Effect of multiple fortification on the bioavailability of minerals in wheat meal bread

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Abstract Bioavailability of calcium, iron and zinc as calcium carbonate (CaCO_3), ferrous sulfate (FeSO_4) and zinc sulphate (ZnSO_4) @ 1,000, 40 and 20 mg kg^{-1} respectively from fortified bread of 72% extraction straight grade flour was assessed. Fortified bread diets were fed to 64 female Sprague-Dawley Albino rats for a period of 28 days. The retention of Ca, Fe and Zn was measured in plasma, femur and liver tissues of rats. The results showed that the feed intake and live body weight of the experimental animals increased significantly with the time period. The Ca levels in plasma and liver did not change significantly while in femur, Ca retention changed significantly with changing type of the fortificants. Similarly, the results for percent apparent absorption (AA) of Ca also remained unchanged ($P < 0.05$). The Fe and Zn levels were significantly higher in the plasma, liver and femur of rats fed Fe and Zn fortified bread. Interaction of Ca, Fe and Zn

resulted in their decreased bioavailability. However Ca, Fe and Zn absorption was higher in the rats fed triple fortified diet compared with those fed unfortified bread diet. This negative interaction did not appear to be great enough to discourage multiple fortification of flour to address minerals malnutrition in the vulnerable groups.

Keywords Fortification · Calcium · Iron · Zinc · Bioavailability · Flour

Introduction

Micronutrient malnutrition is a serious threat to the health and productivity of more than 2,000 million people worldwide (WHO 1995). Calcium is an essential nutrient required for critical biological functions such as nerve conduction, muscle contraction, cell adhesiveness, mitosis, blood coagulation, glandular secretion and structural support of the skeleton. An adequate intake of calcium reduces the risk of such disorders as osteoporosis, hypertension and possibly colon cancer (Miller and Anderson 1999).

Iron deficiency is the most common micronutrient deficiency globally, affecting between 1.5 and 2 billion people, of whom 500 million have iron deficiency anemia (IDA). Most of these people live in developing countries. IDA reduces a person's ability to perform physical tasks, while anemic laborers demonstrate impaired productivity, lower working capacity and fatigue (Lotfi et al. 1996; Walker 1998). Similarly zinc is also an important nutrient. Globally, nearly half of the population is at risk for low zinc intake (Brown et al. 2001). Its deficiency results in reduced growth rate, impaired resistance to infection, delayed wound healing, and neuro-sensory defects such as taste abnormalities (Prasad 1985; Gracey 1999).

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Multiple fortification of foods is a possible way of addressing deficiencies of two or more micronutrients at the same time in a cost-effective manner (Darnton-Hill and Nalubola 2002; Gibson et al. 1997) and does not pose any risk of substantial losses or any deteriorative effect during storage or baking on native mineral contents in whole wheat flour, a highly preferred food carrier for fortification of minerals in the developing world (Akhtar et al. 2009a).

Cereal products are currently the most frequently used vehicles for calcium, iron and zinc fortification. A major advantage of employing cereal products as a vehicle for Ca, Fe and Zn is the facilitating effect of wheat flour on mineral absorption as compared with other cereal foods (Grewal and Hira 2003; Ahmed et al. 2008). Since, wheat is used as dietary staple in Pakistan where Fe and Zn deficiency is highly prevalent (NNS 2002) therefore, the fortification is a logical intervention strategy for this country.

Since, mineral fortificants exhibited anti fungal effect in wheat flour, therefore the addition of fortificants may be another food safety approach. There would be additional advantage of fortification, particularly in countries where the magnitude of micronutrients deficiency is high and weather conditions are hot and humid (Akhtar et al. 2008).

Similarly, another study by the same researcher (Akhtar et al. 2009b) reported that addition of iron and zinc fortificants to wheat flour did not exhibit any critical alteration in the basic composition of the flours, however, marginal differences in various flour attributes concerning rheology were detected.

The current research work is an endeavor to measure the bioavailability and the extent of interaction among minerals like calcium, iron and zinc obtained in 72% extraction fortified wheat flour bread. The study further focuses to explore the possibilities of using wheat flour as a potential vehicle for the minerals fortification.

Materials and methods

Raw material

Wheat variety (*Iqbal*-2000) purchased from the Department of Agronomy, University of Agriculture, Faisalabad, was milled for the production of straight grade flour for this study. The tempered grains were milled through Quadrumate Senior Mill and the straight grade flour thus obtained was used for the preparation of bread. The procedure for milling of wheat grain was followed as described in AACC (AACC 2000). The fortificants i.e. calcium carbonate (CaCO_3) @ 1,000 ppm, ferrous sulfate (FeSO_4) @ 40 ppm and zinc sulphate (ZnSO_4) @ 20 ppm

were used as sources of calcium, iron and zinc. The food grade calcium fortificant was purchased from the local market. The iron fortificants were obtained from Micronutrient Initiative (MI), Islamabad whereas zinc fortificants were received from Fortitech Inc., New York, USA. The fortificants were used singly and in combination with each other for fortification of straight grade flour.

Preparation of bread diets

The bread was prepared from each fortified flour sample by using procedure of AACC (2000), following straight dough method No.10-10B on the basis of Bakers% as flour 100, yeast 001, salt 001, sugar 003, shortening 005 and deionized water according to water absorption. The ingredients were mixed for 5 min in a Hobart A-200 Mixer (Troy, OH) to form dough and were allowed to ferment at 86 °F (30 °C) and 75% relative humidity (RH) for 180 min. First and second punches were made after 120 and 150 min, respectively. The dough was molded and panned into 100 g (pup loaf) test pans, and final proofing was done for 45 min at 95 °F (35 °C) and 85% RH. The bread was baked at 450 °F (232 °C) for 25 min.

In the finely ground fortified bread flour of 875 g, the casein 60, canola oil 50, CaCO_3 3.5, NaH_2PO_4 2.5, vitamin mixture 1.5, NaCl 7 g/kg and copper @ 3 mg/kg were added to meet the nutritional standards set for rats (Reeves et al. 1993). The fortificants, calcium, iron and zinc were used singly and in combination with each other for fortification of flour. The flours were fortified manually for the preparation of the diets. In the 8 diets formulated, protein 145 g/kg, phytic acid 0.35 g/kg, copper 6.60 mg/kg, magnesium 1.12 g/kg and phosphorus 3.0 g/kg was found.

Animals model

The Sprague-Dawley Albino rats were selected for this study following the bioavailability study on calcium fortified bread conducted by Ranhotra et al. 1999. The 64 female Sprague-Dawley Albino rats were purchased from National Institute of Health (Veterinary Division) Islamabad, Pakistan and brought to the laboratory at University of Agriculture, Faisalabad. The rats were housed individually in stainless steel metabolic cages. The cages were placed in a room with controlled temperature (22–24 °C), RH (40–60%) with lightening for 8–12 h. They were used in the experiments at 7–9 week of age (148–159 g). The animals were divided into eight similar groups. Diets were fed to the animals for a period of 28 days and records of diet intake were maintained daily throughout the study. All animals were pair-fed thus nutrients intake, was identical among all groups during each test period. Deionized water was offered ad libitum. Animals were weighed weekly.

During both test periods (throughout 2 or 4 weeks), faeces and urine from each rat were collected from the trays underneath the cages. To minimize contamination from urine and spilled diet, faeces were collected daily and were cleaned of adhering diet. They were then air dried, weighed, finely ground, and stored frozen at -20°C for Ca, Fe and Zn determination. Absorption/retention of minerals in plasma, femur and liver tissues were determined according to the procedure adopted by Ranhotra et al. (1999). The protein content in each test diet was determined by Kjeldahl's method as described in AACC (2000) (method No. 46-10). The fat was determined by following the instructions provided in the manufacturer's manual and the procedure described in AACC (2000), (method No. 30-10). The fortified test diets were determined for phytic acid contents by the method described by Haug and Lantsch (1983).

Tissue sampling

After 2 and 4 weeks on test diets, rats were anaesthetized with sodium pentobarbital (40 mg/kg), and blood was drawn from the abdominal aorta. The clotted blood was centrifuged, and the plasma obtained was analyzed for Ca, Fe and Zn. The liver and left femurs were removed, cleaned of adhering tissues, dried and stored at -20°C for Ca, Fe and Zn determination.

Minerals determination

The concentration of Ca was determined after dry mineralization of tissue, feed and faeces samples (10 h at 500°C). Ash was extracted with 5 M-HCl and made up to an appropriate volume with LaCl_3 solution (1 g/L). A sample weighing 0.25 g dried sample was dry-ashed and acidification at 130°C in $\text{HNO}_3\text{--H}_2\text{O}_2$, until discoloration, to determine Fe and Zn levels in tissue, feed and faeces. Final dilutions were made in 0.14 M- HNO_3 . Plasma Ca concentrations were measured after a 50-fold dilution in LaCl_3 solution. Mineral concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer 420, Norwalk, CT, USA). The % AA was calculated as follows: apparent absorption (%) = (daily mineral intake (mg/d)—daily faecal mineral excretion (mg/d)) $\times 100$ /daily mineral intake (mg/d).

Statistical analysis

The data obtained were analyzed statistically to assess the changes in various parameters by using analysis of variance and DMR test for means separation by Minitab Software Package Version 14.0 (Minitab, Inc., State College, PA, USA).

Results and discussion

Effect on body weight and feed intake

The feed intake and live body weight of the experimental animals increased significantly with the time period (Table 1). While the live-weight gain of the experimental animals was significantly decreased with the time period. The feed intake, live body weight and live-weight gain of the experimental animals was not significantly influenced by changing the fortificants (Table 2).

Effect of fortified bread on retention of calcium in different body parts

At both intervals, plasma Ca levels did not differ significantly. Compared to the initial level of 1.04 ± 0.06 mg/l in week two, plasma Ca levels declined slightly (1.02 ± 0.03 mg/l) by week four (Table 3). The difference in liver Ca level between 2nd and 4th week was also non significant. The level of calcium in plasma and liver remained in the normal range throughout the study period because a very efficient homeostatic mechanism keeps plasma Ca levels in the normal range (Miller 1989). There was significant difference of time period on calcium content in femur of rats fed fortified bread at the 2nd and 4th week. At week four, the Ca was significantly higher (224.50 ± 14.0 mg/g) in femur of rats than week two (213.25 ± 15.29 mg/g). The variation in fortificants exerted significant effect on retention of calcium content in femur of rats (Table 3). The calcium was observed to be retained maximum in the femur of rats which were fed on diet containing calcium alone (T_2) followed by the diets containing $\text{CaCO}_3 + \text{FeSO}_4$ (T_5), $\text{CaCO}_3 + \text{ZnSO}_4$ (T_6) and $\text{CaCO}_3 + \text{FeSO}_4 + \text{ZnSO}_4$ (T_8). The calcium retention was found to be significantly the lowest in the femur of rats

Table 1 Effect of feeding time on body weight, feed intake and gain in body weight of female rats fed fortified bread

Physiological parameters	Weeks				
	0	1	2	3	4
Mean body weight (g)	153.57e	187.41d	210.85c	228.46b	239.50a
Gain in body weight (g)	—	33.84a	23.44b	17.61c	11.04d
Mean Feed Intake/week (g)	—	86.01d	97.58c	105.15b	111.36a

Values are means ($n=8$ rats).
Values in a column not sharing alphabets are different at $P<0.05$.

Table 2 Effect of treatment on body weight, feed intake and gain in body weight of female rats fed fortified bread

Physiological parameters	Treatments							
	Control	CaCO ₃	FeSO ₄	ZnSO ₄	CaCO ₃ + FeSO ₄	CaCO ₃ + ZnSO ₄	FeSO ₄ + ZnSO ₄	CaCO ₃ + FeSO ₄ + ZnSO ₄
Mean body weight (g)	199.32a	203.06a	202.06a	203.04a	205.02a	207.00a	205.12a	207.06a
Gain in body weight (g)	21.98a	20.63a	20.55a	21.98a	20.10a	21.63a	21.95a	23.05a
Mean Feed Intake/week (g)	98.85a	99.70a	99.73a	98.15a	104.88a	99.25a	101.90a	97.78a

Values are means ($n=8$ rats). Values in a column not sharing alphabets are different at $P<0.05$.

which were fed with the diets containing FeSO₄ and ZnSO₄ alone or in combination (T₃, T₄ and T₇). The results further demonstrated that the diets in which calcium was supplemented either in combination with iron or zinc exhibited less calcium retention in femur and the diets containing calcium alone (T₂) showed higher values for calcium in femur of the rats.

Effect of fortified bread on retention of iron in different body parts

Iron was retained more in liver at the 4th week while in plasma and femur, the iron content did not change significantly during the entire course of study (Table 3). The iron in plasma, liver and femur ranged from 2.09 ± 0.05 to 2.86 ± 0.09 $\mu\text{g/ml}$, 185 ± 6.99 to 237 ± 15.66 $\mu\text{g/g}$ and 42.5 ± 1.72 to 50.5 ± 1.66 $\mu\text{g/g}$, respectively. The iron content in plasma, liver and femur was found to be significantly the highest in rats fed diet containing FeSO₄ fortified bread (diet C). Iron contents were significantly lower in the plasma, liver and femur of rats fed diets containing CaCO₃ (T₂, T₅ and T₆). The diets which contained iron either in combination with calcium or zinc exhibited less iron retention and the diets which contained iron alone showed higher levels for iron in plasma, liver and femur of rats. These results suggested that there was a strong interaction of calcium and zinc with iron in reducing the retention of iron in plasma, liver and femur parts of the rats fed different combinations of calcium, iron and zinc fortificants. When iron fortificant was given with calcium (diet E, the iron retention significantly decreased (2.49 ± 0.08) in plasma as compared to iron fortificant in combination with zinc (diet G) fortificant (2.56 ± 0.08). The results manifested that calcium has more detrimental effect on iron retention than zinc in plasma. The same trend was observed in liver and femur of rats fed diet E and G. Hence, iron interacted more severely with calcium than zinc. The Fe retention was also significantly higher (2.45 ± 0.07 $\mu\text{g/ml}$ and 218 ± 9.35 $\mu\text{g/g}$) in plasma and liver of rats fed triple fortified diet (H) than the rats fed unfortified bread diet A (2.09 ± 0.05 $\mu\text{g/ml}$ and 192 ± 16.18 $\mu\text{g/g}$).

Effect of fortified bread on retention of zinc in different body parts

There were non significant difference of time period on zinc content in liver and femur of rats fed fortified bread at the 2nd and 4th week (Table 3) however the zinc content were found to be significantly the highest in plasma of rats during 4th week as compared to the 2nd week of the study. The zinc was retained maximum in the plasma of rats (Table 3) which were fed with the diet containing ZnSO₄ (T₄) followed by the diet containing CaCO₃+ZnSO₄ (T₆), FeSO₄+ZnSO₄ (T₇) and CaCO₃+FeSO₄+ZnSO₄ (T₈). The zinc retention was found to be significantly the lowest in the rats fed with the diet containing FeSO₄ (T₃) and CaCO₃+FeSO₄ (T₅) with a non significant difference between each other. The results further demonstrated that the diet which contained zinc either in combination with calcium or iron exhibited less zinc retention in plasma and liver and the diet which contained zinc alone (T₄) showed higher concentration for zinc in plasma and liver of rats. The effect of fortificants of calcium, iron and zinc on the zinc content in femur of rats was non significant. These results manifested an interaction of calcium and iron with zinc resulting in significant reduction of zinc in plasma and liver parts of the rats, fed different combinations of calcium, iron and zinc fortificants. The zinc content in plasma, liver and femur ranged from 1.30 ± 0.08 to 1.48 ± 0.06 $\mu\text{g/ml}$, 98 ± 7.94 to 140.5 ± 7.83 $\mu\text{g/g}$ and 183 ± 7.39 to 197.5 ± 6.44 $\mu\text{g/g}$, respectively. The Zn retention was higher (1.40 ± 0.09 $\mu\text{g/ml}$) in plasma of rats fed with triple fortified diet than the rats fed with unfortified bread diet (1.36 ± 0.05 $\mu\text{g/ml}$) with no significant difference. Similarly liver Zn content were also higher in the rats fed with triple fortified diet than control diet but femoral zinc content were lower in rats fed triple fortified diet than unfortified one.

Bioavailability of calcium, iron and zinc

The results for bioavailability of calcium shown in Table 4 suggested that calcium intake and excretion was higher in the rats fed diet containing calcium with either combination

Table 3 Ca, Fe and Zn in plasma ($\mu\text{g/ml}$), liver ($\mu\text{g/g}$) and femur ($\mu\text{g/g}$) of female rats fed fortified bread

Ca in different body parts										
Body Parts	Week	Control	CaCO ₃	FeSO ₄	ZnSO ₄	CaCO ₃ + FeSO ₄	CaCO ₃ + ZnSO ₄	FeSO ₄ + ZnSO ₄	CaCO ₃ + FeSO ₄ + ZnSO ₄	Mean
Plasma	2	1.01±0.03	1.1±0.02	1.02±0.03	1.04±0.05	1.07±0.07	0.99±0.09	1±0.04	1.06±0.04	1.04±0.06a
	4	1.01±0.03	1.05±0.03	1±0.01	1.03±0.02	1.04±0.03	1±0.03	0.98±0.03	1.04±0.03	1.02±0.03a
	Mean	1.01±0.03a	1.07±0.04a	1.01±0.02a	1.03±0.03a	1.06±0.05a	0.99±0.06a	0.99±0.03a	1.05±0.03a	
Liver	2	80±6.50	82±4.10	81±2.80	79±2.90	84±4.10	81±6.90	80±3.10	81±2.80	81.00±3.97a
	4	82±2.50	82±5.40	80±7.20	80±6.80	83±8.30	81±7.80	82±8.20	82±2.70	81.50±5.52a
	Mean	81±4.54a	82±4.29a	80.5±4.92a	79.5±4.71a	83.5±5.88a	81±6.59a	81±5.65a	81.5±2.52a	
Femur	2	205±10.90	223±7.30	203±21.60	205±16.20	221±13.30	224±13.70	206±11.20	219±20.0	213.25±15.29b
	4	217±12.60	235±12.5	217±15.60	217±10.70	231±21.0	234±12.90	215±8.80	230±9.90	224.50±14.0a
	Mean	211±12.42b	229±11.27a	210±18.51b	211±13.93b	226±16.65ab	229±13.10a	210.5±10.27b	224.5±15.35ab	
Plasma	2	2.11±0.05	2.19±0.06	2.83±0.04	2.39±0.16	2.47±0.06	2.05±0.09	2.57±0.06	2.43±0.03	2.38±0.26a
	4	2.08±0.06	2.26±0.05	2.89±0.12	2.48±0.12	2.52±0.1	2.13±0.04	2.55±0.1	2.48±0.1	2.42±0.26a
	Mean	2.09±0.05c	2.22±0.06d	2.86±0.09a	2.43±0.14c	2.49±0.08bc	2.09±0.08 e	2.56±0.08b	2.45±0.07c	
Liver	2	189±4.70	184±8.30	233±9.70	192±11.50	207±7.20	191±6.00	231±11.30	212±5.70	204.87±19.6b
	4	195±24.60	186±7.10	241±21.70	210±10.50	215±17.2	187±6.90	238±8.40	225±7.70	212.12±24.1a
	Mean	192±16.18de	185±6.99e	237±15.66a	201±13.94 cd	211±12.58bc	189±6.18de	234.5±9.70a	218.5±9.35b	
Femur	2	49±1.30	45±1.60	50±1.60	43±2.10	46±1.20	44±1.20	49±2.20	49±1.60	46.87±2.92a
	4	50±1.50	45±0.70	51±1.90	42±1.50	47±1.60	44±2.00	50±1.80	50±1.00	47.37±3.48a
	Mean	49.5±1.37a	45±1.11bc	50.5±1.66a	42.5±1.72d	46.5±1.38b	44±1.48 cd	49.5±1.88a	49.5±1.31a	
Plasma	2	1.33±0.04	1.32±0.04	1.3±0.03	1.43±0.03	1.29±0.11	1.43±0.05	1.38±0.04	1.39±0.13	1.36±0.08b
	4	1.4±0.03	1.31±0.06	1.31±0.05	1.53±0.03	1.31±0.05	1.5±0.05	1.4±0.07	1.42±0.04	1.40±0.09a
	Mean	1.36±0.05cde	1.31±0.05de	1.30±0.04e	1.48±0.06a	1.30±0.08e	1.46±0.06ab	1.39±0.05bcd	1.40±0.09abc	
Liver	2	107±10.20	98±9.10	97±10.60	141±11.90	101±4.60	130±5.60	134±5.10	125±2.90	116.62±18.29a
	4	110±8.60	100±4.10	99±6.50	140±3.30	103±4.70	131±6.90	136±1.60	121±4.60	117.50±16.61a
	Mean	108.5±8.60d	99±6.41e	98±7.94e	140.5±7.83a	102±4.30de	130.5±5.65bc	135±3.55ab	123±4.08c	
Femur	2	196±7.80	190±12.90	193±9.70	195±6.10	180±8.70	194±11.70	197±8.20	192±8.20	192.12±9.37a
	4	199±6.00	191±11.80	193±9.10	194±7.70	186±5.80	195±9.10	196±13.50	193±2.80	193.37±8.22a
	Mean	197.5±6.44a	190.5±11.07a	193±8.41a	194.5±6.24a	183±7.39a	194.5±9.39a	196.5±10.01a	192.5±5.51a	

Means (±SD) carrying similar alphabets in a row or a column do not differ significantly ($p<0.05$)

Table 4 Effect of fortified bread on Ca, Fe and Zn intake, excretion, absorption ($\mu\text{g/day}$) and % absorption in female rats

	Control	CaCO ₃	FeSO ₄	ZnSO ₄	CaCO ₃ +FeSO ₄	CaCO ₃ +ZnSO ₄	FeSO ₄ +ZnSO ₄	CaCO ₃ +FeSO ₄ +ZnSO ₄
Ca bioavailability								
Intake	118.06 $\pm$ 3.12d	132.81 $\pm$ 3.51b	118.93 $\pm$ 3.15d	118.03 $\pm$ 3.12d	148.09 $\pm$ 3.92a	136.13 $\pm$ 3.60b	126.55 $\pm$ 3.35c	134.78 $\pm$ 3.57b
Excretion	64.94 $\pm$ 1.72 cd	65.08 $\pm$ 1.70de	64.22 $\pm$ 1.70de	61.38 $\pm$ 1.62e	79.97 $\pm$ 2.12a	68.07 $\pm$ 1.80c	72.13 $\pm$ 1.91b	71.43 $\pm$ 1.89b
I-E	53.13 $\pm$ 1.41d	67.73 $\pm$ 1.79a	54.71 $\pm$ 1.45 cd	56.66 $\pm$ 1.50c	68.12 $\pm$ 1.80a	68.07 $\pm$ 1.80a	54.42 $\pm$ 1.44 cd	63.35 $\pm$ 1.68b
%AA	45 $\pm$ 1.19de	51 $\pm$ 1.35a	46 $\pm$ 1.22 cd	48b $\pm$ 1.27c	46 $\pm$ 1.22 cd	50 $\pm$ 1.32ab	43 $\pm$ 1.14e	47 $\pm$ 1.24 cd
Fe bioavailability								
Intake	440.63 $\pm$ 34.44b	446.09 $\pm$ 30.47b	1051.87 $\pm$ 105.08a	426.87 $\pm$ 39.02b	1189.42 $\pm$ 105.03a	436.81 $\pm$ 26.68b	1148.08 $\pm$ 92.74a	1075.07 $\pm$ 197.13a
Excretion	198.28 $\pm$ 8.74d	240.88 $\pm$ 21.1d	389.19 $\pm$ 42.46c	221.97 $\pm$ 18.22d	594.71 $\pm$ 87.63a	244.61 $\pm$ 12.77d	447.75 $\pm$ 37.65bc	537.54 $\pm$ 163ab
I-E	242.35 $\pm$ 26.42c	205.20 $\pm$ 20.68c	662.68 $\pm$ 85.48ab	204.89 $\pm$ 15.46c	594.71 $\pm$ 60.84ab	192.19 $\pm$ 13c	700.33 $\pm$ 47.41a	537.54 $\pm$ 70.57b
%AA	55 $\pm$ 7.6ab	46 $\pm$ 6.49bc	63 $\pm$ 6.97a	48 $\pm$ 2.96bc	50 $\pm$ 4.82bc	44 $\pm$ 4.82c	61 $\pm$ 6.1a	50 $\pm$ 1.52bc
Zn bioavailability								
Intake	248.96 $\pm$ 27.14b	240.92 $\pm$ 15.18b	241.77 $\pm$ 29.52b	552.11 $\pm$ 69.9a	261.87 $\pm$ 11.49b	555.65 $\pm$ 60.68a	592.00 $\pm$ 25.46a	570.49 $\pm$ 74.16a
Excretion	139.41 $\pm$ 15.69d	146.96 $\pm$ 10.35d	135.39 $\pm$ 9.49d	276.05 $\pm$ 16.05c	162.36 $\pm$ 10.58d	311.17 $\pm$ 25.51b	355.20 $\pm$ 21.98a	330.88 $\pm$ 18.06ab
I-E	109.54 $\pm$ 5.15c	93.96 $\pm$ 8.95c	106.38 $\pm$ 10.53c	276.05 $\pm$ 25.81a	99.51 $\pm$ 12.9c	244.48 $\pm$ 30.31ab	236.80 $\pm$ 30.24b	239.61 $\pm$ 18.88ab
%AA	44 $\pm$ 0.62b	39 $\pm$ 1.28b	44 $\pm$ 4.25b	50 $\pm$ 3.57a	38 $\pm$ 2.02b	44 $\pm$ 4.17b	40 $\pm$ 3.57 b	42 $\pm$ 3.03b

Means ($\pm$ SD) carrying similar alphabets in a row do not differ significantly ($p < 0.05$)

of calcium (diet B, E, F and H). The absorption of calcium was significantly higher from the diets supplemented with CaCO₃ (B, E, F and H) and significantly lower calcium absorption in the rats that were not fed diets containing with extrinsically added CaCO₃ (A, C, D and G). The diets supplemented with CaCO₃ (B) exhibited significantly the highest% AA of calcium followed by the diets containing CaCO₃+ZnSO₄ (F), ZnSO₄ (D) and CaCO₃+FeSO₄+ZnSO₄ (H). The significantly lowest% AA of calcium was exhibited by the diets comprising FeSO₄+ZnSO₄ (G).

The variation in fortificants resulted in significant differences for iron intake of rats fed fortified bread (Table 4). Iron was consumed significantly higher from the diets supplemented with FeSO₄ or its any combination with other salt (diet C, E, G and H) and significantly lower in the rats that were not fed extrinsic FeSO₄ (diet A, B, D and F). But the excretion of iron was significantly higher in the rats fed diet containing CaCO₃+FeSO₄ (E) than the diet containing CaCO₃+ZnSO₄ (F). Similarly, the absorption of iron changed with the difference in combination of fortificant with iron. The absorption of iron was significantly higher in the group of rats fed with the diet containing FeSO₄ (alone) fortified bread (diet C) or in combination with zinc (diet G). The diets supplemented with FeSO₄ (C) exhibited significantly the highest% AA of iron followed by the diets containing FeSO₄+ZnSO₄ (G), unfortified diet (A) and CaCO₃+FeSO₄+ZnSO₄ (H). While significantly the lowest% AA of iron was exhibited by the diets comprising of CaCO₃+ ZnSO₄ (F).

The variation in fortificants resulted in significant differences for zinc intake of rats fed fortified bread (Table 4). The zinc was consumed significantly higher from the diets supplemented with ZnSO₄ or its combination with other fortificants (diet D, F, G and H) and significantly lower in the rats that were not fed extrinsic ZnSO₄ (diet A, B, D and F). The excretion of zinc was higher with the diet containing FeSO₄+ZnSO₄ (G). The absorption of zinc was significantly higher from the diets supplemented with ZnSO₄ (diet D, F, G and H) and significantly lower zinc absorption in the rats that were not fed diets containing extrinsically added ZnSO₄ (A, B, C and E). The diets supplemented with ZnSO₄ (D) exhibited significantly the highest% AA of zinc while significantly the lowest% AA of zinc was exhibited by the diets comprising CaCO₃ (B).

The results from this study validated that mineral absorption was higher from the triple fortified diets of bread as compared to unfortified diets.

Discussion

In the plasma and liver of rats, the Ca concentration did not change with the time period and Ca supplemented diets.

These findings illustrated the effectiveness of Ca homeostasis (Windisch and Kirchgessner 1999), which is mainly regulated by the parathyroid hormone, calcitonin and calcitriol over a wide range of calcium intake. But femur is the best indicator of Ca status (Ranhotra et al. 1999) in which the significant change was observed with time period and Ca fortified bread diets. The Ca supplemented diets increased Ca level in femur of rats. Similarly iron and zinc fortified diets contributed more iron and zinc in the plasma, liver and femur of rats (Table 3) but where the extrinsic calcium was supplemented with iron or zinc fortificants, it decreased the retention of iron and zinc in all the tested organs of the rats. The bioavailability of Ca decreased more (46%) from the diets which were supplemented with Fe than the diets supplemented with Zn (50%). Hence, iron caused more detrimental effect on Ca absorption than Zn. The calcium absorption was 63.35 ± 1.68 mg/day from the triple fortified diet while the calcium was absorbed 53.13 ± 1.41 mg/day from the unfortified diet of bread (Table 4). Therefore, multiple fortified diets contributed more calcium to the rats.

The absorption of iron was significantly higher (537.54 ± 70.57 µg/day) from the triple fortified diet as compared to the unfortified diet (242.35 ± 26.42 µg/day) but % AA of iron was higher in unfortified diet (55%) when compared with triple fortified diet (50%). The iron was more bioavailable when the diet was supplemented with extrinsic FeSO₄ in alone as compared to the other diets in which iron was supplemented with combination of zinc or calcium fortificants.

Similarly the absorption of zinc was significantly higher (239.61 ± 18.88 µg/day) from the triple fortified diet as compared to the unfortified diet (109.54 ± 5.15 µg/day) but % AA of zinc was higher in unfortified diet (44%) when compared with triple fortified diet (42%). Similarly, higher bioavailability of zinc was observed when the diet was fortified with extrinsic ZnSO₄ alone as compared to the diets containing zinc fortificant in the presence of iron or calcium fortificants (Table 4).

Low apparent iron and zinc absorption as well as low femur iron and zinc concentrations were measured in rats fed diet supplemented with CaCO₃. The observations in the present study can possibly be explained by a reduction of zinc availability due to antagonistic Ca–Zn-interactions. Similar results were reported in other studies (Windisch and Kirchgessner 1999; Mendoza et al. 2004) indicating that higher dietary calcium was marginally associated with lower zinc absorption. Several investigators reported that calcium may interfere with the absorption of iron and zinc (Hallberg et al. 1992; Fordyce et al. 1987). Previous studies in animals and humans have demonstrated that calcium reduces iron absorption (Fordyce et al. 1987; Dawson-Hughes et al. 1986; Cook et al. 1991).

Several studies (Solomons 1986; Solomons et al. 1983; Meadows et al. 1983) demonstrated that iron supplementa-

tion lowered plasma zinc concentrations, but plasma zinc concentration is not considered to be a good index of body zinc. Sandstrom (2001) observed negative effects of iron supplementation on indices of zinc and vice versa. Another study (Sandstrom et al. 1987) indicated no inhibitory effect on zinc absorption when iron and zinc were given with a meal of rice and meat sauce. Walker et al. (2005) manifested that iron did not appear to have a negative effect on serum zinc concentrations; if there is an effect, it is small. Iron and zinc appear to be highly bioavailable from foods made from fortified flour, but zinc sulfate co-fortification may have a detrimental effect on iron absorption (Herman et al. 2002).

Several researchers have measured iron absorption from bread and other flour products. It is not easy to compare our results to previous studies because iron absorption is affected by differences in the dose of iron, the form in which it is added, the amount of flour, the bran (Brune et al. 1992) and the phytic acid content of the dough. These findings are in accordance to those reported by others who found a better iron bioavailability from diets with high iron content (Ologunde et al. 1994).

Phytic acid is also a potent inhibitor of calcium, iron and zinc but it is well established that rats have a high capacity to hydrolyse phytic acid, compared with human subjects who have practically no intestinal phytase activity (Iqbal et al. 1994). The calcium–phytate–zinc interactions may adversely affect zinc bioavailability in growing rats. The supplementation of calcium may decrease in the apparent absorption of Fe and Zn. Uthus and Zaslavsky (2001) reported that the interaction between iron and zinc is nutritionally important and dietary iron affects the response of many blood parameters to dietary zinc. Wieringa et al. (2007) demonstrated that combined supplementation reduced prevalence of anemia by 21% and zinc deficiency by 10% but was less effective than supplementation with either iron (28% reduction in anemia) or zinc alone (18% reduction in zinc deficiency). They also emphasized that combined supplementation of iron and zinc was safe and effective in reducing the high prevalence of anemia and iron and zinc deficiencies. Zinc supplementation may negatively affect iron status but iron supplementation does not seem to affect zinc status. This was supported in a recent study (Akhtar et al. 2010) demonstrating that ingestion of ZnSO₄ resulted in the highest absorption of zinc when given alone and/or with iron sources and the presence of zinc in the diet (wheat flour) might have an antagonistic effect on iron absorption in rats.

Conclusion

The results exhibited an interaction of calcium, iron and zinc with each other in fortified bread. It is concluded from the

study that Ca, Fe and Zn fortified diets were relatively higher in absorption of Ca, Fe and Zn as compared to unfortified bread diet. Hence multiple fortification of wheat flour with Ca, Fe and Zn can be recommended to help the targeted population and it is a possible mean of addressing deficiencies of two or more micronutrients in a cost effective manner.

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