

Short Communication

Effect of ascorbic acid rich, micro-nutrient fortified supplement on the iron bioavailability of ferric pyrophosphate from a milk based beverage in Indian school children

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Background and Objectives: Nutritional anemia is a significant public health issue with 50-80% prevalence in Indian children. Fortification of food, specifically milk, with iron is a potential approach to increase dietary iron intake. Ferric pyrophosphate [Fe₄(P₂O₇)₃] is organoleptically neutral and is less soluble in acid medium and, further, has low bioavailability in milk. However, since ascorbic acid is a potent enhancer of iron absorption, the co-administration of ascorbic acid with Fe₄(P₂O₇)₃ might enhance the absorption of iron. We evaluated the effect of ascorbic acid on iron absorption from a Fe₄(P₂O₇)₃ and an ascorbic acid fortified milk beverage with respect to milk fortified with Fe₄(P₂O₇)₃ alone. **Methods and Study Design:** A double-blind, two-way crossover, randomized study was conducted in 25 mildly anemic children. The test group received milk fortified with beverage powder containing 7 mg isotopically labeled iron (⁵⁷Fe/⁵⁸Fe) as Fe₄(P₂O₇)₃, equimolar proportions of ascorbic acid and 200 mg of calcium whereas control group received milk fortified with energy, calcium and iron equivalent beverage powder. Fractional iron absorption was measured by erythrocyte incorporation of stable isotopes of iron (⁵⁷Fe/⁵⁸Fe) in both the groups. **Results:** The fractional iron absorption from the control drink was 0.80% (95% CI: 0.57, 1.12). Fortifying the milk with an equimolar amount of ascorbic acid increased the fractional iron absorption almost 2-fold to 1.58% (95% CI: 1.13, 2.22). **Conclusions:** The presence of ascorbic acid in an equimolar ratio with that of iron from Fe₄(P₂O₇)₃ salt in milk as a fortificant enhanced iron absorption when compared to milk fortified with only Fe₄(P₂O₇)₃.

Key Words: ferric pyrophosphate, micronutrient, milk, iron bioavailability, stable isotope

INTRODUCTION

Chronic deficiency of essential micronutrients and vitamins affect billions of people worldwide and is estimated that nearly 7% of global disease is attributed to the lack of these nutrients.¹ School children are particularly vulnerable to deficiencies of micronutrients with detrimental effects of impaired intellectual and psychomotor development, poor physical growth and increased morbidity from infectious diseases. According to the WHO it is estimated that approximately 59% of Indian children have hemoglobin concentration less than 11.0 gm/dL and 60% of children suffers from anemia in Karnataka, India.^{2,3} The high prevalence of nutritional anemia is often multifactorial which includes low dietary intake of micronutrients, poor bioavailability of iron due to high content of phytic acid, polyphenols and tannins in the diet.⁴ One of the effective strategies to increase the iron density in the diet would be through food fortification with iron salts that are organoleptically suitable.

Milk being nutritious is found to be low in iron content, although on its fortification with iron might serve as a drink that not only bridges the micronutrient gap, but also provides macronutrients. However, the iron fortification of milk is challenging, not only due to the competing effects of calcium in the milk, but also because most of the iron salts have low solubility and unacceptable organoleptic changes, resulting in poor iron intake and absorption. The bioavailability of ferrous sulfate (FeSO₄) is highest among iron salt but it has unacceptable organoleptic

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properties.² $\text{Fe}_4(\text{P}_2\text{O}_7)_3$, is creamish white in color, sparingly soluble in water and poorly soluble in acid medium.⁵ Thus it does not exhibit any organoleptic property when added to food. Studies have shown that compared to FeSO_4 , $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ has poor bioavailability.⁶ While earlier studies have demonstrated varying effects of milk on iron absorption, the addition of ascorbic acid to a FeSO_4 fortified beverage in a molar ratio of 2:1 was shown to overcome the inhibitory effect on iron absorption by calcium.⁷⁻⁹ However, the effect of ascorbic acid on iron absorption specifically from $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ in milk matrix has been largely unexplored. The aim of this study was to evaluate the enhancing effect of an equimolar dose of ascorbic acid on iron absorption from $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ fortified milk based beverage in mildly anemic school children.

METHODS

The protocol of the study was reviewed and approved by the St. John's Medical College and Hospital Institutional Ethical Review Board, Bangalore, INDIA (Study Reference No: 167 / 2012) and it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Subject anonymity and confidentiality was strictly maintained.

Elemental ^{58}Fe and ^{57}Fe isotopes (Chemgas, France) were dissolved in 6 N HCl in two different sets to synthesize isotopically labeled FeCl_3 (aq) and the pH of the solution was adjusted in the range of 3-4 using ammonium hydroxide. This solution of labeled FeCl_3 was used to synthesize $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ as follows; 50 ml of labeled 0.23 M FeCl_3 (aq) solution was added to 55 ml of 0.17 M $\text{Na}_4\text{P}_2\text{O}_7$ solution (aq). A creamish white precipitate of $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ was obtained which was then washed with distilled water to remove adherent FeCl_3 and $\text{Na}_4\text{P}_2\text{O}_7$ and subsequently filtered and air dried. The iron contents of the synthesised $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ were estimated and doses of ^{57}Fe and ^{58}Fe salt were pre-weighed for administration, so as to have 3 mg of iron in each dose.

School children aged 7 to 10 years, were recruited from Resurrection School, Bangalore with prior consent from the parent and oral assent from the child. The inclusion criteria for anthropometry were z-scores of height-for-age of ≥ -2 to 0 and BMI for the age of ≥ -2 to 0. Subjects with severe anemia (haemoglobin < 8 g/dL), elevated C-reactive protein (CRP) concentration (> 8 mg/L), history of allergy/intolerance to any food, participation in another clinical study and recent history of serious infections, injuries and/ or surgeries were excluded from the study. The enrolled children of both genders were divided in to test and the control group. The test group received a micronutrient fortified drink containing ascorbic acid (30 mg), iron (7 mg), calcium (200 mg) and other micronutrients, while the control group received only a calcium and iron matched drink. Isotopically labelled ferric pyrophosphate ($^{57}\text{Fe}/^{58}\text{Fe}$) containing 3 mg of elemental iron was added to drink in both the groups to measure fractional iron absorption (Table 1).

The study design and protocol was followed as described earlier.¹⁰ Briefly, the study children were randomly assigned to receive either the test or the control drink on day 1. The subjects received the second drink on day 2

after being randomly crossed-over. The respective fortified beverage powder was mixed in lukewarm milk in a non-metallic drinking glass and the pre-weighed doses of ^{57}Fe and ^{58}Fe labeled $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ salt was added on consecutive days to the control and the test group respectively.

Blood samples were collected at the start and end of the study (Day 0 and Day 14) from each subject. A complete hemogram was analyzed within 24 h of sampling, using an automated hematology analyzer, ABX Pentra 60 c + Counter (Horiba ABX Inc., USA,) plasma ferritin was measured by electrochemiluminescence assay (Elecys 2010, Roche, Switzerland). Plasma soluble transferrin receptor and CRP was measured by immunoturbidimetry (Roche/Hitachi 902) using an automated clinical chemistry analyzer (Roche Diagnostics Mannheim, USA). Iron absorption from the test and control drinks were estimated based on erythrocyte incorporation of stable isotope labels 14 d later using thermal ionization mass spectrometry (TIMS).

Fractional iron absorption was measured as described earlier.¹¹ Briefly, whole blood samples were ashed and subjected to acid digestion, followed by separation of the iron by anion-exchange chromatography (200–400 mesh Ag 1-X8, Bio-Rad, California, USA) and a solvent/solvent extraction step into diethyl ether. Iron isotopic ratios were then measured by negative-TIMS with a multi-collector system for simultaneous ion beam detection. The amount of circulating isotopic label was calculated as the product of the shift in the iron isotopic ratio and the amount of circulating iron in the blood. Circulating iron was calculated from an estimate of blood volume.¹² Iron incorporation into red blood cells was assumed to be 80 % of the absorbed iron. A commercially available Fe standard (IRMM 014; European Commission, Joint Research Centre, Institute of Reference Material and Measurements, Geel, Belgium) was used as the reference standard for the entire analysis with an internal precision of 0.0286% and 0.0677%, and an external precision of

Table 1. Composition of test and control study products

Nutrients	Test product (27 g)	Control product (27 g)
Vitamin A (μg)	200	-
Vitamin D (μg)	1.25	-
Vitamin E (mg)	1.5	-
Vitamin B-1 (mg)	0.35	-
Vitamin B-2 (mg)	0.8	-
Niacin (mg)	4.5	-
Vitamin B-6 (mg)	1.0	-
Folic acid (μg)	100	-
Vitamin B-12 (mg)	0.5	-
Vitamin C (mg)	30	-
Calcium (mg)	200	200
Iron (mg)	7.0	7.0
Iodine (μg)	37.5	-
Zinc (mg)	1.15	-
Selenium (μg)	10.0	-
Copper (mg)	0.175	-

Isotopically labelled ferric pyrophosphate (containing 3mg of elemental $^{57}\text{Fe}/^{58}\text{Fe}$) was added to the drink in both the control and the test groups.

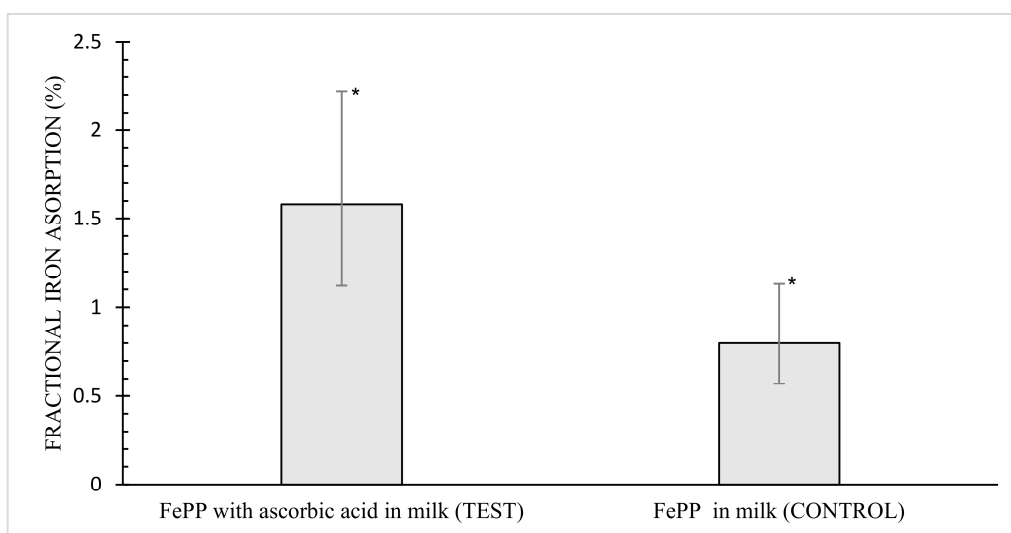


Figure 1. Comparison of iron absorption from the micronutrient fortified drink and the control drink. FePP: Ferric Pyrophosphate. Adjusted mean from ANOVA of log transformed data with factors for treatment, subject (sequence), period, sequence and gender. Back transformed data are presented as mean. Error bars represent 95% CI. *Significant difference at $p < 0.05$.

0.000012 and 0.030052 for $^{57}\text{Fe}/^{56}\text{Fe}$ and $^{58}\text{Fe}/^{56}\text{Fe}$ ratios respectively. Fractional iron absorption of ^{57}Fe and ^{58}Fe were calculated, to give a direct measure of the iron uptake from each of the study treatments.

Descriptive statistics of demographic and baseline characteristics were tabulated. The fractional iron absorption (expressed as % iron absorbed) from each of the products were log transformed. It was observed that the iron absorption values were positively skewed even after log transformation. A non-linear mixed model analysis with factors for sequence, product, gender and subject within sequence considered as random effects was performed by fitting a t distribution for the residual. This PROC GLIMMIX program in SAS was used for this analysis. For each treatment group, the least squares mean and corresponding 95% confidence interval were back-transformed and reported. The difference in least square means was back transformed to obtain a mean iron absorption ratio, which is presented with a 95% confidence interval (95% CI). Two-sided treatment comparison tests were performed at the 5% significance level.

RESULTS

Among 25 children, 24 completed the study and one was withdrawn from the study due to symptoms of gastritis prior to administration of the study products. The mean age, height, weight, BMI and the hemoglobin concentration of the subjects were 8.5 years, 123.6 ± 7.9 cm, 21.7 ± 3.4 kg, 14.1 ± 0.8 kg/m² and 10.1 ± 0.9 gm/dL respectively.

The total amount of iron contained in the drink was 10 mg [isotopically labeled (3 mg) and fortified (7 mg)], representing about over 50% of the RDA of 16 mg for this age group.¹³ The fractional iron absorption in the control group was 0.80% (95% CI: 0.57, 1.12) with no significant gender difference ($p = 0.693$). The fractional iron absorption from the test drink was 1.58% (95% CI: 1.13, 2.22) with no significant gender difference ($p = 0.586$). The treatment ratio between the test and the control group was 1.94 (95% CI: 1.47, 2.67; $p < 0.001$). In effect, the addition of ascorbic acid doubled the basal iron

absorption (Figure 1). Quantitatively, an average of 80 μg and 158 μg out of 10 mg of iron from the drink was absorbed in the control and the test group respectively.

The mean baseline ferritin level of the subjects was 28.9 ± 17.2 $\mu\text{g/L}$. Based on the WHO ferritin criteria for iron stores in children, 21 subjects out of the 24 subjects enrolled for the study were iron replete, while 3 subjects were iron deplete. The fractional iron absorption was related to iron status of the children, represented by their baseline serum ferritin levels, in both the control ($r = -0.42$, $p = 0.03$) and the test group ($r = -0.52$, $p = 0.01$). The fractional iron absorption was also weakly related to the baseline hemoglobin status in the control ($r = -0.127$, $p = 0.554$) and in the test group ($r = -0.251$, $p = 0.237$). However, these did not meet the limits of significance.

DISCUSSION

This study demonstrated that $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ and ascorbic acid fortified milk based beverage powder with ascorbic acid and iron in the ratio of 1:1 had almost 2-fold increase in iron absorption when compared to its control. A similar study by Fidler et al reported 2.6 fold increase in iron absorption from $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ with ascorbic acid: iron in the ratio of 4:1 in a milk based infant cereal with respect to the control group.¹⁴ One of the reasons for the observed decrease in the iron absorption in the present study might be because of low concentration of ascorbic acid in the food matrix, the milk in this study. The amount of ascorbic acid added into the milk in the study was limited by regulatory guidelines that restrict the amount of any added nutrient to less than 1 RDA. The iron absorption of 1.58% from $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ in the present study was comparable with that of 1.3% bioavailability of $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ in a study by Davidsson et al where ascorbic acid was administered in 3:1 molar ratio with iron.¹⁵

Ascorbic acid is a strong reducing agent which reduces ferric to ferrous state, thereby increasing the iron absorption.¹⁶ However, a 2-fold increase in iron absorption with only 1:1 molar ratio of iron: ascorbic acid in the present study could be due to the differences in the solubility of the fortificants, their interactions with the food matrix as

well as the existing low level of iron absorption from $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ in this population.

In absolute terms, approximately 0.158 mg of iron was absorbed from the milk based drink. On consumption of this milk twice a day would provide 0.316 mg of iron /day, a proportion of 0.775 mg of total iron required for school children per day making up to ~40% of the daily requirement.¹³ In effect, this would suitably fortify the daily iron intake in children, since our dietary surveys in school children (unpublished) have shown daily dietary intakes of iron to be in the range of 6-10 mg, which, with a bioavailability of about 5%, would provide 60-70% of the iron requirements; the remainder could come from a fortified intake such as milk, as observed in the present study.

$\text{Fe}_4(\text{P}_2\text{O}_7)_3$ being insoluble in water is least reactive when added to foods and has been used to fortify infant cereal and chocolate drinks.¹⁷ Reducing the particle size of $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ to an average size of 0.3 μm and mixing with emulsifiers, has reported to be a good fortificant with high relative bioavailability of iron with respect to FeSO_4 .¹⁸ However, use of micronized $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ in large scale fortification programs is limited due to high cost of manufacturing micronized $\text{Fe}_4(\text{P}_2\text{O}_7)_3$.

In this context, using non-micronized $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ as a fortificant in the milk powder along with ascorbic acid was able to meet 40% of the daily iron need which is significant from a public health perspective. The added ascorbic acid, in equimolar concentrations with iron, was able to enhance iron absorption even in the presence of dietary calcium, which is known to inhibit the bioavailability of iron.¹⁹ Equally, the test drink was also co-fortified with several micronutrients; these are known to modulate iron absorption and utilization.²⁰ A limitation of this study relates to this co-fortification, since an additional group that was provided with ascorbic acid alone would have been able to precisely quantify its enhancing effect on iron absorption. However, the present study still provides important data on the use of ascorbic acid as an enhancer of iron absorption from a milk based beverage, and could inform the design of scaled up efficacy trials that evaluate iron fortification of milk in field settings.

In conclusion, this study shows that iron absorption from a milk based drink can be enhanced by the addition of ascorbic acid in equimolar ratio to iron. This result needs to be further evaluated as part of a randomized controlled trial to assess the efficacy of $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ and ascorbic acid in reducing the burden of iron deficiency and anemia in school children when administered in the long term.

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AUTHOR DISCLOSURES

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