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Biofortification of Rice with Zinc: Assessment of the Relative Bioavailability of Zinc in a Caco-2 Cell Model and Suckling Rat Pups

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ABSTRACT: Staple foods, such as rice, can now be enriched in micronutrients through conventional breeding (i.e., biofortification) to enhance dietary intake of vulnerable populations. The objectives of this study were (1) to establish a rapid, high capacity Caco-2 cell model to determine the relative bioavailability of zinc (Zn) from samples of staple food breeding lines for potential use as a guideline for selection/breeding and (2) to determine the relative bioavailability of Zn from conventional rice varieties and one Zn-biofortified type. Polished or undermilled, parboiled rice samples were digested in vitro with pepsin and pH adjustment, and by pancreatic enzymes. Zn uptake from digested samples was measured in Caco-2 cells in culture. A previously validated rat pup model was also used to assess Zn absorption in vivo, using gastric intubation and ^{65}Zn labeling. Pups were killed after 6 h, and radioactivity in tissues and in small intestine perfusate and cecum-colon contents was used to measure Zn bioavailability. A biofortified rice variety contained substantially more Zn than conventional varieties, with no change in phytate content. Absorbed Zn (μg/g rice) was significantly higher from the new variety in both the in vitro Caco-2 cell model (2.1-fold) and the rat pup model (2.0-fold). Results from the two models were highly correlated, particularly for the polished samples. Biofortification of rice with Zn results in significantly increased Zn uptake in both models. Since results from the Caco-2 cell model correlated well with those from rat pups, this cell model is likely to predict results in human populations and can be used for screening purposes.

KEYWORDS: *biofortification, Caco-2 cells, rat pups, zinc, zinc bioavailability*

INTRODUCTION

Zinc (Zn) deficiency is common in developing countries and is attributed to low intake of Zn combined with low bioavailability due to the presence of phytate, an inhibitor of Zn absorption, in major staple foods such as rice, legumes, and cereals.[1,2] Zn deficiency can cause stunting in children, impair immune function, and adversely affect pregnancy outcome in women.[1,3] The recent Lancet series on maternal and child undernutrition concluded that Zn deficiency is responsible for ~4% of the worldwide morbidity and mortality of young children.[4] Thus, there is a strong need for increasing the intake of bioavailable forms of Zn in many populations.[5]

Various strategies have been proposed to prevent Zn deficiency in at-risk populations. Zn supplementation is one approach, but the infrastructure required for successful implementation is often lacking, and compliance may be poor. Fortification of foods commonly consumed in the target population is also used, but this requires centralized processing facilities and that the taste of the foods consumed is not adversely affected. Food diversification and changes in food preparation methods may also be used to improve the intake and bioavailability of micronutrients, but may cause problems with acceptability. Another approach is to use biofortification, i.e., improving the content of micronutrients in staple foods, to enhance the total intake of micronutrients.[5−7]

New varieties of rice with increased contents of Zn are now being developed using conventional breeding techniques. The trait for high grain Zn content can be back-crossed into local varieties, and adapted for regional conditions, e.g., soil type, fertilizer use, and water availability.[8] Once such varieties have been developed and found to have increased Zn contents, it becomes important to evaluate whether the additional Zn content is at least equally bioavailable to the native Zn content. This can be done using Zn absorption studies in humans and/or long-term feeding studies in the appropriate population. However, such studies are expensive and lengthy, and hence a very limited number of new varieties can be investigated by such methods. Rapid and inexpensive methods for assessing Zn bioavailability from a larger number of potential breeding lines are therefore needed.

We have previously developed a suckling rat pup model to study Zn absorption from various diets,[9,10] and we have also developed an in vitro Caco-2 cell model to assess Zn uptake.[11] While the former method allows a number of varieties to be compared, the cell model is less work intensive and quicker and allows a large number of breeding lines to be compared in a single experiment. In this study, we used both models to evaluate Zn bioavailability from a recently developed high-Zn variety and four conventional varieties that are widely cultivated in Bangladesh. If results from the two methods are well correlated, the in vitro cell model may be valid to use for preliminary assessment of Zn bioavailability in humans, as results from the rat pup model have been shown to correlate well with Zn absorption studies in human adults.[9,12]

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MATERIALS AND METHODS

Establishment of the Caco-2 Cell Model. Caco-2 cells (American Type Culture Collection, Manassas, VA) were used between the 35th and 45th passages and cultured in minimal essential medium (GIBCO, Gaithersburg, MD) containing 10% fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin and streptomycin (10 units/mL and 1 mg/mL, respectively) at 37 °C with constant humidity in a 5% CO_2−95% air atmosphere. Cells were seeded into 24-well plates at a density of 2.5×10^5 cells/well, and all experiments were conducted at postconfluence (day 14 after seeding) allowing for proper tight-junction formation.[13] To determine the effects of Zn and phytate content on Zn uptake, two Zn concentrations (1 μM or 5 μM of Zn as $ZnSO_4$, Sigma) and two phytate concentrations (0 μM or 10 μM of sodium phytate, Sigma) were used to treat Caco-2 cells in radiolabeled serum free medium (0.1 μCi of $^{65}ZnCl_2$/well), and the radiolabeled medium was equilibrated with test solutions for 16 h. On the day of the experiment, postconfluent cells were washed with PBS and treated with radiolabeled test solutions for 1 h at 37 °C. The medium was aspirated, and cells were extensively washed with ice-cold PBS containing 1 mM EDTA and then solubilized with 1 N NaOH. Radioactivity associated with the cell fraction and medium was measured in a gamma counter (Gamma 8500, Beckman, Irvine, CA). Cellular ^{65}Zn uptake was determined by quantifying radioactivity in the cell fraction in a gamma scintillation counter.

In Vitro Digestion. Test rice varieties were subjected to in vitro digestion, which was a modification of the procedure originally developed by Rudloff and Lönnerdal.[14] One gram of rice powder was prepared at a concentration of 0.1 g/mL in deionized water (95 °C). The pH of the solution was lowered to either 4.0 or 2.0 with 1 N HCl. Upon acidification, 2% pepsin (porcine, 4,200 U/mg, Sigma) in 0.01 N HCl was added at a 1:12.5 ratio of pepsin to protein, and the mixture was incubated in the dark with shaking at 140 rpm for 30 min to simulate gastric digestion. To simulate intestinal digestion, the pH was adjusted to 7.0 with 1 N $NaHCO_3$, followed by addition of 0.4% pancreatin (porcine, 8 × USP, Sigma) at a 1:62.5 ratio to protein. Samples were incubated in the dark with shaking at 140 rpm for 60 min, followed by immediate storage at −20 °C to quench digestive activity. Two different pHs were used for the pepsin digestion as previous research indicated a low pH (pH 2) in the stomach of adults, while more recent studies suggest that pH 4 may more accurately reflect the pH of ingested food throughout the gastric phase of digestion.[15,16]

Optimization of the Caco-2 Cell Model. Two time points (30 and 60 min) were used for gastric and intestinal digestion, respectively, and this was optimized after evaluation of completeness of digestion by polyacrylamide gel electrophoresis (PAGE). Incubation for 60 min was found to result in virtually complete digestion at both pH 2 and pH 4 (clear solutions) and was thus used for the experiments (Figure 1).

To evaluate potential toxicity of the digested rice samples in Caco-2 cells, the viability of postconfluent cells was determined using an assay based on the ability of living cells to convert dissolved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan. After treatment with different dilution ratios of rice solutions and serum-free medium (1:1. 1:3, and 1:9) for 24 h, cell viability was assessed using the MTT assay (Roche Applied Science, Germany) following the manufacturer's protocol.

To determine the effects of incubation time on Zn uptake by Caco-2 cells, postconfluent cells were incubated with the same samples for 1, 4, 6, 8, or 24 h, and Zn uptake was analyzed.

Composition of Rice Samples and Diet Preparation. Five rice varieties were tested for relative Zn bioavailability when prepared in a manner likely to be used by the target population in Bangladesh. Four of the varieties represented some of the most highly produced varieties in Bangladesh (i.e., BR-28, BR-29, BR-11, and Paijam), and one represented a Zn-biofortified line (IR-68-1-44) developed at the International Rice Research Institute (Los Baños, Philippines). All rice samples were produced and handled by the Bangladesh Rice Research Institute (BRRI) in Gazipur, Bangladesh. Samples of each variety were hand-harvested, parboiled, dried, dehulled, and then either polished or undermilled using a trace element-free test mill. Although most commercial white rice is polished, in rural areas rice is more likely to be undermilled. Further, the comparison between highly polished and undermilled rice helps to distinguish whether any potential modifiers of Zn absorption are located in the endosperm or the aleurone layer. Samples were ground to powder and sent to UC Davis. Rice samples were prepared under trace element-free conditions to avoid contaminant sources of Zn.

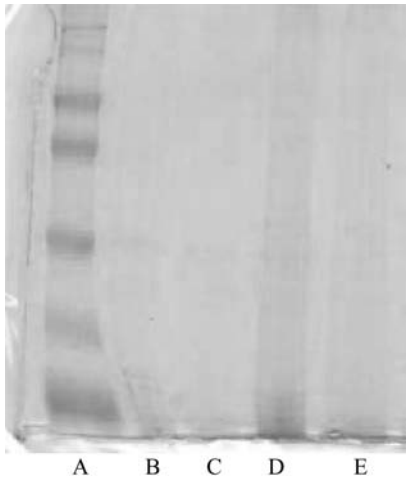

Figure 1. Completeness of different in vitro digestion conditions was evaluated by polyacrylamide gel electrophoresis (PAGE). Incubation for 60 min was found to result in virtually complete digestion at both pH 2 and pH 4 and was thus used for the experiments. A: original (undigested) sample. B: 30 min, pH 2. C: 60 min, pH 2. D: 30 min, pH 4. E: 60 min, pH 4.

The Zn, Fe, Mg, Mn, and Ca content of rice samples was determined by inductively coupled plasma atomic emission spectrometry (ARL 3580B, ARL, Ecublens, Switzerland) following nitric/perchloric acid digestion[17] at Waite Analytical Services, University of Adelaide, Australia. Crude protein content was determined by a micro-Kjeldahl method at BRRI, and phytate in the form of *myo*-inositol hexaphosphate (IP-6) was determined by Dionex liquid chromatography (Dionex Corporation, Sunnyvale, CA, USA) at the School of Biological Sciences, Flinders University, Adelaide, Australia. Nutrient composition of the samples is shown in Table 1.

Test Solutions. To determine effects of phytate content of the rice samples on Zn uptake, phytate solutions at various concentrations (0, 87.5, 175, 350, 525, 875, 1400, and 1750 μM, Sigma) were prepared and Zn ($ZnSO_4$, Sigma), Fe ($FeSO_4$, Sigma), Mn ($MnCl_2$, Sigma), Ca ($CaSO_4$, Sigma), Mg ($MgSO_4$, Sigma), and BSA (bovine serum albumin, Sigma) were added. All test solutions contained 35 μM Zn, 25 μM Fe, 40 μM Mn, 220 μM Ca, 4.6 mM Mg, and 8 g of BSA/L, concentrations that were designed based on the mean values of the rice varieties tested. Phytate/Zn molar ratios were 0, 2.5:1, 5:1, 10:1, 20:1, 30:1, 40:1, and 50:1 at each phytate concentration tested.

Rat Pup Model. The rat study was approved by Animal Research Services at the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Sprague−Dawley rats with litters of 10−12 pups were obtained commercially (Charles River, Wilmington, MA). Rats were maintained in polycarbonate cages with wood shavings in a temperature controlled facility with 12 h dark:light cycle and allowed to consume purified, deionized water and standard rat chow (Ralston Purina, St. Louis, MO) ad libitum. Rats were acclimatized to their environment three days before the day of the experiment. On day 20 postpartum, litters were separated from their dams for 6 h before gastric intubation, and randomly assigned to different rice groups (n = 10/group). Rice samples were prepared and radiolabeled 16 h prior to intubation. We have previously shown that extrinsic zinc (^{65}Zn) exchanges with intrinsic (cold) zinc in both milk and plant materials during in vitro digestion.[18] Pups were intubated with 0.5 mL of radiolabeled diet (0.1 μCi of ^{65}Zn/pup). After 6 h intubation, pups

Table 1. Composition of Rice Varieties[a]

processing	variety	protein (%)	Zn (mg/kg)	Phy[b] (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Phy/Zn (molar ratio)
undermilled	BR-29	5.91	21.9	10289	14	28.0	111	1235	46.2
	BR-28	7.20	21.2	11186	14	28.8	108	1300	51.9
	BR-11	7.39	17.9	8141	12	22.1	80	1130	44.6
	IR68144	11.87	42.5	12932	21	30.5	131	1465	29.9
	Paijam	8.05	20.2	10174	16	23.6	98	1455	49.4
polished	BR-29	5.88	18.8	7987	13	20.7	82	885	41.7
	BR-28	6.91	18.1	7337	11	20.5	77	895	39.8
	BR-11	7.29	14.5	5098	10	15.3	53	780	34.5
	IR68144	11.83	35.5	6907	15	20.1	78	875	19.2
	Paijam	7.80	17.6	8204	12	18.2	75	1060	45.8

[a]Values are means, n = 2 or 3. Dry weight basis. [b]Phytate.

were killed, and tissues were collected. The small intestine was perfused with 2 × 3 mL of saline, and the perfusate was collected. Radioactivity in stomach, perfused intestine, perfusate, liver, carcass, and cecum-colon was measured by gamma counting (Gamma 8500, Beckman, Irvine, CA). Absorbed Zn was expressed as fractional absorption and calculated as radioactivity in carcass, liver, kidney, and perfused small intestine as a percentage of total recovery.

Caco-2 Cell Model. Digested rice samples were prepared, diluted with medium (1:1), and radiolabeled 16 h prior to uptake experiments. On the day of the experiment, postconfluent Caco-2 cells were washed with PBS and treated with digested test diets radiolabeled with a tracer dose of ^{65}Zn (0.1 μCi of $^{65}ZnCl_2$/well) for 6 h at 37 °C. The medium was aspirated, and cells were extensively washed with ice-cold PBS containing 1 mM EDTA and then solubilized with 1 N NaOH. Radioactivity associated with the cell fraction and medium was measured in the gamma counter. Cell protein was assessed using the Bradford assay. Data are expressed as μmol of Zn accumulated/g of cell protein.

Four rice samples (undermilled and polished BR-29, Paijam) were used to evaluate Zn uptake at different concentrations of Zn. Rice solutions (0.1 g/mL) were incubated with 0-, 1-, 2-, and 100-fold the initial Zn concentration of the rice samples and the same amount of ^{65}Zn (0.1 μCi) for 16 h (for equilibration). The next day, Zn uptake assay was performed in triplicate using 24-well plates with Caco-2 cells cultured for 14 days. Cells were incubated with rice mixtures for 6 h in a 37 °C, 5% CO_2 cell incubator. Then, rice mixture medium was removed. Cells were washed 3 times with ice-cold PBS containing 1 mM EDTA and harvested with 1 M NaOH. Cell and rice mixture medium radioactivity was counted in the gamma counter. Data are indicated as nmol of Zn.

Statistics. Statistical comparisons between rice groups were analyzed by one-way ANOVA and post-tested by Tukey test using Prism 3.0 (GraphPad Software, San Diego, CA). Data are presented as means ± SD, and significant effects of diet were determined at $p < 0.05$.

RESULTS

Effects of Zn and Phytate Contents in Solutions on Zn Uptake by Caco-2 Cells. Zn uptake by Caco-2 cells increased with addition of high concentration of Zn (HZ, 5 μM; 1.7-fold of LZ) compared to low Zn concentration (LZ, 1 μM), and this trend was not affected by the addition of phytate in the solution (1.9-fold of LZ + Phy; Figure 2). Phytate (10 μM) significantly decreased Zn uptake (~20% of relative controls) by Caco-2 cells at both high and low Zn conditions.

Composition of the Rice Varieties Studied. The Zn content of the conventional Bangladeshi rice varieties used in this study varied between 14 and 19 mg/kg and was within the expected range for polished rice, i.e., ~16 mg/kg in polished rice,[19] whereas the Zn biofortified variety from IRRI contained 35 mg/kg of Zn in polished form (Table 1). Although the Zn content of the conventional varieties was within the normal range, the contents of other minerals were generally higher than

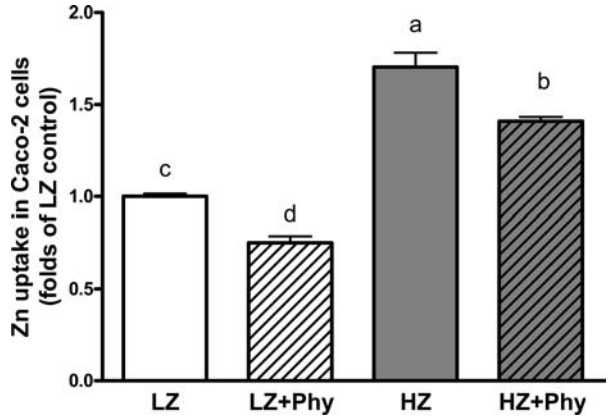

Figure 2. Effects of Zn and phytate content on Zn uptake in Caco-2 cells. Zn uptake by Caco-2 cells increased with addition of high concentration of Zn (HZ, 5 μM) compared to low Zn concentration (LZ, 1 μM). This trend was not affected by the addition of phytate (Phy) (10 μM) in the solution, but phytate significantly decreased Zn uptake at both high and low Zn conditions. Values are means ± SD, n = 3.

has been reported for rice. This is at least in part attributable to the parboiling process as soluble nutrients are believed to pass from the husk into the endosperm. It is unlikely to be due to contamination from the external environment because verified, trace-element free precautions were used throughout sample preparation and the aluminum (Al) content of the samples was not elevated (i.e., >5−10 μg/g); Al content is a useful indicator of trace element contamination from soil and dust in grains because Al is not absorbed by plants.[20]

The crude protein content of the conventional Bangladeshi rice varieties (6−8%) was similar to that reported for Philippine rice varieties,[21] whereas the new variety was higher in protein (11.8%). Phytate in the Zn biofortified variety (6.9 mg/g) was similar to that in the conventional varieties (5−8 mg/g). The phytate content, however, was higher than that reported elsewhere for nonparboiled rice (e.g., China, 0.55−1.85 mg/g;[22] Thailand, 0.41−2.34 mg/g[23]). The phytate content of the same polished rice samples without parboiling indicated that mean phytate content was 3.58 ± 1.81 mg/g, which is closer to, but still higher than, the ranges noted for other studies above. Phytate content is known to vary substantially with variety, location, growing conditions, and phosphorus content of the soil, and it is possible that the conditions on which the rice for this study were grown favored the uptake and incorporation of phosphorus into grain phytate.[24] The substantial increase in Zn content resulted in a lower phytate:Zn molar ratio in the biofortified rice variety (19:1) as compared to the conventional varieties (35 to 46:1).

Optimization of Zn Uptake in the Caco-2 Cell Model. To determine if the digested rice samples affected viability of the Caco-2 cells, the MTT assay was used to determine potential toxicity. There were no significant differences in cell viability for treatments with different concentrations of digested rice samples (data not shown). Moreover, increased Zn uptake was found with increasing incubation time. Zn uptake by Caco-2 cells was significantly higher at 4 and 6 h of incubation as compared to 1 h of incubation (Figure 3). However, there

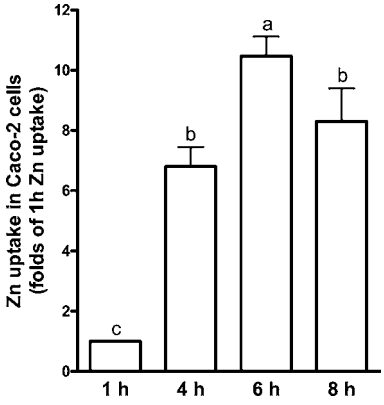

Figure 3. Effects of incubation time on Zn uptake in Caco-2 cells. Cells were incubated with rice digest for varying time points. Zn uptake was significantly higher at 4 and 6 h of incubation as compared to 1 h of incubation. As there was no significant increase at 8 h of incubation as compared to 6 h of incubation, 6 h of incubation was chosen for testing rice varieties. Values are means ± SD, n = 3−4.

was no significant increase at 8 h of incubation as compared to 6 h of incubation, suggesting that 6 h of incubation is adequate for testing rice varieties without cellular toxicity.

Zn Uptake by Caco-2 Cells. To evaluate the response in Zn uptake at various Zn concentrations, Zn was added to 4 rice samples at 1, 2, and 100 times the original concentrations. Mean Zn uptake (nmol) from undermilled BR-29 (21.9 mg of Zn/kg) was 1.0-, 1.4-, 2.1-, and 90-fold for 0-, 1-, 2-, and 100-fold concentrations of Zn, respectively. Means for undermilled Paijam (20.2 mg of Zn/kg) were 1.0-, 2.2-, 3.5-, and 118-fold, respectively. Means for polished BR-29 (18.8 mg of Zn/kg) were 1.0-, 1.3-, 2.4-, and 81-fold, respectively, and from polished Paijam (17.6 mg of Zn/kg) 1.0-, 1.7-, 2.7-, and 89-fold, respectively. This demonstrates that Zn uptake as measured by ^{65}Zn reflected Zn concentrations in the rice samples. Measurement of cell Zn uptake by AAS showed similar results (data not shown).

In the cell model, the means of radioactivity levels (percentage) present in the cell layer exposed to rice varieties (both undermilled and polished) digested at two different pHs were 3.5 ± 0.9% and 1.9 ± 0.2% for pH 2 and pH 4, respectively. Zn uptake from rice varieties in Caco-2 cells is also presented as μmol of Zn/g of cellular protein. Cellular Zn uptake from rice varieties ranged from 0.39 to 1.01 μmol of Zn/g of cellular protein at pH 2 and 0.20 to 0.53 μmol of Zn/g of cellular protein at pH 4. We thus found higher Zn uptake values for rice varieties using an in vitro digestion condition of pH 2 (Figure 4A) than at pH 4 (Figure 4B). IR68144 had the highest value among all rice varieties in both undermilled and polished forms.

Zn Absorption in Rat Pups. Six hours after intubation, the mean radioactivity (percentage) present in the tissues of pups receiving different rice varieties was 0.8, 5.4, 2.8, 10.7, 27.3, and

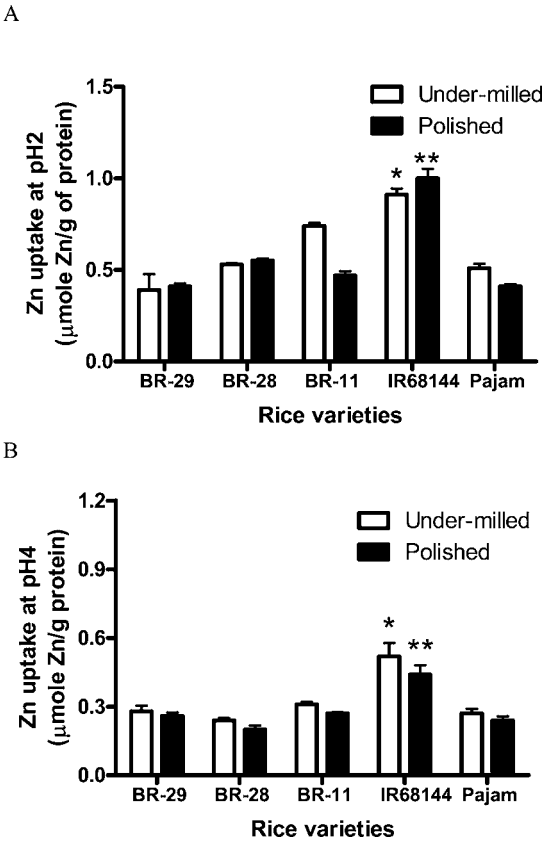

Figure 4. Amount of Zn uptake by Caco-2 cells with different conditions for in vitro digestion. Zinc uptake from rice varieties digested at pH 2 and pH 4, respectively, presented as μmol of Zn/g of cellular protein. Higher amounts of Zn were absorbed from samples digested at pH 2 (A) than at pH 4 (B).

53.0% in stomach, perfused small intestine, perfusate, liver, carcass, and cecum-colon, respectively (Table 2). This means that, after 6 h, the samples had cleared the stomach and small intestine (low numbers) and that unabsorbed Zn had reached the cecum-colon. No stool had been passed by this time. There were no significant differences between rice varieties for any single tissue (ANOVA). Perfused small intestine, liver, and carcass values were pooled to represent whole body uptake of Zn. Mean whole body uptake from the rice varieties varied from 36% to 59% (Table 3). Zn bioavailability from the rice varieties is also presented as μg of Zn per gram of rice powder. The mean amounts of bioavailable Zn from the rice varieties ranged from 5.74 to 16.78 μg Zn/g of rice, while IR68144 had the highest value of all rice varieties for both undermilled and polished forms in the rat pups (Figure 5). No significant differences of Zn bioavailability between undermilled rice varieties and polished ones were observed in this study.

Effects of Phytate Concentration on Zn Uptake in Rat Pups. Zinc uptake from aqueous solutions in the rat pup model was reduced by phytate, but there were no significant differences in Zn absorption between phytate/Zn molar ratios from 10 to 50 (Figure 6). Similar results were observed in the Caco-2 cell model, in which we found a dose-responsive decrease in Zn uptake for phytate/Zn molar ratios of 2.5, 5, 10, and 20 (Figure 6). At phytate/Zn molar ratios of 30, 40, and 50, Zn uptake was not significantly lower than at a phytate/Zn molar ratio of 20. Two-way ANOVA showed no interaction

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Table 2. Tissue Distribution of ^{65}Zn in Rat Pups Postabsorption[a]

processing	variety	stomach (%)	perfusate (%)	perfused small intestine (%)	cecum-colon (%)	liver (%)	carcass (%)
undermilled	BR-29	0.7 ± 0.2	1.9 ± 1.0	5.8 ± 2.0	54.2 ± 17.2	9.8 ± 3.7	27.6 ± 11.7
	BR-28	0.7 ± 0.1	2.7 ± 0.5	5.4 ± 0.9	50.1 ± 9.4	11.6 ± 2.2	29.5 ± 6.5
	BR-11	0.6 ± 0.2	1.9 ± 1.0	4.3 ± 1.1	61.6 ± 12.4	9.3 ± 2.7	22.7 ± 8.4
	IR68144	0.8 ± 0.3	3.7 ± 2.7	5.2 ± 1.1	56.1 ± 10.1	9.7 ± 2.9	24.5 ± 6.3
	Paijam	0.8 ± 0.2	2.6 ± 1.3	5.3 ± 1.4	53.6 ± 13.0	10.7 ± 2.9	26.9 ± 8.6
polished	BR-29	0.8 ± 0.2	2.7 ± 0.8	5.8 ± 1.5	50.2 ± 6.7	12.0 ± 2.1	28.5 ± 3.1
	BR-28	1.2 ± 0.6	3.1 ± 1.1	7.4 ± 1.9	36.6 ± 14.2	14.7 ± 3.7	37.1 ± 9.3
	BR-11	0.7 ± 0.2	2.9 ± 0.7	4.9 ± 2.0	56.9 ± 15.8	9.7 ± 3.9	24.8 ± 9.6
	IR68144	0.7 ± 0.2	3.2 ± 2.1	4.8 ± 1.3	54.3 ± 11.7	10.6 ± 7.5	26.5 ± 7.5
	Paijam	0.8 ± 0.2	3.2 ± 1.6	5.4 ± 2.2	51.1 ± 16.6	10.8 ± 3.8	28.7 ± 10.3

[a]Values are means ± SD, n = 6−10.

Table 3. Total Zn Absorbed by Rat Pups[a]

		PD14 pups		PD20 pups	
processing	variety	%	μg of Zn/g of rice	%	μg of Zn/g of rice
undermilled	BR-29	86 ± 15	18.8 ± 3.31 b	43 ± 17	9.4 ± 3.65 b
	BR-28	82 ± 14	17.4 ± 2.95 bc	47 ± 9	9.8 ± 1.95 b
	BR-11	62 ± 6	11.2 ± 1.00 d	36 ± 12	6.5 ± 2.06 b
	IR68144	64 ± 11	27.2 ± 4.61 a	40 ± 10	16.7 ± 4.18 a
	Paijam	63 ± 20	12.7 ± 4.03 cd	43 ± 12	8.7 ± 2.46 b
polished	BR-29	88 ± 12 a	17.4 ± 0.61 b	46 ± 6	8.7 ± 1.16 bc
	BR-28	63 ± 21 ab	11.5 ± 3.85 bc	59 ± 14	10.7 ± 2.48 b
	BR-11	69 ± 16 ab	10.0 ± 2.35 c	40 ± 15	5.7 ± 2.23 c
	IR68144	72 ± 26 ab	25.6 ± 9.06 a	42 ± 11	14.8 ± 3.74 a
	Paijam	59 ± 9 b	10.3 ± 1.61 c	45 ± 16	7.9 ± 2.81 bc

[a]Values are means ± SD, n = 6−10. Values with different letters a−d differ, $P < 0.05$.

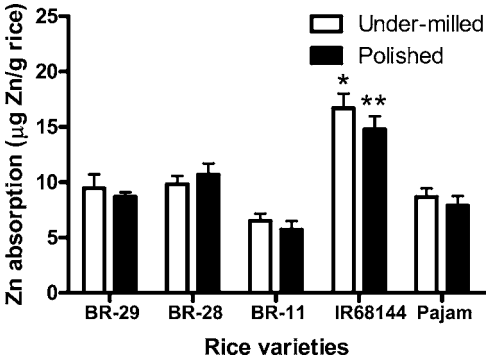

Figure 5. Amount of Zn absorbed in rat pups at day 20. Pups were intubated with radiolabeled solutions of the rice varieties and killed 6 h after intubation. Absorbed Zn was assessed by taking the sum of perfused small intestine, liver, and carcass, then converted into amounts of Zn absorbed in picomoles.

between the data sets from the rat and cell models, meaning that the curves are parallel and thus yield similar results.

Correlation between Zn Absorption in Rat Pups and Zn Uptake by Caco-2 Cells. Zn absorption in rat pups and Zn uptake in Caco-2 cells using an in vitro digestion condition of pH 2 were positively correlated for all rice varieties (Figure 7; r = 0.53, $p < 0.05$). While polished rice samples showed a significant correlation (r = 0.79, $p < 0.05$), no significant correlation was found for undermilled rice samples (r = 0.32, p = 0.32). Although no significant linear relationship was detected between Zn absorption in rat pups and Zn uptake in Caco-2 cells using an in vitro digestion condition of pH 4 for polished rice samples (Figure 7; r = 0.65, p = 0.10), there was a significant relationship when data from both undermilled and polished rice samples were pooled (r = 0.72, $P < 0.005$). Zn absorption in rat pups and Zn uptake in Caco-2 cells using an in vitro digestion condition of pH 4 in undermilled rice samples were positively correlated (Figure 7; r = 0.78, $p < 0.05$).

DISCUSSION

These results confirm that the additional Zn present in the biofortified rice variety was equally biologically available for cellular uptake or absorption of Zn from conventional Bangladeshi rice varieties. For undermilled and polished rice, the percent Zn absorption in rat pups was 39% and 42%, respectively. This fell within the ranges observed for the conventional varieties, which were 36−43% and 39−46% for undermilled and polished rice samples, respectively. The percent uptake of Zn from undermilled and polished rice from the Zn biofortified rice (1.80 and 1.97%, respectively) by Caco-2 cells was also within the range of that determined for the conventional rice varieties (1.51−2.25% and 1.61−2.25%, respectively). As a result, the net amount of Zn taken up by Caco-2 cells or absorbed by the rat pups was twice as high as that from the conventional varieties and thus proportional to the net Zn content of the rice. Thus, the major conclusions derived from both of these models were similar.

In our study, we used extrinsic labeling with ^{65}Zn. It has been shown previously that extrinsic ^{65}Zn equilibrates with intrinsic Zn in complex food matrices of animal and plant origin after in vitro digestion.[9,18] In addition, extrinsic labeling of foods has resulted in results similar to those obtained from the same food intrinsically labeled with Zn in both animal and human studies.[25,26] Further, Zn absorption studies using ^{65}Zn have been shown to yield results similar to those obtained by bone Zn uptake.[19] As mentioned earlier, we have used the Caco-2 cell model successfully for

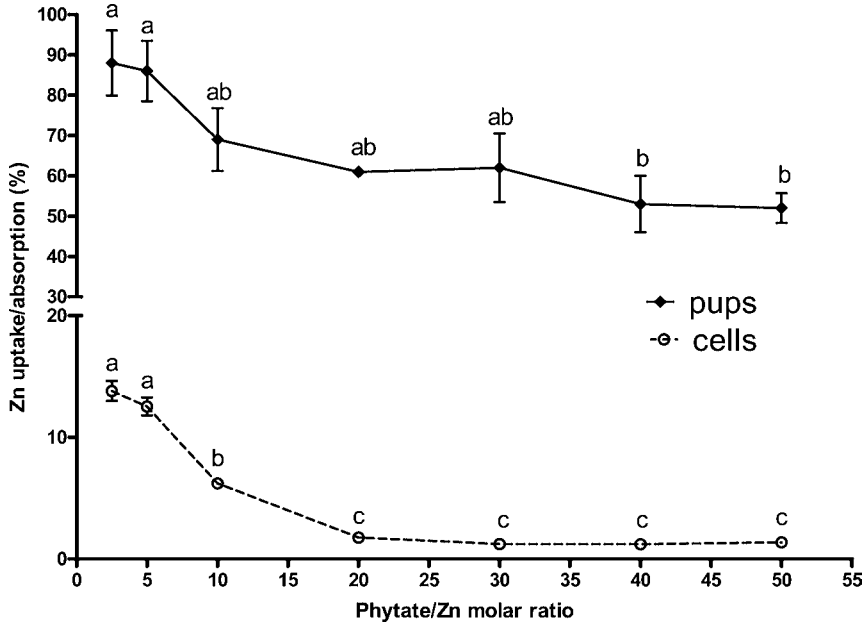

Figure 6. Effects of phytate on Zn absorption in rat pups and Zn uptake in Caco-2 cells. Pups were intubated with radiolabeled solutions with different phytate/Zn molar ratios and killed 6 h after intubation. Zn absorption was measured as described in Figure 5. Caco-2 cells were incubated with the same solutions, and Zn uptake was assessed after 6 h. The two curves were found to be very similar. Values are means ± SD, n = 3−6.

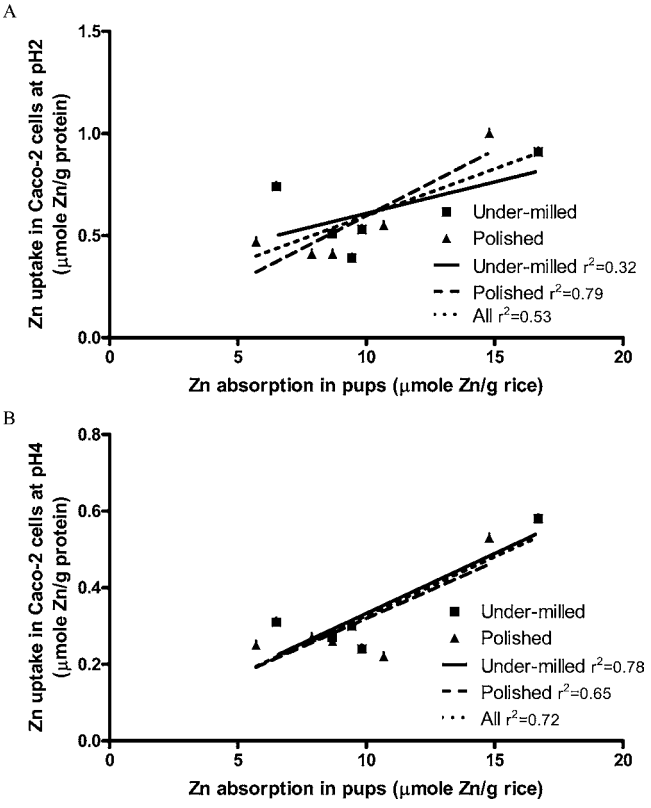

Figure 7. Correlation between Zn absorption in rat pups and Zn uptake in Caco-2 cells using different conditions for in vitro digestion. Results from the in vivo rat pup model were plotted against results from in vitro digestion followed by Caco-2 cell uptake. A: digestion at pH 2. B: digestion at pH 4.

assessing Zn uptake from casein phosphopeptides in the presence and absence of phytate.[11] It has also been used by Han et al.[27] and Kim et al.[28] to assess Zn uptake from inositol phosphates and polyphenols, respectively, and by Etcheverry et al.[29] to study the effect of human milk and infant formula on Zn bioavailability. Further, Sreenivasulu et al. have found that Zn uptake by Caco-2 cells predicts the direction of response to dietary ligands[30] and that metallothionein is less reliable an indicator as its expression is affected not only by Zn but also by other dietary components.[31]

Zn absorption has been shown to be affected not only by dietary Zn content but also by dietary factors such as phytate, casein, amino acids, iron, cadmium, and other low-molecular-weight ions.[2] Although the amount of Zn in a meal is inversely correlated with fractional Zn absorption (%), net Zn absorption is positively affected by dietary Zn content itself. Intestinal perfusion studies in humans have shown linear increases in Zn absorption with solutions containing increasing Zn concentrations.[32] The major finding in our study was that the higher amount of Zn in the biofortified rice resulted in larger net absorption of Zn in the rat pups, suggesting that the amount of total Zn is the major dietary factor determining Zn absorption from these rice varieties. This is supported by a study by Hunt et al.,[20] who assessed the bioavailability of Zn from rice by growth, bone Zn and Zn-65 retention in Zn deficient rats. However, they used adult rats, which have intestinal phytase activity, whereas we used suckling rat pups which have no phytase activity. It is also supported by a study by Rosado et al.,[33] who found larger net absorption of Zn in women consuming Zn biofortified wheat.

Among those factors known to have either positive or negative effects on Zn absorption,[2] phytate is the primary inhibitory factor found in plants, and its content is negatively correlated with Zn bioavailability in rats.[34] Moreover, significantly lower Zn absorption in human adults was observed with the addition of phytate to both cow milk and soy formula.[12] However, effects of phytate on Zn absorption from the rice varieties tested were not observed in our study. In the present study, we found that phytate content was moderately elevated in the biofortified rice with increased Zn content. This increase in phytate did not affect Zn absorption, which might be due to the lack of an increase in the phytate/Zn molar ratio.

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A dose-dependent inhibitory effect of sodium phytate on Zn absorption from single test meals in humans has been found at a phytate/Zn molar ratio from 2.9 to 11.4,[35] but there were no significant differences in Zn absorption found for phytate/Zn molar ratios of 11.4, 16.0, 20.0, and 28.6. Similar results were obtained in our study, in which a dose-dependent inhibitory effect of phytate on Zn absorption was only found for phytate/Zn molar ratios below 10, and Zn absorption was not further affected by a ratio above 20. Although this dose−response effect of phytate has not been studied systematically using methods that measure Zn absorption from total diets by the dual isotope tracer ratio technique, some studies using this method suggest a diminished inhibitory effect of phytate at higher phytate:Zn molar ratios compared to lower ratios. For example, in a study of Zn absorption from low phytate maize,[36] fractional absorption of Zn was not significantly different between study diets with a phytate:Zn molar ratio of 28 and 37 (13.5% and 15.1%, respectively) but was significantly higher with diets with phytate:Zn molar ratios of 7 or 17 (38.3% and 28.5%, respectively). Further, a trivariate predictive model of Zn absorption based on dietary Zn and phytate content also suggests a much smaller inhibitory effect occurring between phytate:Zn molar ratios of 10 and 20 than between 0 and 10.[37]

Since the phytate/Zn molar ratios of our rice varieties ranged from 19 to 52, an effect of phytate content on Zn absorption was not observed in our study, and this is consistent with observations from the human study using labeled test meals.[35] Zn is not the only cation bound by phytate, and other divalent cations in the rice samples may have a potentiating effect on the formation of phytate−mineral complex. Furthermore, the lack of association between phytate and Zn absorption from our rice samples suggests that the phytate effect seemed to reach a plateau at lower phytate/Zn ratios than found in the rice samples. Our data suggest that the inhibitory effect of phytate on Zn absorption from the rice varieties was maximal; it is possible that the inhibitory effect is neutralized with the presence of other divalent cations, including Zn.

Furthermore, studies conducted in human adults have shown that not only the amount of total phytate but varying degrees of phosphorylation of phytate in a meal affect Zn bioavailability differently.[38] Different ester forms of phytate affect Zn absorption in the suckling rat pups differently.[10] Hexa- and pentaphosphates of inositol have strong inhibitory effects, while no significant effects are observed for tetra- and triphosphate inositol. The lack of correlation between Zn absorption and phytate content in the rice varieties might be due to differences in phytate composition and their contribution to the decrease in Zn bioavailability.

Results from the established Caco-2 cell (in vitro) model were correlated to results from the rat pup (in vivo), allowing the former to be used for screening of a larger variety of staple food varieties. However, the correlations of the animal model and cell line model were specifically observed for certain digestion conditions and different milling processes. The compositions of undermilled rice samples were different from the polished samples; not only the Zn content but higher amounts of phytate and other minerals were determined in the undermilled rice samples, and the contribution of each ester form of phytate may also vary. Similar inhibitory results of hexa- and pentaphosphates of inositol are observed in Caco-2 cells[27] as in the rat pup model,[10] but inositol tetra- and triphosphate have different effects on Zn uptake and rate of transport. This suggests that pancreatic and biliary ligands in vivo might affect the solubility of inositol phosphates, which is diminished in the cell model; intracellular hydrolysis of the higher inositol phosphates is also emphasized to be considered as a factor. Moreover, the ternary interactions between minerals, phytate, and protein are complex, and are affected by both pH and the concentration of other ions,[39] suggesting that the lack of correlation between rat and cell models at some pH conditions might be due to such interactions in the rice samples. Nonetheless, the primary results of this study are valid and the two methods indicate a similar trend across a wide range of phytate:Zn molar ratios (Figure 6).

In summary, the higher amount of Zn in the biofortified rice resulted in higher relative bioavailability of Zn and larger net absorption of Zn in the rat pups and Zn uptake by the cell model. Results from the established Caco-2 cell (in vitro) model were correlated to results from the rat pup (in vivo) allowing the Caco-2 cell method to be used for screening of a large variety of Zn biofortified staple food varieties. The validity of the Caco-2 cell model for this purpose was demonstrated by comparing the difference in bioavailability to results from the rat pup model; however, the conditions for the in vitro digestion should be considered and chosen carefully.

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Notes

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