

Long-Term Consequences of Iron-Fortified Flour Consumption in Nonanemic Men

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Key Words

Iron flour fortification · Oxidative stress · Iron overload · Iron

Abstract

Background/Aims: Despite the advantages of fortifying flour with iron, there are still special concerns regarding the possible adverse effects of the extra iron consumed by nonanemic individuals. This study aimed to investigate the oxidative stress and iron status following 8 and 16 months of consumption of iron-fortified flour in nonanemic men. **Methods:** In a before-and-after intervention study, 78 nonanemic apparently healthy 40- to 65-year-old men were randomly selected from Semnan, in the northeast of Iran. Data were collected at three time points. Evaluation of oxidative stress biomarkers as well as the assessment of iron status was performed in all three stages. After baseline data collection, the flour fortification program was started with 30 mg/kg iron as ferrous sulfate. **Results:** After 16 months, serum iron levels had significantly increased from $102.9 \pm 31.5 \mu\text{g/dl}$ (baseline) to $117.2 \pm 29.8 \mu\text{g/dl}$ ($p < 0.001$). The mean total antioxidant capacity ($1.71 \pm 0.10 \mu\text{M}$) was significantly lower than that at baseline ($1.83 \pm 0.17 \mu\text{M}$; $p < 0.01$). Among other

oxidative stress biomarkers, only superoxide dismutase and glutathione peroxidase activity increased significantly compared to the beginning of the study ($p < 0.001$ and $p < 0.001$, respectively). The results of this study did not show any symptoms of iron overload after 8 and 16 months. **Conclusions:** Our data did not support the safety of flour fortification with 30 mg/kg iron as ferrous sulfate as a community-based approach to control iron deficiency in nonanemic healthy men.

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Introduction

In many countries, fortified foods are available on the market to help prevent marginal deficiencies of basic nutrient needs in risk situations for individuals as well as for certain population groups. Flour fortification with iron is now a feasible and cost-effective strategy to reduce the prevalence of iron deficiency in many countries all over the world, even in developed countries [1].

In spite of implementing iron supplementation, nutritional education and public health measures for more

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than two decades, iron deficiency and the resulting anemia is still one of the most common nutritional problems in the Islamic republic of Iran [2]. The extent and severity of anemia and iron deficiency, as well as the health and economic consequences, point to the need for prompt and efficient interventions [2, 3]. It is suggested that food fortification might be an inexpensive, simple and effective way to control and prevent iron deficiency and its related anemia in many countries [4, 5]. Bread consumption is high in most countries of the eastern Mediterranean region. Flour fortification offers an opportunity to deliver efficacious levels of iron to reduce the prevalence of iron deficiency and anemia and to cover a large part of the vulnerable population at a low cost [5]. For this reason, developing a national flour fortification plan was considered one of the priorities of community nutrition programs in Iran. Despite the advantages of this program [5], questions were raised by some nutritionists of steering committees and faculties from the beginning of the intervention regarding the potential hazards of adding iron to flour for those individuals who do not suffer from anemia or iron deficiency. Considering the naturally occurring iron in flour, the total amount of iron could sometimes be about 40–80 mg/kg after fortification, which is about two times more than what we expect from the fortification program. As the National Food Consumption Survey showed varying iron intakes from 3.6 to 68.9 mg/day among different age and sex groups in the country, the wide range of iron intake in the community is another concern [6]. Likewise, even in developing countries, sections of the community with moderate and high socioeconomical backgrounds show adequate or high intakes of heme iron, which is easily absorbed and can enhance the absorption of non-heme iron in the diet and therefore can lead to iron overload or other kinds of related problems in genetically susceptible individuals [7, 8]. In addition, uncertainty exists about the ability of healthy individuals to maintain normal levels of body iron with an iron-rich diet or with chronic administration of supplemental iron [9]. Iron is recognized as a potent pro-oxidant and a necessary catalyst for the formation of reactive oxygen species in biological systems [10]. Iron is also known to catalyze the generation of hydroxyl radicals from superoxide anions and to increase oxidative stress, which in turn increases the free iron concentration [9–12]. This study was designed to investigate the oxidative stress and iron overload following 16 months of flour fortification with iron among apparently healthy non-anemic men.

Materials and Methods

Among the 31 provinces of the Islamic republic of Iran, Semnan, with a low prevalence of anemia and iron deficiency, was selected [13]. In a before-and-after intervention study, 78 non-anemic apparently healthy 40- to 65-year-old men were randomly selected. A medical history was obtained from each subject prior to enrolment. Subjects were selected if they were nonsmoking, nonanemic (hemoglobin ≥ 13 g/dl), not intending to move from their city in the next 16 months, had no surgical history, had not followed a special diet for the 2 months prior to the study and had no clinical organic diseases. The volunteers had not taken iron, multivitamin supplementation or any routine medication for 2 months prior to the study. In the preliminary interview, the subjects were informed of the objective of the study. After they had agreed to participate, all volunteers signed a prepared informed written consent. Then, an appointment was arranged with each individual for a meeting on another day to collect the required data. The study design was approved by the ethical committee of the National Nutrition and Food Technology Research Institute and the Iranian Ministry of Health and Medical Education. Baseline data from this study have been published elsewhere [14]. Data gathering was conducted at three time points, namely at baseline, after 8 months and after 16 months.

Demographic and anthropometric data, including weight, height and body mass index (BMI), were recorded on special information forms.

The study also included dietary assessment using 24-hour dietary recall for 3 days (including one weekend) and a quantitative Food Frequency Questionnaire including 113 items, which was validated by the Center for Endocrinological and Metabolism Research of the Shahid Beheshti University of Medical Sciences, Tehran, Iran [15]. The Food Frequency Questionnaire was completed by a face-to-face interview with the aid of a food album to estimate portion sizes [16]. Dietary data were collected by a trained dietician. Data were translated into energy and nutrients using Food Dorosti Processor II software, which had been modified for Iranian foods. Following baseline data collection, the flour fortification program was started in Semnan with 30 mg/kg iron as ferrous sulfate, and all participants were followed for 16 months. It should be noted that the same subjects who took part in the first phase participated in the second and third phases (participants were paired), and they were allowed to maintain their regular diets. Laboratory analyses of blood samples were performed for all subjects at all three stages of the study.

Blood Sampling

A 10-ml fasting venous blood sample was collected from each individual and divided into two acid-washed tubes, one with and one without anticoagulant. The anticoagulated blood was used to measure hemoglobin and hematocrit. Sera from clot samples were recovered after 1 h at room temperature followed by centrifugation at 2,500 g at room temperature for 20 min. In order to avoid repeated serum defrosting, serum samples were divided into aliquots and kept at -80°C awaiting further analysis.

Laboratory Investigations

Hemoglobin and hematocrit were determined by an automatic cell counter (Orphee, Mythic, France). Serum levels of ferritin were measured using an immunoturbidimetry assay (Pars-

Azmun, Iran) by an autoanalyzer (Hitachi 912, Boehringer Mannheim, Germany).

Serum iron was measured using the Ferene method (Pars-Azmun). Serum transferrin receptor was measured using a kit from DRG Diagnostics (Germany). This assay is based on the microplate sandwich enzyme immunoassay technique using two different monoclonal antibodies specific for serum transferrin receptor. Malondialdehyde levels in serum were measured as an index of lipid peroxidation according to the thiobarbituric acid spectrophotometric method of Satoh [17], with minor modifications. Total antioxidant capacity (TAC) was determined using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfate) as a reagent. The original 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfate) solution is colorless and turns to green-blue after adding potassium persulfate and forms cation radicals. Maximum absorption occurs at 734 nm using the spectrophotometric method.

The superoxide dismutase (SOD) activity of serum was measured with Cayman's SOD assay kit (Cayman Chemical Company, USA). This method utilizes a tetrazolium salt for detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Serum glutathione peroxidase (GPx) activity was evaluated with Cayman's GPx assay kit (Cayman Chemical Company). Cayman's GPx assay measures GPx activity indirectly by a coupled reaction with glutathione reductase [18]. Oxidized glutathione, produced upon the reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The Mercodia Oxidized LDL ELISA kit (Mercodia, Sweden) was used for quantitative measurement of oxidized low-density lipoprotein. Protein carbonyls were measured using the OxiSelect Protein Carbonyl ELISA kit (Cell Biolabs, USA) following the protocol provided by the manufacturer.

Analysis of Data

Data are expressed as means and standard deviations. If the distribution of the variables was not normal, data were expressed as medians and interquartile range. Repeated-measures analysis of variance (ANOVA) was used to identify any difference among groups at the three time points. Multiple comparisons among time periods were made using the Bonferroni and Dunnett post hoc tests.

Data analysis was performed using SPSS software, version 14. A significance level of $p < 0.05$ was considered statistically significant.

Quality Control and Quality Assurance

There are two flour factories (Omid-Semnan and Ard-va-Sabouse-Semnan) that provide all the flour needs of the bakeries in this city. Using two microfeeders in each one, we fortified all kinds of flour. We organized a workshop to train laboratory managers of the flour factories. They were trained to take at least 2–3 fortified flour samples every day. Using the spot test [19], they were asked to check the fortification process in the factory (as quality control). The Central Food and Nutrition Lab (CFNL) in the province also paid regular visits to the flour factories and took some samples of fortified flour for quantitative tests to detect the exact amount of iron in the fortified flour (as quality assurance). In the CFNL, the iron content was assayed using a spectrophotometric method (AACC 40-41B). This method determines iron

content through reaction with orthophenanthroline and spectrophotometric measurement [19]. In terms of the iron level in flour (or bread), we expected to have four levels of fortification, as follows: low, defined as 25–39.9 mg/kg iron; good, defined as 40–65.9 mg/kg; acceptable, defined as 66–79.9 mg/kg, and high, defined as ≥ 80 mg/kg [2]. The only flour that was distributed in the community for intake was that with good and acceptable levels, and the rest of the flour, which had low and high levels, was sent back to the flour factory for adjustment of iron content. The average iron level in bread after 8 and 16 months was 61.6 and 59.6 mg/kg, respectively. To ensure that the observations were true and due to flour fortification, we also took some regular fortified flour samples from bakeries and sent them to an accredited laboratory to check the exact amount of iron and compared these results with the results of the CFNL as a double check.

Flour Fortification Process

Among 90 flour samples from bakeries, the percentages of low, good, acceptable and high levels of fortification as defined above were 5, 88, 6 and 1%, respectively. The median (interquartile range) iron levels in flour after 8 and 16 months of flour fortification were 61.6 (18.6) mg/kg and 59.6 (15.9) mg/kg, respectively, which differed significantly ($p < 0.05$) from the baseline level of 29.8 (9.1) mg/kg. The results of fortified flour samples showed that flour was being fortified with 30 mg/kg iron and the coverage of fortified flour was 100% in this city.

Results

Mean values of BMI and systolic and diastolic blood pressure of the participants were constant throughout the study (table 1). ANOVA did not show any statistically significant differences in BMI and systolic and diastolic blood pressure in the three phases of the study.

Mean values of hemoglobin, ferritin, serum iron, total iron-binding capacity, serum transferrin receptor, oxidative stress biomarkers and nutrient intake at baseline and after 8 months are shown in tables 1, 2 and 3. There were no significant differences in iron status, oxidative stress biomarkers and nutrient intake with the exception of iron, the intake of which was significantly higher than baseline after 8 months ($p < 0.001$).

In the second 8 months of flour fortification in Semnan, iron intake showed significant differences ($p < 0.001$) compared with baseline (table 3). Among iron status biomarkers, only serum levels of iron significantly increased ($p < 0.001$; table 1). Serum levels of TAC, which is an oxidative stress biomarker, decreased significantly after 16 months compared with baseline ($p < 0.05$; table 2). Among other oxidative stress biomarkers, only SOD and GPx activities increased significantly ($p < 0.001$ and $p < 0.05$, respectively; table 2).

Table 1. Comparison of BMI, systolic and diastolic blood pressure and iron status after 8 and 16 months of flour fortification among nonanemic men

Variable	Baseline	After 8 months	After 16 months	p value
BMI	26.9 ± 5.2	26.8 ± 5.6	26.6 ± 5.4	NS
Systolic blood pressure, mm Hg	119.9 ± 13.2	121.0 ± 11.7	118.7 ± 12.8	NS
Diastolic blood pressure, mm Hg	75.3 ± 8.4	75.2 ± 13.5	76.4 ± 10.8	NS
Hemoglobin, g/dl	14.2 ± 0.7	14.3 ± 0.8	14.5 ± 0.8	NS
Serum ferritin, ng/ml	143.0 ± 65.0	146.1 ± 72.8	148.0 ± 40.3	NS
Serum iron, µg/dl	102.9 ± 31.5	109.7 ± 31.6	117.0 ± 29.8 ^{a, b}	<0.001
TIBC, µg/dl	327.1 ± 78.8	320.8 ± 64.8	332.9 ± 37.3	NS
TS, %	30.5 ± 9.3	31.5 ± 10.5	33.8 ± 8.8	NS
Serum transferrin receptor, µg/ml	1.2 ± 0.5	1.1 ± 0.5	1.2 ± 0.4	NS

Values are shown as means ± SD. ^a Significantly different to the value at baseline; ^b significantly different to the value after 8 months. TIBC = Total iron-binding capacity; TS = ■■■■; NS = not significant.

Table 2. Comparison of oxidative stress biomarkers after 8 and 16 months of flour fortification among nonanemic men

Variable	Baseline	After 8 months	After 16 months	p value
Malondialdehyde, µM	3.0 ± 1.0	3.1 ± 0.8	3.2 ± 0.7	NS
TAC, µM	1.83 ± 0.17	1.75 ± 0.16	1.71 ± 0.10 ^a	<0.05
SOD, U/ml	0.56 ± 0.24	0.62 ± 0.10	0.67 ± 0.12 ^a	<0.001
GPx, U/ml/min	176.6 ± 38.1	192.0 ± 49.3	200.0 ± 46.7 ^a	<0.05
Protein carbonyl, nmol/mg	2.21 ± 0.58	2.27 ± 1.4	2.18 ± 1.2	NS
Oxidized LDL, µM	185.1 ± 64.2	193.6 ± 65.0	201.0 ± 70.6	NS

Values are shown as means ± SD. ^a Significantly different to the value at baseline. LDL = Low-density lipoprotein; NS = not significant.

Table 3. Intake of iron, energy and some nutrients related to oxidative stress status

Variable	Baseline	After 8 months	After 16 months	p value
Energy, kcal	2,207.7 ± 652.8	2,158.5 ± 600.3	2,277.3 ± 705.1	NS
Protein, g/day	66.9 ± 21.5	65.7 ± 24.0	72.1 ± 23.8	NS
Fat, g/day	66.7 ± 21.0	66.7 ± 21.0	68.5 ± 22.3	NS
Polyunsaturated fatty acids, g	22.3 ± 4.9	22.9 ± 5.2	23.6 ± 5.4	NS
Vitamin C, mg	90.5 ± 26.1	93.2 ± 28.3	97.5 ± 30.1	NS
Vitamin A, µg	694.5 ± 238	687.5 ± 223	671.5 ± 225	NS
Vitamin E, mg	9.2 ± 3.5	10.4 ± 2.9	10.9 ± 3.9	NS
Iron, mg	12.5 ± 3.1	15.4 ± 6.0 ^a	15.6 ± 5.0 ^a	<0.001
Zinc, ■■■■	7.83 ± 1.9	7.78 ± 2.0	7.71 ± 1.9	NS
Copper, ■■■■	1.91 ± 0.4	1.86 ± 0.5	1.81 ± 0.4	NS

Values are shown as means ± SD. ^a Significantly different to the value at baseline. NS = Not significant.

Discussion

Fortifying flour with iron has been used to enhance iron intake as an efficient and inexpensive approach for reducing the prevalence of iron deficiency in many countries [20]. However, this method has been abolished, even in some developed countries, due to the probable negative effects of iron overload [21]. Concerns regarding the negative effects of iron have been raised after considerable progress was made in recognizing iron metabolism and its role in generation of active oxygen species [22]. The highest tolerable iron intake is 45 mg/day [4]. Although it is nearly impossible to reach this level by adding 30 mg of iron to 1 kg of flour, there is evidence of iron-related oxidative stress with even lower doses in the long term [23].

Evidence suggests that both iron deficiency and high levels of iron stores are risk factors for cardiovascular diseases and cancer [22–25].

To our knowledge, this is the first time that the effect of the consumption of fortified flour (bread) on iron status and oxidative stress has been studied. It must be stressed that the main focus of our study was on the impact of iron intake from fortified flour (bread) on iron parameters and oxidative stress biomarkers in nonanemic apparently healthy men after a short and a long period.

In this study based on the Food Consumption Survey, the mean bread intake in Semnan was about 280–320 g/day [6], but our consumption data showed that the mean bread intake among our sample was 212 g/day; therefore, about 6–7 mg of iron was added to the daily food basket due to flour fortification. The mean iron intake from diet increased significantly after 8 and 16 months ($p < 0.05$). The total iron intake after 8 and 16 months was 15.4 and 15.6 mg, of which 6.2 and 6.0 mg, respectively, were from flour.

Transferrin saturation and serum ferritin were used to determine iron overload in our study. In this regard, ferritin levels more than 300 ng/ml and transferrin saturation higher than 55% were considered to indicate iron overload. Previous studies showed that chronic daily ingestion of bioavailable iron (as ferrous sulfate) in clear excess of the upper tolerable intake level (45 mg/day) can induce iron overload.

This study did not show iron overload in participants after 8 and 16 months of intervention, but there was a positive trend in nearly all parameters of iron status suggestive of increased iron retention in the body. Therefore, as the study of Rehema et al. [23] showed, it is possible that consumption of fortified bread by nonanemic indi-

viduals for a longer period causes iron overload as a result of increased iron retention. This condition is rare and has not yet been seen even in flour fortification programs with iron.

Several *in vivo*, *in vitro* and animal studies have shown the correlation of iron intake and oxidative stress indicators [26, 27], but there are few human studies in this regard [28, 29]. While some studies have shown negative effects of iron on oxidative stress biomarkers, the incidence of some kinds of cancers and the risk of acute myocardial infarction [28–30], other studies have shown a positive role of iron and its related factors, such as ferritin, in the human body [31–34]. For that reason, we measured several parameters of oxidative stress in our study sample.

The significant increase in TAC and also SOD and GPx activity in our study sample, which occurred after 16 months of fortification with 30 mg/kg iron in men, indicated a mild (or slight) oxidative stress level.

It should be noted that with regard to the importance and the specific role of iron in anemic individuals and the necessity of controlling anemia to prevent its adverse outcomes, side effects of iron supplements and fortification with iron in developing oxidative stress are usually ignored [35, 36].

Also, in several studies, the relationship between the level of iron stores and oxidative stress has been shown [12, 37]. These studies have indicated that the amount of iron stores and levels of plasma iron have a positive and significant relationship with the increase in fat peroxidation and oxidative stress. In the most recent of these studies, Choi et al. [38], in a nested case-control study, showed that high iron intake can increase oxidative stress in the body, which in turn increases the risk of prostate cancer. Age is also an important factor in the antioxidant defense status, and there is a positive relationship between age and oxidative stress status [39, 40].

The present study had some limitations, as does any intervention study. We had to fortify all kinds of bread in the community to make sure that all the bread that our volunteers ate was fortified. We had to use ferrous sulfate, but its high bioavailability can induce iron absorption and oxidative stress as well. The main reasons for using ferrous sulfate were as follows: the shelf life of flour in Iran is less than 1 month (3 weeks); we produce ferrous sulfate inside the country, and the price of ferrous sulfate is very low compared to other fortificants.

It is the authors' belief that to witness a better effect of a fortification program as a diet-based intervention at the community level, special attention should be paid to

some sections of the population who are not anemic and do not need extra iron, especially when ferrous sulfate is used as the fortificant.

Conclusion

It can be concluded that although iron intake through the flour fortification program did not affect oxidative stress indicators after 8 months, significant changes in serum iron and also significant changes in some oxidative stress indicators after 16 months of implementing the flour fortification program imply that this program has some side effects in the long term. Regular amounts of daily flour consumption, the burden of iron deficiency, regular monitoring and the safe amount of iron to be added to flour must be taken into consideration before imple-

menting iron fortification in a population. We also recommend that not all kinds of flour in the country should be fortified.

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The authors declare that they have no competing interests.

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