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Biofortification of Pearl Millet with Iron and Zinc in a Randomized Controlled Trial Increases Absorption of These Minerals above Physiologic Requirements in Young Children[1–3]

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Abstract

Millet is unusually drought resistant and consequently there is a progressive increase in the use of these grains as a human food staple, especially in large areas of India and sub-Saharan Africa. The purpose of this study was to determine the absorption of iron and zinc from pearl millet biofortified with 2 micronutrients that are typically deficient in nonfortified, plant-based diets globally. The study was undertaken in 40 children aged 2 y in Karnataka, India ($n = 21$ test/19 controls). Three test meals providing ~84 ± 17 g dry pearl millet flour were fed on a single day for zinc and 2 d for iron between 0900 and 1600 h. The quantities of zinc and iron absorbed were measured with established stable isotope extrinsic labeling techniques and analyses of duplicate diets. The mean (± SD) quantities of iron absorbed from test and control groups were 0.67 ± 0.48 and 0.23 ± 0.15 mg/d, respectively ($P < 0.001$). The quantities of zinc absorbed were 0.95 ± 0.47 and 0.67 ± 0.24 mg/d, respectively ($P = 0.03$). These data did not include absorption of the modest quantities of iron and zinc contained in snacks eaten before and after the 3 test meals. In conclusion, quantities of both iron and zinc absorbed when iron and zinc biofortified pearl millet is fed to children aged 2 y as the major food staple is more than adequate to meet the physiological requirements for these micronutrients. J. Nutr. 143: 1489–1493, 2013.

Introduction

Though not one of the world's most abundant or widely consumed grains, pearl millet does provide a major food staple for millions of people in the western parts of India and in Africa, notably in the Sahel countries currently threatened by the advancing Sahara desert. Pearl millet and other millet grains survive in arid, hot environments in which more widely used cereal grains would not thrive. Hence, its production and importance is growing concurrently with global warming. Already, it accounts for >50% of total cereal grain consumption in some communities in India, especially in Maharashtra, Gujarat, and Rajasthan, where the rural poor heavily depend on this crop (1). Pearl millet is also widely used in Northern Karnataka, where this study was undertaken, in Belgaum.

The bioavailability of zinc and iron from pearl millet was compared with that of other important grain food staples in India in a carefully designed trial reported in 1999 (2). Absorption and liver amounts of both micronutrients from pearl millet and wheat were superior to those from sorghum and rice. Weight gain was highest in the pearl millet group. This study was undertaken in mice before a human trial, which appeared not to have been undertaken by this group. An in vitro study simulating intestinal digestion (3) showed that iron and zinc bioavailability from whole pearl millet flour were significantly improved by phytate degradation, but the authors cautioned that tannins chelate a high proportion of iron and zinc in the grain hulls. The authors could find no published evidence of human studies of bioavailability of iron or zinc from pearl millet.

Biofortification can still be regarded as a new tool in the reduction of micronutrient malnutrition (4), which is more universally acceptable if achieved by traditional plant breeding techniques. The primary specific objective of this study was to compare the quantity of zinc and iron absorbed from iron- and zinc-biofortified pearl millet with that of control grain in young children who were receiving one of these grains as their primary food and the only grain for an entire day. The quantities absorbed were also compared with the physiological requirements for zinc and iron in this age group (5).

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[3] This trial is registered at ClinicalTrials.gov as NCT01783067.
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Experimental Design and Methods

Study design. This was a collaborative study between Jawaharlal Nehru Medical Center (JNMC)[6], Belgaum, India and the Section of Nutrition, Department of Pediatrics, University of Colorado Denver (UCD). Collaborators in Colorado were responsible for isotope preparation, project training and site visits, and all laboratory analyses except for the hematological assays performed at JNMC. The research team at JNMC was responsible for all aspects of the integrity of the human studies and data collection.

The study was a double-blinded, randomized, controlled investigation of iron and zinc absorption from 3 test meals/d in which either iron- and zinc-biofortified or control pearl millet comprised the only source of grain and virtually the only source of iron and zinc. HarvestPlus provided the grain for the study in 4 color-coded bags; 2 colors were assigned to each type of millet and investigators were unaware of the code throughout the study. Participants were randomized in a single block to 1 of 4 color codes using a parallel manner with a 1:1 allocation ratio. The randomization list was generated at UCD and provided to the study investigators at JNMC. As each participant was enrolled, the study investigators consulted the list and assigned the next available randomization from the list to the participant.

Fractional absorption of these minerals was determined with stable isotope labels of zinc and iron (6–8). The total iron and zinc intakes from the meals were determined by mineral analyses of duplicate meals. Total absorption of zinc and iron from the meals was calculated by multiplying the fractional absorption by the amount of total iron and zinc in duplicate meals. The meals were fed by the mothers under staff supervision after accurate weighing of every step in food preparation and consumption. These 3 meals were fed in the large kitchen of JNMC community student quarters between 0900 and 1600 h. The participants ate small snacks at home before and after the study period; the food intake from the pre-breakfast and post-dinner snacks were recorded and duplicate diets were collected and analyzed, but these foods were not labeled with stable isotopes; they were generally low in zinc and iron content.

Test meals extrinsically labeled (9) with a zinc stable isotope were given for 1 d (study day 1) and with an iron stable isotope for 2 d (study days 1 and 2). Pearl millet was fed daily for a week prior to the metabolic studies, but intake quantities were not recorded.

Participants. All participants were recruited from the poor rural community of Kineye, Belgaum, in Northern Karnataka between February 2011 and January 2012. The age of the children ranged between 22 and 35 mo. The community is strictly vegetarian, but the usual diet does include buffalo milk, other dairy products, and eggs. Breast feeding extends through the second year in this population, but none of the participants were breast feeding at the time of enrollment. Mothers of young children were identified through the records of the Primary Health Center and recruited by the project coordinators. Informed consent was obtained from all mothers pursuant to the ethical guidelines of the JNMC internal review board before undergoing any study procedures.

Participants were apparently healthy at enrollment, for the week before, and during the metabolic study. However, iron deficiency and iron deficiency anemia are common at this age and thalassemia is present in the community. To determine study eligibility, potential participants were first screened for anemia by measuring hemoglobin (Hb) with a Hemocue Hb301 system. If Hb results were >8 g/dL, participants proceeded to a secondary screening to measure ferritin and thalassemia Hb. Participants with ferritin <12 mg/L, negative for thalassemia, and with birth weight >2500 g were enrolled in the study, randomized to 1 of 4 color groups (2 colors for control and 2 for biofortified pearl millet), and assigned a study participant number.

Power and sample size. Group sample sizes of 20 and 20 provided 90% power to detect an effect size of −0.2 between the 2 groups assuming SDs of 0.2 and an α of 0.05 using a 2-sided, 2-sample t test.

Preparation and administration of iron and zinc stable isotopes. ^{67}Zn and ^{70}Zn isotope dose solutions were prepared from enriched zinc oxide (Trace Sciences International) as previously described (10).

Enriched ^{58}Fe in the form of elemental iron were also obtained from Trace Sciences International. The iron (~50 mg) was dissolved in 4 mL of 0.4 mol/L H_2SO_4 and the solution was heated at 80°C on a heating block until dissolved. Ten μL of concentrated H_2SO_4 was then added while the solution continued to be heated until the solution turned red. The isotope solution was then diluted to 600 μg Fe/mL using triply deionized water. To prevent the formation of ferric hydroxide, 6.25 mL of 2 mol/L H_2SO_4H was added and the solution left at RT overnight. Individual doses of ~2 mL of isotope solution were then dispensed into sealed empty vials. Solution was tested for iron concentration, pyrogens, and sterility as above.

Oral administration of accurately measured concentrations of tracer solutions was initiated at approximately the mid-point of each of the pearl millet meals on study days 1 and 2. The zinc label was ~150 μg ^{70}Zn (accurately measured) administered by research staff with each of 3 main pearl millet meals on d 1. Approximately ~1200 μg ^{58}Fe (accurately measured) was equally divided and administered with all 3 meals on study days 1 and 2. Iron doses were administered for 2 d to maintain the labeled iron at <10% of intrinsic iron in the food.

During the afternoon of d 2, a sterile, accurately measured quantity (~0.8 mg) of ^{67}Zn was i.v. administered into an antecubital vein using a 3-way stop cock for normal saline rinses.

Description of test meals: preparation, administration, and collection of duplicate test meals. Test meals were prepared on site for each individual using an accurately weighed quantity of ~70 g (35 g for feeding and 35 g for duplicate diet) of recently ground pearl millet flour from the color-coded container to which the participant had been randomized. Meals were provided as sweetened porridge (sheera, primarily breakfast), savory porridge (uppama), or flat breads (roti). Savory spices added to the uppama and roti were zinc and iron free; these foods were primarily eaten at lunch or dinner and the recipes were modeled after local, commonly consumed foods. The weighed dry millet was added to water in a preweighed saucepan and the whole was reweighed after cooking. An aliquot was transferred to the child's preweighed bowl, reweighed, and then weighed again to determine plate waste at the conclusion of each test meal. Meals were fed by the participants' mothers and the family would stay in the ample study quarters for the test days. On each of the 2 test meal days, a duplicate aliquot equal to the amount of the 3 test meals eaten by the child was transferred to a metal-free container and frozen at −20°C until further processing and analyses. Likewise on each day, a duplicate aliquot of any additional foods consumed before or after the test meals was combined in a second container.

Sample collections. Research staff assisted mothers in collecting a clean, baseline, spot urine sample on d 1 prior to any isotope administration. Mothers continued to collect morning and evening spot urine samples in their homes on d 5–9; these samples were picked up by research staff on a daily basis and stored at −20°C at JNMC until shipment to UCD for analyses.

A 5-mL heparinized blood sample was collected at screening and on d 16. Plasma was removed within 30 min of collection and the RBCs were washed twice with normal saline and frozen at −20°C at JNMC until shipment to UCD for measurement of ^{58}Fe erythrocyte incorporation. At the time of the i.v. zinc stable isotope infusion on d 2, a drop of blood was collected via the infusion for Hb assay using a Hemocue system.

Sample analyses. Duplicate dietary samples were homogenized in a preweighed blender with additional water added as necessary to make a smooth puree. The blender and homogenate were weighed to determine the total weight of the homogenate and then the sample was divided into 3 weighed aliquots, which were shipped to UCD for zinc and iron analyses. Duplicate aliquots were wet- and dry-digested prior to reconstitution in 0.1 mol/L HCl for total zinc and iron analyses determined by flame atomic absorption spectrophotometry. Dietary zinc and iron intakes from the test meals for each day were calculated using the duplicate diet homogenate weights. The phytate content in the whole grain was measured using HPLC.

[6] Abbreviations used: FAZ, fractional absorption of zinc; Hb, hemoglobin; JNMC, Jawaharlal Nehru Medical Center; UCD, University of Colorado Denver.

Urine samples were digested using a MARs microwave digestion system (CEM) (11) prior to performing a chelation procedure to purify the zinc for isotope analyses (10,12). Zinc isotope ratios (^{67}Zn:^{66}Zn, ^{70}Zn:^{66}Zn) were measured using inductively coupled plasma MS (10) and were converted to percent enrichment (defined as isotopic zinc/total zinc).

Erythrocyte samples were digested and iron was separated from other minerals by column chromatography. The ratio of ^{58}Fe:^{54}Fe was measured by inductively coupled plasma mass spectrometry. For each participant, the erythrocyte sample collected on d 16 was analyzed immediately next to its baseline sample. The ^{58}Fe:^{54}Fe ratio for each baseline erythrocyte sample was compared with the ratio derived from representative isotopic composition (13) of elemental iron to calculate a correction factor, which was then used to correct the ^{58}Fe:^{54}Fe ratio of the corresponding d 16 sample. Enrichment was calculated from the corrected iron isotope ratios, taking into account the contribution to each individual isotope measurement from all sources of iron, enriched and natural, in the sample. Enrichment is defined as for zinc.

Data processing. Fractional absorption of zinc (FAZ) was determined by a dual isotope tracer technique based on isotopic enrichments in urine from orally and i.v. administered isotopes (6,8). Total absorbed zinc (mg/d) from the test meals was calculated by multiplying total intake of zinc in the test meals by FAZ.

The single iron isotope technique was used to measure fractional absorption of iron (7). The quantity of administered enriched ^{58}Fe incorporated into the erythrocytes was calculated using ^{58}Fe enrichment of the RBCs on d 16 and the following equation:

$$^{58}\mathrm{Fe}_{(\mathrm{inc})} = {}^{58}\mathrm{En} \times \mathrm{Fe}_{(\mathrm{circ})},$$

where $\mathrm{Fe}_{(\mathrm{circ})}$ is the quantity of total circulating Fe (mg) on d 16 estimated as:

$$\mathrm{Fe}_{(\mathrm{circ})} = \mathrm{Vol}_{\mathrm{blood}} \times \mathrm{Hb} \times \cdot 3.47$$

and $\mathrm{Vol}_{\mathrm{blood}}$ is the blood volume estimated by:

$$\log\mathrm{Vol}_{\mathrm{blood}} = [0.6459 \times \log(\mathrm{kg\,wt})] + [0.002743 \times \mathrm{cm\,ht}] + 2.0324)$$

FIA, assuming 80% of absorbed iron is incorporated into erythrocytes, was estimated as: $^{58}\mathrm{Fe}_{(\mathrm{inc})}$/(amount of ^{58}Fe enriched dose × 0.8). The total absorbed iron (mg/d) from the test meals was calculated by multiplying total intake of iron in the test meals by FIA.

Data analyses. The means for dietary intakes and total absorbed zinc and iron were compared across groups using *t* tests using GraphPad Prism version 5.00 for Windows (GraphPad Software). Data are presented as means ± SDs unless otherwise noted. All comparisons were considered significant at $P < 0.05$.

Results

Forty-four children (21 male, 23 female) with a mean age of 28.8 ± 3.5 mo enrolled in the study. Four participants withdrew consent prior to the first day of the study. Sample collection was incomplete in 2 participants from each group, leading to missing data for fractional absorption of iron ($n = 3$) and FAZ ($n = 1$). Demographic characteristics did not significantly differ between the 2 groups (**Table 1**). Only one participant had received any vitamin and/or mineral supplement prior to the study.

All of the children were iron deficient (defined as ferritin <12 μg/L) with a mean ferritin concentration of 5.8 ± 3.0 μg/L and Hb of 9.9 ± 1.0 g/L (**Table 2**). Mean plasma zinc concentrations did not differ between groups; 6 participants had values <65 μg/dL. Mean C-reactive protein (CRP) and α-1-glycoprotein (AGP) were not different between groups (Table 2); no participants had CRP values >5 mg/L and only 2 had slightly elevated AGP values (>120 mg/dL). Presence of thalassemia was an exclusionary criteria; hence, no participants had this condition. No adverse effects were observed during this study.

TABLE 1 Characteristics of the study population[1]

	Biofortified ($n = 21$)	Control[2] ($n = 19$)
Age, *mo*	28 ± 4	29 ± 3
Birth weight, *kg*	2.5 ± 0.6	2.6 ± 0.5
Gender, *M/F*	12/9	8/11
Weight, *kg*	10.7 ± 0.9	10.8 ± 1.4
Height, *cm*	79.9 ± 20.4	84.0 ± 2.8
HAZ	−1.67 ± 1.09	−2.10 ± 1.30
WAZ	−1.47 ± 0.83	−1.50 ± 1.11
WHZ	−0.70 ± 0.84	−0.57 ± 1.32
BMI	14.4 ± 3.9	15.3 ± 1.9
Maternal education, *y*	2.3 ± 0.8	2.7 ± 0.5
Paternal education, *y*	2.6 ± 0.7	2.4 ± 0.78
Household income, *INR/mo*	3237 ± 1456	3929 ± 2503

[1] HAZ, height-for-age Z-score; INR, Indian rupees; WAZ, weight-for-age Z-score; WHZ, weight-for-height Z-score.

The mean quantity of peal millet flour consumed was 84 ± 17 g/d, with no significant difference between d 1 and 2. The quantity of iron in this pearl millet exceeded the EAR for iron at this age of 3.0 mg Fe/d (5) despite the intake of grain being less than the intended 100 g dry grain/d.

The grain concentrations of iron, zinc, and phytate are given in **Table 3**. The quantity of iron absorbed from the iron- and zinc-biofortified pearl millet test meals fed in 1 d was significantly and substantially greater than that from the control nonbiofortified grain (Table 3). The absorption of iron from the test meals exceeded the physiological requirement of 0.54 mg/d for this age group (5). The quantity of zinc absorbed from the 3 biofortified test meals on study day 1 was also significantly and substantially greater than that from identical meals prepared from the control nonbiofortified pearl millet grain (Table 3). The absorption of zinc from the test meals also exceeded the estimated physiological requirement of 2.5 mg/d zinc (5).

Additional iron and zinc was ingested from early-morning and late-evening snacks (Table 3). These additional foods increased the total daily zinc intake by 13 ± 6% and 22 ± 13% in the biofortified and control groups, respectively. Likewise, the daily iron intakes were increased by 21 ± 13% and 32 ± 23%, respectively. The absorption of iron and zinc from these snacks was not measured.

TABLE 2 Biochemical data in children assigned to received either biofortified or control pearl millet as a staple grain in test meals[1]

	Biofortified ($n = 21$)	Control ($n = 19$)	*P* value[2]
Hb, *g/dL*	10.0 ± 1.0	9.9 ± 1.0	0.73
Ferritin, *μg/L*	5.9 ± 3.3	5.6 ± 2.8	0.78
CRP, *mg/L*	2.3 ± 0.5	2.4 ± 0.8	0.68
AGP, *mg/dL*	79.4 ± 24.9	75.9 ± 15.5	0.62
Plasma zinc, *μg/dL*	78.5 ± 17.4	75.6 ± 11.5	0.55

[1] Values are means ± SDs. AGP, α-1-glycoprotein; CRP, C-reactive protein; Hb, hemoglobin.
[2] 2-tailed Student's *t* test.

TABLE 3 Zinc and iron concentrations of pearl millet and dietary intake and absorption in children of zinc and iron from biofortified or control pearl millet test meals[1]

	Biofortified (n = 21)	Control (n = 19)	P value[2]	Effect size	95% CI
Grain zinc, $\mu g/g$	84.1 ± 4.9	43.7 ± 5.2	<0.0001		
Dietary zinc,[3] *mg/d*	5.8 ± 2.1	3.3 ± 1.1	<0.0001		(1.4, 3.6)
FAZ	0.17 ± 0.08	0.20 ± 0.04[4]	0.15		(−0.07, 0.01)
Absorbed zinc,[3] *mg/d*	1.0 ± 0.5	0.7 ± 0.2[3]	0.03	0.74	(0.05, 0.55)
Grain iron, $\mu g/g$	124 ± 17.0	46.5 ± 5.0	<0.0001		
Dietary iron,[3] *mg/d*	7.7 ± 1.8	4.1 ± 1.1	<0.0001		(2.6, 4.6)
Fractional absorption of iron	0.09 ± 0.08[5]	0.06 ± 0.04[6]	0.11		(−0.02, 0.06)
Absorbed iron,[3] *mg/d*	0.7 ± 0.5	0.2 ± 0.2	<0.0001	1.19	(0.11, 0.49)
Grain phytate,[7] *mg/g*	7.5 ± 0.3	10.3 ± 0.8	0.05		

[1] Values are means ± SDs. FAZ, fractional absorption of zinc.
[2] 2-tailed Student's t test.
[3] Quantity in 3 test meals/d.
[4] n = 18.
[5] n = 19.
[6] n = 18.
[7] n = 2.

Discussion

Dietary factors have major effects on the efficiency of absorption of iron from vegetarian meals (14). Notable inhibitory factors are phytate and polyphenols. The major dietary inhibitory factor for zinc is phytate, which is present in the husk but also after decortication. Phytate also inhibits iron absorption. The inhibitory effect of tannins is more clearly defined with iron (15) and it appears debatable (3,15) whether tannins have a significant effect on zinc absorption. The husk of this small grain is notable for its high content of tannin, which is responsible for its purple coloration. Traditionally, the grain is vigorously shaken in a sieve and to the extent possible separated husks are removed by hand. Fermentation and cooking, but not other food preparation procedures, reduce the anti-nutrient effects of pearl millet. Ascorbate enhances iron absorption in the presence of tannins not only by its reducing properties but by decreasing the chelating properties of tannins. The phytate:zinc molar ratio declined from 24:1 in the control grain to 9:1 in the biofortified grain. The corresponding figures for iron were 19:1 and 5:1, respectively. Reliable measurements of phytate in the test meals were not available due to sample deterioration during transit.

The mean plasma zinc concentration of the participants was not indicative of moderate or severe zinc deficiency. However, plasma zinc is not a sensitive biomarker of zinc status (16). The regular inclusion of buffalo milk in the diet of these children is expected to have had a favorable impact on their zinc (but not iron) status. However, the mean zinc absorption was less than the estimated physiological requirements, indicating an intake of bioavailable zinc that was less than optimal and could have contributed to the very poor linear growth of these children (17).

Much remains to be learned about the quantitative effects of these factors, especially in young children. These considerations emphasize the value of absorption measurements as a first stage in the evaluation of the potential benefits of biofortification of a grain that provides a major food staple. The results of this study indicate that, in contrast to control grain, the absorption of iron from this biofortified pearl millet is adequate to meet the estimated physiological requirements for iron at this age. For zinc, absorption increased from less than that required to match physiological requirements to a comfortable excess beyond this requirement. The quantities of pearl millet consumed were readily accepted by children in India at the age of 2 y. These results are even more encouraging when taking into consideration that these data were derived from only 3 of the 5 meals/snacks typically consumed. Although the other meals (in early morning and before retiring) were typically relatively low in iron and zinc, this was not always so and it is reasonable to conclude that if it had been feasible to label and quantitatively collect duplicates of these additional meals that the daily absorption of iron and zinc would have been even more favorable.

These results and the ready acceptance of quantities of pearl millet adequate to meet physiological requirements by very young rural children in Karnataka hold excellent promise for poor, rural-based families and merit priority attention to studies of their effectiveness in populations both in India and Africa for whom pearl millet is currently a food staple, with the potential for its use to expand, especially in drought-threatened areas.

In conclusion, the enhanced concentrations of iron and zinc in pearl millet resulting from biofortification, primarily with the goal of increasing iron concentration, are bioavailable and are sufficient to meet the physiological requirements for these 2 important micronutrients in very young children when consumed as a primary food staple.

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