Fortification of flours with folic acid reduces homocysteine levels in Brazilian women
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Received 10 May 2011; revised 6 September 2011; accepted 12 October 2011

Abstract

Our hypothesis is that the fortification of flour with folic acid contributes to the reduction of plasma homocysteine (Hcy). We conducted a cross-sectional study covering 2 periods, before and after fortification (2002-2003 and 2008-2009, respectively), to assess the influence of the consumption of corn and wheat flours prefortification and postfortification with folic acid on Hcy levels and other biomarkers. In the total, 93 women (38 prefortification and 55 postfortification) were included. Levels of lipids and glucose, total Hcy and serum folate, and cobalamin were determined using commercial kits by colorimetric method, competitive immunoassay, and chemiluminescence, respectively. The participants’ average age was 48.1 ± 9.5 years for the prefortification group and 39.1 ± 4.1 years for the postfortification group (P < .001) but adjusted statistical tests by age. Both groups presented obesity class I. In the prefortification group, 71.1% (n = 27) of women had a dietary intake of folate, which was lower than the current recommended for adults (<400 μg/d), whereas in the postfortification group, only 16.4% (n = 9) of women had lower intakes than recommended. In the prefortification group, 42.1% (n = 16) of women had hyperhomocysteinemia (>10 mmol/L) compared with only 9.1% (n = 5) in the postfortification group. Moreover, statistically significant differences were found between the 2 groups in total cholesterol, high-density lipoprotein, triglycerides, and dietary fiber. Our findings corroborate the hypothesis that fortification of wheat and corn flours with folic acid can possibly be associated with lower concentrations of plasma Hcy, providing probable greater cardiovascular protection in this group.

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Keywords: Hyperhomocysteinemia; Folic acid; Cardiovascular disease; Obesity; Human
Abbreviations: BMI, body mass index; CVD, cardiovascular disease; FFQ, food frequency questionnaire; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein; MS, metabolic syndrome.

1. Introduction

It is estimated that folic acid can reduce the risk of ischemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24%. Although the causal association between homocysteine (Hcy), folate, and stroke cannot be deduced from epidemiological observations, available data reinforce the hypothesis that folic acid fortification helps to reduce mortality from stroke by at least the level of primary prevention [1,2]. Because of the lower bioavailability of folic acid from food, it is unlikely that only a diet could be sufficient to increase the plasma concentrations of folate and reduce the
and wheat flours with folic acid and iron (150 μg/100 g of product) to increase the daily intake of this vitamin in the general population, with emphasis on women of reproductive age [8]. In Brazil, this fortification is performed, the bioavailability of this vitamin for intestinal absorption, when in the form of supplements or fortified food, is approximately 85%, whereas for dietary folate, the bioavailability is approximately 50% [5].

Folate deficiency affects a substantial proportion of the population, especially adolescents, the institutionalized elderly, and people of lower classes [6]. In addition, the folate seems to react with some medications such as antacids, oral contraceptives, anticonvulsants, aspirin, and its derivatives, which increases gastric pH, forming complexes poorly absorbed by decreasing the bioavailability of this vitamin [7].

The US Food and Drug Administration implemented in 1998, a program to fortify whole flour and cereal products with folic acid (140 μg/100 g of product) to increase the daily intake of this vitamin in the general population, with emphasis on women of reproductive age [8]. In Brazil, this practice was adopted in June 2004 following a resolution of the National Agency for Sanitary Vigilance to fortify corn and wheat flours with folic acid and iron (150 μg and 4.2 mg of 100 g of flours, respectively) [9].

Although the rules of mandatory fortification of wheat and corn flours with folic acid were approved in Brazil, research conducted by Soeiro et al [10] showed that concentrations of folic acid were lower in samples of wheat flours. However, corn flours presented extremely high values than that recommended in the Brazilian legislation. In addition, poor homogenization of folic acid in enriched flours was observed [10].

In a population that consumes folic acid supplements or has a diet fortified with this vitamin, the upper reference limit of Hcy is usually 20% to 25% lower than in unfortified populations. A study investigating the association between hyperhomocysteinemia and cardiovascular disease (CVD), serum folate, and cobalamin should also be analyzed [11].

The effectiveness of folic acid fortification in improving folate status has already been shown to be quite striking, with a dramatic increase in blood measurements of folate and a substantial decrease in plasma Hcy levels in the United States [12].

In Brazil, no study has been conducted comparing the plasma concentrations of Hcy before and after the fortification of flour with folic acid in women with metabolic syndrome (MS). Thus, the hypothesis of this research is that the fortification of flour with folic acid contributes to the reduction of plasma Hcy levels. Therefore, the objective of this study is to assess the effect of the consumption of corn and wheat flours fortified with folic acid on Hcy levels and other biomarkers in women by a cross-sectional study covering 2 periods, prefortification and postfortification in Brazil.

2. Methods and materials

2.1. Subjects

A cross-sectional study was conducted in which participants were recruited from 2 different stages: prefortification (2002-2003) and postfortification (2008-2009) of flours with folic acid.

The study was performed with patients of the Nutritional Ambulatory Care of the Federal University of Rio de Janeiro, Brazil, where overweight patients with chronic diseases were being treated. They belonged to the lowest social classes and were residents in several cities in the state of Rio de Janeiro. Only women with MS were selected for this study because they surpassed men in rates of cardiovascular mortality in Brazil.

The 38 volunteers from the prefortification stage were selected from a study with 93 individuals, which were designed to assess the factors associated with Hcy levels in individuals with and without MS [13]. From this study, only women who met the inclusion criteria were included in this research. The 55 volunteers from the postfortification stage were selected from a study of 133 women. This study was designed to assess the association between concentrations of Hcy and biomarkers of MS. Included were those who filled out a food frequency questionnaire (FFQ) [14]; the others were excluded.

In both studies, after the screening, the explanation of the research was given to the patients who met the eligibility criteria. They signed a statement of consent and filled out a general information questionnaire. Subsequently, blood draw and anthropometric measurements were performed. Lastly, the FFQ was applied.

2.2. Subject criteria

Inclusion criteria were the following: women nutritionally diagnosed as overweight, obesity classes 1 and 2, with body mass index (BMI) from 25.0 to 39.9 kg/m² [15], of any race, and aged between 23 and 59 years. Those women with clinical diagnosis of diabetes mellitus, who used vitamin supplements, with presence of renal, liver, or heart failure, and who were pregnant and lactating were excluded.

General information about age, smoking habit, socioeconomic status, family history, medical history, and the use of supplements and drugs were obtained through a questionnaire developed by the researchers.

2.3. Nutrient intake

The usual dietary intakes of folic acid, cobalamin, pyridoxine, cholesterol, fiber, alcohol, and coffee were assessed through the FFQ (which contains 81 food items) [14] because these can influence Hcy levels in accordance to scientific literature.
In the prefortification group, food not fortified with folic acid was not considered in the analysis of the FFQ, whereas in the postfortification group, fortified food with folic acid was considered. Nutrient analysis was performed using the program “The Food Processor” (Esha Research, Salem, Mass., USA) [16], adapted to the Brazilian reality.

The evaluation of the folic acid content in the prefortification group, selected in the program Food Processor food, was not fortified with this vitamin. For the postfortification group, we used food fortified with folic acid.

2.4. Clinical and anthropometric measurements

Body weight (kilograms) and height (meters) were measured using the Filizola platform scale and a Filizola vertical stadiometer, respectively [17]. Body mass index was calculated as weight divided by square height (kg/m²) [15]. Waist circumference was measured at the midpoint between the last rib and the iliac crest using an inelastic metric tape [18]. The pressure levels were measured with a mercury sphygmomanometer [19].

2.5. Biochemical measurements

Blood samples were drawn after a 12-hour overnight fast and were placed into tubes that did not contain anticoagulant or with ethylene diamine tetra-acetic acid (Vacutainer, Becton Dickinson, NJ, USA). Aliquots of serum and plasma samples were obtained by centrifugation at 4000 rpm for 15 minutes (Centrifuge Excelsa Baby I; Fanem, São Paulo, Brazil) and stored at −20°C until analysis. The analyses of the prefortification group were performed at the end of the blood draw from all participants in 2003, and the analyses of the postfortification group were performed at the end of the blood draw of all participants in 2009.

Serum concentrations of glucose [20], triglycerides [21], high-density lipoprotein cholesterol (HDL-C) [22], and total cholesterol [23] were determined by enzymatic method, according to the manufacturer’s instructions (CELM and KATAL kits; Katal Biotechnologica, Ind, Com, Ltda, Minas Gerais, Brazil, and CELM-Cia; Equipadora de Laboratorios, Moderneros-Sao Paulo, Brazil). The following values were considered normal as indicated by the manufacturers: glucose less than 100 μg/dL, triglycerides less than 150 mg/dL, HDL-C greater than 50 mg/dL, and total cholesterol less than 200 mg/dL. Low-density lipoprotein cholesterol (LDL-C) was calculated [24], the ideal reference value being less than 100 mg/dL.

Plasma concentrations of cobalamin were determined by automated chemiluminescence method, using the IMMULITE 2000 kit (Siemens AG, Munich, Germany), with normal values greater than 120 pmol/L [25]. Concentrations of plasma folate and Hcy were determined by competitive immunoassay [26] with the IMMULITE kit, with the values greater than 7 nmol/L and less than 10 nmol/L, respectively, which were considered as appropriate values [25,27].

2.6. Ethical aspects

The women were fully informed about all the procedures before they signed a statement of consent. The protocols of both studies (CEP: 017/03 and CEP: 017/08, respectively) were approved by the Research Ethics Committee of Clementino Fraga Filho University Hospital at the Federal University of Rio de Janeiro.

2.7. Statistical analyses

Data are presented as means, SDs, medians, P_{25}, and P_{75}. The groups were compared using the Mann-Whitney U test. To verify a statistically significant association between categorical variables according to the classical cutoffs, the χ² test was used to compare the 2 groups. In addition, we carried out the adjustment for age for the Hcy, cobalamin, dietary, and serum folate by linear regression. The Spearman correlation was calculated between continuous variables in both groups. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 17.0 (SPSS, Inc, Chicago, Ill, USA) [28]. Differences were considered significant at P < .05.

3. Results

A total of 93 women (38 prefortification and 55 postfortification) were included in the study. The participants’ average age was 48.1 ± 9.5 years, with a median of 51 years, in the prefortification group, and 39.1 ± 4.1 years, with a median of 40 years, in the postfortification group (P < .001).

Both groups were obese class I [15], characterized by the accumulation of visceral fat [29], with average BMI of 31.9 and 32.8 kg/m² and waist circumference of 100.7 and 101.6 cm in the prefortification and postfortification groups, respectively.

Table 1 shows biochemical and dietary variables in prefortification and postfortification of flours with folic acid and the percentages of the adequacy in the same variables. In the prefortification group, 71.1% (n = 27) of the women had a lower dietary intake of folate than the current recommended for adults (<400 μg/d), whereas in the postfortification group, only 16.4% (n = 9) of the women had lower intakes than recommended [30]. In the prefortification group, 42.1% (n = 16) of the women had hyperhomocysteinemia (>10 μmol/L) [27], against only 9.1% (n = 5) in the postfortification group. Differences were also found between the 2 groups for the following continuous variables: total cholesterol, HDL-C, triglycerides, and dietary fiber.

Table 2 shows the Spearman rank correlation coefficient for clinical and dietary characteristics in relation to variable Hcy, with significant correlations marked in bold. In the prefortification group, plasma concentrations of Hcy correlated positively with age. In the postfortification group, total Hcy levels were negatively correlated with HDL-C and
The difference of the mean adjusted by age in the main variables, prefortification and postfortification of flours with folic acid, is presented on Table 3. The mean of plasma Hcy levels, when adjusted by age, was 2.5 mmol/L lower in the postfortification group in relation to the prefortification group, with statistically significant difference between groups.

Fig. 1 shows the correlation between Hcy with folate intake prefortification and postfortification of flours, with plasma Hcy levels decreasing proportionally to increased intake of folate in both groups.

Fig. 2 shows the correlation of Hcy levels with plasma folate prefortification and postfortification of flours. Plasma Hcy decreased with increasing plasma concentrations of folate, but the reduction of Hcy was more accentuated in the postfortification group.

4. Discussion

Because of the lower bioavailability of folic acid from food, it is unlikely that only a diet could be sufficient to increase the plasma concentrations of folate and reduce the concentration of Hcy [3].

US folic acid fortification of enriched cereal grain products is credited with an increase in blood folate concentrations and in reducing the risk of cardiovascular disease and stroke [31]; however, more research is needed to know for sure. Along with these benefits, concerns have also been raised about the potential for adverse effects of high levels of folic acid, such as increased risks of certain cancers, the exacerbation of neurologic effects from exposure to very high doses of folic acid, and cognitive decline [31,32].

Although folic acid is safe and almost free of toxicity, fortification with folic acid might mask symptoms of
cobalamin deficiency primarily in the elderly population, contributing to the development of progressive and irreversible neurologic damage. This problem could be solved with double fortification with folate and cobalamin [33], but in Brazil, the fortification was made with folic acid and iron.

The previous reports explain the discrepancy between the epidemiological observations that suggest a reduction of cardiovascular risk in North America after folic acid fortification and an emerging body of evidence that folic acid supplementation may enhance the development and progression of adverse effects on most of the population that are not the main target of the fortification [31].

In our study of both groups, we found a significant positive correlation between the consumption of folate, pyridoxine, and dietary fiber, which were also the results observed in a Norwegian study that included 5812 men and women, in which the consumption of these vitamins was positively correlated with the intake of vegetables, fruits, fiber, and most vitamins [34].

Data of Pesquisa de Orçamentos Familiares 2008-2009 [35] show an increase of 9.52% in fruit consumption and 10% in consumption of vegetables by the Brazilian population, compared with the period 2002 to 2003, which corresponds to the groups prefortification and postfortification, corroborating the significant increase in the dietary intake of folate, pyridoxine, and fiber found in the postfortification group. Our findings show that after fortification, 83.6% of volunteers had an adequate dietary intake of folate in contrast to only 28.9% in the prefortification group, a fact that indicates the beneficial effect of fortification.

In the postfortification group, 98.2% and 92.7% of volunteers showed adequate plasmatic concentrations of cobalamin and folate, respectively, whereas in the prefortification group, these percentages were 72.4% and 80.6%, respectively. Similar results were found in a study of children in the United States, which showed that after fortification, the intake of cereals ready for consumption or supplements containing folic acid increased the daily intake and serum concentrations of folate and cobalamin [36]. Selhub et al [37], in a study on the US population before and after fortification, showed that Hcy levels do not generally decrease with increasing concentrations of folate among persons with low serum cobalamin. On the other hand, an intervention study conducted with healthy male subjects showed that a system of fortification with 200 μg/d of folic acid, which can be achieved by food fortification, would be effective in reducing Hcy level [38].

### Table 3

<table>
<thead>
<tr>
<th>Variables</th>
<th>Prefortification (n = 38)</th>
<th>Postfortification (n = 55)</th>
<th>Prefortification vs postfortification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Age unadjusted mean difference, SD</td>
</tr>
<tr>
<td>Hcy, μmol/L</td>
<td>9.5 2.7</td>
<td>6.1 3.6</td>
<td>−3.4 −2.5</td>
</tr>
<tr>
<td>Plasma folate, nmol/L</td>
<td>15.7 10.7</td>
<td>12.1 3.8</td>
<td>102.8 0.1</td>
</tr>
<tr>
<td>Plasma cobalamin, pmol/L</td>
<td>362.3 257.9</td>
<td>465.0 362.3</td>
<td></td>
</tr>
<tr>
<td>Dietary folate, μg/d</td>
<td>317.0 136.9</td>
<td>641.7 248.7</td>
<td></td>
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</tbody>
</table>

*P < .05, significant difference between groups.*
We did not observe association between Hcy concentrations and the practice of physical activity; the same result was observed in another study in adults of both sexes, without any chronic illness, in Greece [39].

Observational study in humans showed an inverse correlation between the concentrations of Hcy and HDL-C, an inverse association between HDL-C and CVD, and a positive correlation between Hcy and CVD [40]. The results of the present study showed an inverse correlation, although not significant, between Hcy concentration and the concentration of HDL-C, possibly because the sample was smaller. The differences between the 2 groups in concentrations of total cholesterol, HDL-C, triglycerides, and dietary fiber suggest greater cardiovascular protection in the postfortification group, possibly due to an increased consumption of food rich in fiber.

Nevertheless, it is necessary to point out some limitations of this research. The studies from which the selected women were included were not developed for this purpose and the members of the 2 groups were not the same. However, all procedures were performed with the same technique, equipment, and by the same researchers in both groups. The women from the prefortification group were much older than those from the postfortification group. The Hcy difference found in both groups could have resulted from age difference. To minimize the limitation of age difference between the 2 groups, the main study variables were adjusted by age. The analysis of folate in erythrocytes was not carried out due to unavailability of the analysis in both groups.

In conclusion, our findings corroborate the hypothesis that fortification of wheat and corn flours with folic acid can be possibly associated with lower concentrations of plasma Hcy, providing probable greater cardiovascular protection in the postfortification group. Further studies are needed to monitor the optimum amount of folic acid to be used for fortification and verify whether these programs will result in decreased cardiovascular risk in the future.

Acknowledgment

The authors thank the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (Research funding agency of the State of Rio de Janeiro) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (government agency linked to the Brazilian Ministry of Education in charge of promoting high standards for postgraduate courses in Brazil) for financial support.

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