The Journal of Nutrition
Nuclear Techniques in Nutrition Research

Higher Extrusion Temperature Induces Greater Formation of Less Digestible Type V and Retrograded Starch in Iron-Fortified Rice Grains But Does Not Affect Iron Bioavailability: Stable Isotope Studies in Young Women

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ABSTRACT

Background: Hot extrusion is widely used to produce iron-fortified rice, but heating may increase resistant starch and thereby decrease iron bioavailability. Cold-extruded iron-fortified rice may have higher bioavailability but has higher iron losses during cooking. Thus, warm extrusion could have nutritional benefits, but this has not been tested. Whether the addition of citric acid (CA) and trisodium citrate (TSC) counteracts any detrimental effect of high-extrusion temperature on iron bioavailability is unclear.

Objectives: Our aim was to assess the effects of varying processing temperatures on the starch microstructure of extruded iron-fortified rice and resulting iron solubility and iron bioavailability.

Methods: We produced extruded iron-fortified rice grains at cold, warm, and hot temperatures (40°C, 70°C, and 90°C), with and without CA/TSC at a molar ratio of iron to CA/TSC of 1:0.3:5.5. We characterized starch microstructure using small- and wide-angle X-ray scattering and differential scanning calorimetry, assessed color over 6 mo, and measured in vitro iron solubility. In standardized rice and vegetable test meals consumed by young women ($n = 22$; mean age: 23 y; geometric mean plasma ferritin: 29.3 μg/L), we measured iron absorption from the fortified rice grains intrinsically labeled with ^{57}ferric pyrophosphate (^{57}FePP), compared with ferrous sulfate ($^{58}FeSO_4$) solution added extrinsically to the meals.

Results: Warm and hot extrusion altered starch morphology from native type A to type V and increased retrograded starch. However, extrusion temperature did not significantly affect iron solubility or iron bioavailability. The geometric mean fractional iron absorption of iron from fortified rice extruded with CA/TSC (8.2%; 95% CI: 7.9%, 11.0%) was more than twice that from extruded rice without CA/TSC (3.0%; 95% CI: 2.7%, 3.4%; $P < 0.001$).

Conclusions: Higher extrusion temperatures did not affect iron bioavailability from extruded rice in young women, but co-extrusion of CA/TSC with FePP sharply increased iron absorption independently from extrusion temperature. This trial is registered at www.clinicaltrials.gov as NCT03703726. *J Nutr* 2022;00:1–8.

Keywords: iron deficiency, iron absorption, bioavailability, solubility, rice, extrusion, fortification, resistant starch, citric acid, women

Introduction

Anemia is estimated to affect almost 2 billion people globally and iron deficiency is considered its most common cause (1). Polished rice has very low native iron content, and the prevalence of iron deficiency is high among populations that consume rice as a staple food, especially in Southeast Asia and Africa (2). Therefore, fortifying rice with iron could be an effective and sustainable means of increasing iron intake and

Manuscript received September 24, 2021. Initial review completed November 22, 2021. Revision accepted December 21, 2021.
First published online December 30, 2021; doi: https://doi.org/10.1093/jn/nxab435.

reducing iron deficiency anemia in rice-consuming countries (3).

Ferric pyrophosphate (FePP) is commonly used for rice fortification as it is organoleptically inert and causes a minimal color change in extruded grains (3, 4). Co-extrusion with citric acid (CA) and trisodium citrate (TSC) increases iron bioavailability from FePP-fortified extruded rice while maintaining organoleptic acceptability (5–7). Extrusion is an effective technology to process rice flour and fortify it with iron (8–10), with extrusion temperatures typically categorized as cold (30–40°C), warm (60–80°C), or hot (70–110°C). Warm extrusion is conducted at intermediate temperature ranges and results in partial gelatinization and retrogradation processes (10) upon heating and cooling, respectively.

For nutritional purposes, starch is divided into rapidly digestible starch, slowly digestible starch, and resistant starch (11), based on its behavior when exposed to digestive enzymes. Resistant starch refers to starch and starch degradation products that resist small intestinal digestion and enter the large bowel largely intact (12). In rice, type A starch is the naturally occurring polymorphic form, which can be transformed into type V when heated and recrystallized (13). While the type A structure consists of left-handed double helices packed parallel in the crystalline lattice, the type V structure is characterized by left-handed single helices packed in an antiparallel fashion. During the heating process, type A starch swells in the water while the semi-crystalline polysaccharides become an amorphous gel, which can evolve towards a new semi-crystalline starch structure—or the so-called retrograded starch—upon cooling or aging. This retrograded starch can contain different compositions of the 2 starch forms: type A and type V. Extrusion processes increase the fraction of resistant starch (retrograded starch and starch type V polymorphism), and both are less susceptible to breakdown by pancreatic α-amylase (13–17). Iron absorption occurs mainly in the proximal duodenum (18) and the presence of slowly digestible or resistant starch might reduce iron release from extruded grains, and thereby reduce iron bioavailability. We previously reported cold-extruded fortified rice to have higher iron bioavailability compared with hot-extruded rice (8). This finding was associated with an increase in poorly water-soluble V-type fraction of starch in hot extrusion. On the other hand, cold-extruded rice has higher mineral losses during cooking and allows for lower flexibility in rice-shape manufacturing (8, 10). To our knowledge, warm extrusion has not yet been investigated for iron-fortified extruded rice and could offer some advantage over hot- and cold-extruded rice. We, therefore, aimed to assess the interaction of extrusion temperature (cold, warm, and hot) and the presence of CA/TSC on the starch matrix and iron absorption from iron-fortified rice. Our hypotheses were as follows: *1*) there would be a significant difference in iron absorption from cold, warm, and hot extruded rice, with lower bioavailability predicted by an increase in less readily digestible starch with increasing extrusion temperatures, and *2*) this difference in bioavailability would be offset by the presence of the iron-absorption enhancer CA/TSC.

This study was funded by the Laboratory of Human Nutrition of the ETH Zürich.
Author disclosures: The authors report no conflicts of interest.

Supplemental Tables 1–3 and Supplemental Figures 1–4 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

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Abbreviations used: AGP, α-1 acid glycoprotein; CA, citric acid; CRP, C-reactive protein; ΔE, delta E; DSC, differential scanning calorimetry; FAFe, fractional iron absorption; FePP, ferric pyrophosphate; $FeSO_4$, ferrous sulfate; J, Joule; MΩ-cm, megaohms-centimeter; PF, plasma ferritin; RVB, relative bioavailability; RH, relative humidity; TSC, trisodium citrate; RBV, relative bioavailability; SAX, small-angle X-ray scattering; T_m, melting temperature; T_r, recovery temperature; WAXS, wide-angle X-ray scattering; ZnO, zinc oxide; $ZnSO_4$, zinc sulfate; ΔH_m, melting enthalpy; ΔH_r, recovery enthalpy.

Methods

Materials and chemicals

We obtained long-grain rice flour from La Riseria SA, Taverne, Switzerland; zinc sulfate ($ZnSO_4$) and zinc oxide (ZnO) from Dr. Paul Lohmann GmbH, Emmertal, Germany; and CA/TSC were from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. We prepared an in-house vitamin mix (in powder form) by mixing the individual vitamins obtained from Hänseler AG, Herisau, Switzerland. To achieve nutrient concentrations for rice fortification suggested by de Pee et al. (19), the bulk of 20 g mixed vitamins powder was prepared using 0.01% vitamin A palmitate 250 S/N, 6.2% thiamin mononitrate, 65.0% niacinamide, 6.0% pyridoxine hydrochloride, 1.3% folic acid, 10.0% of 0.1% cyanocobalamin, and 11.5% long-grain rice flour. This vitamin mix was stored in a plastic zip-lock bag at 4°C until further usage. Ultrapure water of 18 megaohms-centimeter (MΩ-cm) was used in all experiments.

Production of extruded fortified rice

For the human absorption study, stable isotope–labeled ^{57}FePP was prepared by Dr. Paul Lohmann GmbH from elemental enriched iron (95.8% ^{57}Fe enrichment; Chemgas) using a scaled-down process as used for the synthesis of their commercial FePP. The extruded rice contained 2 mg labeled iron per gram of extrudate. We mixed long-grain rice flour (290 g) with 2.31 g ^{57}FePP, 1.93 g $ZnSO_4$, and 6.0 g of the vitamin mix, and divided the mixture into 3 batches before extrusion, as previously described (5). The barrel temperature of cold, warm, and hot extrusion was controlled at 40°C, 70°C, and 90°C, respectively. We produced batches with the addition of CA and TSC by adding 0.62 g CA, and 17.4 g TSC to 272 g rice flour, whereas the amount of other ingredients remained the same. After extrusion, we air-dried the 6 batches of each formulation overnight to reach a moisture content of 10%, and then stored the dried samples in polyethylene zip-lock bags.

We produced batches for assessment of starch structure and color stability with identical procedures as above but using 4 mg of natural isotopic iron of FePP. We used ZnO as a substitute for $ZnSO_4$. In the investigation of starch structure, we aimed to determine the effect of extrusion temperatures (cold, warm, hot) and ingredients (i.e., FePP and ZnO), solubilizing agent (CA/TSC), and lubricant (monoglyceride) on the starch structure. We prepared 12 samples as shown in **Supplemental Table 1** and used native basmati rice and unfortified extruded rice as references.

Physiochemical properties of extruded rice

Small- and wide-angle X-ray scattering.

We performed small- and wide-angle X-ray scattering (SAXS and WAXS, respectively) experiments using a Bruker AXS Micro equipped (Bruker) with a micro-focused beam (50 W, 50 kV, 1 mA) with the $\lambda_{CuK\alpha}$ = 0.15418 nm radiation to obtain direct information on the scattering patterns. The scattering intensities were collected by a Dectris 2D Pilatus 100K X-ray detector (83.8 cm × 33.5 cm, 172-μm resolution). An effective scattering vector range of 0.1 nm^{-1} < q < 25 nm^{-1} was obtained, where q is the scattering wave vector defined as $q = 4\pi \sin \theta/\lambda_{CuK\alpha}$ with a scattering angle of 2θ. Entire single rice kernels were measured at room temperature. Deconvolution of the WAXS scattering profile allows for the evaluation of the degree of crystallinity, which is defined as the ratio between the total area corresponding to the crystalline diffraction peaks and the total area under the scattering curve, $\chi = A_{crystalline}/A_{total}$. Moreover, the presence of diffraction peaks at specific q values allows for the unambiguous assignment of the starch polymorphism and the determination of the unit cell (20). The evaluation of the SAXS scattering peak and the adjustment of this peak by the para-crystalline model (21) allows for the evaluation of the semi-crystalline lamellar distance and size domain.

Differential scanning calorimetry.

We used a differential scanning calorimeter (Mettler Toledo DSC1 STAR[e]) to determine the thermal properties of extruded rice grains. Extruded rice grains (1 g) were milled and automatically sieved through a 1-mm sieve size prior to measurement. Milled samples (5 mg) were mixed with 50 mg water and hermetically sealed in metal pans. The pans were placed at room temperature for 12 h. Samples were scanned at a heating/cooling rate of 5°C/min from 25 to 120°C and back under a nitrogen atmosphere. The major parameters of the differential scanning calorimetry (DSC) thermograms were described as melting temperature (T_m), melting enthalpy (ΔH_m), recovery temperature (T_r), and recovery enthalpy (ΔH_r). The degree of retrogradation was calculated from the ratio ΔH_r to $\Sigma\Delta H_m$. Analyses were performed in duplicate.

To investigate the effect of storage temperatures, 5 g iron-fortified cold and hot extruded rice grain in the presence of CA/TSC were weighed into a 50-mL centrifugal tube with 10 mL water. We cooked the samples in a 100°C water bath for 20 min. Thereafter, we weighed 10 mg cooked rice to a DSC aluminum pan. We placed 2 sets of each sample into aliquots and stored in the refridgerator and at room temperature, respectively, and measured after 24 h.

In vitro iron solubility

We measured in vitro solubility in triplicate using amylase and pepsin enzymes, as previously described (5) and modified from Miller et al. (22). Relative solubility was expressed as the solubility of the compound divided by the solubility of the reference sample containing $FeSO_4$, as recently described (5).

Color measurements

We performed a storage trial to investigate the color stability of the iron-fortified extruded rice in 2 different conditions: *1*) 40°C with 75% relative humidity and *2*) 28°C and 35% relative humidity. Samples were stored for 6 mo in transparent polyethylene zip-locked bags. We performed color measurements in 9 replicates with a Minolta Chromameter CR-210 (Minolta Camera Co.), as previously reported (7). We measured the color weekly during the first month, and then monthly for 6 mo.

Human iron absorption study

Test meal preparation.

The test meals and the reference meal contained 50 g uncooked rice. To reach a fortification concentration of 80 μg Fe/g, we set the mixing ratio of natural jasmine rice to fortified extruded rice as 25:1. All test meals consisted of 48 g uncooked jasmine rice and ∼2 g uncooked extruded rice. We combined raw jasmine rice and extruded rice together with 100 g water as single portions in individual glass bowls for each participant and cooked as previously described (5). Reference meals contained 50 g uncooked jasmine rice without extruded rice, but prior to consumption, 4.00 mg (± 0.04) of iron in the form of a labeled $^{58}FeSO_4$ solution, which was produced from enriched elemental iron (99.90% ^{58}Fe enrichment; Chemgas) as previously described (23), was added. We added 30 g standardized vegetable sauce prepared as previously reported (24) to each cooked rice meal, and provided 300 mL water with the meals.

Test meal analysis.

Test characterization included measuring dietary iron absorption inhibitors phytic acid and polyphenols, which were determined as previously described (25, 26). As zinc has been reported to affect iron absorption in rice (27), we analyzed the iron and zinc content of jasmine rice, extruded rice, and vegetable sauce by atomic absorption spectrophotometry (Agilent Technologies AA240Z) after mineralization by microwave-assisted digestion (MLS TurboWave; MLS GmbH) in nitric acid.

TABLE 1 Baseline anthropometric, iron status, and inflammatory variables of the female study participants[1]

Variables	Values
Age, y	22.9 ± 3.4
Weight, kg	59.4 ± 4.8
Height, m	1.7 ± 0.1
BMI, kg/m^2	21.3 ± 1.4
Hemoglobin, g/L	13.6 ± 0.8
Plasma ferritin, μg/L	29.3 (20.8,41.2)
Serum C-reactive protein, mg/L	1.8 (1.3,2.4)
Soluble transferrin receptor, mg/L	5.7 (5.2,6.3)
α-1 Acid glycoprotein, g/L	1.4 (1.4,1.5)

[1]Values are arithmetic means ± SDs or geometric means (95% CIs); $n = 22$

Participants

We calculated that a sample size of 22 would allow us to detect an intrasubject difference in fractional iron absorption (FAFe) of 30% between the administration meals with a β of 0.8, α of 0.05, and an SD of 0.23 units in the log-transformed FAFe, including a 20% attrition for potential dropouts. We enrolled 22 women from students and staff of ETH Zurich, Switzerland. The inclusion criteria were as follows: *1*) age 18 to 40 y, *2*) body weight <65 kg, *3*) not anemic (hemoglobin >12.0 g/dL), *4*) apparently healthy with no chronic diseases or medication (except for oral contraceptives), *5*) nonsmokers, *6*) no blood donation or large blood loss in the previous 4 mo, and *7*) not pregnant or lactating. The baseline characteristics of the subjects are shown in **Table 1**. We obtained informed written consent from all participants. The ethics committee of the canton of Zurich reviewed and approved the study (KEK-ZH-Nr. 2018–009,559); the trial was registered at clinicaltrials.gov as NCT03703726.

Study procedures

We performed the study between September 2018 and December 2018 at the Laboratory of Human Nutrition in Zurich, Switzerland. The study was a single-blind, randomized, crossover trial with each woman serving as her own control. Each woman consumed 7 different isotopically labeled test meals, which were produced with cold, warm and hot extrusion with ^{57}FePP alone or with the addition of CA/TSC. The reference meal consisted of natural jasmine rice fortified with a $^{58}FeSO_4$-solution, added just before consumption. Test meals were served with 2 wk between meals (**Figure 1**), with the reference meal given 1 d prior to 1 of the test meals. The order of the test meal and the reference meal administration was randomized. The total duration of the study was 86 d. We assessed iron absorption by measuring erythrocyte isotopic incorporation of the labels 14 d after administration.

During screening (1 to 2 wk before the first meal administration), we measured body weight and height, collected 8 mL blood to measure hemoglobin, plasma ferritin (PF), and C-reactive protein (CRP) and performed a pregnancy test. On administration days, participants consumed the test meal under close supervision (28) in the morning between 07:00 and 08:00, after an overnight fast. Depending on the order of the reference meal administration, the study started with a blood sampling and the reference meal on day 1, or on day 2, with a blood sampling and the first test meal. On days 16, 30, 44, 58, and 72, participants received their second, third, fourth, fifth and sixth test meals, respectively, after undergoing venipuncture for measurements of hemoglobin, PF, CRP, α-1 acid glycoprotein (AGP), and isotopic iron composition following the same procedure as on day 2. Depending on the randomization schedule, participants received the reference meal either on day 1, 15, 29, 43, 57, or 71. On day 86, 14 d after the last test meal administration, whole-blood samples were collected to measure hemoglobin, CRP, AGP, and isotopic iron composition.

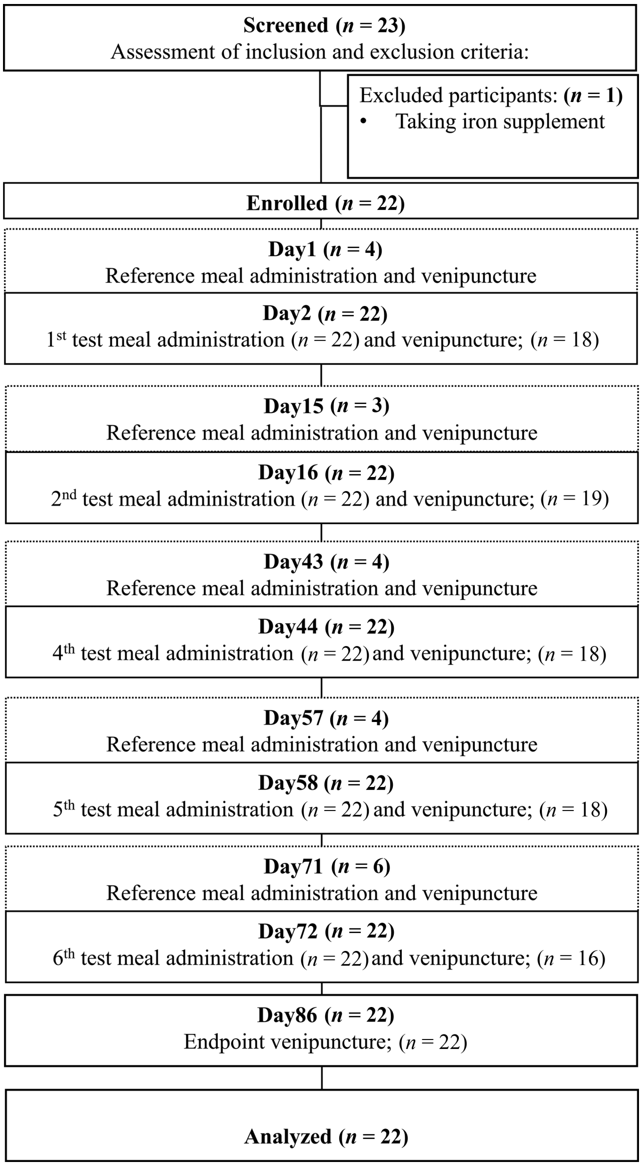

FIGURE 1 Study overview of the human iron absorption study. Six test meals and 1 reference meal were randomly administered to healthy Swiss women ($n = 22$).

Blood analysis

Hemoglobin was measured in whole blood on the day of collection by using a Sysmex XE_5000 (Sysmex Corporation). Anemia was defined as hemoglobin <12 g/dL (29). Iron deficiency was defined as PF <15 mg/L and iron deficiency anemia as hemoglobin <12 g/dL and PF <15 mg/L (29). Reference CRP concentrations for healthy individuals are <5 mg/L (30). We measured the isotopic composition in whole-blood samples and calculated iron absorption as previously described (31).

Statistical analysis

We analyzed data using SPSS (version 24.0, 2016; SPSS Inc.) and Microsoft Excel (2016; Microsoft Corporation). We checked the data for normality by means of Q-Q plots and histograms. Normally distributed data are expressed as means and SDs. Non–normally distributed data were log-transformed prior to analysis, and data were antilog and reported as geometric means (95% CI). We compared FAFe within participants from 7 different test meals. We used repeated-measures ANOVA. We calculated relative bioavailability (RBV) from each meal based on the geometric mean FAFe (%) of the test meals relative to FAFe of the reference meal for each subject. We calculated the concentrations of iron, zinc, and PA of the composite meal based on the means from the analysis of single components (natural jasmine rice, extruded rice, and vegetable sauce; $n = 3$). We used 1-factor ANOVA to assess the difference of in vitro fractional solubility or in vitro relative solubility of 7 different test meals ($n = 3$). We used Wilcoxon signed-rank to assess the difference of iron-fortified extruded rice prior to and after 6 mo storage period. Two-sided P values < 0.05 were considered statistically significant.

Results

Crystalline structure

We performed SAXS experiments to measure the lamellar distance in the semi-crystalline starch domain. SAXS patterns of all extruded samples were similar (**Supplemental Figure 1**), with a peak at the scattering wave vector $q_{max} = 0.72$ nm^{-1}, which corresponds to the lamellar d-spacing of 8.7 nm, and with a correlation length of $\xi = 17$ nm (twice the lamellar distance). The native basmati rice and the long-grain rice flour used for the extruded samples showed a peak at $q_{max} = 0.71$ nm^{-1}, a lamellar distance of $d = 8.9$ nm, and a correlation length of $\xi = 26$ nm (4 times the lamellar distance). These findings indicated that the lamellar d-spacing remained constant in all extrusion temperatures, and that the extrusion ingredients did not alter the lamellar spacing, but the number of lamellae in the crystalline domain decreased due to the extrusion process. We determined the long-range ordered structure of starch by WAXS, and the overall degree of starch crystallinity, the type of starch polymorphism, and the lattice parameter of the unit cell of starch helices. Native basmati rice and the long-grain rice flour presented a characteristic type A starch polymorphism (peaks at $q = 11.1$, 12.4, 13.2, and 16.5 nm^{-1}) together with a small fraction of ∼ 5% type B starch. We found polymorphic mixtures of type A and type V starch in all extruded samples, with the 4 characteristic type V peaks at $q = 6.9$, 9.7, 11.1, and 14.3 nm^{-1} (**Supplemental Figure 2**). Therefore, extrusion introduced some transformation from type A (monoclinic) to type V (orthorhombic) starch, and the higher the extrusion temperature, the higher the percentage of type V starch present in the sample (**Table 2** and **Supplemental Table 2**). The degree of crystallinity (χ) obtained from the WAXS analysis showed that extrusion temperature and ingredients slightly decreased the degree of starch crystallinity (Supplemental Table 2) when compared with the native basmati rice ($\chi = 53\%$). Rice flour showed a degree of crystallinity of $\chi = 42\%$ and a type A and type V content of 87% and 13%, respectively, showing that milling rice decreased the crystallinity and partially transformed type A into type V starch.

Thermal properties

We performed DSC experiments to determine the thermal properties of native basmati rice and extruded rice samples. All samples showed 2 endothermic peaks corresponding to the gelatinization of starch (**Supplemental Figure 3** and **Supplemental Table 3**). Native basmati rice showed a major peak at 76°C and a minor peak at 96°C, corresponding to the gelatinization of type A (95%) and type B (5%) starch, respectively. Based on the polymorphisms detected by WAXS analysis, extruded rice showed the first peak at ∼70°C and the second one at ∼100°C that corresponded to the gelatinization of type A and type V retrograded starch, respectively, with the latter formed after cooling. The enthalpy value, ΔH_m, corresponding to this order-to-disorder transition ranges from 0.5 to 2.0 Joule (J)/g,

TABLE 2 Retrograded starch and type V starch in different samples with different processing temperatures[1]

Sample	Retrograded starch,[2] %				Type V,[3] %			
		Extruded rice[4]				Extruded rice[4]		
	Control	Cold	Warm	Hot	Control	Cold	Warm	Hot
Basmati rice	17 ± 7	N/A	N/A	N/A	0	N/A	N/A	N/A
Long-grain rice flour	18 ± 6	N/A	N/A	N/A	13	N/A	N/A	N/A
Nonfortified extruded rice	N/A	54 ± 3	59 ± 12	65 ± 15	N/A	68	80	92
Iron-fortified rice with CA/TSC[5]	N/A	18 ± 1	33 ± 23	60 ± 30	N/A	45	55	85
Iron-fortified rice without CA/TSC[6]	N/A	37 ± 10	37 ± 23	42 ± 16	N/A	57	70	89

[1]CA, citric acid; DSC, differential scanning calorimetry; N/A, not applicable; TSC, trisodium citrate; WAXS, wide-angle X-ray scattering.
[2]Values are arithmetic mean ± SD, $n = 2$. The values were calculated from the ratio between the recovery enthalpy (ΔH_r) evaluated upon cooling the sample and the total melting enthalpy ($\Sigma\Delta H_m$) evaluated upon heating in the DSC experiments.
[3]Values are percentages, $n = 1$. The values were calculated from the ratio between the amount of type V crystallinity and the total degree of crystallinity defined from WAXS.
[4]Extrusion temperature: cold temperature (40°C), warm temperature (70°C), and hot temperature (90°C).
[5]Rice extruded with ferric pyrophosphate, zinc oxide, and CA/TSC.
[6]Rice extruded with ferric pyrophosphate and zinc oxide.

which required less energy than native basmati rice with an enthalpic value of 8.6 J/g. Upon cooling, the gelatinized native basmati rice and long-grain rice flour showed exothermic peaks at ~76°C and 73°C, which are attributed to the retrogradation of native starch and milled rice, respectively. The associated disorder-to-order transition enthalpy, ΔH_r, ranged between –0.5 to –1.8 J/g for all extruded samples and for native basmati rice. Hot-extruded rice samples had a larger starch retrogradation degree compared with warm- and cold-extruded samples, as shown in the ratio between the recovery enthalpy and the total melting enthalpy (ΔH_r:$\Sigma\Delta H_m$) in Table 2. Iron-fortified extruded rice (retrograded) in the presence of CA/TSC showed 18%, 33%, and 60% of retrograded starch in cold, warm, and hot temperature conditions, respectively. We found the lowest amount of retrograded starch in native basmati rice (17%) and long-grain rice flour (18%).

In vitro iron solubility

Native jasmine rice with $FeSO_4$ added as a solution after cooking showed higher fractional in vitro solubility (80%) than iron-fortified rice with no addition of CA/TSC (~4%; $P < 0.001$) and with CA/TSC (~35 to 39%; $P < 0.001$; **Figure 2**). The addition of CA/TSC increased the relative iron solubility by 8-fold compared with no addition of CA/TSC ($P < 0.001$). Extrusion temperature did not affect the relative solubility of iron in both groups of samples with and without CA/TSC (Figure 2).

Color stability

Additional CA/TSC significantly increased delta E (△E) (~13) of iron-fortified extruded rice compared with the samples with no CA/TSC (ΔE~5) ($P < 0.001$) (**Figure 3**). Storage conditions did not further enhance ΔE score of iron-extruded rice with and without CA/TSC compared with nonfortified extruded rice during 6 mo of storage ($P = 0.630$). There was no effect of monoglycerides added in iron-fortified rice with and without CA/TSC (data not shown).

Iron bioavailability

Co-fortification of iron-fortified rice with CA and TSC (CA/TSC) increased FAFe, and the geometric mean fractional iron absorption of iron from fortified rice extruded with CA/TSC (8.2%; 95% CI: 7.9%, 11.0%) was more than twice that from extruded without CA/TSC (3.0%; 95% CI: 2.7%, 3.4%; $P < 0.001$). Extrusion temperatures did not significantly affect FAFe (Figure 2) or RBV. For rice samples without CA/TSC and produced using cold temperature, FAFe (3.1%; 95% CI: 2.6%, 3.8%) was not different from rice produced at warm (3.0%; 95% CI: 2.5%, 3.6%) and hot (3.0%; 95% CI: 2.5%, 3.7%) temperatures ($P = 0.832$). For rice samples with CA/TSC, warm-fortified extruded rice showed a value of FAFe of 9.4% (95% CI: 7.6%, 11.6%), which was similar to cold (8.8%; 95% CI: 6.5%, 12.0%) or hot (9.2%; 95% CI: 7.0%, 12.0%) extruded rice or with the reference meal containing FeSO (9.2%; 95% CI: 6.9%, 12.0%; $P = 0.78$) (Figure 2). Extrusion temperature did not affect RVB with and without CA/TSC: at cold, warm, and hot temperatures, RBV was 97%, 102%, and 100% ($P = 0.667$) and 34%, 33%, and 33% ($P = 0.827$), respectively.

Discussion

The main finding of this study is that extrusion temperatures did not significantly affect the bioavailability of FePP from extruded rice with or without CA/TSC, even though, compared with cold extrusion, warm and hot extrusion had an increased fraction of retrograded starch, as well as type V starch. These data suggest that while medium to high extrusion temperatures are associated with the presence of resistant starch, it appears that the starch gelatinization process during cooking before consumption may reverse this transition, and this allows for equivalent iron release and absorption from the different extruded kinds of rice (32).

This finding differs from the previous finding of Hackl et al. (8), reporting FAFe of FePP-fortified cold-extruded rice (2.2%) to be 64% greater than that of hot-extruded rice (1.2%). Our study shows comparable FAFe from cold-, warm-, and hot-extruded rice in samples without CA/TSC (~4% in all extrusion temperatures). This difference may be due to several factors. First, a different zinc-fortification compound was used in the study by Hackl et al. (ZnO), whereas $ZnSO_4$ was used in our study. The varying effect of ZnO and $ZnSO_4$ co-fortified with FePP in extruded rice was previously reported (6): $ZnSO_4$ showed no detrimental effect on iron absorption, whereas iron absorption from FePP co-fortified with $ZnSO_4$ was 1.6-fold greater than that of rice co-fortified with ZnO. Second, test meals of both studies were cooked the evening before administration, and meals were kept at 4°C overnight in the study by Hackl et al. (8), whereas the meals in our study were kept at room temperature post-cooking (covered with a lid to prevent drying and secondary microbiological contamination),

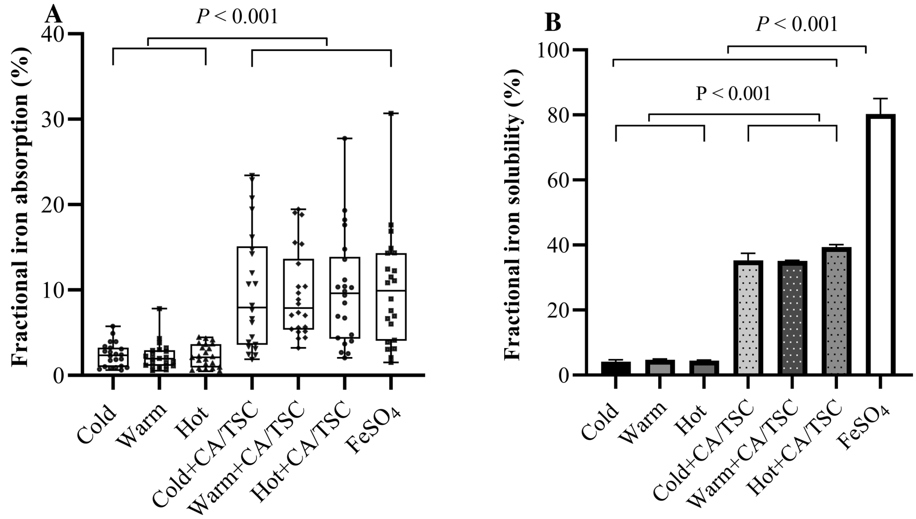

FIGURE 2 (A) Boxplots showing fractional iron absorption (%) in young women ($n = 22$) from 7 different iron-fortified rice meals containing 4 mg isotopically labeled FePP (^{57}FePP). Rice was either with or without CA and TSC and also contained $ZnSO_4$ and a vitamin premix. Molar ratios for iron:zinc:CA/TSC are 1:1:0.3:5.5 for samples with CA/TSC and 1:1:0:0 for samples without CA/TSC. Extrusion temperatures were as follows: cold: 40°C; warm: 70°C; hot: 90°C. Horizontal lines show medians, the whiskers describe the IQRs, dots represent individual values. We assessed the extrusion temperatures or CA/TSC effects from the test meals on absorption by repeated-measures ANOVA. (B) Bars represents the mean iron solubility ($n = 3$). Error bars with whiskers indicate SDs. We assessed the effect of extrusion temperatures or CA/TSC effects from the test meals iron solubility by 1-factor ANOVA. CA, citric acid; FePP, ferric pyrophosphate; $FeSO_4$, ferrous sulfate; TSC, trisodium citrate; $ZnSO_4$, zinc sulfate.

a procedure that may more closely mimic real-life conditions (where rice generally is eaten immediately after cooking) and that may affect starch polymorphism to a lesser extent. In contrast, heating and cooling cycles and low-temperature storage are known to increase starch retrogradation, and these processes can be used to produce instant rice with low digestibility, which partly resists amylolysis (11, 33–35). This is supported by our DSC measurements, indicating iron-fortified cold- and hot-extruded rice in the presence of CA/TSC cooked and stored at 5°C overnight showed higher melting enthalpy values (2.12 and 2.21 J/g, respectively) compared with that of the rice stored at room temperature overnight (0.01 and 0.11 J/g, respectively) (**Supplemental Figure 4**). Thus, cold storage temperature may modify FePP-extruded rice starch to be more densely packed and thus be less digestible. In the study by Hackl et al. (8), meals were briefly heated in a microwave oven before consumption, and this may not have been sufficient to fully reverse starch retrogradation introduced by overnight cold storage for the rice produced with hot extrusion, which has a higher degree of retrograded starch post-extrusion (Table 2). Thus, it is likely that a meal cooked, cooled, stored at low temperature, and only mildly reheated may contain more resistant starch, which would be less digestible and result in lower iron bioavailability from extruded fortified rice. Although the inconsistency between the results of Hackl et al. (8) and the current study may be due to a different preparation method,

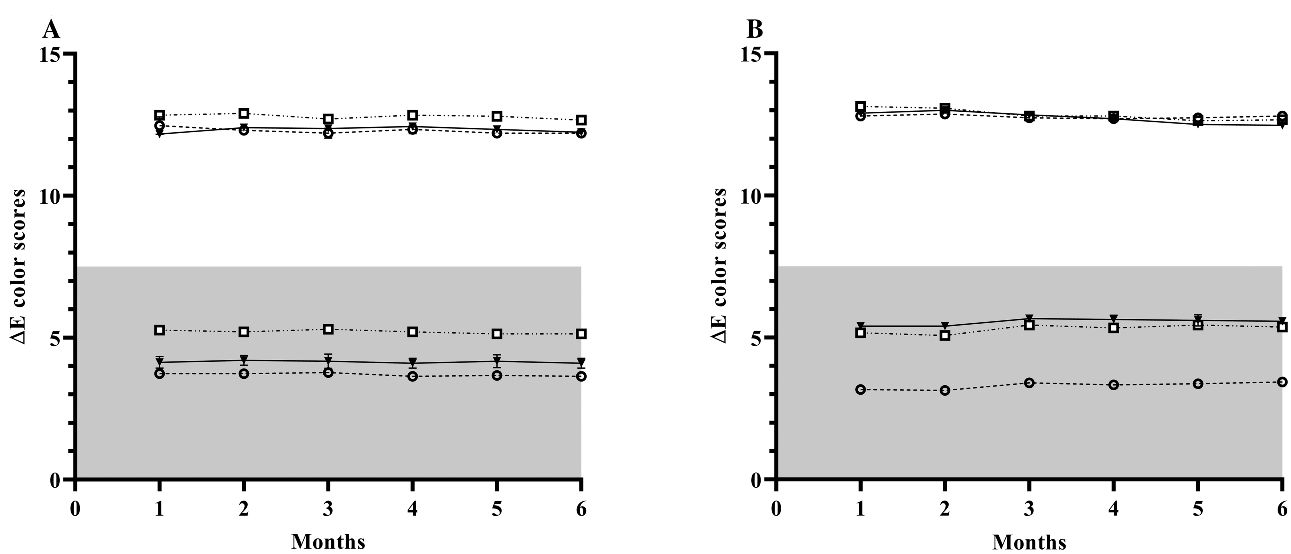

FIGURE 3 Color difference relative to unfortified extruded rice delta E (ΔE) of 6-mo storage trials. Iron-fortified extruded rice without CA/TSC (gray region) and with CA/TSC (white region). Panel A shows the results at 20°C and 62% relative humidity (RH), panel B at 35°C and 73% RH. Cold extrusion (40°C); warm extrusion (70°C); hot extrusion (90°C). $n = 3$. CA, citric acid; TSC, trisodium citrate.

TABLE 3 Composition of the extruded iron-fortified rice in study meals[1]

Sample	Without CA/TSC			With CA/TSC			Reference
	Cold	Warm	Hot	Cold	Warm	Hot	
Barrel temperature, °C	30–40	60–70	80–90	30–40	60–70	80–90	N/A
Solubilizing agents	N/A	N/A	N/A	CA/TSC	CA/TSC	CA7TSC	N/A
Total Fe, mg/meal	4.0 ± 0.01	4.0 ± 0.01	4.0 ± 0.01	4.0 ± 0.01	4.0 ± 0.01	4.0 ± 0.03	4.0 ± 0.02
Total Zn, mg/meal	4.9 ± 0.04	4.9 ± 0.02	4.9 ± 0.03	5.0 ± 0.04	4.9 ± 0.03	5.0 ± 0.03	N/A
Fe:Zn:CA:TSC,[2] mol:mol:mol:mol	1: 1: 0: 0	1: 1: 0: 0	1: 1: 0: 0	1: 1: 0.3: 5.5	1: 1: 0.3: 5.5	1: 1: 0.3: 5.5	N/A

[1]Values are arithmetic means ± SDs unless otherwise indicated; $n = 3$. CA, citric acid; N/A, not applicable; TSC, trisodium citrate.
[2]Molar ratio in fortified rice.

together the findings from the 2 studies suggest that cold storage of hot-extruded iron-fortified rice may reduce iron absorption from the rice and should not be recommended.

In this study, we confirm the enhancing effect of CA/TSC on solubility and bioavailability of iron from FePP in extruded rice (5, 7); however, FAFe and RVB in our study were higher than previously reported in 2 studies (6, 8). This is likely due to a higher molar ratio of iron to CA/TSC in our study (1:0.3:5.5; **Table 3**), compared with the ratio of 1:0.1:2.1 used in a previous study in women (6). Although the ratio used in our study was similar to that used in a previous study in Ghanaian children, in the Ghana study the test meals contained inhibitors of iron absorption and the children had greater inflammation than our healthier population, and these factors likely contributed to the reduced bioavailability in that study (8).

During rice extrusion, the combined effect of temperature, moisture, shear, and screw speed alters the starch structure. Our data suggest that an extrusion temperature above the starch melting point created a higher fraction of resistant starch. Results from SAXS experiments showed that the size of the semi-crystalline domains (ξ) containing the starch lamellae in the extruded rice samples were decreased by ~10 nm compared with native basmati rice and long-grain rice flour. This finding agrees with WAXS results showing that extrusion temperature and ingredients slightly decreased the degree of crystallinity of the extruded samples. This can be explained by the breakdown of intermolecular hydrogen bonds of starch molecules during the gelatinization process of extrusion, which subsequently disrupted crystalline structure (36). Moreover, the extrusion temperature transformed the crystalline structure of starch from a native type A, which is normally found in cereals, to the amylose-lipid complexed type V. This was confirmed by the 2 main melting temperatures at 70°C and 100°C, respectively, in DSC measurements for extruded rice. Moreover, our DSC data show that warm and hot extrusion produced a greater fraction of retrograded starch than that of cold extrusion. Starch usually gelatinizes during the extrusion process, which thoroughly disrupts starch granules, and we verified this point by measuring the melting enthalpy (ΔH), which reflects the degree of gelatinization. Extruded rice starch has lower enthalpy compared with the native sample as a result of gelatinization (37).

We did not find an effect on starch physicochemical properties of the presence of monoglycerides, CA/TSC, iron, or zinc. Although previously reported when rice was extruded with citric acid and monoglycerides (14, 38), we could not confirm this finding, likely because we added only a small amount of additives. The addition of iron and CA/TSC reduced brightness ($L*$) but increased the greenish value (negative $a*$) of the extruded rice and contributed to greater ΔE compared with extruded rice with no enhancers. It is likely that the in situ generation of soluble-citrate complexes during extrusion may alter the rice color, as previously reported (8). However, the color of fortified extruded rice with and without CA/TSC was stable over 6 mo of storage at low and high temperature and relative humidity.

The strengths of our study are as follows: *1*) the measurement of iron absorption from intrinsically labeled fortified rice using stable isotopic labels; *2*) the thorough characterization of starch structure and physiochemical proprieties; *3*) the in vivo and in vitro studies investigating the bioavailability of warm-extruded rice, compared with cold and hot extrusion; and *4*) to more closely mimic real-life conditions in rice preparation, we did not cool the rice after cooking for an overnight period, a procedure that may affect iron bioavailability from extruded rice. Our study also has limitations: *1*) the study of the starch structure was done in a single batch of extruded rice; therefore, we cannot provide data on batch-to-batch starch structure variability; *2*) the standardized meal contained negligible amounts of phytic acid, which can inhibit iron absorption (39) and may not fully be generalizable to settings where legumes and whole grains are consumed along with rice. However, CA/TSC can enhance iron bioavailability from extruded iron-fortified rice even in the presence of moderate amounts of dietary phytic acid (7).

In summary, compared with cold extrusion, hot and warm extrusion of iron-fortified rice altered starch crystallinity and polymorphism, as well as the semi-crystalline domain size of extruded rice starch, and produced a higher fraction of resistant starch. However, iron bioavailability was similar to rice produced using the 3 extrusion temperatures. Thus, hot, warm, and cold extrusion can be recommended for iron-fortified extruded rice. However, a disadvantage of cold extrusion is a high loss of minerals when rice is cooked with excess water (8). Therefore, hot and warm extrusion likely are preferable fortification techniques in settings where rice is pretreated in water before cooking, or where excess water is discarded after cooking.

Acknowledgments

We thank Hänseler AG (Herisau, Switzerland) donated the vitamins used in this study. We thank Daniele Liberto, Adam Krzystek, and Timo Christ (ETH Zurich) for their laboratory and analytical support. The authors' responsibilities were as follows—PS, DM and MBZ: designed the clinical study; PS: conducted the clinical study; PS and AS-F: performed the experiments related to starch microstructure; PS, AS-F, DM, and CZ: analyzed the data and performed the statistical analyses; PS, DM, and AS-F: wrote the first draft of the manuscript; and all authors: participated in the data interpretation and edited, read, and approved the final manuscript.

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