Public Health Nutrition: 8(6), 572–581 DOI: 10.1079/PHN2004708

Haematological response to haem iron or ferrous sulphate mixed with refried black beans in moderately anaemic Guatemalan pre-school children

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Submitted 12 November 2003: Accepted 15 June 2004

Abstract

Objective: Combating iron deficiency in toddlers with iron-fortified food has proved difficult in countries with phytate-rich diets. For this purpose, a new haem iron preparation was developed. The study compared changes in iron status after administration of refried beans with beans fortified with a haem iron preparation or ferrous sulphate ($FeSO_4$).

Design: In a masked, stratified-randomised intervention trial, children received five 156-g cans of refried black beans per week for 10 consecutive weeks. The beans-only (control), $FeSO_4$ and haem iron groups were offered a cumulative dose of 155 mg, 1625 mg and 1700 mg of iron from the bean intervention, respectively. Haemoglobin (Hb) and ferritin concentrations were determined at baseline and after 5 and 10 weeks. Compliance was examined weekly.

Setting: A low-income community in Guatemala City.

Subjects: One hundred and ten children aged 12–36 months with initial Hb values between 100 and 115 $g\,l^{-1}$.

Results: The cumulative intake of beans was approximately 80% of that offered, signifying an additional ~1300 mg of either haem or inorganic iron in the corresponding treatment groups over 10 weeks. Hb concentrations increased by the order of 7.3–11.4 $g\,l^{-1}$ during the intervention, but without significant differences across treatments. Average ferritin concentrations were unaffected by treatment assignment. However, *post hoc* analysis by subgroups of initial high ferritin and initial low ferritin found the Hb increments after 10 weeks in the haem iron group (13.1 ± 7.7 $g\,l^{-1}$) to be significantly greater than the respective increases (6.8 ± 11.2 and 6.4 ± 8.5 $g\,l^{-1}$) in the inorganic iron and beans-only groups.

Conclusions: Canned refried beans are a candidate vehicle for fortificant iron. Given the improved colour and organoleptic properties imparted by haem iron added to refried beans, its additional potential for benefiting the iron status of consumers with iron deficiency may recommend it over $FeSO_4$.

Keywords
Anaemia
Haem iron
Serum ferritin
Black beans
Guatemala

Nutritional anaemia, primarily due to deficiency of iron, is the most widespread of all nutrient deficiency syndromes[1]. Part of its significance to the young child's health is the risk of permanent deficits in cognitive performance[2–4]. The predisposition to iron deficiency and iron-deficiency anaemia (IDA) is based on (1) excessive demand due to rapid growth and situations of excessive blood loss, e.g. via menstrual bleeding in women of childbearing age or intestinal bleeding for those living in areas endemic for hookworm, whipworm or amoeba; and (2) the poor bioavailability of the nutrient. IDA is endemic to Guatemala[5]. In school-aged children, the rate of anaemia varies between 8 and 10%[6,7]. Field teams led by the Institute of Nutrition of Central America and Panama documented a nation-wide prevalence of anaemia of 26% in children aged between 1 and 5 years[8]. The Center for Studies of Sensory Impairment, Ageing and Metabolism (CeSSIAM) found a 60% prevalence of low ferritin concentrations and an anaemia rate of 40% in pre-school children from various sites across the republic[7]. The numerous inhibitory substances in traditional plant-based diets, however, constitute the primary determinant of poor iron stores in most areas with endemic IDA[1,9].

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Iron found in the protoporphyrin haem ring, e.g. in haemoglobin (Hb) and myoglobin, is more biologically available than the inorganic (non-haem) iron from edible plants and storage forms in animal tissue[10]. Bioavailability of 25% has been assumed for dietary haem iron[11]. More recently, it was documented that dietary calcium inhibits absorption of haem iron[12,13]. About a decade ago, in Chile, the idea to create an iron fortificant from haem iron in bovine blood, obtained as a by-product of beef production, was explored in food technology studies and intervention trials[14–17]. The Chilean food vehicles were infant cereals and cookies. This has been extended more recently with a report on food technology developments of a haem iron-fortified weaning food in Spain[18] and the confirmation of its iron bioavailability from the UK[19]. A new production process (US Patent 6 217 932 B1) splits haem from globin. The process reduces the volume needed to supply a given amount of haem considerably and was used in this study.

A staple protein source of the Guatemalan diet is the black variety of the common bean (*Phaseolus vulgaris*). Given the bean's underlying colour, the black pigmentation conveyed by haem iron blends in well with this legume. Beans are high in phytic acid and dietary fibre, compromising the bioavailability of inorganic iron. Hence, the aforementioned characteristics of haem iron would theoretically favour differentially better absorption than with ferrous sulphate ($FeSO_4$) when used as a fortificant in black beans. In the present study, we report the results of a community intervention trial to compare the relative efficacy of various iron sources in black beans to increase average Hb concentrations and serum ferritin at 5 and 10 weeks.

Population, materials and methods

Study design

The study was a double-masked, stratified and randomised, controlled community-based intervention trial, designed to compare the contribution of the iron content of three different preparations of refried beans on the haematological status and iron reserves of infants. Two of the bean offerings had fortification iron, one in the form of an inorganic salt ($FeSO_4$) and the other in the form of haem from bovine blood.

Population and subjects

The study was conducted in a low-income peri-urban settlement, Ciudad Peronia, 9 km south of the centre of Guatemala City. At an altitude of 1502 m above sea level with an average annual temperature of 22°C, the site is a new settlement with a mixture of informal and more formally constructed houses, the majority of which have dirt floors. Potable water and sewage disposal are precarious. At any given moment, over a third of preschool children are ill with symptoms of a presumptive infectious illness[20]. Children of both sexes, ranging in age from 12 to 36 months, i.e. an age group with a high susceptibility to anaemia[7], were recruited. Screening tests of Hb concentration were performed, and children were eligible if their Hb value was in the range of 100 to 115 g l^{-1} as measured by a portable haemoglobinometer (COMPUR Mini-Lab system; Bayer Diagnostics, Munich, Germany).

Inclusion criteria included being in the specified age and Hb range, and being from a family willing to give informed consent and abide by the dietary instructions involved in the 5-day-per-week bean administration. Exclusion criteria included the recent use of vitamin or mineral preparations containing iron, recent surgery, diagnosed chronic gastric or intestinal diseases, or chronic infections. Children were also excluded if they used supplements during the intervention, if at any point the parents made the decision to withdraw them from the study, or when they moved from the house of residence and re-localisation was not possible.

The protocol was approved by the Human Subjects Committee of CeSSIAM. For the screening of Hb concentrations verbal informed consent was obtained. For formal enrolment involving anthelmintic treatment, sampling for ferritin, serial blood sampling and the food distribution intervention, informed written consent was obtained from the parents or legal guardians after the nature, purpose, risks and benefits of the study had been explained in Spanish. Subjects' families were compensated for their participation with supplementary iron if their children were severely or persistently anaemic, gifts valued at US$4.00 over the 10 weeks of contact, and the benefits of a deworming treatment with an anthelmintic.

All children found eligible for the study were given presumptive anthelmintic therapy. Any child who was found to have Hb concentration of <100 g l^{-1} was excluded from the study and was offered a therapeutic course of $FeSO_4$-containing syrup (Iberol 500; Abbott Laboratories, Columbus, OH, USA). Similarly, any child with Hb concentration of <115 g l^{-1} who did not accept enrolment or was excluded for whatever reason was also offered therapeutic iron.

In sample size calculations, based on the variance in Hb in studies among children of similar age in metropolitan Guatemala City[21], we estimated that groups of about 30 children would provide statistical power to detect a differential increase in Hb concentration of 12.5% in one or more treatment group(s) with α of 0.05 and power $(1-\beta)$ of 0.80.

Each individual subject was randomly assigned to a treatment group (yellow, red, green cans). Randomisation was performed in two blocks (one for each neighbourhood) according to the number of children enrolled on the date of assignment; the framework for randomisation was controlled, sequentially, for sex, age and Hb concentration. The weekly regimen was to be five cans to be

given throughout the course of the day's meals, every weekday from Monday to Friday, and only to the eligible target child in the family. The preparation and serving of the beans were left at the discretion of the mother or caretaker. Consumption of the beans was monitored by a weekly home visit to interview the mother who, in addition, was asked to register the daily leftovers from each can on a pictorial form especially designed for semi-quantitative registration. At the conclusion of the trial, all children who had not reached a Hb concentration of 115 g l^{-1} were offered a therapeutic course of $FeSO_4$-containing elixir (Iberol).

Blood collection

Capillary blood was used exclusively. For finger-prick extraction, the volar surface of the distal phalanx of a finger, previously prepared with isopropyl alcohol, was punctured with a sterile, stainless steel lancet. If only screening for Hb value was performed, a simple disposable lancet and microcapillary tubes were used. If the procedure involved ferritin or a combined ferritin and Hb determination, a microtube was employed to collect blood samples. A sample of about 300 μl of capillary blood from the distal phalanx of a finger was obtained in a Microtainer amber tube with serum separator (Becton-Dickinson, Franklin Lakes, NJ, USA).

Measurement of haematological indices and serum ferritin

Hb was measured using the COMPUR Mini-Lab system (Bayer Diagnostics), with a manufacturer's reported precision of a coefficient of variation (CV) of 0.8%. A fresh drop of capillary blood was conducted by capillary action into the chamber of a special reagent cuvette (Bayer Diagnostics) and Hb was registered digitally and recorded in units of g l^{-1}. The plasma was stored frozen at −20°C until shipment to the Pennsylvania State University. Ferritin was determined using a commercial test kit (Diagnostic Products Corp., Los Angeles, CA, USA). All assays included internal and external quality control standards (International Haematology Standards). All samples from all three phases of sampling were analysed in the same runs, and all three samples from a given subject were included in the same assay. As determined in our laboratory, the CV was $<8\%$ for between-assay and $<4\%$ for within-assay comparison. The data are reported in μg l^{-1}.

Mass treatment of intestinal parasites

Prior to participation in the study, the children were treated with 400 mg of albendazole (Cystospar; Intecfa, Guatemala City, Guatemala) to reduce or eliminate any infection with helminths of small intestinal residence, specifically *Ascaris lumbricoides*, which are endemic in Guatemala City children[22].

Community-level refried bean distribution

The basic recipe of a commercial preparation of *P. vulgaris*, refried black beans (Malher Sucs Cía y Ltd, Guatemala City, Guatemala), was the basis of our intervention food vehicle. This is sold in 156-g cans and contains cooked beans mixed with water and sunflower oil, seasoned with onion powder, garlic powder and salt. Its presentation is as a dense paste.

Three batches of 2400 tins of coded black beans were prepared and made ready for the study at the production facilities of Malher in El Tejar, Guatemala. One group was the basic, unaltered recipe of the standard commercial product. Prior chemical analysis revealed an average of 2.4 mg of intrinsic iron per 100 g of edible portion or 3.7 mg per can. A product fortified with inorganic iron was prepared with the calculated addition of 20 mg of elemental iron per 100 g of beans by the addition of hydrated $FeSO_4$ (Merck, Darmstadt, Germany). This was aimed to produce 156-g cans with 31.2 mg of fortification iron and an overall content of 34.9 mg including the intrinsic iron.

For the haem iron-fortified product, a batch of about 8 kg of haem-rich product was prepared in the pilot plant of the Fraunhofer Institute in Freising, Germany, from bovine blood. The patented production process (world-wide registration at the Deutsche Patentamt: international registration number, WO 98/28 302; US registration, 6 217 932 B1) includes smooth acidic hydrolysis of the Hb and separation of the iron-rich haem aggregates by centrifugation. After freeze-drying, the product was determined by analysis to have 15 mg Fe per g of final powder. The haem iron-fortified cans of beans were produced by adding 1.33 g of the haem powder to 100 g of beans. The goal was to produce the same iron fortification that, with the intrinsic iron content, would total approximately 35.0 mg Fe per can in haem iron-fortified as well as in inorganic iron-fortified beans.

Added iron was evenly distributed within the cans and between cans, as checked in three samples from the top, middle and bottom of each of 40 tins. Iron concentration was determined by atomic emission spectrometry (Atom-Comp, Series 800, Jarrell Ash; Fisher Scientific, Waltham, MA, USA) after wet digestion under pressure (170°C, 2.5 h; ~300 mg refried beans dissolved in 1 ml nitric acid (Suprapure®; Merck); calibration standard: Spex Industries, Metuchen, NY, USA). The mean quantity of iron recovered per can in beans-only, $FeSO_4$- and haem-fortified tins was 3.1, 32.5 and 34.0 mg Fe, respectively. A panel rated the taste of haem-fortified beans better than without fortification, whereas $FeSO_4$ fortification gave a slightly metallic taste (Solomons NW, unpublished observations, 1999).

Data and statistical analysis

The descriptive statistics of arithmetic mean (or geometric mean, as indicated) and standard deviation were calculated. The *a priori*, main-effect hypothesis was that

fortification would lead to a higher increment in Hb and ferritin concentrations than that seen in the beans-only group. The non-parametric Kruskal–Wallis test was used to test for significant differences among means and increments across the three groups in terms of Hb and ferritin concentration. We used the non-parametric paired Wilcoxon test to examine the significance of intra-individual increments in these analytes after 5 and 10 weeks. Calculations were made with a statistical software program (SAS 6.12; SAS Inc., Cary, NC, USA).

Post hoc analysis tested the hypothesis that responses in Hb and ferritin would be greater after consumption of iron-fortified beans in the subgroup of children with low initial ferritin values ($< 20\,\mu g\,l^{-1}$) compared with those with higher initial ferritin values ($\geq 20\,\mu g\,l^{-1}$). For this purpose, the three intervention groups were divided according to the $20\,\mu g\,l^{-1}$ serum ferritin criterion into high and low ferritin subgroups. The same statistical procedures were repeated for *post hoc* subgroup analysis as had been done for the *a priori* analysis. A probability of 0.05 was established as the level of significance for hypothesis testing.

Results

Demographic characteristics

A total of 391 children were screened for the study. Of these, 277 (70%) had Hb concentration $> 115\,g\,l^{-1}$ and, hence, were not considered to have the potential to improve in response to additional iron. Four children (1%) had Hb concentration $< 100\,g\,l^{-1}$ and hence, for ethical reasons, were excluded from the intervention. They received therapeutic doses of iron elixir. The remaining 110 (28%) had Hb concentration within the range sought, and were potentially eligible for enrolment. No child refused to join the study or was excluded on the basis of one or another exclusion criterion, leaving all 110 children to begin the study. The attrition over the study was a cumulative 11.8% ($n = 13$), with four of 37 children lost on follow-up in the placebo group, four of 37 lost in the $FeSO_4$ cohort, and five of 36 constituted the attrition from the haem iron group. Table 1 compares the characteristics at baseline of the children across the three groups.

Consumption of refried beans and of bean-derived iron

Compliance monitoring was conducted by having the fieldworker distribute pre-diagrammed forms in which each of the five days of a trial week were represented by the picture of a can divided horizontally into four, equal segments. The mothers or caretakers were requested and instructed to estimate the amount of the content of every day's can that was consumed, by filling in the corresponding number of quarter-cans on the form. Forms were collected on Fridays when the next week's ration of cans was distributed in the homes. Unfortunately, compliance with filling out the forms was inconsistent. Some women were faithful to the point of 100% return, whereas others completed only one or two of the 10 weeks, and eight mothers provided no record at all. Therefore, to estimate average compliance, we built assumption models based on the data collected. If we assumed, when a mother did not pass in a form, that her child had received no beans and no bean iron, the net compliance would be estimated at about 50%. However, daily presence in the community and discussions between fieldworkers and mothers strongly suggested that mothers were, indeed, feeding the children from the cans while omitting the recording step on the forms. Hence, we built a series of more optimistic models based on the assumption that, when the reports were not filed, the mothers were in fact feeding beans at the same efficiency as when they had filed a form. Among the models we used were the following:

1. the feeding rate for non-filed days was equivalent to the global mean of all weeks reported by all reporting mothers;
2. the feeding rate for non-filed weeks was equivalent to the group-wise mean of the specific treatment; and
3. for individual children, the feeding rate was the same as for the weeks in which they had filed a report form.

All three proxy models generated a similar pattern. Using the average group-wise efficiency model, the placebo group was estimated to have received, on average, 77.7% of the beans or 120 mg of additional iron; the haem iron group 79.5% of the beans or 1352 mg of iron; and the $FeSO_4$ group received 80.6% or 1319 mg of iron.

Distribution of haematological indices and response to refried bean consumption

To test the primary hypothesis about whether fortification with haem iron or $FeSO_4$ influenced body iron repletion, the data were analysed as illustrated in Table 2. Mean arithmetic Hb concentration increased significantly after 5 and 10 weeks when analysed for those subjects who were

Table 1 Demographic characteristics of the anaemic pre-school children at the beginning of the study (age range, 12–36 months)

	Beans only	Haem-fortified	$FeSO_4$-fortified	Whole group	Whole group minus drop-outs
Number	37	36	37	110	97
Males	18 (49%)	19 (53%)	20 (54%)	57 (52%)	51 (53%)
Females	19 (51%)	17 (47%)	17 (46%)	53 (48%)	46 (47%)
Age (months)	19.9 ± 6.6	20.8 ± 6.9	21.2 ± 7.5	20.9 ± 7.1	20.8 ± 7.0

available for both respective blood samplings (within-column comparison; paired Wilcoxon test, $z < 0.05$). There were, however, no significant differences among the average increments across the three bean intervention groups (across-row comparison; Kruskal–Wallis test, $P > 0.05$; Table 2). Plasma ferritin concentrations did not increase significantly in response to either 5 or 10 weeks of intervention with iron-fortified beans (within-column comparison; paired Wilcoxon test, $z > 0.05$). The inter-individual variance was high with respect to ferritin as illustrated by the wide standard deviation intervals. The means decreased numerically in the beans-only group after 5 and 10 weeks and in the $FeSO_4$ group after 10 weeks, but not significantly (Kruskal–Wallis test, $P > 0.05$; Table 2).

At baseline, 55–58% of the children in the different intervention groups were iron-deficient with plasma ferritin concentrations $< 20\ \mu g\ l^{-1}$, whereas the remaining fraction was marginally to fully iron-replete with plasma ferritin $> 20\ \mu g\ l^{-1}$. Therefore in Tables 3 and 4, the three dietary groups were subdivided according to initially low ($< 20\ \mu g\ l^{-1}$) or high ($> 20\ \mu g\ l^{-1}$) plasma ferritin concentration. This refinement offers three options for comparison in *post hoc* analysis:

1. time-dependent comparison (using the paired Wilcoxon test) to determine the significance of the change – increment or decrement – with time;
2. within-column (vertical) comparison (using Kruskal–Wallis test) to assess the differences between subgroups, subdivided by ferritin level, within a treatment assignment; and
3. across-row (horizontal) comparison (using the Kruskal–Wallis test) to assess the differences within a ferritin subgroup to the different intervention treatments.

Using this scheme, the upper panel of Table 3 shows the absolute Hb concentrations at baseline and throughout the study within the framework of the high and low ferritin subgroups in the *post hoc* analysis. As seen from the *P*-values in the final column for across-row comparison, no differences existed by treatment group assignment either at baseline or at any intervention interval, although the Hb value for the haem iron group at 10 weeks had a tendency ($P = 0.08$) to be greater. As can be seen from the *P*-values, except for the *post hoc* separation of the $FeSO_4$ group at the baseline time point, division by ferritin status revealed no difference in haematological status at any point.

The lower panel of Table 3 provides six subgroup-based values for 5-week and 10-week interval changes in Hb concentration. The paired Wilcoxon test was applied to each of these 12 within-subject changes. With the exception of the changes for low ($z = 0.37$) and high ferritin subgroups ($z = 0.07$) on the unfortified beans through 5 weeks, and the low ferritin beans-only subgroup ($z = 0.06$) through 10 weeks, the time-dependent changes in Hb were generally statistically significant increments, including the beans-only high ferritin group at week 10 ($z = 0.0001$). For the haem iron subgroups, the Hb increments shown in the lower panel of Table 3 were significant at 5 weeks ($z = 0.01$) and 10 weeks ($z = 0.0001$) in the low ferritin subgroup, as were

Table 2 Haemoglobin (Hb) and ferritin concentrations in anaemic children fed with refried beans fortified with haem or $FeSO_4$: comparison of entire groups (*n* for each group in parentheses)

Time (week)	Beans only	Haem-fortified	$FeSO_4$-fortified	Across-row *P*-value
Hb concentration ($g\ l^{-1}$)				
0	109 ± 5 ($n = 31$)	109 ± 5 ($n = 31$)	109 ± 5 ($n = 31$)	($P = 0.87$)
5	113 ± 11 ($n = 19$)	120 ± 12 ($n = 21$)	117 ± 11 ($n = 24$)	($P = 0.42$)
10	116 ± 12 ($n = 30$)	120 ± 9 ($n = 30$)	117 ± 11 ($n = 31$)	($P = 0.24$)
Hb increment ($g\ l^{-1}$)				
5	5.1 ± 10.5 ($n = 19$) $z = 4.8 \times 10^{-2}$	10.5 ± 11.3 ($n = 21$) $z = 7.7 \times 10^{-4}$	7.7 ± 9.4 ($n = 24$) $z = 7.4 \times 10^{-3}$	($P = 0.46$)
10	7.3 ± 10.0 ($n = 30$) $z = 5.3 \times 10^{-4}$	11.4 ± 9.3 ($n = 30$) $z = 1.8 \times 10^{-6}$	8.3 ± 9.3 ($n = 31$) $z = 2.1 \times 10^{-4}$	($P = 0.23$)
Ferritin concentration ($\mu g\ l^{-1}$)				
0	15.7 (6.1, 40.6) ($n = 31$)	14.7 (5.3, 40.8) ($n = 31$)	12.0 (4.1, 35.7) ($n = 32$)	($P = 0.89$)
5	14.1 (6.2, 32.1) ($n = 24$)	16.0 (7.1, 35.6) ($n = 26$)	13.0 (4.3, 38.9) ($n = 29$)	($P = 0.78$)
10	10.9 (3.7, 32.7) ($n = 28$)	15.3 (5.9, 39.6) ($n = 30$)	10.1 (3.5, 28.8) ($n = 31$)	($P = 0.22$)
Change in ferritin concentration ($\mu g\ l^{-1}$)				
5	−5.1 ± 15.2 ($n = 24$) $z = 0.60$	1.3 ± 9.6 ($n = 26$) $z = 0.38$	0.9 ± 9.5 ($n = 29$) $z = 0.80$	($P = 0.23$)
10	−6.1 ± 16.1 ($n = 28$) $z = 0.31$	0.7 ± 11.3 ($n = 30$) $z = 0.56$	−3.1 ± 12.6 ($n = 31$) $z = 0.53$	($P = 0.14$)

The top portion of the upper panel shows the arithmetic means of Hb concentrations (± standard deviation) for the three groups of anaemic children, as randomly assigned to the bean treatments, including the number (in parentheses) analysed at each interval. The *P*-value in the final column represents the results of the across-row comparison using the Kruskal–Wallis test. The time-dependent increments in Hb concentration are similarly shown in the lower portion of the upper panel, with the across-row comparison expressed as the *P*-value in the final column. Shown below each value of Hb change is the *z*-value from the paired Wilcoxon test for comparison of within-subject values from baseline to 5 weeks, and from baseline to 10 weeks. The top portion of the lower panel illustrates the geometric means (lower and upper confidence intervals) for the corresponding serum ferritin concentrations, and the lower portion the time-dependent changes in ferritin concentration. The format for statistical comparison is the same as in corresponding portions of the upper panel.

Table 3 Haemoglobin (Hb): *post hoc* analysis of subgroups according to low ($< 20\,\mu g\,l^{-1}$) and high ($>20\,\mu g\,l^{-1}$) ferritin values at baseline (mean ± standard deviation, *n* for each group in parentheses)

Time (week)		Beans only	Haem-fortified	$FeSO_4$-fortified	Across-row *P*-value
Hb concentration ($g\,l^{-1}$)					
0	Low ferritin	108 ± 5 ($n = 18$)	108 ± 5 ($n = 18$)	107 ± 5 ($n = 17$)*	($P = 0.71$)
	High ferritin	110 ± 4 ($n = 13$)	111 ± 4 ($n = 13$)	111 ± 4 ($n = 13$)	($P = 0.72$)
		$P = 0.41$	$P = 0.08$	$P = 0.03$	
5	Low ferritin	112 ± 14 ($n = 11$)	120 ± 12 ($n = 11$)	115 ± 13 ($n = 13$)	($P = 0.38$)
	High ferritin	115 ± 5 ($n = 8$)	119 ± 12 ($n = 10$)	118 ± 8 ($n = 11$)	($P = 0.59$)
		$P = 0.77$	$P = 0.97$	$P = 0.26$	
10	Low ferritin	115 ± 14 ($n = 17$)	121 ± 9 ($n = 18$)	113 ± 10 ($n = 17$)	($P = 0.08$)
	High ferritin	118 ± 8 ($n = 13$)	119 ± 10 ($n = 12$)	121 ± 9 ($n = 14$)	($P = 0.72$)
		$P = 0.39$	$P = 0.36$	$P = 0.051$	
Hb increment ($g\,l^{-1}$)					
5	Low ferritin	4.1 ± 12.1 ($n = 11$)	11.8 ± 11.7 ($n = 11$)	8.2 ± 11.5 ($n = 13$)	($P = 0.41$)
	High ferritin	6.5 ± 8.4 ($n = 8$)	9.0 ± 11.2 ($n = 10$)	7.0 ± 6.4 ($n = 11$)	($P = 0.93$)
		$P = 0.65$	$P = 0.78$	$P = 0.98$	
10	Low ferritin	6.8 ± 11.2 ($n = 17$)[a]	13.1 ± 7.7 ($n = 18$)[b]	6.4 ± 8.5 ($n = 17$)[a]	($P = 0.04$)
	High ferritin	8.0 ± 8.7 ($n = 13$)	8.8 ± 11.3 ($n = 12$)	10.6 ± 9.5 ($n = 14$)	($P = 0.90$)
		$P = 0.62$	$P = 0.13$	$P = 0.39$	

The upper panel shows the absolute Hb concentrations at baseline and throughout the study within the framework of the high and low ferritin subgroups in the *post hoc* analysis. The *P*-values in the final column represent the results of across-row comparison using the Kruskal–Wallis test. The *P*-values for within-column comparison of ferritin subgroups by study interval and treatment assignment showed significance for the $FeSO_4$ group at baseline (*). The lower panel shows the arithmetic means with respect to the time-dependent changes in the Hb values by *post hoc* subgroups for each of the treatment groups and time intervals. Comparisons were made for within-subgroup changes in ferritin at 5- and 10-week intervals for each subgroup by treatment assignment by means of the paired Wilcoxon test (data not shown, but discussed in the text). The within-column comparison between ferritin subgroups for time-dependent changes showed no significance (Kruskal–Wallis test). The *P*-values in the last column express the across-row comparison within subgroups across assigned treatments by means of the Kruskal–Wallis test. For the third row, values not sharing the same superscript were significantly different ($P < 0.05$).

Table 4 Ferritin: *post hoc* analysis of subgroups according to low ($>20\,\mu g\,l^{-1}$) and high ($<20\,\mu g\,l^{-1}$) ferritin values at baseline (upper panel: geometric mean, lower and upper confidence intervals and *n* for each group in parentheses; lower panel: arithmetic mean ± standard deviation and *n* for each group in parentheses)

Time (week)		Beans only	Haem-fortified	$FeSO_4$-fortified	Across-row *P*-value
Ferritin concentration ($\mu g\,l^{-1}$)					
0	Low ferritin	7.8 (4.4, 14.1) ($n = 18$)	6.8 (3.6, 12.9) ($n = 18$)	5.5 (2.5, 11.9) ($n = 18$)	($P = 0.53$)
	High ferritin	37.1 (23.1, 59.6) ($n = 13$)*	38.3 (27.2, 53.8) ($n = 13$)*	32.9 (24.0, 45.2) ($n = 14$)*	($P = 0.51$)
		$P = 3 \times 10^{-6}$	$P = 3 \times 10^{-6}$	$P = 2 \times 10^{-6}$	
5	Low ferritin	7.5 (4.1, 13.8) ($n = 12$)	10.6 (5.1, 21.7) ($n = 16$)	6.3 (2.4, 16.2) ($n = 16$)	($P = 0.20$)
	High ferritin	26.6 (17.6, 40.1) ($n = 12$)*	30.9 (21.2, 44.9) ($n = 10$)*	31.4 (20.9, 47.2) ($n = 13$)*	($P = 0.51$)
		$P = 1.2 \times 10^{-4}$	$P = 5.9 \times 10^{-4}$	$P = 4.4 \times 10^{-5}$	
10	Low ferritin	5.4 (2.2, 13.5) ($n = 15$)	9.0 (3.9, 20.6) ($n = 18$)	5.6 (2.2, 14.0) ($n = 18$)	($P = 0.15$)
	High ferritin	24.5 (12.8, 46.6) ($n = 13$)*	33.9 (22.3, 51.6) ($n = 12$)*	22.9 (13.3, 39.4) ($n = 13$)*	($P = 0.51$)
		$P = 2.3 \times 10^{-4}$	$P = 8.8 \times 10^{-5}$	$P = 1.4 \times 10^{-4}$	
Change in ferritin concentration ($\mu g\,l^{-1}$)					
5	Low ferritin	0.3 ± 3.0 ($n = 12$)	5.3 ± 6.9 ($n = 16$)	2.6 ± 8.2 ($n = 16$)	($P = 0.65$)
	High ferritin	−10.5 ± 23.3 ($n = 12$)	−5.2 ± 10.2 ($n = 10$)*	−1.2 ± 11.0 ($n = 13$)	($P = 0.73$)
		$P = 0.09$	$P = 0.01$	$P = 0.06$	
10	Low ferritin	−1.0 ± 4.8 ($n = 15$)	4.7 ± 6.3 ($n = 18$)	1.4 ± 8.1 ($n = 18$)	($P = 0.06$)
	High ferritin	−11.9 ± 22.1 ($n = 13$)	−5.3 ± 14.6 ($n = 12$)*	−9.3 ± 15.1 ($n = 13$)*	($P = 0.83$)
		$P = 0.33$	$P = 0.04$	$P = 0.04$	

As shown in the upper panel for absolute ferritin concentrations and by virtue of the selection of subgroups, assigned *post hoc* by their initial ferritin values, there were highly significant differences at baseline in the geometric mean concentrations in each treatment arm for the within-column comparisons at baseline as assessed by the Kruskal–Wallis test; these differences persisted for within-column comparisons within treatment groups at 5 and 10 weeks into the intervention, as well. The *P*-values in the final column show significance levels for across-row comparisons by the Kruskal–Wallis test for ferritin subgroups across treatment group at baseline or through the intervention. The lower panel shows the arithmetic means with respect to the time-dependent changes in the ferritin values by *post hoc* subgroups for each of the treatment groups and time intervals. Comparisons were made for within-subgroup changes in ferritin at 5- and 10-week intervals for each subgroup by treatment assignment by means of the paired Wilcoxon test (data not shown); *z*-values ranged from 0.06 to 0.93, all not significant. The *P*-values below the couplets of data by interval × treatment show the within-column comparison between ferritin subgroups for time-dependent changes; the *P*-values in the last column express the across-row comparison within subgroups across assigned treatments, both by the Kruskal–Wallis test.

the respective increments at 5 weeks ($z = 0.03$) and 10 weeks ($z = 0.005$) in the high ferritin subgroup. For the children consuming beans enhanced with $FeSO_4$, the Hb increments in Table 3 were also significant in the low ferritin group at 5 weeks ($z = 0.05$) and 10 weeks ($z = 0.05$) and in the high ferritin group at 5 weeks ($z = 0.0002$) and 10 weeks ($z = 0.003$). More importantly, in the across-row comparison among treatment groups,

the Kruskal–Wallis test showed the increase in Hb in the low ferritin group after 10 weeks of bean consumption to be significantly greater than in the beans-only or $FeSO_4$ treatment groups ($P = 0.04$).

As would be expected, there was a significant difference in the absolute ferritin concentrations in the subgroups sub-selected for their initial ferritin status using the Kruskal–Wallis test; these differences persisted throughout the entire study (Table 4, upper panel). When analysed by assignment to treatment group (across-row comparison by Kruskal–Wallis test), however, we found no differences in absolute ferritin concentration in either high- or low-ferritin subjects at any interval of the study (Table 4, upper panel). None of the six *post hoc* subgroups manifested a significant within-group increment or decrement ($z > 0.5$) by the paired Wilcoxon test (data not shown). As shown in the lower panel of Table 4, however, the time-dependent change in ferritin concentrations for the initially high ferritin groups was negative whereas that for the low ferritin groups was generally positive during the intervention (with the exception of the beans-only group, which also trended down in ferritin from zero time to 10 weeks). In the two groups receiving added iron in their beans, this contrast between ferritin groups was generally significantly different within a column. Moreover, the across-row comparison of ferritin changes showed a tendency for the haem iron increment to be higher, almost achieving significance ($P = 0.06$) by the Kruskal–Wallis test in terms of inter-treatment differences in ferritin changes.

Discussion

Anaemia remains a public health problem in Mesoamerica, with the consequences of reduced physical work capacity[23] and impaired cognitive development when encountered in early childhood[2–4,24]. This presumption motivated the double-masked community intervention trial presented here. Haem iron as a fortificant was compared with $FeSO_4$ and a placebo treatment for the restoration of Hb and the incrementing of circulating ferritin as an index of iron stores.

Bovine Hb has been used as a source of iron to fortify infant cereals[15,16], toddler weaning foods[18], biscuits[14] and cookies/cakes for the school-aged population[17]. The impact of a haem-based iron preparation on anaemia in infants[15,16] and juvenile populations was investigated in Chile[17]. In both instances, it proved superior to comparative treatments in restoring Hb levels and reversing anaemia, although in the latter study the baseline rate of anaemia was low to begin with. Guatemalan children have far more serious anaemia problems than those living in Chile in the late 1980s. The low-meat, high-fibre, high-phytate, high-tannin and high-calcium cuisine of the traditional Guatemalan culture posed a much more severe challenge to an iron fortificant than the more refined fare of Chile. Such inhibitory factors seem in major part responsible for the endemicity of IDA in Guatemala[9], and inorganic iron salts are much more susceptible to such inhibitors, calcium being an exception. Therefore, testing the impact of haem iron as food fortificant seemed the most appropriate approach to be explored. Haemoglobin is a macromolecule with a weight of 65 458 Da, 94% of which is globulin. It thus is a very bulky carrier for iron. Therefore, a simple and cheap method to split haem from the globin moiety was developed[25]. Haem iron was supplemented in the form of this novel haem iron preparation.

Although Hb concentrations increased significantly after 5 and 10 weeks of intervention, there were no significant differences in these increments between beans-only children and those children who were offered 30 plus mg Fe per day added as haem iron or as $FeSO_4$ to their daily diet (Table 2). The increased Hb values in the beans-only subjects may be consequence of antiparasitic treatment or due to the statistical phenomenon of 'regression to the mean'. A marginal advantage of haem is suggested by the restriction of significant Hb increases to the haem-fortified group after 5 weeks of intervention. However, the primary hypothesis that food fortification with haem iron will improve anaemia in the average anaemic Guatemalan child is clearly rejected by these findings. The marginally different results suggest that inhibition of iron absorption by phytate, tannin and especially by calcium in food is, indeed, difficult to overcome. This result is quite distinct from that in comparable age groups studied by Schultink *et al.*[26] in Indonesia with away-from-meal iron, where there was a time-dependent build-up of differential ferritin stores with a daily dose of supplemental iron. The Guatemalan diet is a barrier to dietary iron utilisation. Analyses of tortillas in Guatemala[27] show that 100 g of edible portion contains 108 mg of calcium. Correspondingly, Belizán and Villar[28] rated the average daily calcium intake for rural Guatemalan adults to be among the highest in the world. Hence, despite haem iron's higher intrinsic bioavailability, the one dietary inhibitor shown to impair its uptake seems to have considerable effect[13,29], although not all studies have confirmed the calcium inhibition of iron uptake[30]. Differences in duration of interactions and observations possibly account for these discrepancies. Still, this thesis offers a consistent explanation for both the slightly better performance of haem iron and the overall poor performance of fortificant iron with this community-level trial. A longer-term study with the same methodology would possibly have shown more definite results.

Plasma ferritin concentrations at the beginning of the study varied widely. Because chronic inflammation and chronic diseases were carefully excluded during enrolment, this finding suggests substantial differences in the state of iron repletion. Based on this observation we performed *post hoc* subgroup analyses in children with initial ferritin values

$<20\,\mu g\,l^{-1}$. This is the cut-off value for iron stores that are low enough to increase intestinal non-haem iron absorption[31]. The remaining children had initially adequate iron reserves (ferritin $>20\,\mu g\,l^{-1}$). Ferritin concentrations decreased in children with high initial ferritin values, whereas they increased in almost all children with low ferritin values at baseline, the differences between the subgroups being significant in both intervention groups after 10 weeks (Table 4). The most striking finding was that children with initially low ferritin concentrations showed significantly higher Hb increments after 10 weeks of intervention with haem-fortified beans than with beans only or beans fortified with $FeSO_4$ (Table 3).

Noting that this study used capillary blood for sampling, owing to its greater acceptability and for other ethical considerations, raises an issue concerning imprecision in the assessment of haematological variables with finger-prick samples. According to Morris *et al.*[32], capillary blood Hb measurements suffer from greater within-subject variation, to the point that misclassification errors can occur with each determination. For the present study, the implication is that our choice of a finger-prick method might have reduced the power to detect true differential changes in haematological status across treatment groups.

In anaemia with low initial plasma ferritin, absorbed food iron was used preferentially to replete Hb and haem was a marginally better source of iron to do so. Iron absorbed in excess of direct functional demand seems to have repleted the stores. On the other hand, sufficient iron stores seem to have been used for haem synthesis during growth, as high initial ferritin content decreased in parallel to significant increases in Hb after 10 weeks (see *z*-values in Table 3). Such appropriate utilisation of iron for haem synthesis is not observed in anaemia of inflammation. Therefore, one may assume that changes in plasma ferritin represent changes in iron stores and do not indicate chronic inflammation or increased levels of infection. Accordingly, no difference in the propensity of gastrointestinal or respiratory infections was observed between the groups during staff visits. If increased plasma ferritin concentration represents increased iron stores and increased Hb represents increased iron utilisation, both findings strongly argue for a superior bioavailability of iron from haem-fortified beans. However, statistical power in the iron-deficient subgroup was sufficient to prove this notion only for Hb increments, whereas it fell short to do so for Hb concentrations ($P = 0.08$; Table 3). The sample size calculations were based on analysis of entire treatment groups, and for technical reasons, Hb assays were not completely available from some participants at the 5-week interval, which limits the statistical power to separate differential response at the subgroup level. Nevertheless, pursuing the *a posteriori* hypothesis of differential response by ferritin status, the data are consistent with a better uptake, utilisation and storage of iron from haem than from inorganic iron. Hence, in a bean-consuming population with a high prevalence of depleted iron stores, promotion of haem-fortified canned beans should have a salutary influence on the iron status of such a populace.

If we estimate the total increment of body iron, i.e. the combined effect on Hb and ferritin repletion, and relate this parameter to incremental iron intake with the supplement, the advantage of haem iron becomes more obvious. However, as the study was not designed for this purpose, some variables, such as body weight increments and the ratio between increases in ferritin concentration and body iron stores in children, were not determined and need best-guess assumptions based on literature data. Still, it may be worthwhile to do such calculations to estimate the net impact of food iron fortification on body iron content (see Appendix), even if they are not entirely based on Guatemalan data. The assumptions made in the Appendix point to additional parameters and variables that are advisable to be examined in future studies.

The possible dissociation of iron nutriture and anaemia is becoming increasingly appreciated. Thus, a study from Ghana found approximately 40% of infants to remain anaemic after 2 months of iron supplementation that increased serum ferritin significantly[33]. In Côte d'Ivoire approximately 50% of anaemic women were not iron-deficient[34]. Inflammation, infection, malarial parasites and haemoglobinopathies may explain part of these observations. According to the authors, another part may also be due to deficiency states for other micronutrients. Accordingly, a Mesoamerican childhood anaemia phenomenon was described by Allen *et al.*[35] in rural Mexico which cannot be explained by iron deficiency. When pre-school children were supplemented with 20 mg Fe daily for 12 months in a longitudinal study, anaemia persisted in about 30% of cases although ferritin levels had normalised. Initial plasma B_{12}, height-for-age and dietary quality predicted the haematopoietic response to iron. The authors suspected a role of chronic undernutrition and of deficiency states for vitamin A, folic acid, and vitamins B_2 and B_{12}[35]. Such dietary factors may also explain the presence of anaemia in the high ferritin subgroups in our present study. Improved utilisation of iron stores in the high ferritin subgroups may be due to an improved overall nutritional situation of the children due simply to their involvement in the trial. For more definitive confirmation of the Hb-restoration response, a more rigid pre-selection of subjects than simple anaemia would be desirable in future studies.

Acknowledgements

We are grateful to Ing. Emilio Maldonado and the staff and administration of Mahler Sucs Co. Ltd, for the production and masking of the refried, black beans. We thank Dr Petra Klassen and Dr Carlos Valdez for their clinical and laboratory efforts in the field, and Dr Alexander Crispin from the Statistical Department of the Ludwig-Maximilians-University for his support in evaluating the data.

We are grateful to the volunteers and mothers of the Ciudad Peronia community for their assistance and participation.

Some of the data presented herein were reported at the meeting of the American Society of Nutritional Sciences at Experimental Biology 2000, held in San Diego, CA.

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Appendix – Comparison of estimated total body iron and estimated increment in total body iron between haem-fortified beans and beans-only groups after 10 weeks of intervention

Means values of Hb and ferritin concentration were taken as the basis for calculation. Total body iron was estimated as the sum of iron bound to Hb plus iron in tissue stores. Blood volume was assumed to be 7% of average body weight, which was taken as 12 kg at an age of 24 months[36], corresponding to an average blood volume of 840 ml. The average weight increment of a child between 2 and 4 years of age was taken as 3 kg[36], corresponding to an average increment in blood volume of 20 ml over 10 weeks. One gram of Hb was assumed to contain 3.4 mg Fe[37]. It was estimated that 1 $\mu g l^{-1}$ of plasma ferritin corresponds to 8 mg of stores iron in adults[38]. When the ratio between plasma ferritin concentration and body iron stores in the tissue is scaled down from a 70-kg adult to 12-kg child, 1 $\mu g l^{-1}$ ferritin corresponds to 1.37 mg of stored iron. Based on these assumptions, total body iron and its distribution between Hb and tissue iron stores at baseline are calculated in Table A1. On the basis of these data, Table A2 estimates the increment in total body iron content during 10 weeks of intervention.

The increment over 10 weeks (penultimate column in Table A2) was about twice as high in children on the haem-fortified diet as compared with the beans-only diet. Children with initially high ferritin values showed much lower increments in total body iron. To compensate for this, they seem to have shifted markedly more iron from stores to the erythron than children with initially low plasma ferritin concentrations (last column in Table A2). On the haem-fortified diet, children even seem to have added iron to their stores, as signified by negative values for iron derived from stores (see * in Table A2).

The estimated values presented here are admittedly crude. No data are available on the ratio between plasma ferritin concentration and body iron stores for children of different ages and values for increments in body weight were extrapolated from tables on average growth rates. Therefore, we present these considerations as an Appendix, i.e. apart from the rest of the study.

However, the mode of calculation used here permits comparison of the impact of the haem intervention on total body iron content, i.e. on the sum of iron bound to Hb and iron stores, after a dietary intervention. This may be a useful approach to be pursued more rigorously in future studies.

Table A1 Total body iron and its distribution between haemoglobin (Hb) and tissue iron stores at baseline

	Hb conc. ($g l^{-1}$)	Hb Fe (mg)	Ferritin conc. ($\mu g l^{-1}$)	Fe stores (mg)	Total body Fe (mg)	Fe bound to Hb (%)	Fe in stores (%)
Beans only							
Low ferritin	115	328.4	7.6	10.4	338.9	97	3
High ferritin	118	336.9	29.3	40.1	377.0	89	11
Haem-fortified							
Low ferritin	121	345.4	11.9	16.3	361.7	95	5
High ferritin	119	339.6	36.3	49.7	389.3	87	13

conc. – concentration.
Hb and ferritin concentrations are the mean of the values actually determined at baseline.

Table A2 Increment in total body iron content during the 10 weeks of intervention

	Hb incr. in 10 weeks ($g l^{-1}$)	Fe in Hb incr. (mg)	Fe in Δbvol (mg)	Δferritin ($\mu g l^{-1}$)	ΔFe stores (mg)	Cumul. Fe incr. in 10 weeks (mg)	Fraction of Hb Fe incr. derived from Fe stores (%)
Beans only							
Low ferritin	6.80	19.4	7.8	−1.0	−1.4	25.8	5
High ferritin	8.00	22.8	8.0	−11.9	−16.3	14.5	53
Haem-fortified							
Low ferritin	13.1	37.4	8.2	+4.7	+6.4	52.0	−14*
High ferritin	8.8	25.1	8.1	−7.3	−7.3	25.9	22

Hb – haemoglobin; incr. – increment; Δbvol – estimated increment in blood volume due to growth in 10 weeks; Δferritin – change in serum ferritin; ΔFe stores – change in body iron stores; cumul. – cumulative.