



## Twice the recommended daily allowance of iron is associated with an increase in plasma $\alpha$ -1 antichymotrypsin concentrations in Guatemalan school-aged children

Francisco J. Rosales, MD, ScD<sup>a,\*</sup>, Yu Kang<sup>a</sup>, Beate Pfeiffer<sup>b,c</sup>,  
Annegret Rau<sup>b,c</sup>, Maria-Eugenia Romero-Abal<sup>b</sup>, Jürgen G. Erhardt<sup>c</sup>,  
Noel W. Solomons<sup>b</sup>, Hans K. Biesalski<sup>c</sup>

<sup>a</sup>Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802, USA

<sup>b</sup>Center for Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM), Guatemala City, Zona 11 Guatemala

<sup>c</sup>Department of Biological Chemistry and Nutrition, University of Hohenheim, Stuttgart, 70593, Germany

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### Abstract

The effects of iron on zinc status, oxidative stress, and inflammation were assessed in a randomized placebo-controlled trial; 66 children aged 8 to 11 years received iron (20 mg/d of elemental iron), zinc (42.5 mg/d of elemental zinc), or iron and zinc combined (20 and 42.5 mg/d, respectively) for 8 weeks. Hemoglobin, plasma ferritin (FT), and zinc concentrations were determined, and oxidative stress was based on plasma  $\alpha$ -tocopherol,  $\beta$ -carotene, and thiobarbituric acid–reactive substance. Inflammation was based on increased  $\alpha$ -1 acid glycoprotein, C-reactive protein, and  $\alpha$ -1 antichymotrypsin (ACT) concentrations. At baseline, 19% of children were iron deficient (FT < 20  $\mu$ g/L) and 69% had hypozincemia (zinc < 10.7  $\mu$ mol/L) being distributed equally among the groups. Supplementation with iron or zinc alone improved, respectively, plasma FT or zinc concentrations (2-factor analysis of covariance,  $P \leq .03$ ), but no treatment interactions were found. Although none of the supplementation strategies was associated with oxidative stress or inflammation (2-factor analysis of covariance,  $P > .05$ ), ACT concentrations increased with iron alone compared with the other supplementation strategies (median test,  $P < .01$ ). The increase in ACT may represent a marker of peripheral activated

\* Corresponding author. Tel.: +1 814 865 5607; fax: +1 814 863 6103.

E-mail address: [fxr5@psu.edu](mailto:fxr5@psu.edu) (F.J. Rosales).

oxidative stress; thus, twice the recommended daily allowance of iron alone warrants concern in augmenting reactive low-molecular-mass iron in nonanemic populations, although combination with zinc may mitigate this phenomenon.

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## 1. Introduction

Iron supports oxidative metabolism. It is essential for gas exchange at tissue and cellular levels through oxygenation of hemoglobin in red cells and myoglobin in skeletal muscle [1]. Like many other transition elements, it possesses unfilled atomic orbitals that allow it to coordinate electron donors and participate in redox processes [2,3]. This advantage that makes iron an excellent catalyst also makes it a potentially hazardous agent because reactions involving oxygen can favor the formation of free radicals [2,3].

Iron has been implicated in the pathology of several chronic degenerative diseases through its enhancement of free radical formation. Epidemiological studies have shown a positive association between ferritin concentrations and myocardial infarction in Finnish men [4], and a strong prediction of 5-year progression of carotid atherosclerosis by serum ferritin concentrations [5]. Excessive deposition of cerebral iron can also contribute to Parkinson's disease [6] and other neurodegenerative diseases such as Alzheimer's disease [7]. Finally, iron overload has been implicated in diabetes, hypertension, and cancer development in people with hemochromatosis [8]; even in those with no genetic predisposition, iron supplementation can enhance the risk for liver disease [9]. Because of its potential oxidative activity, iron concentrations are carefully controlled by iron-regulating proteins, maintaining a labile iron pool that is adequate for metabolic functions but reducing the levels of free iron that might generate free radical chain reactions or oxygen-reactive species [1,10]. Thus, a concern of any iron intervention should be its possible contribution to altering iron's labile pool.

Iron is one of the most abundant elements in the earth's crust, whereas paradoxically, iron deficiency is the world's most common and widespread nutritional disorder [11]. It is estimated that 66% to 80% of the world's population may be iron deficient [11,12]. In Guatemala, iron deficiency anemia (IDA) represents a moderate public health problem [12,13]. Contributing factors include parasitic infections [14] and foods high in inhibitory dietary fiber and phytic acid such as corn tortillas [15,16]. Hence, iron supplementation is an appropriate strategy to reduce iron deficiency and IDA [17].

Nonetheless, iron supplements can antagonize zinc absorption in adults [18]. Solomons [19] postulated that a total dose of >25 mg of iron may produce a measurable effect on zinc absorption. The present study was conducted to assess whether a dose of iron, equivalent to twice the recommended daily allowance (RDA) for school-aged children [20], would affect zinc status or induce oxidative stress and inflammation.

## 2. Methods and materials

We conducted a randomized placebo-controlled trial of iron or zinc alone or in combination. This efficacy study took place in Guatemala City, Guatemala, between February and April 2000. School children from low-income families, aged 8 to 11 years, were enrolled at a local public school with separate morning and afternoon sessions for girls and boys, respectively. Inclusion criteria included usual good health and the absence of chronic diseases. After screening, 77 schoolchildren (40 girls and 37 boys) were enrolled in the study. They were systematically blocked randomized (criteria included age and school session as a proxy for gender) to receive one of 4 assignments: placebo, iron alone, zinc alone, or iron and zinc combined. The sample size of 20 per group was estimated on the basis of detecting a difference in plasma zinc concentration of  $2.2 \mu\text{mol/L}$  [21] with a 1-sided  $\alpha$  of .05 and a power of 88% using the formula for two independent groups. In addition, this sample size provided 80% statistical power in determining a relative increase of 25% in  $\alpha$ -1 acid glycoprotein (AGP) from a reference median of 0.8 g/L for 10 year-olds [22]. AGP is a highly reliable acute phase protein (APP) compared with C-reactive protein (CRP) [22]. Regarding oxidative status, this sample size provided 86% statistical power to assess a relative difference of 35% in thiobarbituric acid-reactive substance (TBARS).

The 20-mg dose of iron was equivalent to twice the RDA ( $2 \times \text{RDA}$ ) for children [20]. This dose was high enough to provide sufficient iron despite the high consumption of tortilla in this population, but was lower than a dose that would impair zinc absorption [19,23,24]. Moreover, zinc supplements were provided in a Zn-Fe ratio of 2:1 to reduce any possible impairment of its absorption by iron [19]. Thus, the iron-alone group received 3.3 mL of syrup containing 20 mg of elemental iron as iron citrate mixed with 50 mL of orange-flavored juice (ie, water, sugar, and artificial flavor). The zinc group received 5.0 mL of syrup containing 42.5 mg of elemental zinc as zinc sulfate mixed with 50 mL of orange-flavored juice, and the combined iron and zinc group received 3.3 mL of syrup containing 20 mg of elemental iron as iron citrate and 5.0 mL containing 42.5 mg of elemental zinc as zinc sulfate mixed with 50 mL of orange-flavored juice. Children were masked to the content of the mixture, which was prepared every day at the school kitchen by one of the investigators, and it was provided daily 2 hours after breakfast. To maintain masking, the investigator never had direct contact with the subjects, the children had no contact with the preparation area, and the drafts were assigned by code number. Immediately after drinking the syrup, children received 50 mL fruit-flavored, nonvitaminized juice beverage in the same cup to rinse the vessel and ensure complete delivery of the dose of syrup. The supplementation period lasted 40 days over an 8-week period. The study protocol was approved by the school boards and their respective principals and by the Human Studies Committee of CeSSIAM. Signed informed consent was obtained from the parents or guardians of the participating children, and the subjects voluntarily assented to being studied.

Blood samples were obtained at baseline before treatment allocation and 8 weeks later. Blood was extracted by venipuncture into heparinized vacutainers, and they were immediately placed in cool boxes at  $6^\circ\text{C}$ . Aliquots of plasma were prepared after

centrifugation of blood samples at 7000g for 10 min at 8°C. Aliquots were frozen at –70°C in CO<sub>2</sub> and transported to the University of Hohenheim, Germany, where all biochemical determinations were done, or to the Pennsylvania State University, University Park, Pa, where immune assays were conducted.

### 2.1. Iron and zinc status determinations

Hemoglobin was measured in Guatemala using a commercial kit (Compur minilab 3, Bayer Diagnostic, Dublin, Ireland). Ferritin was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) method [25]. Iron deficiency was defined based on the general recommendation of a ferritin cutoff value of <20 µg/L [26]. Plasma zinc concentrations were determined by atomic absorption spectroscopy (AAS) using a Perkin-Elmer 1100 (Perkin-Elmer, Burladingen, Germany) with a hollow cathode lamp as described by Perry [27]. Standards were prepared by spiking serum with a known concentration of zinc, 14.1 µmol/L (Qualitrol HSN, Merck Diagnostica, Darmstadt, Germany). The intra-assay variance ranged between 5% and 6% and the interday assay variance ranged between 11% and 12%. Hypozincemia was defined based on plasma zinc concentrations <10.7 µmol/L [28].

### 2.2. Antioxidant status

Antioxidant vitamins such as  $\alpha$ -tocopherol and  $\beta$ -carotene were determined simultaneously by high-performance liquid chromatography after extraction with ethanol/butanol/butylated hydroxytoluene containing internal standards as previously described [29]. The extracts were injected onto a C18 reversed-phase column eluted with acetonitrile-tetrahydrofuran-methanol-1% ammonium acetate (684:220:68:28, vol/vol/vol/vol), and full elution of all the analytes was realized isocratically within 10 minutes with UV detection of  $\beta$ -carotene at 450 nm and for  $\alpha$ -tocopherol at 292 nm.

### 2.3. Oxidant status

Malondialdehyde (MDA), measured as TBARS, was used to determine oxidation status because of its convenience in assessing in vivo lipid peroxidation [30]. The determination of TBARS was performed as previously described by Jentzsch et al [31]. Briefly, this method consists of using high concentration of ethanol/butanol/butylated hydroxytoluene to deplete oxygen and reducing the in vitro lipid peroxidation during TBARS adducts formation. Malondialdehyde standards were prepared by the hydrolysis of 1,1,3,3-tetramethoxypropane, and the MDA stock solution was diluted with ultrapure water to obtain a range of from 0.1 to 2.9 µmol of MDA. Calibration in plasma was done by adding 200 µL of H<sub>3</sub>PO<sub>4</sub> containing different amounts of MDA to pool plasma samples.

### 2.4. Acute phase proteins

Acute phase proteins were measured by single radial immunodiffusion assay as previously reported [32]. Positive APPs included AGP, CRP, and/or  $\alpha$ -1 antichymotrypsin (ACT), and albumin as a negative APP to control for changes in zinc concentrations because 65% of circulating zinc is bound to albumin. Three positive APPs were selected because they differ in

their onset and half-lives in response to an inflammatory stimulus. Antiserum for each protein and calibrators were purchased from DAKO Corporation (Carpinteria, Calif). Calibrators were titrated for each protein. Four different concentrations were selected for each assay. The College of American Pathologist's (Northfield, Ill) reference standard for plasma proteins was used as an external standard for all assays. The accuracy of each radial immunodiffusion assay was checked against two different dilutions of external standards. Quality control was conducted for each plate and the intra- and interassay coefficients of variation were determined before the experiment [32]. The limit of detection is 1.0 mg/L for CRP, 0.03 g/L for ACT, and 0.01 g/L for AGP. Inflammation status was based on one or more APP concentrations being above their respective cutoff value,  $\geq 10$  mg/L for CRP,  $\geq 1.0$  g/L for AGP, and  $\geq 0.6$  g/L for ACT [33,34].

### 2.5. Statistical analysis

Data were analyzed by using SPSS ver. 11.5 software (Chicago, Ill) Exploratory analyses were conducted to determine possible relationships among the variables; Kolmogorov-Smirnov test were done to assess the normality of the main outcomes. Levene statistics were used to determine the homogeneity of their variance, and the spread vs level with Levene test was used to determine the power transformation for achieving equal variances [35]. Nonnormally distributed variables such as ferritin was transformed using  $\log_{10}$ ; AGP, using the square root;  $\beta$ -carotene, using the reciprocal; and  $\alpha$ -tocopherol, using  $\log_{10}$ . When transformations were not appropriate, as in the cases of TBAR and ACT, nonparametric tests were used [35]. Analysis of variance (ANOVA) was used to evaluate differences between the groups at baseline with Tukey correction for multiple comparisons, or the median test, which is applied in the same data situations as an ANOVA only when the data are nonnormally distributed [35], with Bonferroni corrections for number of comparisons, 4 per outcome. For categorical variables, the  $\chi^2$  test was used. In addition, treatment main effects, treatment interactions, and differences for biochemical indicators among supplementation groups were analyzed with a 2-factor analysis of covariance (ANCOVA), where baseline concentrations were entered as a covariate in the model. If treatment interactions were not significant by the 2-factor ANCOVA, the estimated marginal mean difference, with least significant difference adjustment for multiple comparisons, was used to compare each level of a factor.

Recently, Ash et al [36] demonstrated that determining the within-subject difference from baseline to posttreatment, and the between-group differences on the within-subject difference, provides an analysis that is exactly equivalent to a 2-factor repeated-measures ANOVA. Therefore, in the case of TBAR and ACT, the relative change from post- to presupplementation concentrations (ie, the ratio of post- to presupplementation concentrations) was calculated, and the median test was used to measure between-group differences on the relative change ratio.

For categorical variables (ie, iron deficiency, hypozincemia, or inflammation status), the 2-sample test for binomial proportions for matched-pair data (McNemar's test) was used [35]. In a  $2 \times 2$  table, with the columns representing micronutrient or inflammation status at the end of the supplementation trial and the rows representing micronutrient or inflammation

status at baseline, the discordant cells or paired observations that represented improvement in micronutrient status or no inflammation were compared with the discordant cells or paired observations that represented deterioration in micronutrients or inflammation by supplementation strategy. Significance was determined at  $P \leq .05$ .

### 3. Results

Children were followed up for 54 days, during which 12.5% (5/40) of girls and 16% (6/37) of boys missed 5 or more days of classes and did not receive at least 90% of the supplementation dosage; they were excluded from this efficacy analysis. Micronutrient status of children at baseline did not differ among supplementation groups (Table 1). Hemoglobin concentrations ranged from 121 to 182 g/L at baseline; only one child had anemia based on a cutoff level of 121 g/L (anemia in 8- to 12-year old children,

Table 1

Iron and zinc status before and after supplementation with iron alone, zinc alone, or iron and zinc combined in Guatemalan children, 2000<sup>a</sup>

	Experimental groups			
	Placebo	Iron alone	Zinc alone	Iron and zinc combined
Hemoglobin (g/L)				
Baseline (n)	146.5 ± 11.9 (15)	150.7 ± 12.8 (15)	148.5 ± 14.9 (15)	148.4 ± 9.9 (19)
Posttreatment (n)	154.3 ± 14.8 (15)	156.1 ± 17.6 (15)	153.3 ± 10.1 (15)	145.2 ± 13.1 (19)
Ferritin (µg/L) <sup>b</sup>				
Baseline (n)	28.9(21.7-36.8) (14)	34.4(25.9-59.1) (15)	25.4(20.6-33.7) (16)	37.1(21.8-48.9) (18)
Posttreatment (n)	32.7(24.2-50.1) (14)	46.0(29.2-90.1) <sup>c</sup> (15)	38.1(30.3-62.3) (16)	44.0(26.3-79.7) (18)
Iron deficiency <sup>d</sup>				
Baseline (%)	21	13.3	18.8	22.2
Posttreatment (%)	14.3	6.7	25.0	16.7
Zinc (µmol/L)				
Baseline (n)	10.1 ± 1.1 (14)	9.9 ± 1.6 (15)	10.1 ± 1.2 (16)	9.9 ± 1.9 (18)
Posttreatment (n)	11.3 ± 1.7 (14)	11.1 ± 1.9 (15)	13.9 ± 1.9 <sup>c</sup> (16)	14.2 ± 1.9 (18)
Hypozincemia <sup>e</sup>				
Baseline (%)	71.4	60.0	68.8	83.3
Posttreatment (%)	35.7	31.3	0.0 <sup>f</sup>	16.7 <sup>f</sup>
Albumin (g/L)				
Baseline (n)	48.02 ± 2.97 (14)	47.73 ± 3.90 (12)	46.37 ± 3.37 (12)	45.79 ± 2.58 (13)
Posttreatment (n)	46.64 ± 4.45 (12)	49.48 ± 4.08 (11)	47.72 ± 3.05 (12)	46.45 ± 3.88 (15)

<sup>a</sup> Values are mean ± SD. At baseline, none of the variables differ significantly among the groups (one-way ANOVA or  $\chi^2$ ,  $P > .05$ ).

<sup>b</sup> Medians with quartiles in parenthesis.

<sup>c</sup> Two-factor ANCOVA,  $P \leq .03$  for the main effect of iron or zinc alone.

<sup>d</sup> Iron deficiency defined as ferritin concentration <20.0 µg/L.

<sup>e</sup> Hypozincemia defined as zinc concentration ≤10.7 µmol/L.

<sup>f</sup> Test for binomial proportions for matched-pair data (McNemar's test  $P < .001$ ).

hemoglobin level  $\leq 119.0$  g/L + 2.0 g/L for an altitude approximately 4,000 feet above sea level at Guatemala City [12,37]). In this population with a low prevalence of anemia, supplementation with iron alone, zinc alone, or iron and zinc combined did not affect erythropoiesis, ie, hemoglobin concentrations did not increase (Table 1). However, ferritin concentration increased significantly in children receiving iron alone (Table 1). Although plasma ferritin concentrations increased at the end of the study, the percentage of children with iron deficiency did not change from baseline to posttreatment by supplementation strategy (McNemar's matched-pair analysis,  $P > .05$ ).

Plasma zinc concentrations increased from baseline to posttreatment in children receiving zinc alone or in combination with iron (Table 1); however, zinc alone had a significant effect on plasma zinc concentrations (Table 1). In tandem, the prevalence of hypozincemia was reduced with zinc supplements (Table 1). In children receiving zinc alone, none were hypozincemic at the end of the study, and only 17% of those receiving combined iron and zinc remained hypozincemic at the end of the 8 weeks (Table 1, McNemar's test,  $P < .01$ ). Mean albumin concentrations were within normal range, reflecting an adequate protein nutritional status in these children at baseline and post supplementation. In addition, these normal albumin concentrations indicated no effect of inflammation on zinc status (Table 1).

Neither micronutrient deficiencies nor any of the supplementation strategies were associated with a decrease in antioxidant activity (Table 2). At baseline, 69% of the children had low levels of  $\beta$ -carotene, defined as  $<0.3$   $\mu\text{mol/L}$  [38]; however, this proportion was reduced by 50% by the end of the study (data not shown), and  $\beta$ -carotene

Table 2

Distribution of antioxidant and oxidant status before and after supplementation with iron alone, zinc alone, or iron and zinc combined in Guatemalan children, 2000<sup>a</sup>

	Experimental groups			
	Placebo	Iron alone	Zinc alone	Iron and zinc combined
$\beta$ -Carotene ( $\mu\text{mol/L}$ )				
Baseline (n)	0.28(0.22-0.44) (12)	0.23(0.15-0.25) (16)	0.27(0.17-0.38) (13)	0.27(0.17-0.38) (18)
Posttreatment (n)	0.36(0.32-0.52) (12)	0.38(0.26-0.54) (16)	0.43(0.25-0.53) (13)	0.36(0.21-0.62) (18)
$\alpha$ -Tocopherol ( $\mu\text{mol/L}$ )				
Baseline (n)	22.9(19.6-25.9) (14)	21.5(19.8-24.6) (13)	21.0(19.5-22.8) (14)	20.7(18.7-24.1) (18)
Posttreatment (n)	23.6(20.4-28.1) (14)	21.0(19.6-25.2) (13)	22.3(18.2-24.2) (14)	20.9(17.6-28.1) (18)
TBARS ( $\mu\text{mol/L}$ )				
Baseline (n)	0.81(0.38-0.93) (12)	0.47(0.34-0.70) (15)	0.47(0.31-0.61) (15)	0.42(0.37-0.69) (18)
Posttreatment (n)	0.73(0.39-1.06) (12)	0.51(0.31-0.75) (15)	0.49(0.39-0.79) (15)	0.44(0.30-0.71) (18)

<sup>a</sup> Values are medians with quartiles in parenthesis. At baseline, none of the variables differ significantly among the groups (one-way ANOVA or median test  $P \geq .05$ ).

concentrations increased in all the children (Table 2).  $\alpha$ -Tocopherol concentrations were within the normal range for children in this age group [39] at baseline and after supplementation, and TBARS concentration did not differ among the children by supplementation group at baseline or at the end of the study (Table 2).

Mean and median concentrations of APPs did not differ among the groups at baseline (Table 3), and only 13.7% (7/51) of the children had evidence of inflammation based on one or more APP at baseline. The distribution of children with or without inflammation at baseline did not differ between iron-sufficient vs iron-deficient children (Fisher exact test,  $P = 0.21$ ), or between those with or without hypozincemia (Fisher exact test,  $P = 0.32$ ). At the end of the study, 22% (11/50) of the children had biomarker evidence indicative of inflammation; however, there was no significant increase in inflammation during the study by supplementation groups (McNemar's matched-pair analysis,  $P > .31$ ). At the end of the study, mean and median concentrations of APPs did not reach concentrations above their respective cutoff values indicating inflammation. The factorial analysis for CRP and AGP showed no main effect of iron or any treatment interactions; however, ACT concentrations increased significantly in children receiving iron alone (Table 3). The effect of supplementation strategy on the relative change in ACT is illustrated in Fig. 1, in all children (panel A) and after excluding children who had inflammation based on CRP or AGP (panel B). It is important to note that plasma ACT did not reach concentrations

Table 3

Distribution of APPs before and after supplementation with iron alone, zinc alone, or iron and zinc in combination of Guatemalan children, 2000<sup>a</sup>

	Experimental groups			
	Placebo	Iron alone	Zinc alone	Iron and zinc combined
CRP (mg/L)				
Baseline (n)	1.53 ± 5.3 (14)	0.40 ± 1.39 (12)	0.13 ± 0.46 (12)	2.00 ± 3.89 (13)
Posttreatment (n)	0.18 ± 0.62 (12)	0.98 ± 2.30 (11)	2.51 ± 6.30 (12)	3.31 ± 7.35 (15)
$\alpha$ -1 acid glycoprotein (g/L) <sup>b</sup>				
Baseline (n)	0.71(0.58-0.87) (14)	0.80(0.57-0.89) (12)	0.92(0.69-1.0) (11)	0.71(0.61-0.82) (13)
Posttreatment (n)	0.84(0.59-0.96) (12)	0.84(0.69-1.0) (11)	0.78(0.66-0.98) (12)	0.71(0.62-1.0) (15)
$\alpha$ -1 antichymotrypsin (g/L) <sup>b</sup>				
Baseline (n)	0.33(0.30-0.36) (14)	0.33(0.31-0.38) (12)	0.37(.31-0.41) (12)	0.36(0.31-0.41) (13)
Posttreatment (n)	0.35(0.29-0.37) (12)	0.37(0.36-0.46) <sup>c</sup> (11)	0.35(0.29-0.42) (12)	0.35(0.31-0.47) (15)

<sup>a</sup> Values are mean ± SD. At baseline, none of the variables differ significantly among the groups (one-way ANOVA, median test or  $\chi^2$ ,  $P \geq .05$ ).

<sup>b</sup> Medians with quartiles in parenthesis.

<sup>c</sup> Median test for the relative change from pre- to postsupplementation (ie, the ratio of pre- to postconcentrations)  $P < .01$ .

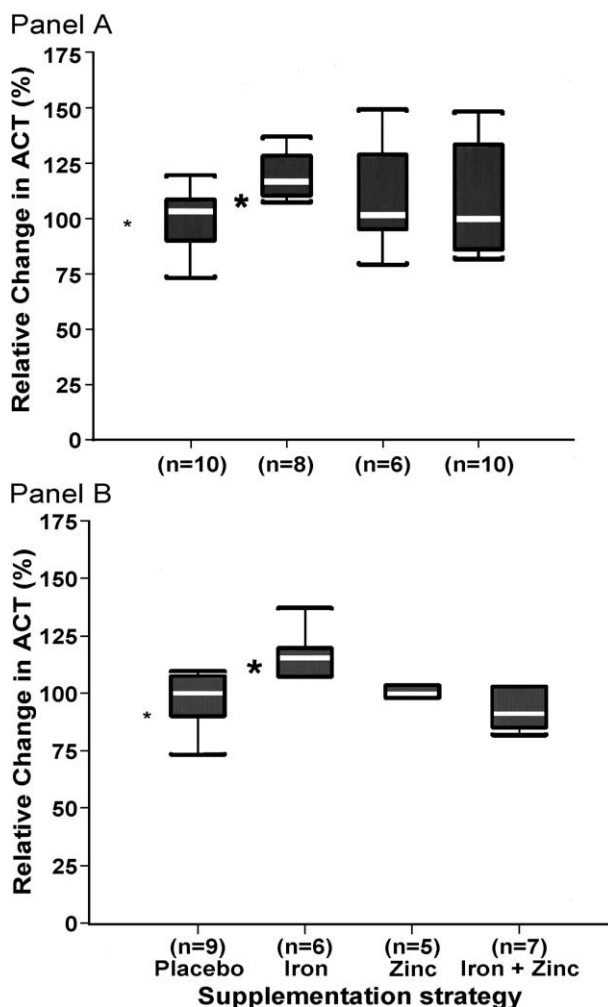


Fig. 1. Box plots of the relative change in  $\alpha$ -1-antichymotrypsin (ACT) from baseline to 8 weeks after supplementation. Panel A presents the relative change in ACT as the ratio of post- to presupplementation concentrations by treatment group. The relative change was significantly higher in children receiving iron alone compared with the other groups. Asterisk indicates  $P < .01$ , median test. Panel B presents the distribution of the relative change in ACT after excluding children with inflammation based on one or more APPs. The relative change remained statistically significant from control. Asterisk indicates  $P = .01$ , median test.

indicative of inflammation, and the only child with inflammation, based on ACT, was in the combined iron and zinc group.

#### 4. Discussion

The 1995 Guatemalan National Micronutrient Survey determined that 26% of school-aged children suffered from IDA [13], making IDA a moderately serious public health problem in this population [12]. In this regard, iron supplementation with 30 to 60 mg/d of elemental iron is recommended in areas where IDA is a public health problem [12,40]. In the present study, a

daily dose of 20 mg of elemental iron was provided to compensate for high consumption of foods rich in chelating substances [14–17]. Negative effects of iron supplementation on indices of zinc and, vice versa, by zinc supplementation on iron have been reported, especially if these 2 minerals are given in the same solution [18,19,23,24]; thus, zinc was dosed in a 2:1 ratio compared with iron. Iron stores increased significantly among children receiving supplements with iron alone in essentially nonanemic children; and zinc alone significantly reduced zinc deficiency in these children. However, there were no treatment interactions on ferritin or zinc concentrations. This might result from the dosage ratio of zinc to iron that we used. Zinc alone was effective in significantly increasing plasma zinc concentrations and alleviating hypozincemia, whereas combined supplementation with iron and zinc had a milder effect in increasing plasma zinc concentration while still reducing significantly the prevalence of hypozincemia. These findings suggest that dosing zinc in a 2:1 ratio along with iron, in the same solution, might partially reduce iron's inhibitory effect on zinc.

The effectiveness and impact of iron supplementation programs have been questioned because of poor compliance and potential risk of overdosing [12]. In addition, iron supplements can potentially induce oxidative stress and contribute to an inflammatory response by producing hydroxyl radicals (OH.) [2]. These radicals can act as an intracellular messenger, via activation of nuclear factor-kappa B, and induce the expression of proinflammatory cytokines and interleukin-6, which is a potent inducer of the synthesis of APPs in the liver [41].

In the present study, we assessed the possibility of  $2 \times$  RDA dosage of iron inducing oxidative stress and inflammation. We found no significant change in plasma  $\alpha$ -tocopherol and TBARS during the supplementation trial. Although we used a TBAR assay that minimized artifactual oxidative degradation of lipids [31], it is noteworthy to recognize that TBARS concentrations are associated with only 60% of lipoperoxidation products in human plasma [42], and thus might underestimate the relative degree of oxidative stress in the present study. Nonetheless, the results suggest that no apparent oxidative stress was associated with iron supplements alone or in combination with zinc. Similarly, mean concentrations of positive APPs did not increase above their cutoff values indicative of inflammation; although some children had evidence of inflammation on concluding the study, there was no association with iron. Based on these findings, we believe that supplementation with iron alone or combined with zinc at the provided doses does not induce an inflammatory response.

Nonetheless, there was a significant increase in ACT concentrations in children receiving iron alone. Evidence suggests a close association between cellular iron content and circulating ACT, insofar as patients with neurodegenerative diseases such as Alzheimer's disease have higher plasma concentrations of ACT than normal age-matched controls [43]. Concomitantly, experimental and clinical studies have shown increased accumulation of iron in brain regions afflicted by this disease [44]. In addition, Northrop-Clewes et al [45] observed a significant increase in ACT concentrations at 3 months of intervention in infants receiving 15 mg of elemental iron daily compared with those receiving placebo.  $\alpha$ -1 Antichymotrypsin possesses a serpin tertiary structure [46], and it is a potent inhibitor, while acting as an APP, of the serine protease cathepsin G, a protein involved in neutrophil activation and chemotaxis [46].

Another physiological function of ACT is its inhibition of neutrophilic superoxide ( $O_2^-$ ) anion production [47]. This activity is mediated by ACT acting on a component of NADPH oxidase complex, an element of the neutrophil respiratory burst [47]. Because toxicity of  $O_2^-$  involves its conversion to OH. radical, a reaction that depends on the amount of catalytic iron available, it is possible that the observed increase in ACT concentration in children receiving iron alone might represent a marker of a peripheral response to activated oxidative stress or its amelioration. This would explain why we did not observe an increase in TBAR or conventional inflammatory biomarkers. In patients with Alzheimer's disease, an increase in ACT concentrations was associated with reduced lipid peroxidation but not with an increase in CRP concentrations [43]. Similarly, in the present study, the increase in ACT in children receiving iron alone was not associated with an increase in APPs. Thus, this increase in ACT warrants concern that daily oral iron supplements may augment reactive low-molecular-mass iron and possibly induce oxidative stress, although combination with zinc may mitigate this phenomenon.

In summary, our results demonstrate that supplementation with iron or zinc alone, at the dosages provided to healthy Guatemalan schoolchildren improved iron stores and zinc status. Moreover, the combination of iron and zinc did not impair the reduction of hypozincemia and was not associated with the induction of oxidative stress. In addition, we have shown that determination of various APPs provides important information in monitoring not only the effect of inflammation on micronutrient status but also the effect of micronutrient supplementation on oxidative stress and inflammation.

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