"Impact of micronutrient fortification of corn flour on iron, zinc and acid folic status of rural Mexican school age children: an efficacy trial"

Final Report

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Received & accepted as technically satisfactory. Followed up w/ proposal to expand fortification to small scale mills. Rejected due to lack of funds & non target country

Introduction

According to the 1999 National Nutrition Survey (1999-NNS) iron deficiency and anemia are public health problems in Mexico. The prevalence of anemia in children < 5 years was 26%, in children 5-11 years of age and in non-pregnant women 20%, and in pregnant women 28%. Iron deficiency, as assessed by percentage of transferrin saturation < 16, was 52% in children < 5 years, 36% in children 5-11 years and 41% in non-pregnant women. Therefore, efforts are underway to improve the iron status of the population. One of the interventions aimed at improving the iron status of the general population is the fortification of wheat and corn flours.

In September 1998 an agreement was signed between representatives from the Mexican government and corn and wheat millers to fortify wheat and lime processed corn flour with iron, zinc, folic acid, riboflavin, niacin, and thiamine in amounts shown in Appendix A. Lime processed corn flour (referred to hereafter simply as corn flour) is used to make *tortillas*, the staple food for millions of Mexican and Central American families. The agreement is not mandatory for corn flour; however, the two companies that produce most of this product started fortifying soon after the agreement was signed and have continued to do so.

Comment: Any documentary proof in an appendix would strengthen the statement. According to Salvador, the voluntary fortification has not been monitored. But maybe the industry has data to share on this

Iron fortification of *tortillas* is a strategy with a high potential for the prevention and control of iron deficiency in Mexico and part of Central America, given its consumption by a large number of people. Therefore, we considered essential to assess the impact of fortified *tortillas* on the micronutrient status of the population. The form of iron used to fortify tortilla is elemental (reduced) iron which is less reactive than alternative iron salts and therefore has virtually no adverse effects on the color and flavor of cereal flours to which it is added.

Comment: H or CO-reduced?

However, reduced iron has low bioavailability. Thus, we expected low impact of the program on iron stratus and decided to compare the impact of reduced iron not only with a control, but also with *tortillas* fortified with a more bioavailable form of iron. Since *tortillas* contain high amounts of phytic acid, a potent inhibitor of iron absorption, we chose FeNaEDTA a fortificant that performs well when added to foods containing high amounts of phytic acid (Ref. **J. Nutr. 133: 3158–3161, 2003.)**

The study was carried out in school age children attending government-run shelter homes for elementary school students in Oaxaca, one of the poorest states in Mexico. These shelter homes were created to provide food, shelter and academic support to children from poor families living in communities without schools, who would have to travel (usually afoot) for hours to reach the closest school. Shelter home services are also available for children living in the village where shelter homes are located as a way to support the poorest families of the village. Children live in the shelter homes and attend the local school from Monday to Friday and return to their homes during the week end. Shelter homes were assigned to the following three treatments: 1) fortified *tortilla* with elemental iron and other micronutrients added under the current standard policy in Mexico (see Appendix A); 2) fortified *tortilla* with FeNaEDTA in amounts equivalent to 30 mg/Kg of iron and the same additional micronutrients as shown in Appendix A ; and c) non-fortified *tortilla*.

This document presents the main results and conclusions. The first section summarizes the design and methods, the second section presents the results and the third section the conclusions.

Design and methods

Characteristics of the sample

The original design of the study considered 21 shelter homes (7 per treatment) on the basis of the prevalence of anemia found in a feasibility study conducted in several shelter homes in the State of Oaxaca. However, the baseline prevalence found in the 21 shelter homes selected was much lower than the prevalence found in the feasibility study; therefore 13 additional shelter homes were included in a second phase to increase the number of anemic children. Altogether, a total of 1786 children in 34 shelter homes participated in the study. The first recruiting phase started in January 2001and included 1132 school children in the original 21 shelter homes. The second phase started in October 2001and included 654 children in 13 additional shelter homes.

Shelter homes were randomly assigned to receive a full free supply of one of the three forms of flours (not fortified, fortified with reduced iron or fortified with FeNaEDTA) during one year. Corn flour was used mostly to prepare *tortillas* but other food preparations such as atole (a gruel-like beverage) or tamales were eventually prepared with the flours. Children ate three daily meals at the shelter homes. During weekends children consumed a free choice diet that did not include fortified *tortillas* at their family houses.

Comment: No habia entrado ya en vigor la fortificacion voluntaria de harina de maiz?

The amount of *tortillas* consumed individually at the shelters was weighed and registered daily. The food composition of the fixed menus offered in the shelter homes were also calculated.

Before, as well as 6 and 12 months after the intervention started, anthropometry and venous blood samples were obtained. Blood samples were transported to a central laboratory for the determination of serum Ferritin, C-reactive protein (CRP) and whole blood folate.

The quantitative measurement of ferritin in serum was done by sandwich immunoassay (ELISA, Opus Behring Laboratories, Westwood, MA, U.S.A.) using commercial kits (Dade Behring Inc, Newark, DE 19714, U.S.A.).

For CRP determination an immunonephelometry method was used (Behring Nephelometer 100 Analyzer, Behring Laboratories, Messer Grisheim Gmbh, Frankfurt/M. W Germany)[1]. In this method the polystyrene particles coated with monoclonal antibodies to CRP are agglutinated when mixed with serum samples containing CRP. The intensity of the scattered light in the nephelometer depends on the CRP content of the sample and therefore the CRP concentration can be determined in comparison with dilutions of a standard of a known concentration (Dade Behring, Marburg, Germany).

Hemoglobin Folate (HF) values were measured using dried blood spots on filter paper by the method described and validated by O'Broin et al. [2-4] In short, blood spots were collected in filter paper (sheet Num 903, S&S, Inc., New York, NY, USA), and allowed to dry in a light-protected environment. Once the sheets were completely dry, they were wrapped in absorbent paper and stored at 4°C in zippered plastic bags containing two desiccant sachets until storage in the central lab at -20°C. Dried spots were extracted by sonication in phosphate/ascorbic acid buffer. Eluates were assayed for folate in whole blood by a microbiological method using *Lactobacillus caseii* as the sensitive organism [5] and for Hb content by a colorimetric method. HF values were calculated by dividing the whole blood-folate concentration by the sample hemoglobin concentration and adjusted to the concentration of Hb in whole blood [2]. In a validation procedure carried out by the authors of the method, HF values correlated well with the erythrocyte folate concentrations of normal Americans measured by conventional methods ($r^2 = 0.99$; $n = 11887$). [2]

Hemoglobin concentrations were measured *in situ* using portable photometers (Hemocue™).

Unadjusted comparisons were made using Generalized Estimating Equations (GEE) for normally distributed variables in order to account that children living in the same home shelter are correlated. For categorical variables, Pearson's Chi-square tests were used for comparisons among treatments and McNemar Chi-square tests for comparisons overtime within treatment group.

Adjusted analyses were conducted to control for basal hemoglobin and ferritin values or categories, for other factors that differed between groups at recruitment and for recruitment phase. Again, Generalized Estimating Equations (GEE) were employed to take into account that children are nested within shelter homes. Linear regression and logistic regression models were performed using hemoglobin and different categories of hemoglobin values as dependent variables, respectively.

Our original expectation was to find larger effects on children who were anemic, particularly on those with iron deficiency anemia. Therefore, interaction terms between treatment and hemoglobin and ferritin categories were tested. The cut-off point used to classify children 5-11 years of age as anemic was 115 g/L. Therefore, the first set of hemoglobin categories were < 115 g/L and ≥ 115 g/L. However, for the three-way interaction between treatment, hemoglobin and ferritin categories, the sample sizes for the resulting treatment groups among iron deficient and anemic were too small. Therefore, a second set of categories, using 120 g/L as the cut-off point (< 120 g/L and ≥ 120 g/L) were employed for the final models.

Differences were considered statistically significant at p values < 0.05 for main effects and < 0.10 for interactions.

Results

The crude mean basal concentrations of hemoglobin (g/L), adjusted only for altitude were 139±14.0 for the group that received flour fortified with reduced iron, 140±14.1 for the group receiving flour with FeNaEDTA and 141±14.3 for the group receiving non-fortified flour. Differences were not statistically significant among the groups (p=0.567). One year after the intervention, all hemoglobin concentration values had decreased: 138±13.8, 135±17.1 and 133±15.2, respectively. The differences were not statistically significant among groups (p=0.600). Comparison of basal and final mean hemoglobin values within treatment groups showed statistically significant reductions for the FeNaEDTA and the non-fortified groups (p<0.001), but not for the reduced iron group (p=0.360). Sample sizes were 272, 293 and 279 for the reduced iron, FeNaEDTA and non-fortified flour, respectively. Only cases with hemoglobin values both at baseline and at the final measurement were used to obtain the means compared.

Comment: Oración incompleta¿?

The crude prevalence of anemia (adjusted only for altitude), in the basal evaluation varied from 10.6% to 13.6% with no significant differences among the three treatment groups (Table 1). After one year of intervention, the prevalence of anemia increased significantly in the FeNaEDTA and in the non-fortified group, but not in the reduced iron group. The prevalence of iron deficiency defined with both criteria (<20 and <12 ng/dL) showed no differences among or within groups (Table 1).

Table 2 presents crude means and standard deviations for hemoglobin (adjusted only for altitude) and ferritin concentrations at basal and final measurements by hemoglobin (less or greater and equal than 120 g/L) and ferritin (less and greater or equal than 20 ng/dL) categories. Statistical tests were only performed in the comparisons among treatment groups

at the basal evaluation. The only differences that were statistically significant were in hemoglobin concentrations in the low-hemoglobin and high-ferritin group. Hemoglobin concentrations tended to increase in the low hemoglobin group and to decrease in the high hemoglobin group from baseline to final evaluation. Likewise, median ferritin concentration tended to increase overtime in the low ferritin groups and to decrease or remain with similar values in most of the high ferritin groups.

In a GEE linear regression model using final hemoglobin concentration as dependent variable, a three-way interaction between treatment, hemoglobin < 120 g/L and Ferritin < 20 ng/dL was statistically significant ($p<0.10$) (Table 3). Among subjects with low hemoglobin (< 120g/L) and low ferritin (<20 ng/dL) concentrations, those receiving FeNaEDTA fortified flour had final hemoglobin concentrations 8 and 12 g/L greater than non-fortified and reduced iron groups, respectively (Figure 1). The difference between the reduced iron and the FeNaEDTA groups ($p=0.016$) was marginally significant after adjusting the significance level for multiple comparisons. In contrast, no significant differences were observed among groups in the groups of children with high hemoglobin concentrations (>120 g/L) or in those with low hemoglobin but high ferritin concentrations. Similar results were obtained for the GEE model using 115 g/L as the cut-off point for hemoglobin. However, the interaction was not statistically significant ($p=0.23$), probably due to small sample sizes (Table A1, Appendix A).

In a logistic regression model, a significant three-way interaction was found between treatment, basal ferritin (at or above or less than 20 ng/dL) and basal hemoglobin category (at or above or less than 120 g/L) on their effects on the probability of low hemoglobin (< 120 g/L) after one year of intervention ($p< 0.10$) (Table 4). Among subjects with low hemoglobin and low ferritin concentrations, those receiving corn flour with FeNaEDTA had a lower probability of anemia (0.24) than those receiving flour with reduced iron or non-fortified flour

(probabilities: 0.50 and 0.65, respectively) (Figure 2). The difference between the FeNaEDTA and the non-fortified group (p=0.014) was marginally significant after adjusting the significance level for multiple comparisons. In contrast, among children with high hemoglobin concentration at baseline and among those with low hemoglobin concentrations and high ferritin concentrations differences among treatment groups were small and were not statistically significant. Similar results were obtained for the model using 115 g/L as the cut-off point for hemoglobin. However, the three-way interaction was not statistically significant (p>0.2), probably due to small sample sizes (Table A2, Appendix A).

Conclusions

A positive impact on hemoglobin concentration of *tortillas* fortified with FeNaEDTA was found after one year of consumption only among children who were iron deficient and had hemoglobin concentrations < 120 g/L at baseline, but there were no apparent effects among children with hemoglobin concentrations ≥ 120 g/L nor in children with low hemoglobin concentrations but high iron concentrations. *Tortillas* fortified with reduced iron had no effect on hemoglobin concentration in any subgroup. These results are consistent from a biological point of view. Greatest effects were expected in the group receiving FeNaEDTA, a form of iron that is bioavailable in the presence of inhibitors such as phytic acid, which is present in high amounts in *tortillas*. Maximum effects were also expected in children with low hemoglobin and ferritin concentrations, the most likely iron deficient group. In the group of children with low hemoglobin concentrations and high iron concentrations, low hemoglobin is not the result of iron deficiency and therefore children do not respond to increased iron intake. Possible causes of low hemoglobin in this group include folate, B-12 or vitamin A

Comment: Do we have figures for PA:Fe ratios for these/similar tortillas?

deficiencies or other non-nutritional causes. Lack of effects in the reduced iron group was expected, given the low bioavailability of that form of iron.

Comment: Does the nutrient composition and intake from the fixed menus at the shelter homes justify this possibility for any/all these nutrients?

The magnitude of the effect of FeNaEDTA fortified *tortillas* on hemoglobin concentration was between 8 g/L and 12 g/L and between 26% and 41% on the prevalence of low hemoglobin relative to the reduced iron and non-fortified *tortillas, respectively*. These effects are biologically important. The study was randomized and double blinded; therefore, results are unlikely to be biased.

Comment: Term "low hemolglobin" chosen because we did not find a similar effect when using the tradicional cut-off values, right?

One of the limitations of the study was that prevalence of anemia at baseline was lower than expected. The ideal population for this study would have been one with higher prevalence of iron deficiency anemia.

Comment: Given the results, simple sizes, and power, is this really a limitation? If we do not have enough power for some of the inferences being made, that would be different. But I think we are ok.

The cut-off point recommended to identify anemic children 5-11 years of age is 115 g/L. This was our first choice of cut-off point. However, the final models presented use <120 g/L instead as the cut off point because sample sizes were small for comparisons among subgroups resulting from the three-way interaction when <115 g/L was used. The fact that models using the <115 cut-off point had similar results although non-statistically significant, suggests that the lack of effect is due simply to the small sample sizes.

In summary, results suggest that *tortillas* made with FeNaEDTA fortified corn flour had positive effects in hemoglobin concentration in children with low hemoglobin and iron concentrations but not in other groups. The fact that *tortillas* fortified with reduced iron had no effects even in the low hemoglobin and low iron group, while FeNaEDTA did, suggests that reduced iron may not be an adequate form of iron fortificant for *tortillas*. FeNaEDTA is potentially a better fortificant for corn flour; however, the higher cost of the fortificant may be a disincentive for its use.

Comment: What happens if we extrapolate these results to the tortilla-eating Mexican population with low hemoglobin and/or low ferritin concentrations? What would be the expected reduction in ID and ID anemia were the corn flour fortified with nothing, reduced iron, or NaFeEDTA?

References

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Table 1. Prevalences[a] of anemia and iron deficiency in school age children in shelter homes with basal and final evaluation.

Prevalence	N	Reduced iron	N	FeNaEDTA	N	Non-fortified	*p-value* [b]
Anemia (%)	272		293		279		
Hemoglobin <115 g/L							
Basal		13.6 (6.6- 20.6)		10.6 (6.9 - 14.2)		11.1 (8.0-14.3)	0.497
Final		12.9 (9.5-16.3)		21.2 [c] (4.4- 37.9)		24.4[c] (15.0- 33.8)	0.002
Hemoglobin <120 g/L							
Basal		20.6 (11.8-29.4)		18.4 (12.7-24.1)		19.4 (15.2-23.5)	0.810
Final		22.1 (17.1-27.0)		28.7 [c] (9.0-49.0)		39.4 [c] (32.2-46.7)	0.001
Iron deficiency (%)	243		278		241		
Ferritin <12 ng/dL							
Basal		10.3 (5.4- 15.2)		13.3 (4.2- 22.4)		10.4 (2.7- 18.1)	0.461
Final		11.1 (5.0- 17.2)		10.4 (1.9- 19.0)		11.2 (0.0- 22.5)	0.953
Ferritin <20 ng/dL							
Basal		37.9 (25.0- 50.7)		36.0 (17.8- 54.0)		38.2 (19.2- 57.2)	0.852
Final		31.3 (24.0- 38.6)		33.8 (20.2- 47.5)		34.0 (13.9- 54.2)	0.770

[a] Prevalences and 95% C.I.
[b] *p*-values of Chi^2 among treatments.
[c] $p<0.05$ for McNemar Chi^2 Test comparing basal and final prevalences within group.

Table 2. Average concentrations of hemoglobin and ferritin serum in school age children in shelte hemoglobin and ferritin categories.

Variables	Treatment	Basal Hemoglobin								
		< 120 g/L						≥ 12		
		Basal ferritin < 20 ng/dL			Basal ferritin ≥ 20 ng/dL			Basal ferritin < 20 ng/dL		
		Basal	N[d]	Final	Basal	N[d]	Final	Basal	N[d]	Final
Hemoglobin (g/L)[b]	Reduced iron	111±8.8	23	115±19.2	109±7.0[•]	33	128±12.8	135±9.3	73	132±10.7
	FeNaEDTA	110±10.0	22	122±20.1	113±5.1[•]	30	125±14.7	135±9.1	79	128±17.1
	Non-fortified	111±7.6	22	120±11.5	114±5.3[•]	31	127±18.4	137±11.0	82	126±15.2
Ferritin (ng/dL)[c]	Reduced iron	11.7 (7.9-15.6)	21	17.4 (11.9-19.9)	28.5 (25.0-39.9)	29	27.3 (16.7-40.0)	15 (12.1-17.4)	71	22.4 (13.6-29.8)
	FeNaEDTA	12.8 (8.9-16)	22	20.6 (13.1-33.5)	37.0 (28.7-45.0)	28	27.6 (20.9-39.4)	13.2 (11.3-15.8)	78	17.8 (12.7-25.0)
	Non-fortified	13.5 (10-15.4)	18	16.8 (8.1-29.9)	26.8 (22-36.1)	27	24.7 (18.5-34.8)	15 (12.5-17.6)	73	22.9 (13.0-33.1)

[a] Only children with basal and final hemoglobin determinations.
[b] mean ± s.d.
[c] median (Percentiles 25 - 75).
[d] For hemoglobin, the sample size is the same for basal and final determinations. For ferritin, sample sizes for basal determinat as for hemoglobin but for final determinations they are smaller.
[•] $p<0.01$ from a GEE model to compare among groups in basal evaluation.

Table 3. Multiple regression model[a] for final Hemoglobin (g/L).

Independent variables	Coefficient	Std. Err.	p-value
Recruitment phase	-4.140	2.607	0.112
Basal Hb g/L	0.270	0.049	0.000
Macrocytosis (MEV > 90 fL)	1.946	1.137	0.087
Treatment 1 (Reduced iron)	5.334	3.396	0.116
Treatment 2 (FeNaEDTA)	2.496	3.307	0.450
Ferritin < 20 ng/dL	-0.371	1.974	0.851
Basal Hb < 120 g/L	7.976	2.868	0.005
Ferritin < 20 ng/dL x Hb < 120 g/L	-7.967	4.144	0.055
Treatment 1 x Ferritin < 20 ng/dL	1.105	2.844	0.698
Treatment 2 x Ferritin < 20 ng/dL	5.314	2.812	0.059
Treatment 1 x Hb < 120 g/L	-3.877	3.743	0.300
Treatment 2 x Hb < 120 g/L	-6.524	3.722	0.080
Treatment 1 x Hb < 120 g/L x Ferritin < 20 ng/dL	-6.562	5.837	0.261
Treatment 2 x Hb < 120 g/L x Ferritin < 20 ng/dL	6.622	5.841	0.257
Constant	90.663	7.186	0.000

[a] GEE Linear Regression Model

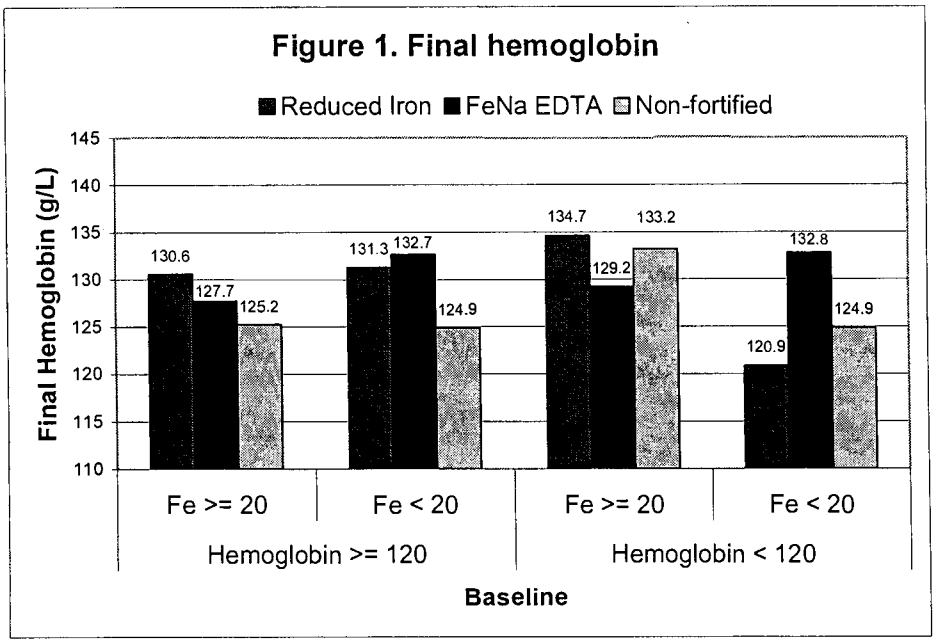
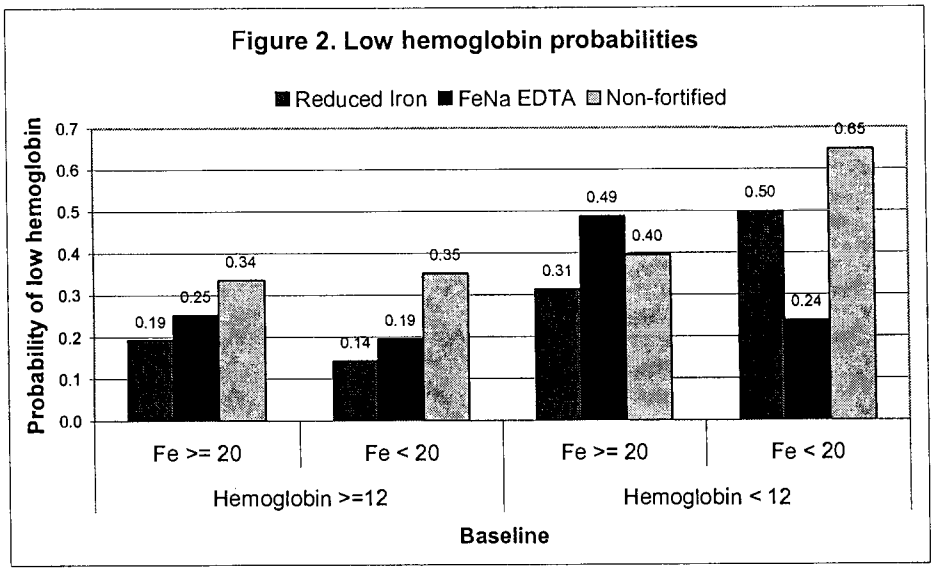


Table 4. Multiple logistic regression model [a] for low hemoglobin (Hb < 120g/L).

Independent variables	Coefficient	Std. Err.	p-value
Recruitment phase	0.513	0.339	0.130
Basal low Hb	0.256	0.389	0.510
Treatment 1 (reduced iron)	-0.756	0.463	0.104
Treatment 2 (FeNaEDTA)	-0.406	0.427	0.344
Ferritin < 20 ng/dL	0.067	0.294	0.819
Ferritin < 20 ng/dL x basal low Hb	0.969	0.621	0.119
Treatment 1 x Ferritin < 20 ng/dL	-0.427	0.488	0.383
Treatment 2 x Ferritin < 20 ng/dL	-0.402	0.450	0.373
Treatment 1 x basal low Hb	0.394	0.574	0.492
Treatment 2 x basal low Hb	0.779	0.550	0.156
Treatment 1 x basal low Hb x Ferritin < 20 ng/dL	0.170	0.903	0.850
Treatment 2 x basal low Hb x Ferritin < 20 ng/dL	-1.748	0.896	0.051
Constant	-0.938	0.352	0.008

[a] GEE logistic regression model.

Figure 2. Low hemoglobin probabilities



Appendix A. Amounts and forms of fortificants added to corn flour under current policy in Mexico.

Micronutrient	Minimum level (mg/kg)	Recommended level (mg/kg)	Maximum level (mg/kg)	Fortificant
Iron	24	30	40	Reduced extrafine (elemental) iron
Thiamin	4.0	5.0	8.0	Thiamin mononitrate
Riboflavin	2.4	3.0	5.0	Riboflavine hidrocloridrate
Niacin	28	35	45	Nicotinamide
Folic acid	0.4	0.5	0.8	Folic acid

Table A1. Multiple regression model [a] for final Hemoglobin (g/L).

Independent variables	Coefficient	Std. Err.	p-value
Recruitment phase	-4.198	2.623	0.110
Basal Hb g/L	0.243	0.045	0.000
Macrocytosis (MEV > 90 fL)	1.932	1.147	0.092
Treatment 1 (Reduced iron)	4.867	3.378	0.150
Treatment 2 (FeNaEDTA)	1.913	3.297	0.562
Ferritin < 20 ng/dL	-1.285	1.927	0.505
Basal Hb < 115 g/L	6.094	3.784	0.107
Ferritin < 20 ng/dL x Hb < 115 g/L	-6.198	5.190	0.232
Treatment 1 x Ferritin < 20 ng/dL	0.338	2.745	0.902
Treatment 2 x Ferritin < 20 ng/dL	6.053	2.742	0.027
Treatment 1 x Hb < 115 g/L	-3.055	4.693	0.515
Treatment 2 x Hb < 115 g/L	-7.316	4.927	0.138
Treatment 1 x Hb < 115 g/L x Ferritin < 20 ng/dL	-6.194	7.118	0.384
Treatment 2 x Hb < 115 g/L x Ferritin < 20 ng/dL	6.036	7.350	0.412
Constant	95.321	6.574	0.000

[a] GEE Linear regression model.

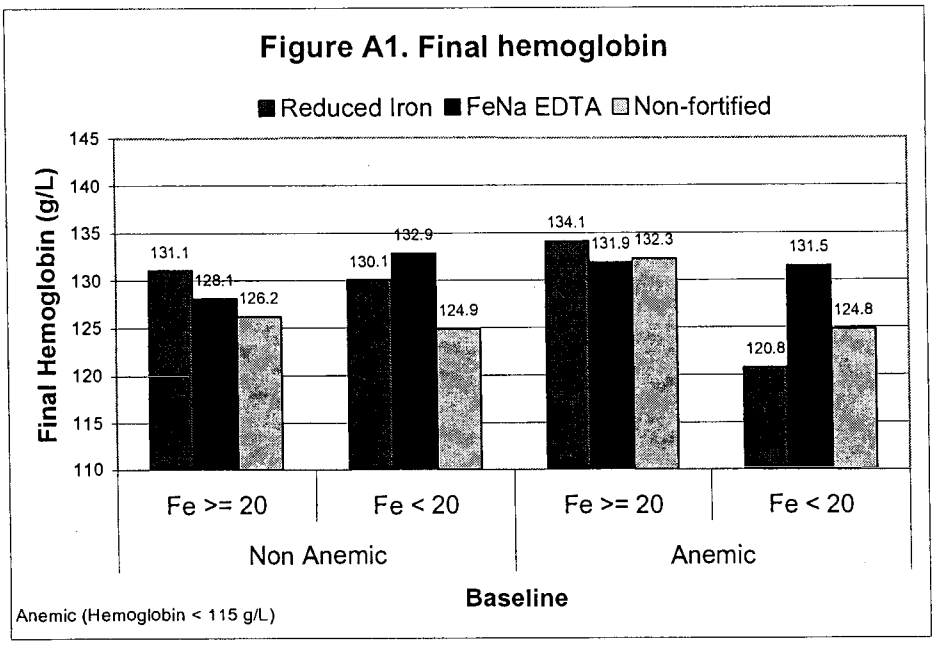


Table A2. Multiple regression model [a] for final anemia (hemoglobin < 115 g/L).

Independent variables	Coefficient	Std. Err.	p-value
Recruitment phase [b]	0.543	0.385	0.159
Basal anemia	0.816	0.550	0.137
Treatment 1 (Reduced iron) [c]	-0.571	0.539	0.290
Treatment 2 (FeNaEDTA) [c]	0.113	0.465	0.808
Ferritin < 20 ng/dL	0.209	0.333	0.529
Ferritin < 20 ng/dL x basal anemia	0.051	0.779	0.947
Treatment 1 x Ferritin < 20 ng/dL	-0.435	0.553	0.432
Treatment 2 x Ferritin < 20 ng/dL	-0.759	0.501	0.131
Treatment 1 x basal anemia	-0.688	0.834	0.410
Treatment 2 x basal anemia	-0.360	0.770	0.640
Treatment 1 x basal anemia x Ferritin < 20 ng/dL	1.526	1.186	0.198
Treatment 2 x basal anemia x Ferritin < 20 ng/dL	-0.576	1.234	0.641
Constant	-1.732	0.412	0.00

[a] GEE logistic regression model.
[b] The recruitment was performed in two phases; the first started in January 2001and included 1132 school age children in 21 shelter homes (recruitment phase=1). The second phase started in October 2001and included 654 school age children in 13 additional shelter homes (recruitment phase=0).
[c] Treatment 3 (Non-fortified flour) was used as reference.