Original Research Article

Dietary iron intakes based on food composition data may underestimate the contribution of potentially exchangeable contaminant iron from soil

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ABSTRACT

Iron intakes calculated from one-day weighed records were compared with those from same day analyzed duplicate diet composites collected from 120 Malawian women living in two rural districts with contrasting soil mineralogy and where threshing may contaminate cereals with soil iron. Soils and diet composites from the two districts were then subjected to a simulated gastrointestinal digestion and iron availability in the digests measured using a Caco-2 cell model. Median analyzed iron intakes (mg/d) were higher ($p < 0.001$) than calculated intakes in both Zombwe (16.6 vs. 10.1 mg/d) and Mikalango (29.6 vs. 19.1 mg/d), attributed to some soil contaminant iron based on high Al and Ti concentrations in diet composites. A small portion of iron in acidic soil from Zombwe, but not Mikalango calcareous soil, was bioavailable, as it induced ferritin expression in the cells, and may have contributed to higher plasma ferritin and total body iron for the Zombwe women reported earlier, despite lower iron intakes. In conclusion, iron intakes calculated from food composition data were underestimated, highlighting the importance of analyzing duplicate diet composites where extraneous contaminant iron from soil is likely. Acidic contaminant soil may make a small but useful contribution to iron nutrition.

1. Introduction

The assessment of iron intakes in low-income countries is most frequently calculated from 24-h recalls or records using food composition data. However this procedure does not consider iron sources extrinsic to the food. The importance of these extrinsic sources of iron in low-income countries has been emphasized by Harvey et al. (2000) because some of this iron may be available for absorption. Sources include contamination of foods from soil, dust, and water; metal fragments from milling; leaching of iron into foods through the use of iron cooking pots (Prinsen Geerligs et al., 2004), and the practice of geophagia.

A recent cross-sectional study of women living in two rural districts in Malawi with contrasting soil mineralogy revealed a low risk of iron deficiency among the women, notwithstanding diets based on unrefined cereals with high concentrations of phytate and negligible intakes of heme iron from cellular animal foods. This suggested that perhaps some contaminant iron joined the common non-heme iron pool (i.e. was exchangeable) and thus was available for absorption.

Therefore we have compared iron intakes of the Malawian women in these two districts, calculated from weighed food records and Malawian food composition data, with the results from the analysis of duplicate diet composites collected on the same day. We hypothesized that the iron intakes based on the analyzed

duplicate diet composites (Siyame et al., 2014) would be higher than those based on the calculated iron intakes presented here which do not account for contaminant iron or any contribution of iron from drinking water. We also examined the potential bioavailability of iron from the composite diets and soils from the two districts using a Caco-2 cell model system (Wawer et al., 2012).

2. Materials and methods

2.1. Study site and participants

Details of the study site, recruitment of participants, and their socio-demographic, anthropometric, and biochemical iron status have been published earlier (Hurst et al., 2013; Siyame et al., 2014). Briefly, a convenience sample of 120 apparently healthy Malawian women aged 18–50 years participated in the study. The women were living in six rural villages in Zombwe Extension Planning Area (EPA) ($n = 60$), characterized by acid soils with a low pH (median 5.2), and six rural villages in Mikalango EPA ($n = 60$), with calcareous Eutric Vertisols (median pH = 7.8). Verbal informed consent was obtained from the traditional authorities in the villages and from the participants, and the study protocol was approved by the National Health Sciences Research Committee of Malawi.

2.2. Assessment of iron intakes from weighed food intakes

One-day weighed food records were collected from the women in their homes by trained research assistants at the same time as weighed duplicate diet composites (including drinking water). Women were instructed not to change their normal dietary pattern during the diet-composite day. Money was given to the women to reimburse them for the cost of the food.

Intakes and major food sources of iron were calculated from the weighed food records using a Malawian food composition database compiled by the investigators (Yeudall et al., 2005). The values for the iron of the major plant-based staples in this food composition database were based on chemical analyses in our laboratory; details have been published earlier (Ferguson et al., 1990). The source of the other values was the WorldFood Dietary Assessment System (Bunch and Murphy, 1997). For composite dishes, recipe data were used and the iron values for raw foods adjusted for changes in retention and yield after cooking by using retention (USDA, 2007) and yield factors (Matthews and Garrison, 1987), where appropriate. Adjustments were made to all added iron values for any differences between the moisture content of the food stated in the Malawian Food Composition Database and the external source value.

2.3. Elemental analyses of duplicate diet composites and soil samples

Calculated iron intakes were compared with iron intakes based on chemical analysis of the duplicate diet composites collected on the same day. Concentrations of aluminum and titanium in the diet composites and iron in the soil samples collected from the two EPAs were also analyzed by inductively coupled plasma spectrometry (ICP-MS). For the analyses, aliquots of the homogenized freeze-dried duplicate diet composites or finely ground soil samples were microwave digested in 3.0 mL of 70% trace analysis grade HNO$_3$ (Fisher Scientific, UK), 2.0 mL H$_2$O$_2$ and 3.0 mL milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd., Loughborough, UK). The acid digests were then analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (X-Series[II], Thermo Fisher Scientific Inc., Waltham, MA, USA). Iron and aluminum were quantified using an external multi-element calibration standard (Claritas-PPT grade

CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA); titanium was determined semi-quantitatively. Internal standards were introduced to the sample stream on a separate line and included Sc (20 µg/L), Rh (10 µg/L), Ge (10 µg/L) and Ir (5 µg/L) in 2% trace analysis grade HNO$_3$ (Fisher Scientific, UK). Two standard reference materials (SRMs) from National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) were included to check on the accuracy and precision of the ICP-MS procedures for iron and aluminum – NIST 1573a (tomato leaves) and NIST 1577c (bovine liver); no certified values were available for titanium. Values for the iron and aluminum content of tomato leaves (NIST 1573a) were: Fe, 328.3 mg/kg (89% recovery); Al 476.1 mg/kg (79% recovery) compared to certified values of Fe, 368 mg/kg and Al, 598 mg/kg. Corresponding values for iron for bovine liver (NIST 1577c) were 185.6 mg/kg (93% recovery) compared to a certified value for Fe of 197.9 mg/kg. Operational sample blanks ($n = 10$) were run to determine limit of detection (LOD; 3*standard deviation, SD) and limit of quantification (LOQ, 10*SD) values in diet composite samples.

2.4. Phytate analysis of duplicate diet composites

Inositol penta-(IP5) and hexa-(IP6) phosphates were determined by a modified method of Lehrfeld (1989). Briefly, inositol phosphates were extracted from aliquots of the freeze-dried powdered diet composites (0.5 g) with 5 mL of 0.67 M HCl (BDH, Aristar). Dried extracts were then reconstituted with 1 mL of distilled deionized water and the inositol phosphates concentrated using a Hypersil column (H30DS-250A, HICHROM, Berkshire, UK). Inositol phosphates were then separated and analyzed in duplicate by high-performance liquid chromatography using a Waters 2690 Separation Module (Waters, Milford, MA, USA) and a differential refractometer (410 Differential Refractometer, Waters, MA, USA). Phytate as IP5 and IP6 concentrations were calculated from regression equations derived from different concentrations of standard solutions and peak areas of the sample. The inter-run coefficient of variation (CV) for the HPLC method was 7.2%. Concentration of combined IP4, IP5 and IP6 in maize flour (72% extraction rate) was 755 mg phytate/100 g (CV = 3.4%) compared to a generated laboratory mean value from other previous studies of 796 mg phytate/100 g of maize flour.

2.5. Cell culture

Unless otherwise stated all chemicals and enzymes were purchased from Sigma–Aldrich (UK). Caco-2 cells (HTB-37; ATCC, USA) were grown in collagen-coated 6-well plates (Greiner, UK) at a density of 4.75×10^4 in 2 mL of Dulbecco's modified Eagle's medium (DMEM media; LGC, UK) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 mL of 5000 u/mL Penicillin/Streptomycillin solution (Gibco, UK) and 5 mL of 100× non-essential amino acids. Medium was replaced every 2 days. Cells between passages 28 and 30 were used for experiments at 13 days post seeding, and 24 h prior to experimentation, cells were switched to serum-free medium (MEM, Invitrogen) supplemented as above with the exception of fetal bovine serum.

2.6. In vitro digestion and preparation of cell monolayers

Composite diet and soil samples were subjected to a simulated gastrointestinal digestion (with the addition of ascorbic acid (AA) at 1:10 or 1:30 iron:AA molar ratio to improve the sensitivity of the model by facilitating uptake of iron into the cells) according to the method of Glahn et al. (1996). Briefly, for the gastric phase of digestion, the samples were exposed to pepsin at pH 2 at 37 °C for 1 h after which the pH was raised

to 6.9–7.0 to inactivate pepsin. Next pancreatin-bile solution was added to imitate the duodenal phase of the digestion, then the samples (digests) were placed on a dialysis membrane (15kDaMWCO, Fisher, UK) situated above the Caco-2 cell monolayer and incubated at 37 °C. After 2 h, the digests were removed, the cells were incubated for a further 22 h, and then harvested according to the method of Glahn et al. (1998) and frozen at −20 °C. After 24 h, the frozen cell lysates were defrosted and the cell suspensions sonicated on ice prior to storage at −20 °C for subsequent analyses of ferritin and protein.

2.7. Ferritin analysis

Caco-2 cell ferritin content was measured in duplicate using a spectroferritin ELISA assay (Ramco, Houston, TX, USA) according to manufacturer instructions. Absorbance was measured using a microplate reader (Omega, BMG Labtech, UK) at 490, 570, and 630 nm. Standard solutions containing 0.3 mL human spleen ferritin calibrated to concentrations of 6, 20, 60, 200, 600 and 2000 ng/mL were applied in duplicate to obtain a standard curve. Uptake of iron by Caco-2 cells was estimated from the ferritin content (ng/mg total protein), a surrogate index of iron availability (Glahn et al., 1998).

2.8. Total protein

A bicinchoninic acid (BCA) protein assay (Pierce, Thermo Scientific, Rockford, IL, USA) was used to measure Caco-2 cell protein. A BCA working solution and standard solutions of Bovine Serum Albumin (BSA) (0, 125, 250, 500, 1000 and 2000 μg/mL) were prepared according to the manufacturer's instructions. The assay was performed with the addition of 10 μL protein standard or 10 μL experimental samples (in duplicate) to 200 μL of BCA working solution in 96-well microplates. Absorbance was read at 562 nm using a microplate reader (Omega, BMG Labtech, UK) after shaking and incubating the microplates at 37 °C for 30 min.

2.9. Statistical analysis

Values in the text for the dietary iron intakes are median (1st and 3rd quartiles) unless otherwise stated. All p values are 2-sided and not adjusted for multiple testing. Distribution of data was checked for normality using the Shapiro-Wilkes test. Differences in median total iron intakes determined by the two dietary methods were assessed using the Mann–Whitney U test, whereas differences in the proportions of the major food sources of iron between the two districts were tested using the two sample test for proportions. Statistical analyses for the dietary intake data were carried out using STATA-11.0 (Stata Corp, College Station, TX, USA). For the cell studies, statistical analyses were performed using SPSS Inc, USA (version 16.0.0). Two-factor ANOVA with Tukey's post hoc tests were conducted to examine pair-wise differences on power-transformed data. Data are presented as mean ± SD. For both dietary and cell studies, differences were considered significant at $p < 0.05$.

3. Results

3.1. Dietary iron intakes

In both districts, median calculated iron intakes (mg/d) were lower ($p < 0.001$) than those based on the analyzed diet composites (Table 1). Both calculated and analyzed iron intakes were lower in Zombwe EPA than in Mikalango EPA ($p < 0.001$). Cereals were the primary source of dietary iron based on the weighed food records, with unrefined maize contributing ~53%,

Table 1
Comparison of iron intakes (median; 1st, 3rd Quartile) calculated from weighed food records and analyzed diet composites collected on the same day, and analyzed levels of aluminum, titanium, and phytate.

	Zombwe EPA	Mikalango EPA	p^a
Calculated Fe intake (mg/d)	10.1 (7.0, 14.9)[b]	19.4 (12.9, 25.3)[b]	<0.001
Analyzed Fe intake (mg/d)	16.6 (10.9, 26.0)[c]	29.6 (15.9, 49.3)[c]	<0.001
Analyzed Al intake (mg/d)	15.6 (11.3, 27.6)	16.7 (10.0, 29.3)	0.827
Analyzed Ti (mg/d)	0.18 (0.12, 0.31)	0.32 (0.22, 0.44)	<0.001
Analyzed phytate (mg/d)	846 (525, 1197)	1564 (1094, 2236)	<0.001

[a] Mann–Whitney U-test.
Superscript letters (b, c) indicate differences ($p < 0.001$) between calculated and corresponding analyzed intakes.

and sorghum and millet providing ~57% of the total iron in Zombwe and Mikalango EPA, respectively. Cellular animal foods (i.e. meat, poultry and fish) contributed less than 3% of iron in each district based on the weighed food records (Table 2). The proportion of iron contributed by beverages was negligible in both districts because tea was the only beverage consumed by the women (Siyame et al., 2014), and our food composition database did not account for any iron that may have been contributed by the drinking water used to prepare the tea.

3.2. Cell studies

There was no ferritin response in Caco-2 cells treated with digests from composite diets, probably because any solubilized iron was bound to phytate, which was high in the analyzed diet composites from both regions (Table 1), and to polyphenols, present in the red sorghum and millet varieties consumed in Mikalango. The composite soil samples had different iron contents: the acidic soils of Zombwe EPA contained 25.84 mg iron/g (dry weight), whereas the calcareous soils of Mikalango EPA contained 47.1 mg iron/g (dry weight). A ferritin response was observed with digests obtained from 0.5 g soil from Zombwe but not from Mikalango EPA ($p < 0.005$) in the presence of ascorbic acid at 1:10 molar ratio (Fig. 1). When the weight of the soils was adjusted to expose the cells to similar quantities of iron from digestates (6.9 mg iron/well), again there was a greater ferritin response with the soil from Zombwe than Mikalango EPA in the presence of AA at 1:30 molar ratio ($p = 0.01$) (Fig. 2). The ferritin response to the same concentration of iron as ferrous gluconate, a highly available form of iron, was approximately 10-fold greater (122 ± 18 ng/mg protein).

4. Discussion

Our findings highlight that calculating iron intakes from the weighed food records using food composition data can significantly underestimate iron intakes, consistent with the earlier report of Beaton (1974). We attribute this discrepancy to the contribution

Table 2
Major food sources of calculated intakes of dietary iron (as %).

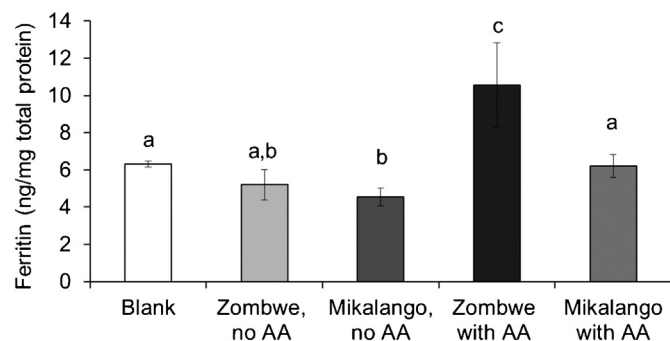|	Zombwe EPA	Mikalango EPA	p^a
Cereal grains	77.0	74.7	0.453
Legumes, nuts and eggs	3.4	15.3	<0.001
Meat, poultry, fish	2.7	1.5	0.248
Vegetables and tubers	13.9	8.5	0.009
Fruit	3.0	0.1	0.002

[a] Two sample test for proportions.

Fig. 1. Ferritin response in Caco-2 cells treated with digests of 0.5 g soil from Zombwe and Mikalango expressed in ng/mg total protein. AA: with or without 1:10 Fe:Ascorbic Acid molar ratio. Number of samples = 5 for Blank, Zombwe no AA and Mikalango no AA treatments; number of samples = 8 for Zombwe with AA and Mikalango with AA treatments. Superscript letters (a, b, c) denote significant differences ($p < 0.05$).

of contaminant iron from soil, based on the concentrations of aluminum and titanium and their positive correlation with the iron concentrations of the analyzed diet composites reported earlier (Siyame et al., 2014). Soils contain high concentrations of both titanium and aluminum, yet their uptake and transport to the edible parts of plants is relatively low resulting in a demonstrably large soil:plant ratio (Chilimba et al., 2011). Hence even minor soil contamination (by mass) of the diet composites will strongly affect their elemental concentrations of iron, aluminum, and titanium. In contrast, aluminum from cooking pots and titanium from food additives (Weir et al., 2012) were not possible sources that could have contributed to the elemental concentrations in the diet composites of these rural subsistence farmers.

Earlier we postulated that the use of traditional threshing sticks contaminated the outer surface of the cereal grains with iron from the soil (Siyame et al., 2014). Small grains such as sorghum and millet, the major cereal staples consumed in the Mikalango EPA, are more susceptible to soil contamination from threshing than maize, as shown by the higher concentrations of both titanium and total iron in the Mikalango diet composites compared to those for the maize-based diets in Zombwe EPA.

The plasma ferritin concentrations and total body iron levels of the Zombwe women were higher (Siyame et al., 2014), despite their lower iron intakes compared to the Mikalango women (Table 1). However, both the intrinsic and extrinsic sources of non-heme iron in the unrefined maize-based diets of the Zombwe
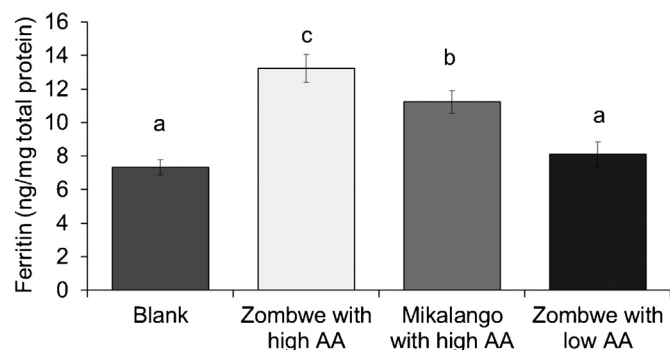
women were likely to be more bioavailable than that of the Mikalango diets which were based on red sorghum and pearl millet with a higher content of phytate (Table 1), and probably polyphenols, although the latter were not analyzed in the duplicate diet composites. In contrast, the proportion of readily absorbable iron from cellular animal protein in the diets of both EPAs was similar and low (i.e. <3%) (Table 2).

The high phytate content of the diets in both regions of Malawi probably explains the lack of ferritin response in the Caco-2 cell model. Our findings indicate that the acidic soils of the Zombwe EPA contained more available iron than the calcareous Eutric Vertisol soils (median pH 7.8) of Mikalango EPA (Fig. 1). Moreover, even when similar concentrations of iron from soil were applied to Caco-2 cells, the digestate from Zombwe EPA elicited a greater ferritin response (Fig. 2), suggesting there may be other modulators of iron availability in the soils, for example differences in iron mineralogy or calcium concentrations. Certainly the proportion of exchangeable contaminant iron in soil is likely to depend in part on soil mineralogy, particularly the solubility of the soil iron at the pH of the small intestine (Lee, 1982).

5. Conclusions

Our findings emphasize that in settings where threshing practices are likely to contribute to extraneous contaminant iron from soil, collection and analysis of weighed duplicate diet composites should be used to determine total iron intakes instead of estimates calculated from food records or recalls and food composition data. The presence of acidic contaminant soil in foods may contribute to dietary iron intake and make a useful contribution to iron nutrition. Future research should investigate the exchangeability of iron from different types of soils in settings where threshing practices are likely to contaminate staple cereal grains.

Fig. 2. Ferritin response (mean ± SD) in Caco-2 cells treated with digests of soil from Zombwe and Mikalango containing 6.9 mg iron/well expressed in ng/mg total protein. AA: with 1:30 (high) or 1:10 (low) Fe:Ascorbic Acid molar ratio. Combined data from 2 separate experiments with $n = 6$ for each treatment in each experiment. Superscript letters (a, b, c) denote significant differences ($p < 0.05$).

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