ELSEVIER

Food Chemistry 80 (2003) 545–550

Food Chemistry

www.elsevier.com/locate/foodchem

In vitro bioavailability of iron from wheat flour fortified with ascorbic acid, EDTA and sodium hexametaphosphate, with or without iron

Binata Nayak, K. Madhavan Nair*

Department of Biophysics, National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania, Hyderabad-500007, Andhra Pradesh, India

Received 5 February 2002; received in revised form 10 July 2002; accepted 10 July 2002

Abstract

A laboratory scale technology was developed to fortify wheat flour with absorption promoters of iron, such as ascorbic acid, disodium ethylenediaminetetraacetic acid (NaEDTA) and with a stabilizer, sodium hexametaphosphate (SHMP), with or without iron. The in vitro bioavailability of iron in food (Indian bread, chapathi) prepared with the wheat flour fortified at 60 mg of iron/kg in the presence (1:1 molar ratio) or absence of the three chemical additives was tested. NaEDTA and ascorbic acid enhanced the in vitro bioavailability of native iron from Indian bread while SHMP had no effect. All three additives showed a trend of enhancing the in vitro bioavailabilty of total iron (native and added iron) from iron fortified chapathis. The predicted bioavailability of iron in man from Indian bread containing ascorbic acid or NaEDTA was twice as high than that with wheat flour alone or that with SHMP (8%). Similar enhancing effects of these two compounds were shown with iron-fortified wheat flour. It is concluded that wheat flour fortified with ascorbic acid or NaEDTA, either with or without iron, can enhance the predicted bioavailability of both native and added iron in man.

Keywords: Fortification; Wheat flour; NaEDTA; Ascorbic acid; Sodium hexametaphosphate; In vitro bioavailability

1. Introduction

Nutritional iron deficiency is a public health problem in developing countries, including India (Seshadri, 1997). Inadequate intake of iron and consumption of foods low in bioavailable iron are identified as the causes of iron deficiency anemia (Rao & Prabhavathi, 1983). In most parts of the world, including India, wheat is the staple food. Fortification of wheat flour with iron is a common strategy in industrialized countries. Successful introduction of mandatory fortification of wheat flour with iron, combined with the consumption of foods rich in iron absorption promoters, such as meat and citrus fruits, helped the developed countries to reduce iron deficiency anemia significantly (Hurrell & Jacobs, 1996). However, in less developed countries, such as India, the bioavailability of iron from a typical cereal–pulse-based diet (3%) is rather low and the intake of foods rich in iron absorption promoters is inadequate (Rao & Prabhavathi, 1983).

The approach of enhancing the bioavailability of native food iron thus seems to be an appropriate strategy that needs to be tested in developing countries. Ascorbic acid is the most potent enhancer of iron absorption, both in its natural form in fruits and vegetables and when added as the free compound (Cook & Monsen, 1977; Hazell & Johnson, 1987). Ascorbic acid increases the bioavailability of all iron fortification compounds as well (Hurrell, 1992). Sodium hexametaphosphate (SHMP) is a polyphosphate, used in the double fortification of salt with iron and iodine. Based on the in vitro studies, the predicted bioavailability of iron from food with SHMP was shown to be better than that without SHMP (Rao & Rao, 1985). However, its inclusion as an iron absorption promoter in fortified foods has not been attempted. Ethylenediaminetetraacetic acid (EDTA) is a chelator and combines stoichiometrically

* Corresponding author. Fax: +91-40-7019074.
E-mail address: nairthayil@hotmail.com (K.M. Nair).

0308-8146/03/$ - see front matter
PII: S0308-8146(02)00341-2

with iron. Ferric iron forms a strong complex with EDTA and has a high stability constant, log K, of 25.1 (West & Sykes, 1960). There is convincing evidence that iron chelated by EDTA is available for absorption (Candela, 1989). Thus fortification of wheat flour with iron absorption promoters, to enhance the native and added iron bioavailability appears to be an attractive strategy. The present study has been undertaken with the objective of evaluating the in vitro bioavailability, of wheat flour fortified with iron and iron absorption promoters.

2. Materials and methods

2.1. Materials

Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), a cost-effective source of iron, was chosen for the study. All the chemicals used were of analytical grade and procured locally, except ascorbic acid, which was from Sigma (St. Louis, USA). SHMP used had a P_2O_5 content of more than 62%.

2.2. Fortification of wheat flour

A single batch of wheat (*Sharbati* variety) was procured from the local market. After ensuring that the wheat was free from contaminant iron (the iron content of washed and unwashed wheat samples were 3.39 ± 0.19 and 3.23 ± 0.13 mg/100 g, respectively), it was powdered in a flourmill. No further processing was done on the flour. An RDA of 28 mg of iron and 460 g of cereals for an Indian adult man was used to derive the level of fortification of wheat flour with iron (Report of the Expert Group of the Indian Council of Medical Research, 2000). Accordingly, the fortification was done at 60 mg of iron per kg of wheat flour. The additions of ascorbic acid, disodium EDTA (NaEDTA) and SHMP (phosphorus) were adjusted to eqimolar concentrations either to native iron or to the total (native + added) iron.

A five times concentrated premix of each of the fortificants ($FeSO_4{\cdot}7H_2O$, ascorbic acid, NaEDTA and SHMP) was prepared in wheat flour base. A two stage dry-mixing (hand procedure) was adopted to obtain a homogeneous preparation. Briefly, the required quantity of the premix was mixed with small amounts of wheat flour until a 1:1 dilution of the premix was achieved (Table 1). Iron contents in these preparations were estimated and they were packed in 500 g polythene bags stored at room temperature for in vitro bioavailability of iron.

2.3. Preparation of Indian bread

Indian bread (chapathi) was made with all the types of fortified wheat flour, separately, using glass-distilled water under identical conditions. Care was taken to avoid iron contamination during preparation. Dough was prepared by hand-mixing of the flour with sufficient water and salt to taste. This was then made into quantities of about 100 g and uniformly spread on a clean wooden surface with a hand held steel roller. This was then put on a hot aluminium pan, first, and then directly on a fire and baked.

Table 1
Formulations of wheat flour fortified with or without iron and iron absorption promoters

Type	Staple	Iron source	Promoter
1	Wheat flour	–	–
2	Wheat flour	–	Ascorbic acid
3	Wheat flour	–	NaEDTA
4	Wheat flour	–	SHMP
5	Wheat flour	$FeSO_4{\cdot}7H_2O$	–
6	Wheat flour	$FeSO_4{\cdot}7H_2O$	Ascorbic acid
7	Wheat flour	$FeSO_4{\cdot}7H_2O$	NaEDTA
8	Wheat flour	$FeSO_4{\cdot}7H_2O$	SHMP

Fortification of the wheat flour was done at 1:1 molar ratios of iron and absorption promoter. In types 4 and 8 the molar ratio of phosphorus content of SHMP was used.

2.4. Estimation of total iron

Iron contents in all the preparations were determined in the mineral solutions of the dry digested samples according to the Wongs method (Oser, 1965). About 5–10 g of the fortified wheat flour, or the food preparation, were ashed at 600 °C in a muffle furnace for 12 h. The residue was treated in tandem with concentrated nitric and hydrochloric acid and evaporated to dryness. The residue thus obtained was dissolved in 5 ml of 6 N HCl and filtered. This process was repeated twice with glass-distilled water. The combined filtrate was made up to 100 ml. To an aliquot of the mineral solution (6.5 ml) 1 ml of 30% sulfuric acid, 1 ml of 7% potassium persulfate and 1.5 ml of 40% potassium thiocyanate were added and the optical density was read at 540 nm in a Spectronic 21 D (Milton Roy). Iron standards (Sigma, St. Louis, USA), ranging from 10 to 50 μg, were also run with each assay set.

2.5. In vitro method of food iron bioavailability

The in vitro bioavailability of iron from Indian bread was estimated by the method described by Rao and Prabhavati (1978). The method involves incubation of duplicates of an 8% (g/v) homogenate (25 ml) of the food preparation in pepsin–HCl (0.5% pepsin in 0.1 N HCl) solution, pH 1.35 (adjusted with distilled H_2O) at 37 °C for 90 min, after which the contents of one set were centrifuged at 3000 rpm for 45 min and the supernatant

filtered and saved for the iron estimation. The pH of the other set was adjusted to 7.5 with 5 N sodium hydroxide and incubated and processed as above to obtain the filtrate. Ionizable iron in the filtrates was estimated by α,α′-dipyridyl method (AOAC, 1965).

2.6. Estimation of ionizable iron

Duplicate aliquots (5 ml) of the filtrates were taken and the volume in one set was made up to 15 ml and served as the extract blank. The other aliquot was treated with 1 ml of 10% hydroxylamine hydrochloride, 5 ml of acetate buffer pH 4.2, 2 ml of 0.1% of α,α′-dipyridyl reagent, and made up to 15 ml with water. The colour developed was measured at 510 nm in the Spectronic 21 D against a reagent blank. Iron standards (1–15 μg) were simultaneously run in the assay. The ionizable iron content in the sample was derived after subtracting the optical density of the extract blank from that of the sample.

2.7. Statistical analysis

Since the study design contained more than two groups, one-way analysis of variance was used to find out the mean difference between the groups. Multiple comparison with the least significant difference (LSD) method was used to assess which combination was different from the rest. A '*P*' value of less than 0.05 was considered significant. A SPSS 10.0 Windows version was used for analyzing the above tests.

3. Results

3.1. Iron content

The endogenous iron content of Indian bread was 3.9 mg/100 g (range 3.5–4.1 mg/100 g) and was not different from that of wheat flour 3.39 ± 0.19. The total iron content of different combinations of iron fortified wheat flours ranged from 9.0 to 9.9 mg/100 g.

3.2. Effect of additives on in vitro bioavailability of native iron from Indian bread

The native iron, ionizable at pH 1.35 with ascorbic acid, NaEDTA and SHMP, was comparable to wheat flour. The percentage of ionizable iron, under gastric pH conditions, was similar for different combinations of Indian bread, i.e. 31.3% for wheat flour and SHMP to 45.2% for wheat flour and NaEDTA (Table 2).

The bioavailability of native iron, measured at pH 7.5, was twice higher ($P < 0.05$) with the Indian bread containing ascorbic acid and NaEDTA than with that containing SHMP and wheat flour (Table 2). The ionizable iron content of bread containing SHMP was similar to that of the control preparation.

3.3. Effect of additives on in vitro bioavailability of total (native and added) iron from Indian bread

Absolute, as well as percentage, values of ionizable iron at pH 1.35 with iron fortified wheat flour and with SHMP were similar. The values were significantly higher ($P < 0.05$) in ascorbic acid-containing iron-fortified bread than in the other combinations ($P < 0.05$). The ionizable iron content at the gastric pH was the lowest ($P < 0.05$) with NaEDTA-containing iron-fortified bread (Table 3).

Although inclusion of chemical additives increased ionizable iron content at the duodenal pH of 7.5, the values were not statistically significant compared to the control iron-fortified Indian bread. About 79% of ionizable iron at the gastric pH, 1.35, was still in the ionizable form at the pH of 7.5 with NaEDTA, while with ascorbic acid it was 37%, SHMP 47% and with the control 32%.

4. Discussion

4.1. General

Ligands which can bind iron to keep it in solution with good stability, at the neutral or alkaline pH of the small intestine, can serve as useful absorption promoters of dietary iron. Ascorbic acid, NaEDTA and SHMP are strong enhancers of non-heme iron absorption (Rao & Rao, 1984; MacPhail, Patel, Bothwell, & Lamparelli, 1994). Therefore, approaches such as enhancing the native or added iron bioavailability from wheat flour with absorption promoters may help in controlling iron deficiency anemia. The maximum enhancing effect of ascorbic acid on iron absorption was reported to be with a molar ratio of iron to ascorbic acid of 1:4 (Stekal et al., 1986). The suggested molar ratio of NaEDTA to iron should be at least 1:1 for it to function as an absorption promoter of iron (MacPhail et al., 1994). In the present study, however, iron absorption additives were tested only at a 1:1 molar ratio, keeping in view the prooxidant properties of ascorbic acid (Almaas, Rootwell, Oyasaeter, & Saugstad, 1997).

Iron content of the wheat flour tested in the present study was around 4.0 mg/100 g. This value is similar to that reported earlier (Gopalan et al., 1989). A two stage dry-mix method was used for wheat flour fortification. The amount of added iron (6.0 mg/100 g) meets 100% of the RDA of iron for an Indian adult man. This is in agreement with the policy of certain developed countries. Many countries have adopted the mandatory or voluntary iron fortification of wheat flour. The levels of iron

Table 2
In vitro bioavailability of native iron from ascorbic acid-, NaEDTA- and SHMP-fortified Indian bread

Type	Combination	Average total iron (mg/100 g)	pH 1.35		pH 7.5	
			Ionizable iron (mg/100 g)	% of total iron	Ionizable iron (mg/100 g)	% of total iron
1	Wheat flour	3.5	1.4a±0.41	38.6a±11.88	0.6a±0.19	15.8a±5.46
2	Wheat flour + Ascorbic acid	3.7	1.6a±0.02	44.0a±0.62	1.3b±0.38	34.4b±10.09
3	Wheat flour + NaEDTA	3.2	1.5a±0.32	45.2a±9.98	1.2b±0.37	38.3b±11.57
4	Wheat flour + SHMP	3.7	1.2a±0.21	31.3a±5.70	0.7a±0.13	17.7a±3.49

$N=3$, values are means ± SD. Mean values in a given column with different letters are statistically significant at $P<0.05$ by one-way ANOVA and post hoc multiple comparison test with LSD.

Table 3
In vitro bioavailability of native and added iron from iron- and ascorbic acid-, NaEDTA- and SHMP-fortified Indian bread

Type	Combination	Average total iron (mg/100 g)	pH 1.35		pH 7.5	
			Ionizable iron (mg/100 g)	% of total iron	Ionizable iron (mg/100 g)	% of total iron
5	Wheat flour + $FeSO_4{\cdot}7H_2O$	9.9	4.7a±0.45	47.9a±4.55	1.5a±0.41	15.4a±4.16
6	Wheat flour + $FeSO_4{\cdot}7H_2O$ Ascorbic acid	9.8	5.9b±0.43	60.6b±4.34	2.2a±0.81	22.0a±8.21
7	Wheat flour + $FeSO_4{\cdot}7H_2O$ + NaEDTA	9.2	2.8c±0.69	30.7c±7.58	2.2a±0.37	24.9a±4.06
8	Wheat flour + $FeSO_4{\cdot}7H_2O$ + SHMP	9.8	4.7a±0.26	47.5a±2.72	2.2a±0.69	22.7a±7.00

$N=3$, values are means ± SD. Mean values in a given column with different letters are statistically significant at $P<0.05$ by one-way ANOVA and post hoc multiple comparison test with LSD.

fortification commonly used in developed countries are 6.5 mg/100 g in Sweden, 4.4 mg/100 g in USA and 1.65 mg/100 g in the UK (Barrett & Ranum, 1985; Hurrell & Jacobs, 1996).

4.2. In vitro bioavailability of iron from Indian bread

Ionizable iron at pH 7.5 has been shown to be useful for predicting food iron bioavailability in humans (Rao & Prabhavathi, 1978). It is assumed that, at gastric pH of 1.35, most of the iron will be in the ionizable form. During the transit of iron to the duodenal pH of 7.5, most of the iron becomes insoluble, unless chelated by dietary components that enhance iron absorption.

4.3. Effect of ascorbic acid, NaEDTA and SHMP on native iron bioavailability

The food iron that is ionizable at the duodenal pH of 7.5 is expected to be available for absorption. The native iron ionizable at pH 7.5 was higher in wheat flour fortified with ascorbic acid (34.4%) or NaEDTA (38.3%) than in wheat flour alone (15.8%) or wheat flour with SHMP (17.7%). Both ascorbic acid and NaEDTA were found to retain more than 80% of the native iron ionizable at the gastric pH available at pH 7.5 as against 50% with controls. With SHMP the value was around 60%. This is in agreement with the strong iron absorption promoting abilities of NaEDTA and ascorbic acid and data obtained from other in vitro studies (Rao & Rao, 1983, 1985)

Inorganic polyphosphate, when added to raw and cooked foods at the 1% level, was shown to increase dietary ionizable iron significantly, i.e. 22–24% ionizable iron in Indian bread containing polyphosphates compared to 8.5% without polyphosphates (Rao & Rao, 1983, 1984). In the present study, the native iron ionizable with SHMP fortified wheat flour was similar to the control preparation and could be due to the low level (0.1% compared to 1%) of this additive tested.

4.4. Effects of ascorbic acid, NaEDTA and SHMP on total (added and native) iron

It is well established that inhibitors, such as phytic acid, tannins and phosphates inhibit absorption of both native and added iron from food (Agte & Joshi, 1997; Martinez-Torres & Layrisse, 1973). Additives, such as ascorbic acid, are known to reverse this inhibitory effect (Derman, Bothwell, & MacPhail, 1980).

Iron-fortified Indian bread containing ascorbic acid had a significant enhancing effect on ionizable iron at pH 1.35 compared with all the other combinations. NaEDTA had very little effect in keeping the native and added iron in ionizable form at this pH. Inclusion of these additives gave higher ionizable iron contents at the duodenal pH of 7.5, though this was statistically not significant (22–25% vs. control 15.4%). Also NaEDTA

was found to retain about 81% of ionizable iron found at the gastric pH, 1.35, in the ionizable form at the duodenal pH of 7.5, while, with ascorbic acid, this was 36%, with SHMP 48% and without any absorption promoters 32%. These results suggest that NaEDTA could be useful to include in iron fortification of wheat flour. This is expected to keep iron in ionizable form and aid in better absorption of native and added iron.

4.5. Predicting iron bioavailability in humans

There are no human studies in India with iron-fortified wheat flour containing iron absorption promoters. Therefore an attempt was made to derive the in vivo availability of iron from Indian bread with or without iron and absorption promoters. Using the equation of Rao and Prabhavathi (1978), the predicted bioavailability of iron from wheat flour bread fortified with ascorbic acid (16.7%) and sodium NaEDTA (18.5%) was found to be twice higher than that with wheat flour alone (7.9%) or with SHMP (8.8%). Similar predicted values were derived for iron-fortified bread combinations also containing absorption promoters. These values are higher than those reported earlier and could be due to the difference in the meals used (MacPhail et al., 1994; Rao, 1994; Rao, Prasad, & Apte, 1972). Recently, an iron absorption enhancing effect of $FeSO_4$-fortified wheat cereal containing NaEDTA (molar ratio 0.7:1) or ascorbic acid (molar ratio 0.6:1) was shown in human subjects (Davidsson, Walczyk, Zavaleta, & Hurrell, 2001; Hurrell, Reddy, Burri, & Cook, 2000).

There are many studies that show the absorption promoting effect of ascorbic acid. Derman et al. (1980) showed that addition of ascorbic acid to ferric pyrophosphate-fortified cereal, up to an ascorbic acid to iron ratio of 4.4:1, progressively increased iron absorption by adult women from 1.6–7.5%. Similarly, a higher mean range of absorption of 5.9 – 11.3% was reported in infants fed a powdered milk formula with addition of ascorbic acid at a concentration of 100 mg/l or higher up to 800 mg/l (Stekal et al., 1986). In a recent study in children, the geometric mean iron absorption of 5% from a low ascorbic acid-containing meal (0.6:1) increased to 8.2% with a 70 mg ascorbic acid (molar ratio 1.6:1)-added meal. A significant effect on the mean fractional incorporation (5.9–9.6%) of absorbed iron into erythrocytes was also demonstrated in infants when the iron:ascorbic acid molar ratio in the infant formula based on soy isolate was increased from 1:1.2 to 1:4.2 (Davidsson et al., 1994). The results of the present in vitro studies with a 1:1 molar ratio of iron to ascorbic acid in Indian bread are in agreement with the in vivo studies reported earlier.

The effectiveness of NaEDTA in enhancing the bioavailability of added iron in Egyptian bread was demonstrated in animals (Whittaker & Vanderveen, 1990). Previous studies in adults and children showed significantly increased iron absorption after addition of sodium NaEDTA, together with ferrous sulfate, to cereal-based meals (Davidsson et al., 2001; MacPhail et al., 1994). In the present study, the predicted bioavailability of iron from Indian bread containing NaEDTA was twice higher than that without NaEDTA, which is in line with the reported results. Currently, NaFeEDTA is considered as a promising iron compound for food fortification programmes because of its high iron bioavailability from meals containing dietary inhibitors of iron absorption (Viteri et al., 1995).

The use of SHMP, at 1% level, as a stabilizer in iron and iodine double fortified salt has shown a 1.6 times higher (6.1% with rice-based meal) iron absorption in adults (Rao, 1994). The predicted bioavailability of iron with SHMP was close to that without, indicating only a limited use of this additive for enhancing iron bioavailability.

From the in vitro studies it can be predicted that wheat flour fortified with ascorbic acid or NaEDTA, with or without iron at a molar ratio of 1:1, enhances the absorption of both native and added iron in humans.

Acknowledgements

Dr. Kamala Krishnaswamy, the Director and Dr. B. Sivakumar, Head of Biophysics Division, are thanked for their useful discussions. We also thank Dr. M. Vishnuvardhan Rao for his help in statistical analysis and Mr. J.S. Acharya for technical help.

References

Agte, V., & Joshi, S. R. (1997). Effect of traditional food processing on phytate degradation in wheat and millets. *Journal of Agriculture and Food Chemistry*, *45*, 1659–1661.

Almaas, R., Rootwell, T., Oyasaeter, S., & Saugstad, O. D. (1997). Ascorbic acid enhances hydroxyl radical formation in iron fortified infant cereals and infant formulas. *European Journal of Pediatrics*, *156*, 488–492.

AOAC. (1965). *Official Methods of Analysis of the Association of Official Agricultural Chemists*. Washington DC: AOAC.

Barrett, F., & Ranum, P. (1985). Wheat and blended cereal foods. In F. M. Clydesdale, & K. L. Weiner (Eds.), *Iron fortification of foods* (pp. 75–109). New York: Academic Press.

Candela, E., Camiacho, M. V., Martinez-Torres, C., Perdamo, J., Mazzarri, G., Acunemo, G., & Layrisse, M. (1989). Iron absorption by humans and swine from Fe (111)-EDTA. Further studies. *Journal of Nutrition*, *114*, 2204–2211.

Cook, J. D., & Monsen, E. R. (1977). Vitamin C, the common cold and iron absorption. *American Journal of Clinical Nutrition*, *38*, 648–659.

Davidsson, L., Galan, P., Kastenmeyer, P., Cherouvier, F., Juillerat, M. A., Hercberg, S., & Hurrell, R. F. (1994). Iron bioavailability studies in infants: the influence of phytic acid and ascorbic acid in infant formulas based on soy isolate. *Pediatric Research*, *36*, 816–822.

Davidsson, L., Walczyk, T., Zavaleta, N., & Hurrell, R. F. (2001). Improving iron absorption from a Peruvian school breakfast meal by adding ascorbic acid or Na_2EDTA. *American Journal of Clinical Nutrition*, *73*, 283–287.

Derman, D. P., Bothwell, T. H., & MacPhail, A. P. (1980). Importance of ascorbic acid in the absorption of iron from infant foods. *Scandinavian Journal of Hematology*, *25*, 193–200.

Gopalan, C., Ramasastry, B.V., & Balasubramanian, S.C. (1989). Nutritive value of Indian foods. Revised and updated in: Rao, B. S. N., Deosthale, Y. G., & Pant, K. C. (Eds.), National Institute of Nutrition. Hyderabad, India: Indian Council of Medical Research (pp. 63–73).

Hazell, T., & Johnson. (1987). In vitro estimation of iron availability from a range of plant foods: influence of phytate, ascorbate and citrate. *British Journal of Nutrition, 57*, 223–233.

Hurrell, R. F., Reddy, M. B., Burri, J., & Cook, J. D. (2000). An evaluation of EDTA compounds for iron fortification of cereal-based foods. *British Journal of Nutrition*, *84*, 903–910.

Hurrell, R. F. (1992). Prospects for improving the iron fortification of foods. In S. Faman, & S. Zlotkin (Eds.), *Nutritional anemias* (pp. 193–208). New York: Raven Press.

Hurrell, R.F., & Jacobs, S. (1996). The role of the food industry in iron nutrition. Iron intake from industrial food products. In: L. Hallberg, & N. Asp, (Eds), *Iron nutrition in health and disease* (pp. 341–346). England: John Libbey & Company Ltd.

MacPhail, A. P., Patel, R. C., Bothwell, T. H., & Lamparelli, R. D. (1994). EDTA and the absorption of iron from food. *American Journal of Clinical Nutrition*, *59*, 644–648.

Martinez-Torres, C., & Layrisse, M. (1973). Nutritional factors in iron deficiency: food iron absorption. In S. T. Callender (Ed.), *Clinics in hematology* (pp. 339). London 2: W.B. Saunders and Co.

Oser, B. L. (1965). *Hawk's physiological chemistry* (14th ed.). New York: The Blakiston Division, McGraw-Hill.

Rao, B. S. N. (1994). Fortification of salt with iron and iodine to control goiter: development of a new formula with good stability and bioavailability of iron and iodine. *Food and Nutrition Bulletin*, *15*, 32–39.

Rao, B. S. N., & Prabhavathi, T. (1978). An *in vitro* method for predicting the bioavailability of iron from foods. *American Journal of Clinical Nutrition*, *31*, 169–175.

Rao, B. S. N., & Prabhavathi, T. (1983). Contaminant iron in foods and its bioavailability predicted by *in vitro* method. *Indian Journal of Medical Research*, *74*, 37–41.

Rao, B. S. N., Prasad, S., & Apte, S. V. (1972). Iron absorption in Indians studied by whole body counting: a comparison of iron compounds used in salt fortification. *British Journal of Hematology*, *22*, 281–286.

Rao, S. K., & Rao, B. S. N. (1983). Studies on iron chelating agents for use as absorption promoter: effects of inorganic polyphosphate on dietary ionizable iron. *Nutrition Report International*, *27*, 1209–1219.

Rao, S. K., & Rao, B. S. N. (1984). Studies on the effect of inorganic polyphosphates on dietary ionizable iron and solubility of other minerals in vitro. *Nutrition Report International*, *29*, 941–948.

Rao, S. K., & Rao, B. S. N. (1985). *In vitro* studies on chelating agents as potential iron absorption promoters. *Food Chemistry*, *17*, 13–23.

Report of the Expert group of the Indian Council of Medical Research. (2000). *Nutrient requirement and recommended dietary allowances for Indians*. Hyderabad, India: National Institute of Nutrition.

Seshadri, S. (1997). Nutritional anemia in South Asia. In S. Gillispie (Ed.), *Malnutrition in South Asia. A regional profile* (pp. 75–124). Regional office for South Asia, Katmandu, Nepal, Rosa Publication No. 5: UNICEF.

Stekal, A., Olivares, M., Pizarro, F., Chadud, P., Lopez, I., & Amer, A. (1986). Absorption of fortification iron from milk formulas in infants. *American Journal of Clinical Nutrition*, *43*, 917–922.

Viteri, F. E., Alvarez, E., Batres, Torun, B., Pineda, O., Mejia, L. A., & Sylvi, J. (1995). Fortification of sugar with iron sodium ethylene diamino tetra acetate (FeNaEDTA) improves iron status in semi rural Guatemalan populations. *American Journal of Clinical Nutrition*, *61*, 1153–1163.

West, T.S., & Sykes, A.S. Diamino-ethane-tetraacetic acid. In: A*nalytical applications of diamino-ethane-tetraacetic acid*. (pp. 9–22). Poole, UK: The British Drug House.

Whittaker, P., & Vanderveen, J. E. (1990). Effect of EDTA on the bioavailability of fortification iron used in Egyptian balady bread. *British Journal of Nutrition*, *63*, 587–595.