Introduction

Adequate folate intake during the periconceptional period (the time just before and just after a woman becomes pregnant) helps protect against a number of congenital malformations, including neural tube defects (NTDs), which are the most notable birth defects due to folate deficiency [1]. Birth defects (congenital anomalies)
are the leading cause of death in babies under 1 year of age. NTDs are the second most common type of birth defect after congenital heart defects [2], and arise during the process of neurulation, between the 17th and 30th post-fertilization days. Interruption of DNA biosynthesis or methylation reactions can prevent the proper closure of the neural tube. Such inhibition could be caused by a simple deficiency of folic acid [3]. Many countries are considering folic acid fortification to ensure sufficient intake of folic acid for women of childbearing age, because many women who plan their pregnancies do not take folic acid supplements in the period advised and many pregnancies are unplanned. A periconceptional increase in the intake of folic acid by women of childbearing age reduces the risk of having an infant with a neural tube defect. Food fortification with folic acid as an intervention seems likely to result in an increased folate intake among populations throughout the world, especially in less-developed countries [4].

Deficiencies of micronutrients (especially iron, zinc, and vitamins A and D) have been reported as one of the most common nutritional problems in Iran [5]. Food fortification along with the other strategies – including supplementation, nutrition education and public health measures – has been considered as a key strategy to prevent and control micronutrient deficiencies by the Ministry of Health and Medical Education. Wheat flour is highly subsidized by the government, and it is quite accessible to the whole population in both urban and rural areas. As a staple food, particularly in large and poor families that have fewer food choices, bread is an appropriate vehicle to be fortified with iron and folic acid in Iran. It is low cost and readily available.

In Iran, several studies have shown a high prevalence of NTDs. In the Khuzestan province in the southwest of Iran, an incidence of 4.2 per 1,000 births has been reported [6]. Also, a prevalence of 5.5 per 1,000 births was reported in Kordestan province in the northwest of Iran [7]. Considering the evidence on the role of folic acid in reducing the occurrence of NTDs and also the available experience, folic acid was added to wheat flour simultaneously with iron. Based on the World Health Organization recommendations, 1.5 ppm folic acid was added to the wheat flour; this is 150 μg per 100 g flour. Mandatory flour fortification with iron and folic acid was implemented in one province from 2001 and expanded simultaneously to the other provinces in Iran. A semi-quantitative test or spot test (AACC method 40–40, iron-qualitative method) is universally used in the flour mills to check whether flour is properly fortified [8]. In Iran, this test is being used at the level of mills to check the fortified flour. Also, a quantitative test is being used by applying the spectrophotometric method to measure the amounts of iron in fortified flour in the provincial food laboratory. The use of a single nutrient in flour as an index of all the micronutrients added by a fortification premix has been advised. Given that the indicator nutrient is within specifications, it follows that all the other micronutrients should be as well, providing that the fortification premix is correct. Iron is the most likely nutrient to be used as an indicator of adequate fortification in government checks [9].

Analysis of folate is not easy due to its multiple forms, lower stability and presence in low concentrations in biological systems, as well as complex extraction and detection techniques. Because of the difficulty and high cost of measuring the amount of folic acid in fortified flour routinely, it was decided to monitor iron as an indicator nutrient in the flour fortification program in Iran. No interventional trial on the efficacy of folic acid fortification has been conducted in Iran. Thus, it is necessary to study the efficacy of folic acid fortification with regard to folate status and NTD prevalence in Iran. In Golestan province in the north of Iran, flour fortification with iron and folic acid was started in 2006. In the present study, the efficacy of folic acid fortification in improving folate status in women of childbearing age and reducing NTD prevalence was studied.

**Methods**

This evaluative study was designed as a population-based study and included three components: one longitudinal hospital-based and two cross-sectional surveys carried out before flour fortification in December 2006 and after flour fortification in December 2008. A group of women of childbearing age, as a representative sample of Golestan province, was studied. This province has a population of about 1.8 million. The results of the second study were compared with baseline data. We included all healthy women aged 15–49 years from urban and rural areas. However, pregnant subjects, those who had taken supplements containing folic acid within the past month or were taking medication known to interfere with folate and vitamin B12 metabolism, and those with a history of chronic disease were excluded from the before and after cross-sectional studies. According to the sampling frame in rural and urban areas, the main cluster households were selected. Each cluster included 10 women aged 15–49 years. If there was not an eligible woman in the randomly selected household, the nearest house was selected to find a woman in this age group. In each selected household, only 1 woman aged 15–49 years old was studied (the first woman whom the interviewer contacted in the household). The sample size was 580 women at baseline, and this was rounded up to 600 in the final study.
Sociodemographic Data Gathering

At the beginning of each interview, the selected woman was provided with explanations about the project. After obtaining written consent, the interview was conducted and the woman was invited to come in the next morning to the rural or urban health center selected for collecting blood samples. All the women were advised to fast overnight (at least 12 h) before blood samples were taken. Information on sociodemographic and health characteristics was collected by trained interviewers with a structured questionnaire. The same questionnaire was completed at baseline and in the final evaluation. Approval for the study was obtained from the ethics committee of the Ministry of Health and Medical Education.

Dietary Assessment

Diet was assessed by a single 24-hour recall. Trained nutritionists from the provincial health center interviewed each subject individually about their food and beverage intake of the previous day, processed food items (if known), and the types and names of manufacturers. The field work covered all days of the week, including weekends. The portion sizes were described in household measures and for processed food items by using the manufacturer’s information. The diet records were coded by one of the principal investigators using an Iranian food composition database [10], modified using the new version of the UK food composition tables 1, 2 [11] for the folate contents of those food items that were not included in the Iranian food composition software. The final dietary intake data were validated in the Department of Nutritional Sciences, University of Vienna, Austria, by comparing the nutrient contents of selected food items, representative of the average Iranian diet, with the food composition data of the Austrian food composition database. The selected 42 food items included major food sources of folate (i.e. leafy green vegetables, fruits, pulses, cereals, dairy/egg products and nuts/seeds). The observed variation in the nutrient content was mostly 10–15% for 27 of these items, such as vegetables, fruits, dairy and bread. In some kinds of pulses (i.e. lentils and beans), the observed variation was up to 30%, but within the expected limits.

Blood Sample Collection

From each woman, a blood sample of about 10 ml was collected. To measure serum concentrations of folate, 5 ml of the blood sample was collected in a test tube, transferred to the provincial laboratory in cold boxes, and centrifuged (2,000 rpm for 15 min) immediately. For homocysteine determination in plasma, 2 ml of the blood sample was collected in a test tube containing 50 μl EDTA. The samples collected in rural areas were immediately centrifuged by a mobile centrifuge (1,700 rpm for 15 min) and transferred in cold boxes to the provincial laboratory, while the other blood samples were centrifuged in the provincial laboratory. Test tubes containing plasma were stored at −20°C and transferred to the National Reference Laboratory in Tehran to measure folate, vitamin B_{12} and homocysteine concentrations.

For the complete blood count, 2 ml whole blood was put in covered tubes containing 50 μl EDTA, and thoroughly stirred. These were transferred as quickly as possible (within 3 h) to the provincial laboratory.

Bread Sample Collection

Samples of bread were collected during the final evaluation in 2008 at the household level and during interviews with the randomly selected women. One sample of bread was collected from the home of the first women in each cluster; in total 60 bread samples were gathered from 60 selected clusters.

NTD Data-Gathering Technique

We established a hospital-based surveillance system in Dez-yani Teaching Hospital in Gorgan, capital city of Golestan province, to register NTDs. This is a referral hospital for neonatal intensive care and gynecological problems with an annual rate of more than 6,500 deliveries, accounting for 25% of annual births in Golestan province and the largest portion of deliveries (80%) in Gorgan district. Patients are usually from moderate-to-low socioeconomic class families. The longitudinal hospital-based study was performed between September 2006 (before flour fortification) and December 2008 (after flour fortification).

In this hospital, two members of staff (registered nurses) were recruited and trained to advise all births and register describe NTDs. Types of NTDs registered were anencephaly, encephalocele and spina bifida, associated or not with other malformations. If two NTD in a newborn occurred concomitantly, the anatomically higher defect was defined. A specially trained clinical geneticist monitored the correct registration of NTDs. Rate of NTDs was defined as the number of NTD cases divided by the total number of live births, stillbirths and pregnancy terminations due to NTDs. Total prevalence rates were calculated as the total number of NTDs per 1,000 births. Time trend analysis of monthly NTD rates was performed. We included all live births and stillbirths at the gestational age of ≥20 weeks and with newborns weighing ≥500 g among women admitted to neonatal intensive care unit of Dez-yani Hospital from September 2006 (before flour fortification) to December 2008 (after flour fortification). We defined NTDs according to the ICD criteria [12]. The design was based on a sample of 13,361 postpartum women after admission for childbirth. Data were collected through interviews with mothers in the immediate postpartum period, as well as by reviewing the patient records of both the mothers and newborn infants. The clinical records of the babies and their mothers were reviewed, and the following data were recorded: antenatal diagnosis and care, age, level of education, usage of folic acid supplement, smoking and type of NTD.

Analytical Methods

Serum folate and vitamin B_{12} levels were measured using the SimultRAC-SNB Radioassay Kit vitamin B_{12}/folate, and the results were validated by the National Reference Laboratory in Tehran. The same method for measuring serum folate and vitamin B_{12} was used in both before and after studies.

Determination of homocysteine concentration in overnight fasting blood plasma was carried out using high-performance liquid chromatography and fluorescence detection [13].

Reference values of Sauberlich [14] were used to define lower limits of adequate serum concentrations of folate and vitamin B_{12}, i.e. for folate <6.7 mmol/l and for vitamin B_{12} <110 pmol/l. An elevated plasma homocysteine concentration was defined as >12 μmol/l (moderate hyperhomocysteinemia) [15]. The recommended dietary allowances of 2.4 μg for vitamin B_{12} and 400 μg for food folate were used to define adequacy of intakes [16].
Reversed-phase ion-pair high-performance liquid chromatography was coupled with detection by UV absorption (280 nm) for separation and quantitation of added folic acid in fortified bread [17].

Statistical Analysis
Collected data on NTDs were analyzed by Stata version 10, and compared using the $\chi^2$ test. The rate ratio and 95% CI were estimated for effect of fortification. Values of $p \leq 0.05$ were considered statistically significant.

SPSS for Windows (version 13, SPSS Inc., Chicago, Ill., USA) was used for all statistical analyses of sociodemographic data, blood tests and measures of folic acid in the bread samples and intake data. Metric data were presented as means, SD and 95% CI. Changes in hematological indices and intake data from baseline before and after flour fortification were assessed using t tests.

Logistic and linear regressions (for numeric variables) were used to adjust for confounders. Statistical significance was set at $p \leq 0.05$.

Results
Completeness of Data
At baseline (pre-fortification period), the sample size was 580. One participant was excluded because of an uncompleted questionnaire. The final numbers analyzed for different types of variables were: 557 women for dietary assessment (22 subjects were excluded due to incompleteness of dietary data), 572 serum samples for folate and vitamin B$_12$ (7 samples were excluded due to clotting) and 561 plasma samples for homocysteine (18 samples excluded due to clotting). At the post-fortification period, the sample size was 600. We analyzed the results of 600 blood samples for serum folate, serum B$_12$ and plasma homocysteine. Dietary intake data were analyzed for 594 completed questionnaires.

Subjects
We compared the sociodemographic characteristics of the women in order to find out whether there were significant differences between their characteristics in the pre-fortification and post-fortification periods. There were no significant differences between baseline and the end between women according to: rural and urban areas ($p = 0.907$), district ($p = 0.847$), education level ($p = 0.429$), employment ($p = 0.193$) or age ($p = 0.474$).

Comparing the other confounding variables (table 1) showed that there were no significant differences between distributions of the women according to age ($p = 0.129$), smoking ($p = 0.147$), passive smoking ($p = 0.396$), history of stomach surgery ($p = 0.474$), contraceptive use ($p = 0.336$), lactation ($p = 0.323$) and marriage status ($p = 0.614$). In contrast, there were significant differences at baseline and after flour fortification in the household size ($p < 0.001$) and husband’s job (% of unemployed; $p < 0.02$).

In the logistic regression model, all biochemical indicators – including serum folate, serum vitamin B$_12$ and plasma homocysteine – were adjusted for those sociodemographic characteristics of the studied women that were found to be significant.

Dietary Intake Findings
The mean daily intakes of folate from natural food in the pre-fortification and post-fortification periods were 198.3 g/day (95% CI 185.4–211.3 g/day) and 200.8 g/day (95% CI 191.9–209.6 g/day), respectively. No significant difference between natural folate intake in the pre- and post-fortification periods was found ($p = 0.741$). Table 2 shows the mean natural food folate intake before and the mean total folate intake (natural folate + folic acid added to bread) after flour fortification. The folate intake increased significantly from 198.3 g/day before flour fortification to 413.7 g/day after flour fortification ($p < 0.001$).

We assessed the mean consumption of bread by women: 246.2 g/day (95% CI 234.2–259.1 g/day) before and 253.8 g/day (95% CI 242.3–265.4 g/day) after flour fortification. There was no significant difference before and after flour fortification ($p = 0.821$). The mean folic acid content for 60 bread samples collected in the post-fortifi-
The percentage of the women with a low folate intake (<400 μg/day) decreased from 93.8% before flour fortification to 52.9% after flour fortification. Conversely, the percentage of women with a folate intake above 400 μg/day increased from 5.6 to 46.8% after flour fortification (fig. 1).

**Biochemical Indicators of Folate Status**

The mean serum folate level at baseline was 13.6 nmol/l (95% CI 12.8–14.3 nmol/l); after flour fortification, this increased significantly (p < 0.001) to 18.1 nmol/l (95% CI 17.4–18.8 nmol/l). The prevalence of low serum folate levels (<6.7 nmol/l) decreased significantly from 14.3% before flour fortification to 2.3% after flour fortification (p = 0.000). In turn, plasma homocysteine was 12.6 μmol/l (95% CI 12.1–13.2 μmol/l) before flour fortification, which decreased significantly to 7.1 μmol/l (95% CI 6.7–7.5 μmol/l) after flour fortification (p < 0.001).

We found a significant reduction in the prevalence of hyperhomocysteinemia, from 38.3 to 7.3% before and after flour fortification (p < 0.001).

Figure 2 shows the distribution curves of serum folate concentrations before and after fortification. This figure shows the clear upward shift in the distribution of serum folate from baseline (before fortification in 2006) to the final evaluation (after fortification in 2008). Serum folate concentration was positively correlated with folate intake the pre- and post-fortification periods (r = 0.084; p < 0.05).

Figure 3 shows the distribution curves of plasma homocysteine. A clear downward shift in the distribution of plasma homocysteine concentrations from baseline to the final evaluation was observed.

**Trend in NTD Prevalence before and after Flour Fortification**

Between September 2006 (before fortification) and December 2008 (after fortification), 35 NTD cases were recorded, of which spina bifida was the most common type of anomaly (51.4%), followed by anencephaly (48.6%).

The overall prevalence of NTDs decreased from 3.16 per 1,000 births including live births and stillbirths before flour fortification (September 2006 to July 2007) to 2.19 per 1,000 births during the full fortification (December 2007 to December 2008) period (rate ratio 0.09, 95% CI 0.042–0.21) – the difference being statistically significant (p < 0.01).

The total annual rate of NTDs significantly declined by 31% (95% CI 26–35%) after the implementation of folic acid fortification (p < 0.01). Figure 3 shows the change in the average rate of NTDs after a period of 28 months (before and after flour fortification).
There were no significant differences between before and after the flour fortification periods with regard to mother’s age ($p = 0.579$), level of education ($p = 0.346$), taking folic acid supplements ($p = 0.481$), smoking ($p = 0.721$) and the incidence of NTDs.

**Discussion**

In this population-based study, we determined whether the response in serum folate and plasma homocysteine as markers of folate status were associated with fortified bread consumption. The results of the present study demonstrate that the mean intake of natural food folate is almost half of the recommended levels, indicating that the women of childbearing age can not reach to the optimal folate intake through diet. The mean daily intake of naturally occurring folate among the women studied (before and after flour fortification) is higher than the folate intake in Dutch women [18], but lower compared to Austrian [19] and Canadian [20] women. However, the folate intake of these Iranian women is similar to that of Finnish women [21].

In our study, the mean folate intake of the women after fortification (413.7 μg/day) is very close to the mean intake of women in Chile after implementing flour fortification with folic acid (427 μg/day) [22].

The results of the present study also indicate that flour fortification with folic acid increased folic acid intake by an average of 226 μg/day due to consumption of fortified bread by women of childbearing age.

The current study shows that there was no significant difference in folate intake from naturally occurring sources before and after flour fortification with folic acid, which suggests that any increase in folate status is the result of folic acid added to bread as part of the mandatory flour fortification policy in Iran. This is further supported by the finding that there was no significant difference

![Figure 2](image-url)
between the amount of bread consumption before and after flour fortification \((p = 0.821)\).

This indicates that an increase in the folate intake is because of the consumption of bread fortified with folic acid. Additionally, due to the non-availability of any other food products fortified with folic acid in Iran and the lack of use of folic acid supplements before pregnancy by women of childbearing age, we can conclude that the wheat bread fortified with folic acid was the main source of this nutrient in the population studied and improved folate intake as well as serum folate.

The results of this study are consistent with those reporting the potential of a mandatory fortification policy to improve folate intake and folate status in women of childbearing age [23] to a similar degree as achieved in this study.

In this study, serum folate and plasma homocysteine levels were used as biomarkers of folate intake and folate status. The results show that due to flour fortification with folic acid, the prevalence rate of low serum folate \(< 6.7 \text{ nmol/l}\) decreased by 84\% in the women.

We found an increase of 4.5 \(\mu\text{g/l}\) in serum folate under mandatory folic acid fortification in Golestan province, which is higher than the increase in serum folate (3.8 \(\mu\text{g/l}\)) under a mandatory folate fortification policy in Canada [24] and lower than in Chile (7.2 \(\mu\text{g/l}\)) [25].

Only those subjects who had fasted \(\geq 12\) h were included in the sample, since folate concentration measured in a fasted state is a better indicator of folate status, and there is evidence that serum folate in a fasting blood sample is as good for assessing folate status as red blood cell folate at the population level [23]. The advantage of measuring serum folate is that it is easier to perform and less expensive.

Folate intake was positively correlated with serum folate concentrations in the pre- and post-fortification periods. Serum folate is an indicator of recent intake [16] and the food intake data were collected through a 24-hour recall, also representing recent intake.

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**Fig. 3.** Prevalence of hyperhomocysteinemia before (a) and after (b) flour fortification with folic acid among the women. Vertical line = Homocysteine levels >12 \(\mu\text{mol/l}\).
Findings of the present study also showed a significant reduction in the mean homocysteine concentrations of the women by 56% (p = 0.000), which is in accordance with the findings of Jacques et al. [26] who reported a reduction of 52%. In contrast, Ganji and Kafai [23] reported a much lower reduction (16.1%) in the mean homocysteine concentrations after folic acid fortification in the USA. Furthermore, our findings showed a considerable reduction in the prevalence rate of hyperhomocysteinemia, from 38.3 to 7.3% in the population studied (88%). This improved folate status remained significant even after adjusting for sociodemographic characteristics of the women.

Serum folate concentrations demonstrate that with increasing folate intake due to fortified bread, there was a significant increase in the proportion of women achieving an optimal folate status (i.e. a serum folate value >6.7 nmol/l) [14].

It is not likely that the observed differences in serum folate status resulted from variations in folate intake unrelated to fortification. We adjusted serum folate values for folate dietary intake, and therefore we are confident that the changes seen in serum folate status can be ascribed to fortification of bread with folic acid. Blood folate levels are still an intermediate outcome, and the real measure of the impact of increased folic acid consumption is the reduction in NTDs rates. The current study shows a 31% reduction in the prevalence rate of NTDs after flour fortification with folic acid. This observation is similar to those reported from other countries where fortification is practiced. The reduction in the NTD rate after implementation of fortification in our study is greater than the 19% reduction in the USA after mandatory fortification [27], but lower than the 78% reduction in Newfoundland [20]. Similar to our results, a 30% reduction in NTDs in Nova Scotia has been found [28].

Thus, our findings support the view expressed by many investigators that mandatory flour fortification with folic acid is the only means of ensuring that all women of reproductive age can benefit in terms of reduced risk of NTDs. Decline in the NTD prevalence rate in our study was also consistent with the observed increase in serum folate levels and folic acid intake in the women of reproductive age after flour fortification with folic acid. Our findings also confirm that mandatory flour fortification in Iran would be effective in reducing NTD risk in women who regularly consume fortified bread.

The limitation of our study is that it was carried out in a health referral center and we did not include spontaneous abortions caused by NTDs, as it was difficult to obtain the data.

In Iran, the folic acid along with iron supplementation program for preventing nutritional anemia is being implemented for pregnant women through the primary health care system. According to this program, pregnant women receive folic acid supplements from the first visit (normally after 3 months of pregnancy), the time after closing neural tube, since almost all of the pregnancies are unplanned. In our longitudinal hospital-based study, 70% of women had taken folic acid supplements during pregnancy (according to the routine supplementation program) from the 3rd month, meaning that folic acid supplementation is not effective for preventing NTDs. To prevent NTDs, it is necessary to take folic acid supplements during the first month of pregnancy [29]. So, the reduction in NTD incidence in the studied women is not because of folic acid supplementation during pregnancy in Iran. Based on the findings of this study, it is concluded that fortification of flour with folic acid results in a significant reduction in NTD prevalence.

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