetabolized folic acid in the milk of even unsupplemented mothers emphasizes the ongoing need to evaluate the potential benefits and risks of long-term consumption of folic acid–fortified foods. Confirmation of these latter findings and assessment of its impact on the bioavailability of folate to the breastfed human infant requires further exploration.

We thank the Motherisk Program at The Hospital for Sick Children, Toronto, for assisting with recruitment and are grateful to all the mothers who participated in the study. We also thank Glynnis Dubois for lactation support and Kelly Sherwood for assisting with study coordination and data collection.
REFERENCES


