

Original Article

Folic acid fortified milk increases blood folate and lowers homocysteine concentration in women of childbearing age

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Daily consumption of 400 µg folic acid prior to conception and during early pregnancy is recommended for the prevention of neural tube defects (NTD). Strategies to increase folic acid consumption include supplements and fortified foods. Milk is consumed by women and can be fortified with folic acid but little is known about the effect of fortified milk on blood folate concentration in women of childbearing age. The objective of this study was to determine whether daily consumption of milk fortified with 375 µg folic acid increases blood folate and lowers homocysteine concentrations in women of childbearing age. Seventy-three non-pregnant women (aged 18-47 y) were randomized to receive either 75 g/d of a fortified or unfortified (control) milk powder for 12 weeks. Women who consumed the folic acid fortified milk had mean (95% CI) red blood cell and plasma folate concentrations that were 539 nmol/L (436, 641) and 35 nmol/L (30, 41) higher, respectively, than in the control group. Women drinking fortified milk had a 14% lower mean plasma homocysteine concentration at week 12 than women consuming the control milk. Daily consumption of fortified milk powder providing 375 µg folic acid increases blood folate and lowers homocysteine concentrations over 12 weeks in women of childbearing age. Daily consumption of fortified milk would be expected to reduce NTD risk.

Key Words: folic acid, milk, NTD, fortification, red blood cell folate, plasma folate, homocysteine, women.

Introduction

Folic acid taken prior to conception and during early pregnancy reduces neural tube defect (NTD) risk.¹⁻³ In a population-based prevention campaign involving 31,960 women living in Hebei province in China, there was a 79% reduction in NTD risk in women taking 400 µg of folic acid per day.³ Public health authorities in several countries recommend that women who could become pregnant consume ~400 µg/d folic acid to prevent NTDs.⁴⁻⁶ Strategies to reduce NTDs with folic acid include supplement use and food fortification. The Canadian, American, and Chilean governments have mandated the fortification of enriched grains with folic acid; moves that have been associated with a temporal decline in NTD rates.⁷⁻⁹ The success of a national mandatory fortification program depends on uniform processing of the staple food. In regions where there is limited centralized control of food manufacturing this might be difficult.

An alternative fortification strategy is the use of foods targeted to women planning a pregnancy. Several companies sell milk fortified with folic acid for use prior to and during pregnancy. These milks when consumed as directed provide approximately 400 µg/d folic acid and may be protective against NTDs. Whether folic acid fortified milk will lower NTD risk is not known. However, in a case-control study of 56,049 women in Ireland, Daly *et al.*, found a graded reduction in NTD rate with increasing

plasma and red blood cell folate concentration.¹⁰ NTD rate was 6.6/1000 births in women in the lowest fifth of red blood cell folate concentration and 0.8/1000 births in women in the highest fifth. Similarly, NTD rate was 3.7 and 0.9/1000 births in the lowest and highest fifth of plasma folate. If fortified milk increases blood folate concentration in women of childbearing age it would be expected to decrease NTD risk.

Plasma homocysteine is a functional indicator of folate status. An elevated circulating homocysteine concentration has been associated with an increased risk of a number of adverse pregnancy outcomes including NTDs,¹¹ recurrent miscarriage and placental abruption.¹² Homocysteine is inversely correlated with blood folate levels and taking folic acid either as a supplement or in fortified food has been shown to lower homocysteine concentration.^{13, 14}

The aim of this study was to determine whether daily consumption of milk fortified with ~400 µg folic acid over 12 weeks increases blood folate concentration and lowers plasma homocysteine concentration in women of childbearing age.

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Participants and Methods

Subject recruitment

Seventy-three female volunteers from Dunedin aged 18–45 years were recruited through advertisements in local newspapers and signs placed around the university. Women were excluded if they consumed vitamin and/or mineral supplements in the previous three months, consumed folic acid or iron fortified foods greater than three times per week during the previous three months, or if they had established chronic disease. We also excluded women who had been pregnant in the previous year, were planning a pregnancy, or had a prior history of a NTD affected pregnancy. Participants were advised to withdraw from the study if they suspected they were pregnant. The Human Ethics Committee of the University of Otago approved the study and all women gave written and informed consent to participate.

Intervention

This was a 12-week, double blind, randomized controlled trial. Women were asked to attend an early morning clinic at the Department of Human Nutrition (Week 0). Women were randomized to one of two treatment groups (fortified or control milk). The women were weighed and had their height measured. Participants were asked to complete a demographic and lifestyle questionnaire. Women were provided with verbal and written instructions on how to prepare the milk powder. The participants were instructed to avoid foods fortified with folic acid and iron for the duration of the study. Women were asked to return to clinic 6 weeks and 12 weeks after baseline and to return any unused milk powder. Participants were asked to complete a diary of their milk consumption and compliance was assessed by dividing the number of servings of milk consumed by the number of possible servings. Participants were asked to complete a three-day weighed diet record between weeks 6 and 9. Energy and nutrient contents of the diets were calculated based on the New Zealand Food Composition Database.¹⁵

Milks

New Zealand Milk Ltd (Auckland, New Zealand) provided the folic acid fortified milk (Anmum™ is a trademark of New Zealand Milk Brands Ltd, Auckland, New Zealand) and the control milk. The control milk powder was a mixture of whole milk and skim milk powders that was blended to match the fat level of the fortified milk (Table 1). Participants were instructed to consume 75g of milk powder daily as 37.5g powder in 200ml water twice daily (morning and evening).

Laboratory assessment

Blood samples were collected at weeks 0, 6 and 12 by venipuncture after a 10–12h overnight fast. Blood was drawn from each participant into tubes containing EDTA. For the whole blood folate analyses an aliquot of whole blood was diluted 10-fold in 1% ascorbic acid to lyse the cells and protect the folates from oxidation. A complete blood count was determined on freshly collected blood using a Cell Dyn 1200 Hematology Analyzer (Abbott

Laboratories, Abbott Park, IL). Plasma was obtained by centrifuging the whole blood for 15 minutes at 1200 x g. An aliquot of plasma was diluted 1 in 20 with 0.5% sodium ascorbate for plasma folate analysis. Blood samples were stored at –80°C until analysed.

Plasma folate and whole blood folate concentrations were determined using the microtiter technique with chloramphenicol resistant *Lactobacillus casei* as the test microorganism.¹⁶ Whole blood standard (National Institute for Biological Standards and Control, UK) with a certified folate concentration of 29.4 nmol/L was used to generate a standard curve. Red blood cell folate was calculated from whole blood folate by subtracting plasma folate and correcting for hematocrit. The intra-assay coefficient of variation was 2.7% for plasma ($N=6$) and 2.4% for whole blood ($N=6$), based upon repeated measurements of pooled samples. Plasma total homocysteine and vitamin B₁₂ were determined using an Abbott IMx analyzer, reagents, calibrators, and controls (Abbott Laboratories, Abbott Park, IL).

Statistical analysis

The study was analysed as intent to treat. Baseline characteristics and compliance between treatment groups was compared using a one-way ANOVA for continuous variables and chi-square analyses for categorical variables. The difference in folate concentration between the treatment group and the control group at week 12 were determined by regression analysis, controlling for baseline values. Plasma homocysteine concentrations were log-transformed to better fit a normal distribution. For homocysteine the difference between the intervention and control groups is expressed as a ratio. We converted this ratio into a difference in homocysteine concentrations. Statistical analyses were performed using Version 10 of SPSS for Macintosh software (SPSS Inc, Chicago).

Results

Of the 73 women randomized to treatment, seven withdrew from the study, five in the fortified milk group and two in the control milk group. Women withdrew for the following reasons; pregnancy ($N=1$), anaemia ($N=1$), gastrointestinal disturbances ($N=2$), dislike of the milk ($N=3$). The majority of participants were non-smoking, young adult women of European ethnicity. Participant characteristics of the two treatment groups at baseline were not different (Table 1). Energy, natural folate, and vitamin B₁₂ intakes estimated between weeks 6 and 9 did not differ between groups. Compliance and weight change were not different between groups ($P > 0.05$).

Plasma and red blood cell folate concentrations at baseline were not different between the two groups (Table 2). Drinking the fortified milk caused red blood cell folate concentrations to rise markedly so that by week 12 the mean concentration was 539 nmol/L (436, 641) higher in women consuming the fortified milk than women consuming the control milk. Mean plasma folate concentration in participants consuming the fortified milk was 35 nmol/L (30, 41) higher at week 12 than in women consuming the control milk. Women consuming the fortified milk had 14% (6,21) mean lower plasma homocysteine

Table 1. Energy, macronutrient, and vitamin composition of placebo and fortified milk powder per 75 g

	Placebo Milk	Fortified Milk
Energy (kJ)	1326	1340
Protein (g)	23.0	24.2
Carbohydrate (g)	36.0	34.4
Fat (g)	8.8	9.0
Folate (DFE)	38	680
Natural folate (μg)	38	38
Folic acid (μg)	0	375
Vitamin B ₁₂ (μg)	2.4	2.4

DFE = dietary folate equivalents [natural folate (μg) + 1.7 x folic acid (μg)]

concentration at week 12 than women consuming the control milk.

Participants receiving the fortified milk were divided into three groups according to baseline red blood cell folate concentrations; these rose by 542, 573, and 402 nmol/L in the three groups from lowest to highest baseline concentrations, respectively (Fig. 1). The correlation between red blood cell folate concentrations at baseline and week 12 was 0.6.

Discussion

To reduce NTD risk, women planning pregnancy are advised to consume 400 μg folic acid per day either as a supplement or fortified food starting before pregnancy until the end of their first trimester.⁴⁻⁶ Taking folic acid increases blood folate concentration. Daly *et al.*,¹⁰ found a graded reduction in NTD risk with increasing blood folate concentrations. We were interested in determining whether daily consumption of milk fortified with 375 μg folic acid increases women's blood folate concentration. Red blood cell and plasma folate concentrations were 539 nmol/L and 35 nmol/L higher, respectively, in women drinking the fortified compared with control milk after 12 weeks. This increase would be expected to decrease NTD risk if these women were to become pregnant.

The red blood cell folate concentrations in women consuming the fortified milk appeared not to have reached a plateau by 12 weeks. The length of time to reach a steady state of red blood folate concentration after increasing folic acid intake is greater than three months.¹⁸ Therefore, it is possible that red cell folate concentrations would have increased further with continued use of fortified milk. This is important because the relationship between NTD and red blood cell folate is continuous with no apparent threshold. Although we did not include a folic acid supplement treated group in our study a comparison of our results with other studies of similar duration and dose of folic acid indicate that

Table 2. Characteristics of study participants in each treatment group. Values are means (95% CI) unless otherwise stated.

	Placebo Milk (N = 37)	Fortified Milk (N = 36)
Baseline characteristic		
Age (years)	28.8 (26.3, 31.3)	28.0 (25.5, 30.6)
Weight (kg)	64.9 (61.2, 68.7)	67.4 (63.2, 71.2)
BMI (kg/m^2)	23.7 (22.4, 25.0)	23.3 (22.9, 25.8)
Smokers, N (%)	1 (8.3)	2 (5.6)
Ethnicity		
European, N (%)	33 (91.7)	29 (78.4)
Asian, N (%)	2 (5.6)	6 (16.2)
Indian, N (%)	1 (2.8)	2 (5.4)
Serum vitamin B ₁₂ (pmol/L)	292 (262, 323)	309 (274, 345)
Daily Dietary intakes (weeks 6-9)		
Energy (MJ)	8.1 (7.3, 8.3)	8.5 (8.0, 9.0)
Folate (DFE)	232 (203, 261)	889 (862, 915)*
Vitamin B ₁₂ (μg)	4.8 (4.4, 5.2)	5.5 (4.5, 6.4)
% Compliance to treatment	96 (95, 98)	95 (92, 98)

* $P < 0.05$ compared to placebo group.

bioavailability of folic acid from milk is good relative to folic acid from supplements. In a recent study we asked women of childbearing age to take a daily supplement containing 400 μg folic acid or placebo.¹⁷ Mean (95% CI) red blood cell and plasma folate concentrations were 411 nmol/L (325-504) and 44 nmol/L (39-49) higher, respectively, in the supplemented women than in the women receiving the placebo. Similarly, Cuskelly *et al.*, randomized women to receive a placebo, a daily supplement containing 400 μg folic acid, or daily consumption of fortified foods containing ~400 μg folic acid for 12 weeks.¹⁹ Mean red blood cell folate concentration increased by 320 nmol/L in the supplemented women and 392 nmol/L in the women consuming the fortified foods.

Red blood cell and plasma folate concentrations fell in the control group by 126 and 6 nmol/L, respectively. This fall was expected because participants were asked not to eat folic acid fortified foods during the study. There is no mandatory folic acid fortification of a staple food in New Zealand, but many breakfast cereals and a few other foods are fortified with folic acid.²⁰ We did not attempt to estimate folic acid intake from fortified foods at baseline.

However, the red blood cell and plasma folate concentrations were similar in both groups at baseline suggesting that dietary intakes did not differ markedly. Presumably the removal of these fortified foods from the participant's diets would have lowered blood folate concentrations to a similar extent in the treated and the control groups. The difference in blood folate concentrations between groups reflects the effect of the fortified milk in the absence of other fortified foods.

Table 3. Mean (95% CI) plasma and red blood cell folate, and homocysteine concentrations during the trial*

	Baseline	Week 6	Week 12	Week 12 adjusted for baseline	Ratio	Adjusted difference [†]
Plasma folate (nmol/L)						
Placebo milk	21 (19, 23)	18 (16, 21)	16 (14, 18)	16 (12, 20)		
Fortified milk	21 (18, 23)	48 (42, 53)	51 (46, 56)	51 (47, 55)		35 (30, 41)
Red blood cell folate (nmol/L)						
Placebo milk	867 (773, 961)	754 (692, 816)	741 (672, 810)	724 (652, 795)		
Fortified milk	834 (754, 913)	1023 (955, 1091)	1245 (1154, 1335)	1262 (1189, 1335)		539 (436, 641)
Plasma homocysteine ($\mu\text{mol/L}$) [‡]						
Placebo milk	9.0 (6.9, 11.2)	-	8.9 (6.8, 11.0)	8.7 (8.2, 9.3)		
Fortified milk	8.6 (6.5, 10.7)	-	7.4 (5.2, 9.5)	7.5 (7.1, 8.0)	0.86 (0.79, 0.94)	

*Placebo group, $N=37$; fortified milk group, $N=36$. [†]Mean difference (95%CI) determined by regression analysis adjusting for baseline values. [‡]Values are geometric means (95% CIs).

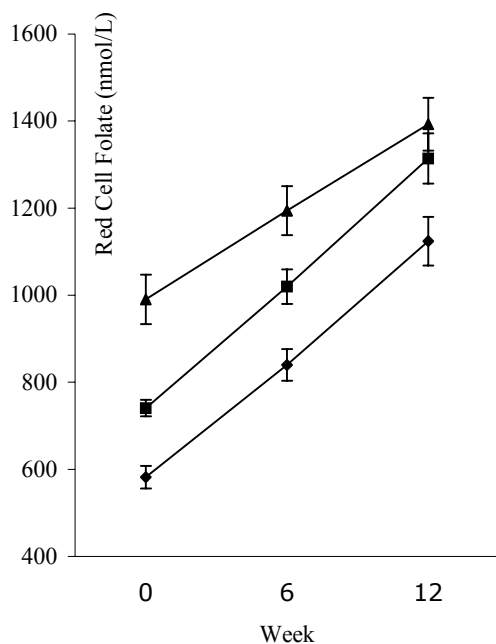


Figure 1. Mean (SE) red blood cell folate in the women receiving folic acid fortified milk by third of baseline red blood cell folate ($N=36$).

It is important to recognize that the study population consisted primarily of New Zealand European women who were recruited from the university and through newspaper advertisements, whereas, the target population for this milk is primarily Asian women of lower socio-economic status. The target population in Asia may have lower folate status. To determine whether folic acid fortified milk raised blood folate concentrations in women irrespective of their initial folate status, we divided the women receiving fortified milk into thirds according to baseline red blood cell folate concentration. Red blood cell folate increased in women consuming the fortified milk in both the lowest (420 - 660 nmol/L) and highest (830 - 1482 nmol/L) third of baseline red blood cell folate (540 cf 403 nmol/L). Therefore, it appears that fortified milk is effective at increasing red blood cell folate in all women regard-less of their initial folate status.

Another well-described effect of increasing folic acid intake is lowering blood homocysteine concentrations. The mean decline in homocysteine concentration in our study in women receiving the fortified milk was 1.2 $\mu\text{mol/L}$ (14%). This reduction is similar to that found in other studies using folic acid supplements in women of childbearing age. We previously reported a 1.4 $\mu\text{mol/L}$ decline in plasma homocysteine in women who took daily folic acid supplements containing 400 μg folic acid for 12 weeks.²¹ Similarly, a 1.4 $\mu\text{mol/L}$ reduction in homocysteine concentration was reported in young women following 12 weeks of daily supplementation with 400 μg folic acid.²² Thus, we have demonstrated that folic acid added to milk increases blood folate and decreases plasma homocysteine concentrations to a similar extent as folic acid provided as supplements.

Randomised controlled trials in the United Kingdom, Hungary and China have convincingly demonstrated the benefits of folic acid supplementation in reducing the risk

of bearing a fetus with a neural tube defect.¹⁻³ Folic acid fortification has increased mean blood folate status in the US and Canada and has been associated with reduced incidence rates of NTD.^{7,8,23,24} Drinking milk providing ~400 μg folic acid per day increases blood folate concentrations. Fortified milk is promoted as a source of folic acid for the prevention of NTDs in several countries. Our study provides reassurance to women that consuming fortified milk before and during early pregnancy will reduce NTD risk.

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