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Effect of Iron Enriched Bread Intake on the Oxidative Stress Indices in Male Wistar Rats

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Abstract

Background: Contrary to the proven benefits of iron, few concerns in producing the oxidative stress is remained problematic.
Objectives: The aim of the study was to evaluate the oxidative stress in the male Wistar rats fed bread supplemented with iron in different doses i.e., 35 (basic), 70 (two fold), 140 (four fold), and 210 mg/kg (six fold) with or without $NaHCO_3$ (250 mg/kg).
Methods: In this experimental study Iron, ceruloplasmin, ferritin, total iron binding capacity (TIBC), albumin, total protein, uric acid and plasma superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), malondialdehyde (MDA), and total antioxidant capacity (TAC), were evaluated in 30 rats at the first and last day of the experiment (day 30). In addition, phytic acid levels were detected in all baked breads. The data were analyzed by ANOVA and t test procedure though SPSS statistical software version 20.
Results: Serum iron level in rats that received basic level of iron plus $NaHCO_3$ decreased significantly in the last day of the trial. Higher level of serum iron was seen in rats that received iron twofold, fourfold and sixfold and rats that received iron fourfold plus $NaHCO_3$. Serum ceruloplasmin and ferritin in groups of rats that received fourfold level of iron plus $NaHCO_3$ and rats that received iron sixfold showed a significant increase ($P \leq 0.05$). Serum total protein and uric acid in rats that received basic level of iron plus $NaHCO_3$ and rats that received twofold level of iron showed a significant decrease. Serum total protein levels in rats that received fourfold level of iron showed a significant decrease. Bread with $NaHCO_3$ showed higher phytic acid levels than other groups.
Conclusions: These results indicate that oxidative stress was not induced, whereas some antioxidant activities were significantly changed in rats that received iron-enriched bread.

Keywords: Bread, Iron, Oxidative Stress, Rats, Wistar

1. Background

In spite of the advantages of iron fortification in some foodstuffs, there are few concerns about diverse effects of producing oxidative stress specially in the normal non-anemic people [1]. Anemia is a major health problem in developing countries. Most cases of anemia are due to iron deficiency [2]. Distribution of nutritional iron deficiency is common worldwide [3]. Thus, in many developing countries competing with 'hidden hunger' for iron is a major goal [4]. Iron deficiency still exists in some countries of the Middle East despite various efforts to control malnutrition of micronutrients [5]. Iron-deficiency anemia is a major nutritional problem in Iran. Adverse effects of anemia on the growth and development of children are very important [6]. Anemia in particular affects the learning and scholastic performance in school girls entering adolescence [7]. Iron deficiency was reported in 78 % and 28% of female and male regular blood donors of Yazd, Iran, respectively; and 55.6% and 16% of these donors had iron deficiency anemia [8]. Increase in iron intake can be achieved either through pharmacological iron supplementation, through food fortification or through biofortification [4]. The best food for fortification is wheat and maize flour [9]. Flour fortification with iron was launched in some provinces of Iran in 2001. Iron fortification program in Iran has had beneficial effects on some indicators of anemia [10]. Bread is one of the major contributors of human diet that plays an important role in the primary energy needs of Iranian families. It is considered as the most important source to fulfill the requirements of energy, protein, minerals and vitamins. In the case of complete fermentation of bread, most nutrients of natural wheat and flour can be metabolized, digested and absorbed by the body. Phytic acid combines with minerals

such as iron and calcium, making them insoluble in water and preventing the absorption of wheat minerals [11]. When bread is made with yeast, the phytic acid content is reduced, which in turn improves people's ability to absorb these nutrients. However, when bread is made with $NaHCO_3$ (baking soda), the phytic acid content is not affected. Bakers in Iran commonly use $NaHCO_3$ instead of yeast [12]. It should be mentioned that the use of iron for healthy people might have side effects associated with Iron excess. Iron overload is believed to generate oxidative stress [13]. Antioxidants are consumed in the reaction with free radicals generated during the oxidative stress. Some researchers evaluated biomarkers of oxidative stress in an apparently healthy population [14]. They found no significant changes in women following eight-month consumption of iron-fortified bread, but in men, total antioxidant capacity showed a significant decrease. In Semnan, a province in Iran, a study was conducted on non-anemic men. Results indicated a reduction of antioxidant capacity and induced oxidative stress in the iron fortificated flour, however, no symptoms of iron overload was observed [15]. Iron is very important in the production of free radicals. Both iron excess and deficiency result in free radical mitochondrial damage. It was revealed that with excess iron supplementation, the intestinal mucosal cells are exposed to unabsorbed iron excess and oxidative stress [16].

2. Objectives

These observations have led us to investigate whether the feeding of male Wistar rats with different doses of the iron supplemented bread was resulted in alteration of antioxidant capacity and induced oxidative stress.

3. Methods

3.1. Animals

Thirty male Wistar rats weighing 200 - 250 g were housed separately in stainless steel cages 25 × 22 × 20 cm in standard rat house (22 - 25°C and 40 - 50% humidity on a 12 hours light-dark cycle). All rats were given access to food and tap water ad libitum.

3.2. Animal Ethics

This experimental study was conducted with the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran (Ref: 1512-1513, 23-1-93). Recommendations of European council directive (86/609/EC) of November 24, 1986 were followed, regarding the guidelines for the protection of animals used for experimental purposes. The study was carried out in fall 2014 in school of veterinary medicine, Shiraz University.

3.3. Treatment Schedule

After one-week acclimatization, rats were divided randomly into 6 groups of 5 animals and specific diets were prepared (iron-enriched bread) daily for 30 days ad-libitum.

3.4. Experimental Groups

Group I: control, iron enriched bread (35 mg/kg), or basic level; Group II: iron in basic level with $NaHCO_3$ (250 mg/kg); Group III: iron enriched bread in twofold (70 mg/kg); Group IV: iron enriched bread in fourfold (140 mg/kg); Group V: iron enriched bread in fourfold and $NaHCO_3$ (250 mg/kg); Group VI: iron enriched bread in sixfold (210 mg/kg). At the end of the experiment (day 30) the rats were anesthetized with Ketamine (50 mg/kg) after withholding food for 12 hours. Blood and fecal samples were taken at the first and at the end of the trial on day 0 and 30 respectively. After sampling, the blood was centrifuged at 750 g for 15 minutes and the serum was kept at -80°C until analysis. The samples with hemolysis were discarded.

3.5. Bread Making

Commercial Sangak wheat flour (93% extraction rate) was used in this study. Sangak is a plain, rectangular, or triangular Iranian traditional whole wheat leavened flatbread. The bread is baked on a bed of small river stones in an oven. For preparing the bread, dough was prepared in one batch. In brief, 39.19 kg of Sangak flour, about 60 l of lukewarm water and 350 g salt were mixed for 12 minutes. After 5 minutes, 150 g instant yeast (Saccharomyces cervisiae, Dez-Maye, Iran) and 17.35 kg of sourdough (12 hrs. aged) were added and mixed for another 2 minutes. Fermented dough was left for 50 minutes for proofing at room temperature (23°C). This dough was manually molded (900 - 950 g each), sheeted and baked in a traditional stove for about 6 min at 350°C. Baked breads were cooled and stored at -20°C and were gradually air-dried at room temperature for about 24 hours and completely dried in a vacuum oven at 70°C for 24 hours to constant weight. Other ingeradiants of bread were salt and active leaven (1% salt, 0.5% yeast and 85% water). For diet preparation, dried breads were finely grounded and ferrous sulfate was added as Iron source as mentioned amounts. Fortified bread was used as thirty-five percent of daily diet of rats [17].

3.6. Phytic Acid Measurement

Phytic acid in the dried samples (2 g) was determined by Lane method (1995) using ion-exchange chromatography, and measured as hexaphosphate equivalents. Calculation of phytic acid was done by the following Formula 1.

$$phyticacid\left(\frac{mg}{g}\right)=\left(\frac{meanK\times A\times 20}{0.282\times 1000}\right) \quad (1)$$

Where A = absorbance, k = standard phosphorus concentration (μg)/A, mean k = k/n, n = number of standards [15].

3.7. Chemical Analysis

3.7.1. Iron, Ceruloplasmin, Ferritin, and Total Iron Binding Capacity (TIBC)

Iron was measured using an atomic absorption spectrophotometer (Shimadzo AA-670, Kyoto, Japan). The activity of ceruloplasmin in serum was measured calorimetrically by the method of Sunderman and Nomoto [18]. Serum level of ferritin was measured by ELISA technique using a commercial kit (Birex, Fars, Iran). TIBC was measured using a commercial kit (Darman Kaveh kit, Iran). TIBC was a colorimetric assay using two reagents sequentially. The first acidic reagent contains an iron-binding dye and excess iron. The pH change causes transferrin to release all iron atoms, these are bound by the iron-binding dye along with the excess iron, producing a colored complex. The second, neutral reagent increases the pH and restores the transferrin's affinity for iron. The transferrin extracts iron from the dye-iron complex and becomes 100% saturated; the remaining excess iron stays bound in the dye-iron complex. The decrease in the absorbance of the colored dye-iron complex is measured spectrophotometrically at 660 nm and is directly proportional to the TIBC of the serum sample.

3.7.2. Albumin, Total Protein and Uric Acid

Albumin and total protein were measured by Bromocresol green methods and Biuret method respectively (Commercial kit; Pars Azmoon, Tehran, Iran) [19]. The Uric Acid Assay Kit was a sensitive quantitative fluorometric assay for measuring uric acid or uricase concentrations. Uric acid reacts with water and oxygen in the presence of the enzyme uricase to produce allantoin and H_2O_2. In the presence of horseradish peroxidase (HRP), a fluorescence probe reacts with H_2O_2 in a 1: 1 stoichiometry to produce a highly fluorescent product. A fluorescence microplate reader with an excitation of 540 nm and an emission of 590 nm can easily read this fluorescent product. Fluorescence values were proportional to the uric acid or uricase levels within the samples, depending on the compound that was being measured. The uric acid or uricase content in unknown samples was determined by comparison with its respective standard curve (Zist Shimi, Tehran, Iran) [20].

3.7.3. Phytic acid and NaHCO3

In bread samples, phytic acid was determined according to the chemical methods and $NaHCO_3$ was determined according to the method of AOAC [21].

3.7.4. Antioxidant Enzymes Activities, Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC)

Superoxide dismutase (SOD) activity was measured with a commercial kit (RANSOD kit, Randox Com, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl) -3-(4-nitrophenol) -5-phenyltetrazolium chloride (INT) to form a red formazan dye. The enzyme activity was then determined by the degree of reaction inhibition, as one unit of SOD corresponded to 50% inhibition of INT reduction under assay condition. GPX activity was measured by a commercial kit (RANSEL kit, Randox Com, UK) based on the method of Paglia and Valentine [22]. GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured at 340 nm. The values of both enzymes were expressed as units/gr of hemoglobin. The activity of catalase (CAT) was determined with the commercial catalase assay kit (Oxford Biomedical Research, Inc., USA) based on the colorimetric method described by Slaughter and O'Brien [23] and the activities of the enzymes were expressed as U/g of hemoglobin. MDA, an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation [24]. The lipid peroxidation level of the RBC membrane was evaluated by means of a modified HPLC method with UV-Visible spectrophotometry based on Lykkesfeldt [25]. The measurement was based on MDA reactions with thiobarbituric acid (TBA) to form colored MDA-TBA adducts and the values were expressed as mmol/L of MDA. The determination TAC in serum by commercial kit (Labor Diagnostika Nord (LDN) Com, Nordhorn, Germany) was based on the reaction of peroxides with peroxidase followed by a color reaction of the chromogenic substrate tetra methyl benzidine. The change in color was measured calorimetrically at 450 nm and expressed as mill moles per liter.

3.8. Statistics

The significance of differences between the treatments was established by the ANOVA and t test procedure of SPSS statistical software (version 20) and using Duncan's multiple range test post hoc. Significance level was set at $P < 0.05$.

4. Results

Serum iron level in rats that received basic level of iron and $NaHCO_3$ decreased significantly in the last day of trial. Significantly higher levels of serum iron were seen in the last day of the trial in rats that received iron twofold, fourfold and sixfold, and rats that received iron fourfold plus $NaHCO_3$. Serum ceruloplasmin and ferritin in groups of rats that received iron fourfold plus $NaHCO_3$ and rats that received iron sixfold were significantly higher in the last day compared to the first day of trial. TIBC decreased in rats that received iron sixfold. Serum total protein levels in groups of rats that received basic level of iron plus $NaHCO_3$ and rats that received iron twofold and fourfold were significantly lower in the last day of trial. Serum uric acid levels in rats that received basic level of iron and $NaHCO_3$ and rats that received twofold level of iron were significantly lower in the last day of trial (Table 1). The mean ($\pm$ SEM) concentrations of SOD, GPx, CAT, MDA and TAC in the serum of rats that received enriched bread and/or $NaHCO_3$ (n = 30) were shown in the Table 2. The concentration of phytic acid in the bread samples of mentioned groups was as follows: group I (241 $\pm$ 17.4mg/100g), group II (290 $\pm$ 11.3 mg/100g), group III (234 $\pm$ 24 mg/100g), group IV (228 $\pm$ 14.08 mg/100g), group V (282 $\pm$ 12.9 mg/100g) and group VI (215 $\pm$ 33.6 mg/100g). In groups of rats that received basic level of iron plus $NaHCO_3$ (290 $\pm$ 11.3 mg/100g) and rats that received fourfold level of iron plus $NaHCO_3$ (282 $\pm$ 12.9 mg/100g) the concentrations of phytic acid were significantly higher compared to other groups that did not receive $NaHCO_3$.

5. Discussion

Results revealed that, with the increase of iron in the diet, serum iron levels increased. Iron in the hemoglobin is in the Fe^{2+} (ferrous) form. Iron is transported by transferrin in the blood or stored intracellularly as ferritin or Fe^{3+} (ferric) form [26]. This increased manner was reversed in the rats that received basic level of iron plus $NaHCO_3$ (group II). An interaction exists between iron and $NaHCO_3$. O'Neil-Cutting and Crosby studied the effect of some antacids on the absorption of iron [27]. They found a negative correlation between $NaHCO_3$ and the ingested iron. In the present study increased ceruloplasmin and ferritin levels were seen parallel with higher levels of iron, which reached statistical significance in rats that received iron in fourfold plus $NaHCO_3$ (group V) and rats that received iron sixfold (group VI). One of the reasons for the increase of the ceruloplasmin and ferritin is related to the oxidation of Fe^{2+} into Fe^{3+}. In fact, ceruloplasmin is a ferroxidase that oxidizes toxic Fe^{2+} to its nontoxic Fe^{3+} form [28]. Ceruloplasmin assists in transport of Fe^{3+} in the plasma in association with transferrin, which can carry iron only in the Fe^{3+} state. Ceruloplasmin is also involved in carrying more than 95% of the total copper in plasma [29]. It must be noted that ceruloplasmin is a major antioxidant in serum based on its ability to inhibit oxidation of lipids induced by inorganic iron. Ceruloplasmin is a scavenger of superoxide anion radicals [30]. An association between elevated ceruloplasmin levels with atherosclerotic disease and both type 1 and 2 diabetes mellitus in humans has been noted [31, 32]. The antioxidant effects of ceruloplasmin could have important implications for various neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease in which iron deposition is known to occur [28]. Ferritin is an intracellular protein that stores iron and releases it in a controlled manner and acts as a buffer against iron deficiency and iron overload. Serum ferritin level correlates with total body iron stores. TIBC measures the blood's capacity to bind iron with transferrin. In fact, TIBC measures transferrin indirectly. Significant decrease in TIBC was observed in rats that received iron sixfold (group VI) in comparison to the first day of trial. The amount of albumin in this study was almost constant. Albumin has several antioxidant properties. In general, albumin constitutes the major plasma protein target of oxidative stress [33]. Total protein was decreased significantly in rats that received basic level of iron plus $NaHCO_3$ (Group III) and rats that received iron twofold and fourfold (Group III and IV) compared to the first day of trial. Total protein consists of albumin and globulins, the amount of albumin was almost constant, therefore, globulin might be decreased in these groups. Iron-binding globulins (transferrin) in serum control the level of free iron in biological fluids with transport of iron to tissues. When iron exceeds transferrin-binding capacity it causes tissue damages by lipid peroxidation and induces injuries in some vital organs such as kidneys, liver, heart and lungs [34]. Lower uric acid level was seen in groups II (basic iron plus $NaHCO_3$) and III (twofold of iron). Causes of low uric acid are numerous. Uric acid is a product of the metabolic breakdown of purine nucleotides. The enzyme xanthine oxidase makes uric acid from xanthine and hypoxanthine. Xanthine oxidase contains iron [35]. Probably, the amount of uric acid is influenced by iron level. In humans, over half the antioxidant capacity of blood plasma comes from uric acid [36]. Uric acid is an indicator of the presence of iron overload [37]. The level of uric acid was not increased. Therefore, there is no evidence of iron overload in all rats that received iron enriched bread. In some situations such as iron overload, oxidative stress generates reactive oxygen species (ROS) which interact by cell membrane lipids. These reactions lead to lipid peroxidation

Table 1. The Mean (± SEM) Concentrations of Iron, Ceruloplasmin, Ferritin, TIBC, Albumin, Total Protein and Uric Acid in the Serum of Rats That Received Enriched Bread and/or $NaHCO_3$ (n = 30)[a]

Group/Day	Iron, μg/dL		Ceruloplasmin, g/L		Ferritin, ng/mL		TIBC, μg/dL		Albumin, g/dL		Total protein, g/dL		Uric Acid, mg/dL	
	0	30	0	30	0	30	0	30	0	30	0	30	0	30
I	176.5 ± 30.16	144.25 ± 23.18	0.08 ± 0.01	0.09 ± 0.01	57.00 ± 19.79	55.25 ± 10.50	1272.75 ± 336.56	569.75 ± 59.47	3.12 ± 0.47	3.05 ± 0.19	7.48 ± 0.95	6.29 ± 1.15	3.21 ± 1.29	3.70 ± 2.44
II	195.40 ± 37.85	100.4[b] ± 36.86	0.08 ± 0.01	0.09 ± 0.01	59.00 ± 14.93	57.20 ± 19.54	1023.75 ± 260.87	866.50 ± 121.76	3.69 ± 0.62	3.38 ± 0.19	7.91 ± 0.16	5.06[b] ± 0.23	5.23 ± 0.57	3.26[b] ± 0.71
III	155.0 ± 22.08	206.0[b] ± 22.33	0.07 ± 0.005	0.08 ± 0.02	45.33 ± 10.96	51.00 ± 19.97	1358.50 ± 120.92	1267.00 ± 28.28	3.66 ± 0.57	3.21 ± 0.30	7.04 ± 1.38	4.58[b] ± 0.54	4.84 ± 2.38	2.24[b] ± 1.11
IV	245.5 ± 91.22	415.75[b] ± 21.4	0.09 ± 0.02	0.11 ± 0.01	58.00 ± 14.0	63.66 ± 18.82	1497.00 ± 729.69	1194.00 ± 104.0	3.32 ± 0.30	3.14 ± 0.46	7.83 ± 1.32	4.61[b] ± 0.42	3.95 ± 0.15	3.06 ± 0.46
V	310.5 ± 50.01	343.0[b] ± 12.9	0.14 ± 0.01	0.17[b] ± 0.01	64.11 ± 31.58	148. 61[b] ± 7.0	1269.00 ± 338.7	913.25 ± 139.09	3.54 ± 0.67	3.69 ± 0.50	8.56 ± 1.44	6.69 ± 2.45	3.86 ± 1.40	4.61 ± 1.33
VI	227.0 ± 36.13	434.2[b] ± 48.85	0.14 ± 0.01	0.21[b] ± 0.02	89.25 ± 12.14	137.25[b] ± 6.65	1224.33 ± 90.78	869.67[b] ± 70.29	3.29 ± 0.64	3.77 ± 0.25	5.82 ± 3.29	6.39 ± 1.99	3.61 ± 1.92	1.70 ± 0.34

[a] Group I, control, received iron enriched bread (35 mg/kg); Group II, received iron enriched bread (35 mg/kg) with 250 mg/kg $NaHCO_3$; Group III: received iron enriched bread (70 mg/kg); Group IV, received iron enriched bread (140 mg/kg); Group V, received iron enriched bread (140 mg/kg) and 250 mg/kg $NaHCO_3$; Group VI, received iron enriched bread (210 mg/kg); TIBC, total iron binding capacity.
[b] Significant difference between first and last day of experiment at the 0.05.

Table 2. The Mean (± SEM) Concentrations of SOD, GPx, CAT, MDA and TAC in the Serum of Rats That Received Enriched Bread and/or $NaHCO_3$ (n = 30)[a]

Group/Day	SOD, U/grHb		GPx, U/grHb		CAT, nmol/mL		MDA, mmol/mL		TAC, mmol/L	
	0	30	0	30	0	30	0	30	0	30
I	1258.70 ± 15.73	1252.12 ± 9.37	32.53 ± 0.63	32.54 ± 0.71	17.95 ± 0.91	18.26 ± 0.29	1.82 ± 0.90	1.98 ± 1.37	2.37 ± 0.36	2.28 ± 0.33
II	1253.53 ± 18.17	1259.68 ± 15.43	33.10 ± 0.17	32.72 ± 0.67	17.75 ± 0.25	17.79 ± 0.68	2.83 ± 0.30	2.06 ± 0.92	2.37 ± 0.07	2.49 ± 0.35
III	1250.66 ± 16.61	1259.96 ± 18.2	32.52 ± 0.7	32.84 ± 1.31	18.45 ± 0.28	18.53 ± 0.36	1.31 ± 0.81	2.41 ± 0.82	2.33 ± 0.59	2.45 ± 0.37
IV	1258.36 ± 17.36	1265.50 ± 18.18	32.34 ± 1.01	32.52 ± 0.30	17.96 ± 0.61	17.63 ± 0.73	2.28 ± 0.86	1.51 ± 0.97	2.28 ± 0.52	2.16 ± 0.27
V	1261.56 ± 8.85	1258.95 ± 15.74	32.52 ± 0.67	32.50 ± 1.10	18.32 ± 0.30	18.38 ± 0.61	2.19 ± 1.31	1.99 ± 0.06	2.60 ± 0.28	2.28 ± 0.38
VI	1258.17 ± 17.91	1246.67 ± 15.79	32.44 ± 1.01	32.53 ± 1.04	18.07 ± 0.63	17.90 ± 0.50	2.28 ± 0.77	2.21 ± 0.71	2.19 ± 0.4	2.58 ± 0.4

[a] Group I, control, received iron enriched bread (35 mg/kg); Group II, received iron enriched bread (35 mg/kg) with 250 mg/kg $NaHCO_3$; Group III, received iron enriched bread (70 mg/kg); Group IV, received iron enriched bread (140 mg/kg); Group V, received iron enriched bread (140 mg/kg) and 250 mg/kg $NaHCO_3$; Group VI, received iron enriched bread (210 mg/kg); SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; MDA, Malate dehydrogenase; TAC, total antioxidant capacity.

and the generation of a cytotoxic product, namely MDA. Because of oxidative stress, some endogenous antioxidant compounds such as SOD, GPx, and CAT will be decreased. TAC provided the cumulative antioxidant status [13, 38, 39]. Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage. It has been proposed that antioxidants would be consumed in the reaction with free radicals [40]. Based on Table 2, no evidence of reducing TAC, SOD, GPx and CAT and subsequent induction of oxidative stress was observed. Phytic acid is a hexaphosphate ester of inositol. It is found in the highest levels in legumes, nuts, and cereal grains. The amount of phytic acid in a food depends on the various processes that are used. Phytic acid can decrease absorption of minerals, such as zinc, iron, calcium, and manganese, so its high intake can cause mineral deficiency [10]. Payan [41] showed that various wheat cultivated in Iran have about 1.0% (1000 mg) phytic acid and in processed wheat, depending on flour extraction rate, different amounts of phytic acid remain. In flours with high extraction rate (percentage by weight of flour milled from the cleaned grain more than 80%) 600 - 700 mg of phytic acid remains, while in low extraction rate flours (percentage by weight of flour milled from the cleaned grain less than 60%), its amount decreases to less than 30 mg. Gargari et al. [11] showed a significant decrease (mean~ 59%) in phytic acid content of flour during bread making. In the present study, mean and standard deviation of phytate in all studied breads was 108.53 ± 67.45 (with 95% confidence interval: 93.75 - 123.31 mg/100g). In the study by Roohani et al. [42], content of phytic acid in Baggette bread samples of Isfahan, Iran was reported to be less than 15 mg/100g. In the present study, wheat flour had high extraction rate and phytic acid levels of bread samples were in the range of 215 - 290 mg/100g. In addition, $NaHCO_3$ used in the bread had increasing effect on the phytic acid level. Phytic acid can be inactivated during baking process. For this, enzymes, called phytases, inactivate during fermentation and thus enhance the nutrient absorption. Phytase can be effective only when it is used with yeast or sourdough and is left for an appropriate length of time and in breads made with baking soda, because of the absence of phytase enzyme, the minerals of bread cannot be digested and absorbed. Yeast is a living and single-celled organism that

converts starch and sugar into alcohol and carbon dioxide during a complex process and makes the digestion easy for the human digestive system through breaking down the long chains of starch [43]. Carbon dioxide and alcohol produced by yeast form bubbles that make the dough rise, and give bread its spongy texture. Moreover, the alcohol and acid produced during fermentation process remove the pathogenic microorganisms from bread and enhance its hygienic production. Having low pH level due to producing acid and colloidal state of gluten can enhance the durability of bread. Another commercial alternative for processing food is to add baking soda to dough which, due to its lack of fermentation causes some disorders in digestion and absorption of effective minerals and bivalent ions, thus leading to gastroenteritis [44]. Using baking soda in bread increases the absorption of heavy metals (e.g. lead and mercury) which in the long-term can create severe disorders [45]. Despite the numerous advantages of yeast, studies have shown that only 45 - 55 percent of the bakeries in Iran actually use industrial yeast or leaven and the amount of baking soda in bread varies from 2% - 47%. A limitation of the study was the relatively small sample size in each group. It remains to be determined whether iron induced alterations in some antioxidant compounds in normal or anemic people and whether this effect of iron fortification is clinically important.

5.1. Conclusions

Regarding the study findings and in comparison with similar researches, there is no evidence of iron overload in all rats from iron-enriched bread in this study. However, some antioxidant compounds such as ceruloplasmin significantly increased and total protein and uric acid were decreased in rats that received iron-enriched bread. In the routine administration of iron supplements, especially in men, some health problems due to iron overload and its consequent oxidative stress must be noticed. The use of iron-fortified breads over long periods requires further investigation.

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Footnotes

Authors' Contribution: Conceived and designed the experiments: Arash Omidi and Hamid Reza Gheisari; performed the experiments: Sharareh Heidari and Hamid Reza Gheisari; analyzed the data: Hamid Reza Gheisari and Arash Omidi; contributed reagents/materials/analysis tools: Saeed Nazifi; wrote the paper: Arash Omidi.

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