Efficacy of highly bioavailable zinc from fortified water: a randomized controlled trial in rural Beninese children

Valeria Galetti,1,8* Prosper Kujinga,4,8 Comlan Evariste S Mitchipke,5 Christophe Zeder,3 Fabian Tay,6 Félicien Tossou,7 Joseph D Hounhouigan,7 Michael B Zimmermann,3,9 and Diego Moretti3,9

1Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland; 2Division of Human Nutrition, Wageningen University, Wageningen, Netherlands; 3Laboratory of Human Nutrition, Faculty of Agricultural Sciences, Abomey-Calavi University, Cotonou, Benin; 4Division of Human Nutrition, Wageningen University, Wageningen, Netherlands; 5Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland; 6Clinical Trials Center, Center for Clinical Research, University Hospital of Zurich, Zurich, Switzerland; and 7Natitingou Health Zone, Ministry of Health, Natitingou, Benin

INTRODUCTION

Zinc deficiency is prevalent in many low- and middle-income countries, and it is mainly due to inadequate dietary intake of absorbable zinc that results from consumption of monotonous plant-based diets (1). A poor zinc status is particularly common in infants, children, and pregnant women because of their increased needs for growth (1). Together with consumption of contaminated water (2), zinc deficiency is a major risk factor for diarrhea because of its important role in immunity (3–5). In turn, diarrhea depletes zinc stores (2, 6). Diarrhea is a leading cause of death in children younger than 5 y and accounts for 11% of total child mortality (~0.8 million deaths/y) worldwide (7). Therapeutic and preventive zinc supplementation has a protective effect on diarrhea (8, 9). Conversely, efficacy studies of zinc fortification have produced mixed results (10). This is likely because of lower zinc bioavailability from staple foods caused by phytic acid—a zinc absorption inhibitor (11, 12).

Water has the advantage of being free of inhibitory factors of absorption. The efficacy of water as a carrier of fluorine (13), iodine (14), and iron (15) was previously shown. On the other hand, no studies have been published on the effect of consuming zinc-fortified water on zinc status indicators or diarrhea. Life-StrawFamily (LSF)10 is a novel point-of-use water ultrafiltration

ABSTRACT

Background: Zinc deficiency and contaminated water are major contributors to diarrhea in developing countries. Food fortification with zinc has not shown clear benefits, possibly because of low zinc absorption from inhibitory food matrices. We used a novel point-of-use water ultrafiltration device configured with glass zinc plates to produce zinc-fortified, potable water.

Objective: The objective was to determine zinc bioavailability from filtered water and the efficacy of zinc-fortified water in improving zinc status.

Design: In a crossover balanced study, we measured fractional zinc absorption (FAZ) from the zinc-fortified water in 18 healthy Swiss adults using zinc stable isotopes and compared it with zinc-fortified maize porridge. We conducted a 20-wk double-blind randomized controlled trial (RCT) in 277 Beninese school children from rural settings who were randomly assigned to receive a daily portion of zinc-fortified filtered water delivering 2.8 mg Zn (Zn+filter), nonfortified filtered water (Filter), or nonfortified nonfiltered water (Pump) from the local improved supply, acting as the control group. The main outcome was plasma zinc concentration (PZn), and the 3 groups were compared by using mixed-effects models. Secondary outcomes were prevalence of zinc deficiency, diarrhea prevalence, and growth.

Results: Geometric mean (−SD, +SD) FAZ was 7-fold higher from fortified water (65.9%; 42.2, 102.4) than from fortified maize (9.1%; 6.0, 13.7; P < 0.001). In the RCT, a significant time-by-treatment effect on PZn (P = 0.026) and on zinc deficiency (P = 0.032) was found; PZn in the Zn+filter group was significantly higher than in the Filter (P = 0.006) and Pump (P = 0.025) groups. We detected no effect on diarrhea or growth, but our study did not have the duration and power to detect such effects.

Conclusions: Consumption of filtered water fortified with a low dose of highly bioavailable zinc is an effective intervention in children from rural African settings. Large community-based trials are needed to assess the effectiveness of zinc-fortified filtered water on diarrhea and growth. These trials were registered at clinicaltrials.gov as NCT01636583 and NCT01790321.

Keywords: Benin, school-age children, water fortification, zinc absorption, zinc fortification

1Supported by Vestergaard, Lausanne, Switzerland.
2Supplemental Table 1 and Supplemental Figures 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.
8These are joint first authors.
9These authors contributed equally to the article.
10Abbreviations used: AGP, α1-acid glycoprotein; CRP, C-reactive protein; ETH, Swiss Federal Institute of Technology; FAZ, fractional zinc absorption; FM+W, zinc-fortified maize porridge consumed with ultrapure water; FW, zinc-fortified water alone; LSF, LifeStrawFamily; M+F, arm with unfortified maize porridge but with zinc-fortified water; MEM, mixed-effects models; PZn, plasma zinc concentration; RCT, randomized controlled trial; SRSS, sparse random serial sampling.
Received June 11, 2015. Accepted for publication August 19, 2015.
First published online October 14, 2015; doi: 10.3945/ajcn.115.117028.
device that purifies water by using a gravity-fed nanopore hollow-fiber filter. Previous laboratory tests have shown that the LSF meets the microbiological performance specifications of the US Environmental Protection Agency and the WHO (16, 17). A lower incidence of diarrhea in groups using the LSF than in control subjects has been reported in 2 community-based randomized controlled trials (RCTs) in the Democratic Republic of Congo (18) and Zambia (19), although no differences in diarrhea cases were reported between intervention and control groups in a cohort study in Kenya (20). We tested a new version of the LSF containing glassy zinc phosphate–based plates that simultaneously filters and fortifies water with zinc and achieves controlled zinc elution in a range of concentrations (1–10 mg/L) relevant for human zinc nutrition. This strategy was recently judged to be a potentially cost-effective approach for zinc delivery in children aged 1–5 y from low-income settings (21). The combined provision of safe drinking water with additional zinc could be an effective approach to improving zinc status and reducing diarrhea in zinc-deficient populations with no access to improved water supplies. In this study, we first measured the bioavailability of zinc from zinc-fortified filtered LSF water. We then assessed the efficacy of zinc-fortified filtered LSF water to deliver zinc to Beninese schoolchildren from rural, low-income settings in a double-blind RCT. The objective was to assess the effect of simultaneous zinc fortification and water filtration on zinc status, with the primary outcome being plasma zinc concentration (PZn). Secondary outcomes were the prevalence of zinc deficiency, diarrhea, and growth.

METHODS

Absorption study

Participants

We performed the trial between June and August 2012 at the Clinical Trials Center of the University Hospital of Zurich, Switzerland. We recruited potential subjects from the local student community and screened 25 individuals; inclusion criteria were as follows: 18–45 y of age, a BMI (in kg/m²) of 19 to 25, no mineral or vitamin supplementation for ≥2 wk before the start and during the study, no major chronic diseases or long-term medication (except for oral contraceptives), no vegan diet, nonsmokers, not pregnant and not lactating, no participation in any other clinical study within the preceding 30 d, and no earlier participation in a study using zinc stable isotopes. We invited 18 subjects to participate in the study; all gave informed written consent. The study protocol was approved by the Cantonal Ethics Commission of the Canton of Zurich (KEK-ZH-No. 2012–0168), and the study conduction adhered to the principles expressed in the Declaration of Helsinki of 1975, as revised in 1983.

Study design

In a single-blind, 3-way crossover, balanced design, we quantified fractional zinc absorption (FAZ) from 3 zinc-fortified meals: 1) a zinc-fortified maize porridge consumed with ultrapure water (18.2 MΩ × cm; FM+W), 2) the same maize porridge but unfortified consumed with zinc-fortified water (M+FW), and 3) zinc-fortified water alone (FW). We designed the 3 meals to each deliver 2 mg Zn per serving. On the day of administration, we added the 67Zn tracer to either the porridge or the water, and the subjects consumed the meals under supervision. Immediately after the feeding, we intravenously infused 0.2 mg 70Zn in 9 mL saline over 5 min; we weighed the syringe before and after infusion and flushed the system (catheter and needle) with 10 mL physiological saline to ensure quantitative isotope administration. Each evening before meal administration, the subjects were instructed to consume no food after 2000 and no beverages after midnight. After meal administration, the subjects fasted for ≥3 h. Thereafter, each of the subjects resumed their usual dietary habits until the evening before the next meal administration. The following meal was administered by using the same procedure and the same tracers >24 d apart to allow for isotope washout. Before and after 96 ± 3 h after each meal administration, we collected a morning urine sample to assess baseline and enriched isotopic composition. The fourth day from isotope administration was identified in a pilot study as being the most suitable time for collection because of the proportional decline of both isotopes in urine, and we used the same procedure in 2 previous studies (22, 23). Baseline samples collected before the second and third test meals accounted for residual enrichment from the previous administration. We calculated the fractional absorption of the 67Zn dose from the test meal by using the oral to intravenous tracers ratio method applied to spot urine samples, as previously described (24).

Randomization and masking

Using spreadsheet software (Excel; Microsoft Office 2010), we randomly assigned the eligible participants to 1 of the 6 possible sequences of administration of the 3 meals (block size of 3) so that each subject consumed all 3 test meals. The 3 meals were labeled “A,” “B,” or “C” and masking was applied to the participant only. The research team, which was responsible for the quantitative addition of isotopic zinc solutions to the meals just before they were administered, was not blinded.

Meal preparation

We prepared the porridge in bulk and accounted for water loss during cooking by adding ultrapure water back to the cooked porridge. Each serving consisted of 5 g sugar, 170 g ultrapure water, and 50 g maize flour. We prepared servings of 225 g wet weight maize porridge in precoded plastic bowls that were frozen to −20°C until the evening before the feeding day. The FM+W meal consisted of a serving of maize porridge containing 330.5 mg phytic acid, 0.56 mg native zinc, 0.44 mg added zinc as ZnSO4, and labeled with 1.00 mg 67Zn as ZnSO4. We served this meal with 300 mL ultrapure water. The M+FW meal was composed of the same porridge (330.5 mg phytic acid and 0.56 mg native zinc) but was unfortified and was served with 300 mL zinc-fortified water containing 0.44 mg Zn and labeled with 1.00 mg 67Zn as ZnSO4. The FW meal was composed of 300 mL zinc-fortified water containing 1.00 mg Zn and labeled with 1.00 mg 67Zn as ZnSO4. We prepared the zinc-fortified water at the needed concentration by diluting water fortified with zinc by the LSF with ultrapure water. Zinc fortification rates for the maize flour and the LSF water were 28.8 and 6.7 ppm, respectively. The FM+W and M+FW meals had a molar ratio of phytic acid to zinc of 16.4.

ZINC-FORTIFIED WATER: BIOAVAILABILITY AND EFFICACY

1239
Preparation of stable isotope labels

We purchased isotopically labeled zinc oxide powders ($^{67}$ZnO and $^{70}$ZnO) from Chemgas. Powders were stored in a glass vial as provided by the supplier at room temperature before preparation. For the test meal label, we prepared isotopically labeled $^{67}$ZnSO$_4$ from $^{70}$ZnO by dissolution in diluted sulfuric acid. The solution was stored at room temperature until used. To prepare the intravenous dose, we converted $^{70}$ZnO to $^{70}$ZnCl with HCl and adjusted to a pH of 6 by adding NaHCO$_3$ and diluted it with physiological saline. The Cantonal Pharmacy of the University Hospital in Zurich prepared the individual intravenous doses of the 9.5-g solution by transferring it to septum-sealed glass vials, where they were sterilized and checked for sterility and pyrogens. Doses were stored at 4°C until used. We used inductively coupled plasma mass spectrometry to determine the concentration of the administered isotopic labels in solution and in the administered zinc fortificant.

Blood sampling and analysis

We drew a 7.5-mL fasting venous whole-blood sample from each study participant into a trace element–free lithium heparin tube (Sarstedt) in the morning of study day 1 for baseline biochemical analyses [PZn; plasma C-reactive protein (CRP)] before the first meal and intravenous dose. Because elevated intravascular pressure causes the outward movement of fluid into the interstitial space, thereby increasing the concentration of serum proteins and zinc, the subjects remained laying for the blood-drawing procedure, and the tourniquet was placed for a standardized length of time (<1 min). The blood samples were refrigerated at 4°C immediately after collection, centrifuged within 1 h (3000 × g for 10 min at room temperature), portioned into aliquots in acid-washed plastic vials (Eppendorf AG), and frozen at −25°C for later analysis of PZn and CRP. We measured PZn by flame atomic absorption spectrometer (AA240FS; Varian Inc.) using a commercial aqueous standard (Titrisol 1.0009953.0001; Merck) for external calibration, and Seronorm Trace Elements Serum L-2 (Sero AS) as reference material, which delivered values within acceptable ranges as specified by the manufacturer. Instrumental parameters were set at 213.9 nm for wavelength, 1 cm for slit width, and no background correction. We measured acute response indicator CRP in duplicate by using an automated chemiluminescent immunoassay system (IMMULITE; Diagnostic Products Corporation) according to the manufacturer’s instructions. We compared the mean PZn concentration (from duplicate analysis) with the suggested sex- and age-specific lower cutoffs indicating zinc deficiency in populations, which for morning fasting blood samples in adults were defined as 74 and 70 μg/dL for males and nonpregnant females, respectively (25).

Urine sampling and analysis

All urine samples were collected into prelabeled zinc-free (acid-washed) polyethylene containers and kept at 4°C until delivered to the Swiss Federal Institute of Technology (ETH), where they were stored at −25°C until analyzed. We applied a human chorionic gonadotropin marker test for pregnancy to each baseline urine sample from the female participants. After the samples were freeze-dried, we mineralized the urine samples by using an HNO$_3$/H$_2$O$_2$ mixture and microwave digestion followed by separation of the sample zinc matrix by anion-exchange chromatography. We performed the isotopic analyses by inductively coupled plasma mass spectrometry, using a high-resolution double focusing magnetic sector field multicollector mass spectrometer (Neptune; Thermo Scientific), in duplicate and under chemical blank monitoring. We measured $^{70}$Zn, $^{66}$Zn and $^{67}$Zn: $^{68}$Zn ratios to determine $^{70}$Zn and $^{67}$Zn enrichment. Enrichment observed in the baseline samples from the second and third meal administration were used as the new natural abundance in subsequent calculations. All laboratory analyses were performed at the Laboratory of Human Nutrition at ETH.

Sample size calculation and statistical analysis

We required 18 eligible subjects to detect a difference in FAZ of 40%, with 80% power at a 0.05 significance level (2-tailed), and taking into account a 20% dropout rate. We based the sample size calculation on the pooled results of 5 previous zinc absorption studies that we performed at the ETH (SD of the log-transformed differences between pairs) (22).

We conducted the data analysis using spreadsheet software (Microsoft Excel). When the data were not normally distributed, the values were logarithmically transformed before the statistical analysis. The difference between meals was assessed by repeated-measures ANOVA with a Bonferroni test for multiple comparisons. Significance was set at $P < 0.05$.

Efficacy trial

Study site and participants

We conducted the study at the primary school of Kotopounga, a rural town in northwestern Benin, where the climate is a subequatorial/tropical savanna with one rainy season (May-June to September-October) and one dry season (October-November to April-May). In this community, plant staples contribute an estimated 87% of the daily zinc intake (26). We invited the parents of children enrolled in the first 3 school years to private meetings, where we explained the study procedures in the local Waama and Otamari languages. Before the screening, parents gave informed consent by either a written signature or a fingerprint. We invited children with an expected endpoint age <11.0 y for a screening at the local health center, where a local nurse performed a physical examination following WHO protocols on integrated management of childhood illness as adapted into national health policy. The exclusion criteria were as follows: severe anemia, major chronic diseases or long-term medication use, and zinc supplementation. A total of 277 children were enrolled. The study protocol was approved by the ETH Ethics Commission (EK 2012-N-47) and by the Ministry of Health of Benin under favorable advice of the Beninese National Ethics Committee for Health Research (No. 029; 19 October 2012). The study conduction adhered to the principles expressed in the Declaration of Helsinki of 1975, as revised in 1983, and an independent Data Safety Monitoring Board was set up to monitor the intervention.

Study design

We conducted a 20-wk double-blind RCT from February to June 2013 to investigate whether consumption of filtered water fortified with low-dose zinc (Zn+filter arm) was beneficial at improving zinc status compared with nonfortified filtered water.
ZINC-FORTIFIED WATER: BIOAVAILABILITY AND EFFICACY

Randomization and masking

Using spreadsheet software (Microsoft Excel), we individually randomized children at enrollment and assigned them to 1 of 3 treatment arms. Randomization was masked, and we used a color code to identify the 3 groups. The code—unknown to the participants, the field assistants, and the research team throughout the duration of the study and the data analysis—was held by a person not involved in the fieldwork, data analysis, or Data Safety Monitoring Board.

Water serving preparation

We freshly sourced trial water each morning from the local borehole pump that supplies the school. After pumping, we either filtered the water via an LSF equipped with a zinc fortification chamber (Zn+filter) or via an LSF with a placebo chamber (Filter) and hygienically stored it in tanks until feeding time. The pump water for the control group (Pump) did not undergo any treatment and was directly poured into the tanks for hygienic storage. To ensure masking, an external teacher was in charge of replacing the arm tags “Zn+filter,” “Filter,” and “Pump” on the storage tanks with color tags according to the defined code. After masking, we prepared water servings by filling color-coded glasses to the 300-mL mark with the corresponding water and covered them with a clean tissue until administration to prevent airborne contamination. During the school break, we handed out color-coded identity photo cards to the study children, who, according to group assignment, collected their water serving, which was consumed under direct supervision. At each administration, we recorded any full, partial, or missing water consumption or re-gurgitated servings on a log. At feeding completion, the color tags were replaced with arm tags. The masking-unmasking procedure was performed on a daily basis in a room inaccessible to the participants, field assistants, and research team.

Monitoring of zinc concentration in water

The zinc concentration in water was monitored daily, both with a rapid assessment method (Aquaquant; Merck) in the field and then after the intervention by flame atomic absorption spectrometry (Varian Inc.). To this purpose, each day we collected 10 mL postfiltration water in acid-washed tubes, stabilized them with 0.1 mol HNO₃/L, and stored them at room temperature with 0.1 mol HNO₃/L, and stored them at room temperature.

Monitoring of zinc concentration in water

The zinc concentration in water was monitored daily, both with a rapid assessment method (Aquaquant; Merck) in the field and then after the intervention by flame atomic absorption spectrometry (Varian Inc.). To this purpose, each day we collected 10 mL postfiltration water in acid-washed tubes, stabilized them with 0.1 mol HNO₃/L, and stored them at room temperature.

### TABLE 1

Baseline characteristics of the Swiss subjects in the absorption study, by sex

<table>
<thead>
<tr>
<th></th>
<th>All (n = 18)</th>
<th>Men (n = 7)</th>
<th>Women (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>24.0 ± 1.7</td>
<td>25.4 ± 1.7</td>
<td>23.2 ± 1.1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.5 ± 1.6</td>
<td>22.3 ± 1.7</td>
<td>21.0 ± 1.4</td>
</tr>
<tr>
<td>Plasma zinc, μg/dL</td>
<td>76.0 ± 11.1</td>
<td>80.3 ± 10.0</td>
<td>73.2 ± 11.4</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>0.2 (0.2–15.6)</td>
<td>0.2 (0.2–10.2)</td>
<td>0.2 (0.2–15.6)</td>
</tr>
</tbody>
</table>

1All values are means ± SDs or medians; ranges in parentheses. Values in a row with different superscript letters are significantly different, P = 0.014 (independent-samples t test).

### TABLE 2

Zinc fractional and total absorption from meals delivering 2 mg Zn in young Swiss adults

<table>
<thead>
<tr>
<th></th>
<th>FM+W (n = 16)</th>
<th>M+FW (n = 17)</th>
<th>FW (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional absorption of zinc, %</td>
<td>9.1 (6.0, 13.7)</td>
<td>9.8 (5.7, 16.7)</td>
<td>65.9 (42.4, 102.4)</td>
</tr>
<tr>
<td>Total absorption of zinc, mg</td>
<td>0.18 (0.12, 0.27)</td>
<td>0.20 (0.11, 0.33)</td>
<td>1.32 (0.85, 2.05)</td>
</tr>
</tbody>
</table>

1All values are geometric means (−SD, +SD). Treatment effect based on repeated-measures ANOVA with Bonferroni test for multiple comparisons. Values in a row with different superscript letters are significantly different, P < 0.001. FM+W, fortified maize porridge and ultrapure water; FW, zinc-fortified water; M+FW, maize porridge and zinc-fortified water.

2Meal composition: 225 g wet weight maize porridge (0.56 mg native zinc, 0.44 mg added zinc of normal isotopic composition as ZnSO₄, 1.00 mg added²⁷Zn as ZnSO₄, 330.5 mg native phytic acid) and 300 mL ultrapure water. Molar ratio of phytic acid to zinc: 16.4.

3Meal composition: 225 g wet weight maize porridge (0.56 mg native zinc, 330.5 mg native phytic acid) and 300 mL zinc-fortified water (0.44 mg added zinc of normal isotopic composition as ZnSO₄, 1.00 mg added²⁷Zn as ZnSO₄). Molar ratio of phytic acid to zinc: 16.4.

4Meal composition: 300 mL zinc-fortified water (1.00 mg added zinc of normal isotopic composition as ZnSO₄, 1.00 mg added²⁷Zn as ZnSO₄). Molar ratio of phytic acid to zinc: nil.
until they were transported to the Laboratory of Human Nutrition at the ETH for analysis.

**Anthropometric assessment**

We measured height to the nearest 0.1 cm using a stable stadiometer and weight to the nearest 0.1 kg using a frequently calibrated Robusta 813 scale (Seca) while the subjects were wearing light indoor clothing and no shoes (30). We calculated height-for-age, weight-for-age, and BMI-for-age $z$ scores using the WHO 2007 package developed for the R statistical programming environment (version 3.0.3) (31) according to the 2007 WHO growth standards for school-age children (32).

**Blood sampling, biochemical analysis, and applied cutoffs**

To avoid exogenous or endogenous zinc contamination during sample collection, handling, and analysis, standardized protocols by the International Zinc Nutrition Consultative Group were followed (33). For the biochemical analyses, we collected 2 morning (mean time: 1024 ± 1 h 18 min) fasting (subjects in nonfasting state were 5%, 4.3%, and 3.3% at baseline, midpoint, and endpoint sampling, respectively) venous whole-blood samples from each study participant directly into trace element–free lithium heparin tubes (for PZn analysis) and EDTA tubes (for analysis of all other biochemical indicators) (Sarstedt). Hemoglobin was assessed with a portable HemoCue 201+ photometer (HemoCue AB). We recorded the time of blood withdrawal, plasma separation and the last previous meal before the venipuncture. Samples were immediately stored in a refrigerated cool box and centrifuged within 1 h (3000 × g for 10 min at room temperature) on an E8F Portafuge portable centrifuge (LW Scientific). Clearly hemolyzed samples were discarded ($n = 11$), and sampling was rescheduled ($n = 10$). Plasma was thus portioned into aliquots in acid-washed test tubes (Eppendorf AG) and transported in a refrigerated cool box to the laboratory unit, where it was stored at −20°C within 5 h. We shipped the specimens to the ETH and to a laboratory in Freiburg, Germany, on dry ice with an express international courier. In Zurich, we measured PZn according to the method used during the zinc absorption study. The mean interassay CV was 2.1% for PZn. In Freiburg, CRP and AGP were analyzed by using a sandwich ELISA (34). Liquicheck Trilevel (Bio-Rad Laboratories Inc.) was used as control material with each run of analysis, and measured values were within acceptable ranges as specified by the manufacturer. The mean interassay CV was 5.2% for CRP and 5.1% for AGP. We coded specimens from all time points and analyzed them without knowledge of the study arm and in a random order. All values represent the mean of an independent duplicate measurement; we reran the analysis if the CV exceeded 5% and removed obvious outliers. We calculated the prevalence of zinc deficiency by using sex- and age-specific PZn lower cutoffs by the International Zinc Nutrition Consultative Group (25). Subclinical inflammation was defined as CRP >5 mg/L and/or AGP >1 g/L. Severe anemia was defined as hemoglobin <7 g/dL (35).

**Diarrhea longitudinal prevalence**

We assessed diarrhea morbidity during 10 follow-up private interviews with each child by recording any self-reported diarrhea cases in the preceding day. Diarrhea was defined according to the WHO definition of ≥3 loose stools within a 24-h period (2) and

![FIGURE 1 Diagram of the children’s participation in the efficacy trial.](https://academic.oup.com/ajcn/article-abstract/102/5/1238/4564426)
evaluated as longitudinal prevalence, i.e., the number of days ill divided by the number of days under observation (36, 37).

**Microbiological analysis of water**

We measured microbiological contamination using Colilert reagents, Quanti-Trays and Quanti-Tray Sealer (IDEXX Laboratories Inc.), a semiquantitative analysis based on the most probable number of total coliforms (as an indicator of environmental contamination), and *Escherichia coli* (as an indicator of fecal contamination).

**Sample size calculation and statistical analysis**

We estimated that 90 children were needed in each group, based on 80% power to detect a PZn difference of 5 μg/dL with a 0.05 significance level (2-tailed) and anticipating a dropout rate of 20%. We based the sample size calculation on the SD of PZn measured in previous studies in school-age children from rural settings conducted by the ETH (38, 39).

We conducted data analysis with the R statistical programming environment (version 3.0.3) using packages nlme and lme4 (31). When data were not normally distributed, values were logarithmically transformed before statistical analysis. We excluded from the analysis any outliers, defined as >3 SDs from the overall mean at baseline or group mean at midpoint and week 20. Values in the text and in the tables are represented as means ± SDs for normally distributed data, medians (IQRs) for nonnormal data, and percentages (95% CIs) for prevalences. The tables and text do not report averaged data from the midpoint assessment because it is difficult to interpret since it originated from sparse assessment. In the absence of the time variable, we tested groupwise differences by using one-factor ANOVA with Bonferroni test for multiple comparisons for continuous variables and Pearson chi-square test for binary outcomes. We assessed the intervention effect over time (time-by-treatment interaction) by fitting linear or nonlinear mixed-effects models (MEMs) and logistic regression MEMs for all 3 time points for continuous and binary outcomes, respectively. Fixed effects of the variance were time, which was defined as the day from intervention start to account for unequal sampling intervals (subject-specific), and treatment, defined as the intervention arm. Subjects were the random component. For the PZn data only, and to best fit the correlation structure of the data, we modeled the fixed effect as a \((t + 1)^{-1}\) function of time \(t\), as known from previous work (40).

As covariates of the model, we included variables that correlated with the dependent variable at baseline or that were reasonably

**TABLE 3**

Plasma zinc concentration and acute phase proteins of children participating in the efficacy trial, by treatment group and stage of study

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>P-main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>All</td>
</tr>
<tr>
<td>Baseline</td>
<td>274</td>
</tr>
<tr>
<td>Week 20</td>
<td>254</td>
</tr>
<tr>
<td>Plasma zinc, μg/dL</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>68.7 (60.7–75.6)</td>
</tr>
<tr>
<td>Week 20</td>
<td>65.3 (58.2–72.5)</td>
</tr>
<tr>
<td>Zinc deficiency, plasma</td>
<td></td>
</tr>
<tr>
<td>less than cutoff, %</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>38.3 (32.6, 44.1)</td>
</tr>
<tr>
<td>Week 20</td>
<td>52.0 (45.8, 58.1)</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.4 (0.2–1.4)</td>
</tr>
<tr>
<td>Week 20</td>
<td>0.5 (0.2–1.4)</td>
</tr>
<tr>
<td>Elevated C-reactive protein, &gt;5 mg/L, %</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>8.0 (4.8, 11.2)</td>
</tr>
<tr>
<td>Week 20</td>
<td>11.0 (7.2, 14.9)</td>
</tr>
<tr>
<td>α1-Acid glycoprotein, g/L</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.9 (0.7–1.1)</td>
</tr>
<tr>
<td>Week 20</td>
<td>0.8 (0.7–1.1)</td>
</tr>
<tr>
<td>Elevated α1-acid glycoprotein, &gt;1 g/L, %</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>35.4 (29.7, 41.1)</td>
</tr>
<tr>
<td>Week 20</td>
<td>30.3 (24.7, 36.0)</td>
</tr>
</tbody>
</table>

1Continuous variables are presented as medians; IQRs in parentheses, whereas prevalences are presented as percentages; 95% CIs in parentheses. Continuous data were analyzed by using a mixed-effects model, and frequencies were analyzed with a logistic regression mixed-effects model. Values in a row with different superscript letters are significantly different, \(P < 0.05\). Filter, nonfortified filtered water; Pump, nonfortified nonfiltered water; Zn+filter, zinc-fortified filtered water.

2Controlled for C-reactive protein and baseline.

3Controlled for age, height-for-age \(z\) score, and \(\alpha_1\)-acid glycoprotein. Sex- and age-specific cutoffs by IZINCG (25).

4Controlled for \(\alpha_1\)-acid glycoprotein.

5Controlled for weight and C-reactive protein.
thought to predict the dependent variable according to current literature. Statistical dependence was tested with the Pearson product-moment correlation coefficient \( r \) for normally distributed data and with Spearman’s rank correlation coefficient \( r_s \) for nonnormal data. To achieve the minimal adequate model, we applied a stepwise backward deletion procedure. We checked normality and homoscedasticity by residuals plots and selected models according to the Akaike Information Criterion for model fit and Bayesian Information Criterion for over-parameterization (41).

Longitudinal prevalences ratios for diarrhea were calculated, and we assessed the time-by-treatment effect on diarrhea as binary variable by using generalized estimating equations to account for the correlation of repeated measures within individuals. We used negative binomial regression with the Wald test and robust covariance estimator to model count variables such as number of visits to health centers. Significance was set at \( P < 0.05 \).

RESULTS

Absorption study

Eighteen subjects were enrolled, two subjects dropped out on the day of the first meal administration, and one was replaced. Another subject developed a cystitis that was judged unrelated to study participation and withdrew from the study after the administration of the second meal. Thus, we administered 50 meals and intravenous doses \([n = 16 \text{ (FM+W)}, n = 17 \text{ (M+FW)}, \text{and } n = 17 \text{ (FW)}] \) and collected 50 pairs of baseline and enriched urine samples. The subjects’ characteristics at baseline are shown in Table 1. The geometric mean (−SD, +SD) FAZ was 65.9\% (42.2, 102.4) from FW, 9.8\% from M+FW, and 9.1\% from FM+W (Table 2). In post hoc comparisons, maize-containing meals showed significantly lower FAZ than FW (\( P < 0.001 \)), but FAZ did not differ significantly between the FM+W and M+FW meals.

Efficacy trial

In total, 382 children were invited to the screening, 331 of whom were eligible (Figure 1). Thirteen subjects did not come to the screening, and 41 were excluded because their school attendance was expected to be low due to the absence of teaching staff. Thus, 277 children were enrolled and 262 completed the study (94\% of those enrolled). At the first and second follow-up visits, 6 and 9 subjects, respectively, were absent, but their remaining data were included in the analysis. During the study, 40,442 portions of 300 mL water were served for consumption during 81 school days: 33,697 were fully consumed, 24 were consumed only partially, and 6654 were not consumed because the child was absent at the time of feeding. The participants’ median (IQR) compliance was 87\% (78–92\%) and did not differ significantly between treatment groups (\( P = 0.341 \)).

Water servings contained a median (IQR) zinc dose of 2.4 (2.0–3.2) mg, resulting in a daily zinc intake of 4.3 (3.5–5.2) mg on school days and, averaged over the entire study, an overall daily zinc intake of 2.8 (0.0–4.5) mg. During the study, we detected a significant time-by-treatment effect on PZn (\( P = 0.026 \)) and an overall decrease in PZn (main effect of time: \( P < 0.001 \)) (Table 3 and Figure 2). Parameter estimates between the groups showed that the PZn in the Zn+filter group over the study period was significantly higher than in the Filter (\( P = 0.012 \)) and in the Pump (\( P = 0.031 \)) groups, whereas it did not differ between the Filter and Pump groups (\( P = 0.759 \)). When CRP was controlled for, the model’s fit improved significantly. Change (\( \Delta \)) in PZn from baseline to the end of the intervention was higher in children with a lower initial PZn (0.55 \( \mu \)g/dL mean increase in PZn for each 1-\( \mu \)g/dL lower initial PZn (Figure 3). The strongest predictor of the magnitude of \( \Delta \)PZn was baseline PZn (\( P < 0.001 \)) and endpoint CRP (\( P < 0.001 \)). Treatment
assignment significantly predicted ΔPZn when baseline zinc status was controlled for ($P = 0.041$).

A significant time-by-treatment effect was found on the prevalence of zinc deficiency ($P = 0.032$), combined with an increase in prevalence over the study period (main effect of time: $P < 0.001$) (Table 3 and Figure 2). Zinc deficiency in the Filter group over the study period was more prevalent than in the Zn+filter ($P = 0.046$) and Pump ($P = 0.014$) groups, whereas no difference was detected between the Zn+filter and the Pump groups ($P = 0.770$). Prevalence of zinc deficiency at baseline differed between groups ($P = 0.021$) and was 50.0% (45 in 90) in the Pump group, 33.3% (32 in 96) in the Filter group, and 31.8% (28 in 88) in the Zn+filter group.

At baseline, median (IQR) CRP and AGP values were 0.4 (0.2–1.4) mg/L and 0.9 (0.7–1.1) g/L, respectively, which indicated infection/inflammation in 8% (22 in 274) and 35.4% (97 in 274) of the subjects, respectively (Table 3). During the intervention, there was an overall increase in CRP concentration (time effect: $P < 0.001$) and in the prevalence of elevated CRP values (time effect: $P = 0.028$). The AGP concentration was not affected by time or treatment.

We detected no significant effect of time ($P = 0.322$), treatment ($P = 0.108$), or interaction of time and treatment ($P = 0.366$) on diarrhea. The longitudinal prevalence ratio for diarrhea in the Zn+filter group compared with the Filter group was 1.15 (95% CI: 0.58, 2.29; $P = 0.73$) and compared with the Pump group was 1.33 (95% CI: 0.64, 2.76; $P = 0.47$). Prevalence in the Filter group did not differ from that in the Pump group (1.16, 95% CI: 0.55, 2.42; $P = 0.72$). There was no treatment effect on the number of visits to the local health centers ($P = 0.268$), although visits increased significantly during the intervention ($P < 0.019$) (Figure 4). No time-by-treatment interaction effect was found on stunting or

**FIGURE 3** Change in plasma zinc concentration from baseline to week 20 as a function of the participant’s baseline plasma zinc concentration, by treatment group. Statistical dependence was tested by using Spearman’s rank correlation coefficient ($r_s$). Coupled baseline-to-endpoint data available for $n = 84$ in the Zn+filter group, $n = 88$ in the Filter group, and $n = 82$ in the Pump group. Filter, nonfortified filtered water; Pump, nonfortified nonfiltered water; Zn+filter, zinc-fortified filtered water.

**FIGURE 4** Number of walk-in visits to the health centers during the efficacy trial, by intervention week and treatment group (A) and by nurse’s diagnosis and treatment group (B). Number of visits to the health centers increased significantly during the intervention (negative binomial regression, $P < 0.019$), but the total number of visits ($n = 15$ in the Zn+filter group, $n = 22$ in the Filter group, and $n = 20$ in the Pump group) did not differ between groups ($P = 0.268$). ALRI, acute lower respiratory tract infection; Filter (or F), nonfortified filtered water; Pump (or P), nonfortified nonfiltered water; Zn+filter (or Z), zinc-fortified filtered water.
underweight prevalence, which were generally highly prevalent (Table 4).

Household water of the families that included the study children was sourced from unprotected wells (n = 8), a borehole equipped with manual pump (n = 5), or a protected well (n = 1), which had >1011.2, 681.7 ± 395.4, and 344.1 coliforms CFU/100 mL, respectively, and 110.6 ± 146.0, 84.1 ± 106.7, and nil E. coli CFU/100 mL, respectively. Water provided during the study from the school pump water sampled at the source had no detectable coliforms or E. coli contamination (n = 14). In storage tanks, where water was stored before servings preparation, coliform count was 2.1 ± 2.6 CFU/100 mL in filtered water (n = 4) and 42.3 ± 83.2 CFU/100 mL in control water (n = 4) with no detectable E. coli.

**DISCUSSION**

Zinc from fortified water eluted from the LSF was highly bioavailable (FAZ = 66%) and was ~7-fold more bioavailable than from zinc-fortified maize. On average, the subjects absorbed 1.32 mg Zn from fortified water, enough to cover 94–132% of their physiological requirement (males and females, respectively). In contrast, when consuming zinc-fortified maize, they absorbed 0.18 mg Zn, covering for only 13–18% of their requirements. Our data highlight the advantage of using water as a fortification vehicle: with no inhibition of zinc absorption from the food matrix, even a low zinc dose (2 mg) can achieve high levels of absorbed zinc. However, this extremely high bioavailability was achieved only when the water was consumed alone, because consumption with an inhibitory maize meal reduced FAZ to 10%.

Our data from the efficacy trial show that consumption of zinc-fortified water at low zinc dosages is an effective strategy for maintaining a higher PZn and a lower prevalence of zinc deficiency among Beninese school children compared with the control. Our findings indicate that PZn can respond to fortification strategies, whereas available evidence on PZn as an indicator of response to fortification trials is inconsistent and mainly negative (10). Trials of food fortification with daily zinc doses comparable with ours (≤5 mg/d) have shown mixed results: some studies have shown beneficial effects on zinc status (42, 43), whereas others have not (44–46). No significant difference in PZn response between the Pump and the Filter groups was found (Table 1), which suggests that microbiological purification of the water did not have an effect on PZn. The microbiological quality of the trial waters was generally high, in contrast with the highly contaminated household water, which probably contributed largely to the amount of daily drinking water.

### TABLE 4

<table>
<thead>
<tr>
<th>Anthropometric measurements of children participating in the efficacy trial, by treatment group and stage of study¹</th>
<th>Treatment group</th>
<th>P-main effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>All</td>
<td>Pump</td>
</tr>
<tr>
<td>Baseline</td>
<td>271</td>
<td>90</td>
</tr>
<tr>
<td>Week 20</td>
<td>249</td>
<td>80</td>
</tr>
<tr>
<td>Height, m²</td>
<td>121.2 ± 10.1</td>
<td>120.5 ± 9.6</td>
</tr>
<tr>
<td>Baseline</td>
<td>123.4 ± 9.9</td>
<td>122.9 ± 9.3</td>
</tr>
<tr>
<td>Week 20</td>
<td>20.4 ± 4.2</td>
<td>20.2 ± 3.9</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>22.9 ± 4.6</td>
<td>22.7 ± 4.0</td>
</tr>
<tr>
<td>Baseline</td>
<td>13.8 ± 1.1</td>
<td>13.8 ± 1.1</td>
</tr>
<tr>
<td>Week 20</td>
<td>14.8 ± 1.1</td>
<td>14.9 ± 1.0</td>
</tr>
<tr>
<td>Height-for-age z score</td>
<td>−1.3 ± 1.1</td>
<td>−1.3 ± 1.2</td>
</tr>
<tr>
<td>Baseline</td>
<td>−1.2 ± 1.1</td>
<td>−1.3 ± 1.1</td>
</tr>
<tr>
<td>Week 20</td>
<td>−1.8 ± 1.0</td>
<td>−1.8 ± 1.0</td>
</tr>
<tr>
<td>Stunted, height-for-age z score &lt; −2 SD, %</td>
<td>21.8 (16.9, 26.7)</td>
<td>20.0 (11.7, 28.3)</td>
</tr>
<tr>
<td>Baseline</td>
<td>18.1 (13.3, 22.9)</td>
<td>16.3 (8.2, 24.3)</td>
</tr>
<tr>
<td>Weight-for-age z score</td>
<td>−1.3 ± 1.0</td>
<td>−1.2 ± 1.0</td>
</tr>
<tr>
<td>Baseline</td>
<td>39.8 (33.4, 46.3)</td>
<td>41.1 (29.8, 52.4)</td>
</tr>
<tr>
<td>Week 20</td>
<td>19.6 (14.1, 25.1)</td>
<td>15.2 (6.5, 23.8)</td>
</tr>
</tbody>
</table>

¹Values are means ± SDs or prevalences (95% CIs). Continuous data were analyzed by using a mixed-effects model, and frequencies were analyzed with a logistic regression mixed-effects model. Filter, nonfortified filtered water; Pump, nonfortified nonfiltered water; Zn+filter, zinc-fortified filtered water.

²Controlled for age and weight.

³Controlled for height and α1-acid glycoprotein.

⁴Controlled for sex, age, and α1-acid glycoprotein.

⁵Controlled for weight.
The differences in PZn between groups in this study were due to a decrease in PZn in the nonzinc arms compared with the zinc arm, in which PZn was maintained at baseline concentrations throughout the intervention. This pattern was likely due to seasonal variation in systemic infections, such as diarrheal diseases and malaria, which typically peak in Subsaharan Africa after transition from the dry to the rainy season (47). Our study was initiated during the dry season, but the intervention coincided with the onset of the main rainy season in northern Benin, which lasted beyond the end of the intervention (Supplemental Figure 1). During the study, we observed a significant increase in the inflammation marker CRP and in the number of walk-in visits to health centers, which reflected an increased occurrence of infection and an increased demand for medical care. Infections, which produce an acute phase response that causes PZn to decrease (29), may have thus reduced PZn in the nonzinc arms, whereas PZn was maintained in the zinc arm and resulted in a highly significant time-by-treatment effect (Table 3 and Figure 2). To adjust for this seasonal effect on PZn, we integrated CRP data as a covariate in the MEM analysis, which improved the model’s fit significantly. This pattern was also visible in the prevalence of zinc deficiency.

Although group allocation was randomized, a significantly higher prevalence of zinc deficiency at baseline was found in the Pump group than in the other 2 groups. Although MEM analysis took into account baseline differences, there may have been residual confounding due to regression to the mean (48). Therefore, children in the Pump group, although they did not receive zinc, might have experienced a greater increase in PZn simply because of a stronger regression to the mean. In fact, the negative correlation of ΔPZn as a function of initial PZn had a slightly steeper slope in the Pump group (−0.565) than in the Zn+filter (−0.492) and the Filter (−0.557) groups. This may, at least partly, explain why the effect of the intervention was less strong in the comparison of the Zn+filter and Pump arms than in the comparison of the Zn+filter and Filter arms.

Previous studies using the LSF reported decreases in diarrhea rates (18, 19), but we did not detect a treatment effect on diarrhea prevalence or growth. However, our study was not powered to detect an effect on these outcomes, and the study duration of 4 mo was likely too short. Also, we recorded self-reported episodes of diarrhea by privately interviewing study children, who may have been too young to provide reliable answers. We also interviewed mothers and caretakers, but children spent most of the day at school and thus may have had symptoms that the mothers did not see.

This was the first time, to our knowledge, that an SRSS technique combined with a MEM analysis has been applied in a zinc fortification efficacy trial. This approach has several advantages: 1) outcome evaluation over the entire study duration by implementation of a sparse midpoint with visualization of kinetic curves; 2) consideration and quantification of the variability generated by confounders that often occur in clinical studies (e.g., subject-specific sampling intervals, subject-specific dosages, and unbalanced or incomplete observations); 3) increased flexibility in sampling schedule with the possibility of rescheduling individuals missing a sampling time point, which may decrease dropouts and increase compliance; and 4) potential reduction of the sample size needed to assess the efficacy of the intervention, by equal statistical power. In this respect, to compare conventional designs (i.e., assessment of 2 time points) with ours (i.e., assessment of 3 time points), we performed an ANCOVA (for between-group variability) controlled for initial PZn by using baseline and endpoint data from our complete data set and on reduced data sets created by performing a 100-fold randomized selection of 90% and 80% observations from the original dataset (Supplemental Table 1 and Supplemental Figure 2). In our simulation, MEM analysis was more sensitive at detecting the intervention effect than was ANCOVA, largely as a result of integration into the model of values from the sparse midpoint, which showed strong time-dependent effects not considered by ANCOVA. We feel that the SRSS technique combined with a MEM analysis is a promising new approach for the evaluation of micronutrient efficacy to increase statistical sensitivity and/or reduce sample size.

In summary, the strengths of our study include its double-blind RCT design, the direct supervision of each child during water consumption to assess compliance, rigorous procedures to avoid exogenous and endogenous zinc contamination of plasma samples for the assessment of plasma zinc concentration, and application of the SRSS technique with MEM data analysis. The duration of intervention, the high bioavailability of aqueous zinc, and the fact that feeding was carried out reasonably away from main meals likely contributed to its success. Limitations include a seasonal effect that likely contributed to the lack of an absolute increase in PZn in the Zn+filter group, group differences in baseline prevalence of zinc deficiency that possibly confounded arms comparison, and difficulty in collecting reliable diarrhea self-reports. Future large community-based trials are needed to confirm our findings, evaluate whether combined water purification and zinc fortification have a synergistic effect on PZn and other functional outcomes, and evaluate the effectiveness and acceptability of the LSF.

We thank Fayçal Sanoussi, Sadia Akondo, Prosper N’Tchagaba, Gaspard Kouagou, Julien Madoske, Patrice Nata, Dieudonné Yeropa, Diane Seda and Annalena Timmer for their help in the field, Natasa Mihajlovic for the PZn analysis, Adam Krzyzek for the zinc isotopic measurements, Jürgen Erhardt for the protein analysis, and Luciano Molinari for statistical advice.

The authors’ responsibilities were as follows—VG, PK, DM, and MBZ: designed the research; VG, PK, and CZ: conducted the absorption trial; F Tay, DM, and MBZ: supervised the absorption trial; F Tong, Fossou, and JDH: supervised the efficacy trial; VG, DM, and MBZ: wrote the manuscript; and all authors: read and approved the final manuscript. The funder provided the LSF for both studies and had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the authors declared any competing interests.

REFERENCES

15. de Almeida CA, De Mello ED, Ramos AP, Joao CA, Joao CR, Dutra-de-Oliveira JE. Assessment of drinking water fortification with iron plus ascorbic acid or ascorbic acid alone in daycare centers as a strategy to control iron-deficiency anemia and iron deficiency: a randomized blind clinical study. J Trop Pediatr 2014;60:40–6.
41. Vraci SI. Model selection and psychological theory: a discussion of the differences between the Akaikes information criterion (AIC) and the Bayesian information criterion (BIC). Psychol Methods 2012;17:228–43.
46. Vraci SI. Model selection and psychological theory: a discussion of the differences between the Akaikes information criterion (AIC) and the Bayesian information criterion (BIC). Psychol Methods 2012;17:228–43.