Bioavailability of iron in multiple fortified milk

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Abstract The objectives of the study were to evaluate the bioavailability of iron in milk fortified with ferric pyrophosphate (FPP) soluble and vitamin A acetate and to establish the role of vitamin A in enhancement of iron absorption. Balance indices *viz.* apparent digestibility coefficient, % retention/intake of iron and haematological parameters *viz.* blood haemoglobin, plasma ferritin, plasma transferrin and iron content in rat livers were analyzed to evaluate iron bioavailability. Anaemia was induced in one group of rats to evaluate the effect of iron status of body on iron absorption from diet. The results of in vivo study showed that feeding of rats with lyophilates of milk fortified with FPP soluble and FPP soluble + vitamin A acetate had a significant effect on the balance indices of the iron as well as on the haematological parameters and iron liver status. The utilization of iron in the body, as indicated by the results of balance indices, haematological parameters and iron status of livers was significantly higher in anaemic rats compared to control group rats. Vitamin A appeared to be playing role in enhancement of iron absorption and utilization in body.

Keywords Milk · Fortification · Iron · Vitamin A · Bioavailability

Introduction

Globally iron deficiency is the most common micronutrient deficiency, affecting between 1.5 and 2 billion people, of whom 500 million have iron deficiency anaemia (Ahmed et al. 2010). National Family health Survey (2007) reported that 79 % in children of 6–35 months of age, 55 % in women of 15–49 years and 24 % in men of 15–49 years of age are prevalent to anaemia in India. National Nutrition Monitoring Bureau (2003) indicated that prevalence of clinical and sub-clinical vitamin A deficiency among pre-school children in India is high.

A direct correlation between serum retinol and haemoglobin levels in women and children has been observed in several surveys (Mejia et al. 1977; Mohanram et al. 1977; Suharno et al. 1993). Anaemia associated with marginal vitamin A deficiency is characterized by a decrease in the serum iron concentration, total iron-binding capacity and transferrin saturation, and with increased storage of iron in liver and spleen. Vitamin A deficiency impairs iron mobilization from stores and has little influence on iron absorption (Amine et al. 1970; Mejia et al. 1979) i.e. iron delivery to the developing erythroid cells appears to be impaired during vitamin A deficiency in the body. 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).

Sun et al. (2010) observed an additive effect of retinol and iron supplementation on iron status. Semba and Bloem (2002) also reported that vitamin A appeared to be involved in the pathogenesis of anaemia through diverse biological

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mechanisms, such as the enhancement of growth and differentiation of erythrocyte progenitor cells, potentiation of immunity to infection and reduction of the anaemia of infection, and mobilization of iron stores from tissues.

Ferric pyrophosphate (FPP) soluble, also known as sodium-citro-ferric pyrophosphate used for milk fortification in the present investigation is a chemical combination of ferric pyrophosphate and sodium citrate. The iron core of FPP soluble is tightly chelated to citrate and pyrophosphate ligands. Unlike the similarly named ferric pyrophosphate, which is essentially insoluble in aqueous solutions, FPP soluble is very soluble in aqueous solutions over a wide range of pH (pH 2–8). Food grade FPP soluble is relatively inexpensive and is allowed as an iron fortificant in Europe (Zhu et al. 2009). There is an unequivocal need for predicting the bioavailability of dietary iron. Bioavailability is affected by diet composition and may be a more important factor in determining the adequacy of diets for iron nutrition than total iron intake (Argyri et al. 2009).

Considering the above facts, the present study was conducted to evaluate the bioavailability of iron using FPP soluble as an iron fortificant for milk and to evaluate the effect of vitamin A on iron absorption and utilization in the body.

Materials and methods

Animals

Male Wistar albino breed rats ($n=48$) recently weaned, aged 3 weeks procured from Central Animal Facility, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India were used in this study. Animal care procedures and experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), National Dairy Research Institute (NDRI), Karnal, Haryana, India.

Experimental design and diets

Figure 1 shows the experimental design used. From the start of the study, the animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature of 20–22 °C, 12 h light–dark cycle and 55–60 % humidity in a well ventilated room in Small Animal House, NDRI, Karnal, India. The rats were given an acclimatization period of 7 days, during which animals adjusted to the metabolic cages and the new diet. Rats were randomly divided into two groups namely normal and anaemic, each group contained 24 rats. Normal group was divided into three groups *viz.* control, iron fortified and iron + vitamin A fortified group. Normal group underwent an experimental period of 21 days, during which, the control group was fed with mixture of synthetic diet (67 %) and milk lyophilate (33 %). Iron fortified

group was fed with synthetic diet (67 %) and iron-fortified milk lyophilate (33 %), and iron + vitamin A-fortified group was fed with synthetic diet (67 %) and iron + vitamin A fortified milk lyophilate (33 %). Blood was collected from normal group at zero day as well as on the 21st day and analysed for hemoglobin content, plasma ferritin content and plasma transferrin content. All the animals were sacrificed at the end of 21 days of experimental period and their livers collected. The other group (anaemic) was fed with low iron diet during pre-experimental period of 21 days to induce anaemia in the rats by omitting iron from the mineral supplement of diets, hemoglobin content was measured at zero day, followed by 21 days of experimental period similar to that of normal group.

The normal group was fed with basal diet/normal diet with normal iron content (45 mg kg⁻¹); while the other group (anaemic) was provided with synthetic anaemic diet with low iron content (5 mg kg⁻¹) to induce dietary iron deficiency as described by Pallarés et al. (1993). Diet and de-ionized water were available *ad libitum* to all rats. The diets containing mineral and vitamin supplements provided during the pre-experimental and experimental period were prepared as given in Table 1 (AOAC 1995).

Collection of blood, urine, faeces and liver

For determination of digestive and metabolic utilisation of iron, balance (absorption-retention) studies were conducted for 3 days during the experimental period for which food intake was measured, and urine and faeces were collected daily. The faeces were lyophilized (Daihan Lab Tech India Pvt. Ltd., Hyderabad, India), weighed and homogenized (The Laboratory Glass Co. (LABCO), Ambala, India). Urine was collected in a 0.5 % v/v HCl solution and was later filtered through Whatman No. 41 filter paper (ashless, Whatman International Ltd., Kent, England). Biological indices, namely apparent digestibility coefficient and % retention/intake of iron were estimated by the method described by Alferez et al. (2006). Blood was collected from the caudal vein after an overnight period of food deprivation (12 h) from this group at zero day of pre-experimental period as well on zero day and the 21st day of experimental period and analyzed for haemoglobin content using cyanomethaemoglobin method (Drabkin and Austin 1932). Plasma was separated from blood by centrifugation at 1500×g at 4 °C for 15 min and stored at -20 °C. Ferritin and transferrin level of blood plasma of rats were measured using highly sensitive two site enzyme linked immunoassay (ELISA) kit (MyBioSource, San Diego, CA, USA). Blood plasma samples were diluted 100 times for ferritin analysis and 200,000 times for transferrin analysis with the diluents provided in the ELISA kit. All the animals were sacrificed at the end of 21 days experimental period. They were first anaesthetised using diethyl ether and then killed

Fig. 1 Experimental design of in vivo study. After a pre-experimental period of 21 days to obtain an anaemic rats group, the animals were allocated to one of the three normal-Fe diets (unfortified, iron fortified and iron + vitamin A fortified). *Blood from caudal vein was withdrawn on zero day of pre-experimental period as well on zero day and the 21st day of experimental period. #Rats were sacrificed to remove the liver. where, *UFL* unfortified milk lyophilates, *IFL* iron fortified milk lyophilates, *IAFL* iron + vitamin A fortified milk lyophilates

by cervical dislocation. After this, the rats were dissected and entire liver was excised, washed with isotonic buffer three times and stored at -20°C .

Preparation of milk lyophilates

Cow milk and buffalo milk were mixed in 1:1 ratio and cream was removed for the preparation of toned milk. The fat and solid-non-fat (SNF) were adjusted to 3.0 and 8.5 %, respectively, by adding cream/skim milk using Pearson square method. Milk samples prepared were control toned milk, toned milk fortified with FPP soluble alone ($@25\text{ mg L}^{-1}$) and FPP soluble ($@25\text{ mg L}^{-1}$) + vitamin A acetate ($@2500\text{ IU L}^{-1}$) and lyophilized to convert it into powder. Lyophilates of the unfortified, FPP soluble fortified and FPP

soluble + vitamin A acetate-fortified milk were mixed with the basal diet in the ratio (1:2) and were fed ad-libitum to respective group of rats. The rats consumed $\sim 10\text{--}15\text{ g}$ diet daily. Unfortified lyophilates + basal diet (A group) contained $1.201\text{ }\mu\text{g}$ vitamin A and 0.48 mg iron per 15 g diet; FPP soluble fortified lyophilates + basal diet (B group) contained $1.201\text{ }\mu\text{g}$ vitamin A and 6.73 mg iron per 15 g diet; and FPP soluble + vitamin A acetate-fortified lyophilates + basal diet (C group) contained $207.25\text{ }\mu\text{g}$ vitamin A and 6.73 mg iron per 15 g diet.

Iron analysis

Iron analysis in faeces and urine was carried by the method described in AOAC (2005) using atomic absorption spectrophotometer (AA-7000, Shimadzu, Tokyo, Japan) flame mode, air-acetylene flame (temperature 2300°C with flow rate 1.5 L/min).

Statistical analysis

Data are expressed as means $\pm$ standard error of mean (SEM). Significance was tested by employing two way analysis of variance (ANOVA) with replication and comparison between means was made by critical difference (CD) value (Snedecor and Cochran 1994). For computation of data, software application programme Microsoft Office Excel 2007 was used.

Results and discussions

It has been recommended that the use of multiple iron status indicators (i.e. plasma ferritin, total iron binding capacity

Table 1 Composition of normal diet for control and anaemic groups

Ingredient	%
Starch	59 for control group 62 for anaemic group
Fat	10
Protein	16
Cellulose	2.5
Sucrose	7.5
Mineral mixture ^a	3.5
Vitamin mixture ^a	1
Choline chloride	0.25
DL methionine	0.3
Final weight was made up to 1 kg with starch	

^a Mineral and vitamin mixture were prepared and mixed according to AOAC (1995)

(TIBC), unsaturated iron binding capacity (UIBC), transferrin saturation and haemoglobin) simultaneously provide a more accurate measure of iron status than any single index (Cook et al. 1976; Kasdan 1996; Looker et al. 1995). Therefore, balance studies, haemoglobin, plasma ferritin, plasma transferrin and liver storage of iron were selected for having an estimate about the body's response towards iron from fortified milk.

Biological indices

Apparent digestibility coefficient and % retention/intake of iron for control group rats was significantly different ($P<0.05$) for all the groups (Table 2 and Fig. 2). Similar trend was observed in case of anaemic group rats (Table 3). In the control and anaemic group rats, % apparent digestibility coefficient and % retention/intake followed the order of Group C>Group B>Group A, which indicated that vitamin A might have played role in enhancement of iron absorption.

Two way ANOVA ($P<0.05$) for the effect of diet and anaemia on iron absorption and retention in body revealed that % retention/intake was higher and significantly different for all anaemic groups compared to control group rats. It was also observed that in both the groups, rats fed with vitamin A-supplemented diet showed significantly higher iron absorption than those fed with either control diet or iron-fortified diet (Fig. 2), which indicated that improvement in iron absorption could be due to inclusion of vitamin A in diet.

Our results are in corroboration with Garcia-Casal et al. (2003) who also observed that addition of vitamin A produced a 1.8 times increment in iron absorption and further reported that addition of vitamin A improved iron absorption by a process at least partially due to the stabilizing effect of this vitamin on iron. However, Bloem (1995) suggested that

Fig. 2 Retention/intake (%) of iron for control and anaemic group rats. ^{a-b}Different lowercase letters denote significant difference ($P<0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500 + FPP25 fortified). ^{A-B}Different uppercase letters denote significant difference ($P<0.05$) between groups (control and anaemic). Error bars show the variations of eight determinations in terms of standard error of mean

vitamin A influences iron metabolism, but this mechanism has not yet been fully elucidated. He reported that it is unlikely for vitamin A to interfere directly with the intestinal absorption of iron, but it may mobilise available iron stores and use them to form haemoglobin. Vitamin A deficiency impaired iron mobilisation from stores and had little influence on iron absorption (Amine et al. 1970; Mejia et al. 1979).

Higher levels of apparent digestibility coefficient and % retention/intake of iron were obtained from the anaemic rats than from their control group rats, for all the diets studied. Our results were in accordance with Alferrez et al. (2006) who reported that the digestive utilisation of iron was greater in anaemic rats than in their controls, regardless of the type of diet consumed. Thus, it can be inferred that there was a greater absorption and utilization of iron in anaemic rats, which might be due to the fact that iron deficiency enhanced the membrane receptors and thus affected and increased iron absorption. Pallarés et al. (1993) also reported increase in apparent digestibility coefficient iron in deficient rats.

Table 2 Iron balance studies of control group rats

Group→ Parameters↓	Control A	Control B	Control C
Food intake (g/d)	10.37±0.14	11.13±0.23	11.73±0.12
Iron intake (mg/d)	0.33±0.01	5.00±0.11	5.27±0.06
Faecal excretion (mg/d)	0.21±0.003	3.12±0.070	3.17±0.040
Absorbed iron (mg/d)	0.121±0.002	1.872±0.038	2.101±0.025
Apparent digestibility coefficient (%)	36.34±0.12 ^a	37.44±0.12 ^b	39.93±0.38 ^c
Urinary iron (mg/d)	0.0013±0.0001	0.0294±0.0011	0.0336±0.0019
Iron balance (mg/d)	0.119±0.002	1.842±0.038	2.068±0.025

^a Data are presented as means±SEM ($n=8$)

^b Means with same superscript letter in a row do not vary significantly ($P<0.05$) from each other

Table 3 Iron balance studies of anaemic group rats

Group→ Parameters↓	Anaemic A	Anaemic B	Anaemic C
Food intake (g/d)	13.93±0.24	14.40±0.27	14.65±0.15
Iron intake (mg/d)	0.446±0.008	6.465±0.123	6.578±0.069
Faecal excretion (mg/d)	0.273±0.005	3.815±0.072	3.745±0.043
Absorbed iron (mg/d)	0.173±0.003	2.649±0.052	2.833±0.027
Apparent digestibility coefficient (%)	38.86±0.16 ^a	40.98±0.08 ^b	43.07±0.10 ^c
Urinary iron (mg/d)	0.0012±0.0004	0.0327±0.0004	0.035±0.0011
Iron balance (mg/d)	0.172±0.003	2.618±0.051	2.798±0.028

^a Data are presented as means±SEM ($n=8$)

^b Means with same superscript letter in a row do not vary significantly ($P<0.05$) from each other

Haemoglobin repletion assay

Haemoglobin (Hb) repletion assay in rodents is considered to be a good indicator of iron bioavailability (Bloem 1995). Two way ANOVA ($P<0.05$) revealed that both the type of diet and anaemia had significant effect on the percentage increase in haemoglobin. % increase in haemoglobin in iron-deficient rats was higher and significantly different than controls for all the three diets assayed, as shown in Fig. 3. The percentage increase in haemoglobin was higher in both the control and the anaemic rats which consumed vitamin A + iron-fortified milk lyophilates and iron-fortified milk lyophilates compared to rats fed with unfortified milk lyophilates. However, % increase in haemoglobin was higher in rats fed with vitamin A + iron-fortified milk lyophilates compared to rats fed with only iron-fortified milk lyophilates suggesting a probable role of vitamin A in haemoglobin formation. The provision of vitamin A to iron deficient rats resulted in a rapid rise in haemoglobin; leading to a conclusion by Koessler et al. (1926) that blood regeneration cannot take place without the presence of vitamin A.

Alferez et al. (2006) reported that feeding the iron deficient rats for 14 days with the iron fortified diet, rats recovered from anaemia as assessed by higher Hb level. Hunter (1978) also found that haemoglobin concentration depleted continually during the first 4–5 weeks of feeding the rats with iron deficient diets. When rats fed with iron deficient diets were switched to the control levels, their haemoglobin levels increased to control levels within first week. Haro-Vicente et al. (2008) also reported that there was an increase in the haemoglobin content after rats were fed with fruit juice fortified with different iron salts for 21 days.

Plasma ferritin content of rats

Ferritin levels are an indicator of the amount of iron utilized from absorbed iron by the body. Two way ANOVA ($P<0.05$)

Fig. 3 Percent increase in haemoglobin content in control and anaemic group rats. ^{a–b}Different lowercase letters denote significant difference ($P<0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500 + FPP25 fortified). ^{A–B}Different uppercase letters denote significant difference ($P<0.05$) between groups (control and anaemic). Error bars show the variations of eight determinations in terms of standard error of mean

revealed that when rats were fed with iron-fortified milk lyophilates and iron + vitamin A-fortified milk lyophilates, the increase in ferritin content was higher for anaemic rats than control group and the difference was statistically significant suggesting that low iron status of anaemic rats lead to an increase in uptake of iron and thereby enhancing iron utilization to ferritin more than the rats having normal iron status (Fig. 4).

Our results were in accordance with Zhu et al. (2009) who studied the effect of food matrix (non fat dry milk powder) on iron bioavailability in combined in vitro digestion and caco-2 cell model. They reported that ferritin formation in caco-2 cells exposed to soluble ferric pyrophosphate was twice the amount of that in cells treated with ferrous sulphate. They also found that iron free non fat dry milk powder alone was shown to be a poor source of bioavailable iron. Adding soluble ferric pyrophosphate significantly increased ferritin formation. Siimes et al. (1974) also found a rapid rise in serum ferritin concentration in the first week of iron treatment of anaemic children.

Rats fed with iron + vitamin A-fortified milk showed significantly higher ($P<0.05$) ferritin content than rats fed with unfortified diet and iron fortified diet. However, Mejia and Chew (1988) reported that vitamin A supplementation produced non significant change in serum ferritin.

Plasma transferrin content of rats

Transferrin is iron-binding blood plasma glycoprotein that controls the level of free iron in biological fluids. Two way ANOVA ($P<0.05$) revealed that percent increase in plasma transferrin content was higher for anaemic group compared to control group and significantly different for all the three diets and the values were significantly different from each other in the respective subgroups (Fig. 5). Conceicao et al. (2001) reported that iron supplementation (Fe^{2+} or Fe^{3+})

Fig. 4 Percent increase in plasma ferritin content in control and anaemic group rats. ^{a–b}Different lowercase letters denote significant difference ($P<0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500 + FPP25 fortified). ^{A–B}Different uppercase letters denote significant difference ($P<0.05$) between groups (control and anaemic). Error bars show the variations of eight determinations in terms of standard error of mean

Fig. 5 Percent increase in plasma transferrin content in control and anaemic group rats. ^{a–b}Different lowercase letters denote significant difference ($P < 0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500 + FPP25 fortified). ^{A–B}Different uppercase letters denote significant difference ($P < 0.05$) between groups (control and anaemic). Error bars show the variations of eight determinations in terms of standard error of mean

normalised the serum transferrin levels in iron-deficient rats more than the control group. Haro-Vicente et al. (2009) reported that transferrin saturation was significantly ($P < 0.05$) higher in groups (infants and toddlers) fed with iron-fortified growing-up milk compared to the control group.

Iron content in liver of rats

Two way ANOVA ($P < 0.05$) revealed that for all the diets studied, there was non significant difference ($P > 0.05$) in iron status of control and anaemic rats (Fig. 6). However, the content was higher in anaemic rats compared to their control counterparts. Haro-Vicente et al. (2008) also reported that the iron content in the liver of iron deficient rats fed with ferrous sulphate and micronized dispersible ferric pyrophosphate fortified fruit juice for 21 days was

Fig. 6 Iron content of liver of control and anaemic group rats. ^{a–b}Different lowercase letters denote significant difference ($P < 0.05$) across sub-groups (unfortified, FPP25 fortified and VA2500 + FPP25 fortified). ^{A–B}Different uppercase letters denote significant difference ($P < 0.05$) between groups (control and anaemic). Error bars show the variations of eight determinations in terms of standard error of mean

significantly higher than control group. However, Alferez et al. (2006) reported that iron concentrations decreased in all the tissues of anaemic rats that were analysed, compared with their controls, regardless of the diet supplied. Similar findings have been reported by Katsuhiko et al. (1991). They concluded that the decrease in iron concentration in the liver is suggestive of the depletion of iron storage in anaemic rats. Hunter (1978) reported that anaemic animals would need to restore their oxidative metabolism (by increasing haemoglobin synthesis) before they would need to replenish their iron stores.

However, in the present study, higher iron content of livers in anaemic rats could be due to the longer repletion time (21 days) as compared to 14 days repletion time in study conducted by Alferez et al. (2006). On the same line as in case of control group, iron status of liver was higher for rats receiving diets fortified with vitamin A + iron. The findings were in support with Alferez et al. (2006) who reported that iron content of liver was higher for the rats fed with goat milk diet (having high vitamin A content than other diets studied).

Zhu et al. (2009) reported that soluble ferric pyrophosphate exhibited higher bioavailability than simple iron salts, such as ferrous sulphate and ferric chloride. Ranhotra et al. (1981) also reported that iron from milk fortified with a water-soluble iron citrate phosphate complex (potassium ferric pyrophosphate) had similar bioavailability as that from ferrous sulphate (99 % vs 100 %); the same was true when iron complex was tested as such (not added to milk). Thus, milk or milk components did not appear to adversely affect the bioavailability of added iron under the conditions used in their experiment.

Conclusions

From the present study, it can be concluded that fortification of milk with ferric pyrophosphate soluble and ferric pyrophosphate soluble + vitamin A acetate had a significant effect on the digestive and metabolic utilization of the iron as well as on the haematological parameters. All the positive effects were more evident in anaemic rats, indicating the dependence of iron bioavailability on the iron status of individual. Similarly, the increase was greater for vitamin A acetate+ferric pyrophosphate soluble fortified group compared to that with only ferric pyrophosphate, suggesting that vitamin A plays role in enhancement of iron absorption. Normal rats also showed an increase in haemoglobin content, plasma ferritin content, plasma transferrin content, percentage digestibility and retention of iron which clearly established that the selected salt has good bioavailability.

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