Ferric pyrophosphate fortified extruded rice increased iron status in rats

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Abstract

Background:

Iron deficiency is the most common micronutrient deficiency worldwide and one of the five prominent causes of years lived with disability in humans, particularly affecting women and children. Ferric pyrophosphate (FePP) and ferric phosphate ($FePO_4$) are commonly used in fortifying rice due to their minimal impacts on its sensory properties, but have both presented with poor iron bioavailability. Particle-size reduction of FePP and addition of ligands such as citric acid (CA) and trisodium citrate (TSC) to extruded FePP-fortified rice have been associated with improved iron bioavailability in iron-fortified rice.

Objective:

To compare the iron outcomes of extruded rice flour formulated with four different iron fortificants in rats.

Methods:

Using the prophylactic-preventative method, 50 male weanling Sprague-Dawley rats aged 20-23 days were randomly distributed into 5 groups and fed ad libitum for a 28-day period. The control group consumed powdered AIN-93G growth diet, while the other four experimental diets were AIN-93G based extruded rice flour diets fortified with micronized FePP (μFePP), a higher FePP concentration, $FePO_4$ with TSC and CA or FePP with TSC and CA at a molar ratio of 1:0.3:5.5 for iron to TSC/CA. Hematological parameters and hepatic iron concentration were assessed for the iron status. The dual energy x-ray absorptiome-

try (DEXA) PIXImus scan was used to assess the bone mineral density (BMD) and body composition of the rats.

Results:

The consumption of FePP, μFePP, FePP+TSC+CA fortified rice flour significantly increased ($p<0.05$) hepatic iron concentration compared to the $FePO_4$+ TSC+CA fortified rice flour and AIN-93G groups. However, there were no significant differences between the hepatic iron concentrations of the FePP groups or the hematological and anthropometric assessments between all groups ($p>0.05$).

Conclusion:

Increased concentration of FePP in extruded fortified rice can improve iron status, and suggests that neither micronizing FePP nor extruding it with TSC and CA improved iron status compared to FePP. The extrusion of $FePO_4$ with TSC and CA did not improve iron status.

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Chapter 1

Introduction

1.1 Rice as a tool for Global Nutrition

Rice is grown in more than 100 countries, with countries in the Asian continent responsible for roughly 90% of the total global production[1]. Paddy rice production has increasingly grown by more than 3-fold from 215 million to 755 million tons between 1961 and 2019[2]. Variations in the grain length, color, thickness, stickiness, aroma and growing conditions/production practices of rice are all capable of impacting the quality and nutrient profiles of the various rice species. Additionally, the regional and cultural preferences of various populations also influence the global market for the different rice species[3].

More than half of the world population consume rice as a staple food, especially in the rice-producing regions of the world. Bangladesh had the highest per capita rice consumption with 269kg per capita per year, and followed by Laos and Cambodia out of 154 countries in 2017[4]. Half the daily energy intake of South India is from refined grains, with 75% being white polished rice[5]. Rice grains are essential nutritional source of carbohydrates and proteins alongside essential micronutrients. The utilization of rice and rice flour is considered one of the most common dietary interventions for people with macronutrient and micronutrient deficiencies and age-related chronic diseases due to its wide consumption[6].

1.2 Anemia

The World Health Organization has described anemia as a hemoglobin (Hb) concentration below 12 g/dl in women and 13 g/dl in men[7]. Anemia represents a common public health challenge characterized by a reduced hemoglobin concentration and/or volume of red blood cells below an acceptable range. It is a common medical complication in critically ill patients, and impairs oxygen transport to body tissues[8]. About 25% of the world's population have been reported to be affected by anemia, with pregnant women and school aged children in Africa and Southeast Asia being the most affected[9;10]. Approximately half the children in developing countries are estimated to be anemic, with sub-Saharan Africa countries like Kenya, Mali and Tanzania having 48.9%, 55.8% and 79.6% children anemic respectively. Anemia can cause several acute and chronic health challenges in children, including impairing their learning performance, psychomotor and cognitive maturity, behavioral and physical growth and elevates morbidity and mortality risks in them[11]. It has also been implicated in the poor intelligent quotient and language coordination in children, and as an indicator of poor health status in a nation[9].

Anemia can be caused by several factors including nutritional deficiencies, chronic infections, inherited blood disorders, obesity, and chronic non-communicable diseases[12;13]. Dietary iron deficiency (ID), inherited blood disorders (sickle cell anemia, thalassemias), malaria, helminthic infestation have been reported as the leading causes of anemia and they vary across different geographical areas, population group, and general environmental settings[14]. Socioeconomic factors including illiteracy, gender norms, poverty, large number of children, high density of inhabitants per room, poor access to public services, such as basic sanitation and electricity, quantitatively and qualitatively inadequate food consumption, among others have also been indicated as predisposing to risks of anemia too[8]. These multiple risk factors are often found coexisting with some essential micronutrients deficiencies viz folic acid, vitamin B12, vitamin A, with iron deficiency being implicated as a major contributor to anemia; and is estimated to cause approximately 50% of all anemia cases[15;16].

1.3 Iron Availability and Absorption

Iron is an integral element for a broad range of biologically important reactions critical for vital cellular functions including hemoglobin synthesis and other hemopoietic activities. It is contained in numerous hemoproteins such as the oxygen transport proteins, heme containing enzymes and many essential non-heme iron proteins catalyzing various reactions and playing a central role in oxygen sensing mechanisms[17;18]. An adequate iron supply to the body and maintenance of its balance is therefore essential for normal human health. Iron is a transition metal existing in two readily reversible redox states of reduced ferrous (Fe (II)) and oxidized ferric (Fe(III)) forms respectively. However, most of iron's biological complexes at physiological oxygen concentrations occur in its stable form of Fe (III) state[19]. The biological function of iron has been reported to be largely due to its chemical properties as a transition metal, and reduction reactions are highly essential in its metabolism. Reduced iron ion is the only form of iron that can be used as a substrate for transmembrane transport of iron, loading and releasing of iron from ferritin and for heme synthesis[20].

An adult human has a total body iron content of 3–5 g ($\approx$ 45 mg / kg for women, $\approx$ 55 mg / kg for men), with most of it occurring in the hemoglobin of peripheral erythrocytes (60–70%). About 20–30% body iron is in ferritin and hemosiderin in both hepatocytes and Kupffer cells as storage/spare iron. The adult male has a stored iron content of around 0.2-0.5g, while children, adolescents and women of childbearing age are almost lacking iron reserve[21]. Some spare body iron is in the muscle myoglobin or incorporated in enzymes, while transferrin typically binds roughly 3 mg of iron[17]. In situations where the amount of cellular iron exceeds the body's immediate need, the excess iron is stored in a bioavailable form as ferritin which is readily deployed whenever the cells become iron deficient[22;23]. Besides functioning as an iron reserve, ferritin also protects the cells from the potentially toxic catalytic reactions of iron[24]. Hemosiderin is a second form of cellular iron storage and an insoluble product of incomplete ferritin degradation by the lysosomes. Due to its ineffective donation of iron, hemosiderin typically plays a protective role in the circulatory system in normal physiological conditions and becomes an iron donor under inflammatory and hypoxic

conditions[25].

The regulation and balance of iron is important for all cell life and is specifically controlled by the intestinal absorption. Changes in body iron stores and demand for iron affect the iron absorption rate in the proximal duodenum accordingly, with iron deficiency causing an increased absorption rate and iron overload resulting in a decreased rate of iron absorption. The mechanisms of iron homeostasis therefore evolved to curb excess iron accumulation and dangerous reactive oxygen species production by recycling body iron and limiting its uptake from the environment[26]. Iron is lost from the body via desquamated skin epithelium, intestinal cells and intestinal secretions[21]. Adult men lose only 1 mg of iron per day, while women of reproductive age lose twice this amount to menstrual bleeding, pregnancy and childbirth. In compensating for this routine loss, about 1–2 mg of iron is absorbed by healthy individuals per day. There is however an increased demand for iron during adolescence due to growth and in pregnancy owing to plasma volume expansion and fetal growth. This therefore alters the daily optimal nutrition of such individuals to around 8–10 mg of iron being required[27]. Iron is present in food as either inorganic or heme iron. Inorganic iron is found in the standard diet; constituting 90% of the total iron contained in food, while heme iron only represents 10% of the dietary iron[28].

Despite iron being the fourth most abundant element in nature, it has a very low bioavailability and the disorders of its homeostasis are amongst the most common human disorders. In spite of iron's low daily requirements, iron deficiency remains the most common nutritional disorder in the world[29]. It is noteworthy that hereditary and acquired iron overload diseases are also present at the opposite end of the iron disorders spectrum.

1.4 Iron Deficiency and Iron Deficiency Anemia

Micronutrients deficiencies are very crucial public health problem, and about 2 billion people worldwide suffer from the subclinical deficiency of micronutrients commonly referred to as hidden hunger. Iron deficiency (ID) is the most common micronutrient deficiency worldwide, particularly affecting pregnant women, infants and young children due to increased

iron demands resulting from the rapid growth in this group[30]. It has been implicated as the leading cause of anemia worldwide[14]. Iron deficiency progresses in three-stages affecting all the body cells. The first stage is the total body iron stores depletion, which leads to the second stage of iron-deficient erythropoiesis and lastly iron deficiency anemia characterized by reduced hemoglobin levels. It often occurs due to inadequate dietary intake or a compromised absorption from inflammation or blood loss[9]. Iron deficiency and iron deficiency anemia therefore result from an imbalance in bioavailable iron absorption from diet and the body's iron requirements[31]. The failure of iron supply to meet the body's demand thus results in iron stores being used up faster than their replacement, and consequently iron depletion.

Iron deficiency can exist without anemia in the absence of iron stores. It is a broader condition preceding anemia or indicating a deficiency in organs/tissues besides erythropoiesis including skeletal muscles and the heart which is dependent on iron for myoglobin and energy production for its mechanical contraction[32]. Due to the high amount of iron utilized for hemoglobin synthesis during daily erythropoiesis of 200 billion erythrocytes, anemia is easily the most evident sign of iron deficiency; and iron deficiency anemia is often readily suggestive of iron deficiency[26]. Iron deficiency anaemia (IDA) is one of the five prominent causes of years lived with disability in humans, and most especially in women. Besides Iron deficiency anemia being majorly classified as a public health challenge that affects young children, women of child-bearing age; its recognition as a clinical condition capable of affecting chronic disease patients and the elderly is becoming more common. Numerous physiological, environmental, pathologic and genetic factors can cause iron deficiency to result in IDA. These etiologies/factors may however differ in the affected patient populations, geographies and specific clinical conditions or they could be found co-existing[33].

The incidence of iron deficiency anemia is increasing globally and is reported as the most common anemia worldwide[14]. Preschool children under 5 years of age, reproductive age and pregnant women are the most affected by iron deficiency anemia, with prevalence rates of about 41.7%, 32.8% and 40.1% respectively. Iron deficiency anemia can result from heavy dependence on vegan diets and some high-risk factors in high-income countries

including malabsorption syndromes and menorrhagia, with iron deficiency/iron deficiency anemia being diagnosed in about two-third of women with menorrhagia[34]. Iron deficiency anemia represents only about 30% of anemia cases in the elderly. Regular blood donation has also been implicated as a cause of iron deficiency anemia too. About 37%-61% of chronic heart failure patients and 24%-85% of chronic kidney disease patients are iron deficient, with the incidence rates increasing as these conditions become more debilitating[33]. There have also been previous reports of iron deficiency in 13–90% of inflammatory bowel disease patients, and its occurrence dependent on the disease activity and severity[35]. Also, 33% - 43% of cancer patients suffer from iron deficiency and iron deficiency anemia respectively, depending on the disease progression, proximity to cancer therapy and poor outcomes in solid tumour patients[36].

1.5 Iron Status Assessment

Iron status can be readily assessed through serum ferritin and the hemoglobin levels respectively[33]. Serum ferritin level is a highly specific and effective test for assessing total body iron stores and is universally available and standardized. Biochemically, a low serum ferritin level in a patient depicts the hallmark of absolute iron deficiency; a total depletion of the body iron stores. However, serum ferritin level is affected by inflammations and infections, resulting in an elevated value[26]. Anemia is diagnosed after confirming a reduction in the hemoglobin concentration via a complete blood count test. The hemoglobin concentration thresholds for anemia do vary according to social demographics, age, body physiology, disease epidemiology and management.

Other laboratory investigations for iron status assessment include serum iron level which represents the iron bound to transferrin, and that is available for erythropoiesis in the bone marrow. The serum iron level depends on the efficient recycling of iron from senescent erythrocytes by tissue macrophages as well as iron absorbed from the diet. Total iron binding capacity (TIBC) is a functional measurement for peripheral transferrin levels and is deduced by adding the serum iron to the unsaturated iron binding capacity[37]. The red blood

cell (RBC) indices are negatively impacted by iron deficiency and iron deficiency anemia, with the red cells gradually becoming microcytic and hypochromic. There is a reduction in the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) and an increase in the red blood cell distribution width (RDW) in iron deficiency anemia. These changes in the red cell indices however appear late in course of iron deficiency anemia, and as such may limit their clinical relevance in iron deficiency[26].

Contrary to the RBC indices, the reticulocyte hemoglobin content (RHC) is an early index of ID as it indicates iron availability for erythropoiesis in the previous 3–4 days. Also, the percentage of hypochromic red cells (% HRC) can also be used to assess recent iron reduction[38]. Soluble transferrin receptor (TfR) is an underutilized laboratory measurement; its levels are increased in iron deficiency and iron deficiency anemia but low during inflammation. It can be useful in the detection of true iron deficiency from inflammatory conditions associated with low serum iron[39]. The measurement of hepcidin level is another useful tool in determining iron deficiency and iron deficiency anemia when expertly applied. Hepcidin level becomes reduced/undetectable in iron deficiency anemia. It is however affected by a lot of factors including circadian rhythm, hepatic and renal function, all of which impacts its routine acceptability in clinical practice[40].

1.6 Management of Iron deficiency and Iron deficiency Anemia

The treatment of ID and IDA is aimed at providing sufficient iron erythropoiesis and replenish the depleted iron stores. This subsequently improves the quality of life, clinical manifestations of the patients, and the prognosis of many chronic disorders. There are two distinct approaches for the management of ID and IDA, viz active iron supplementation approaches in confirmed IDA patients, and prevention strategies targeted at populations at risk[41]. Iron supplementation can be administered either orally or intravenously depending on the patient's clinical situation and preference. However, significant gastrointestinal side

effects comprising constipation and sometimes diarrhoea, a metallic taste, gastric cramping and thick, green, tenacious stool have been reported in approximately 70% of patients taking oral iron. And this markedly reduced the patients' adherence to the oral iron therapy[42]. The universal administration of intravenous iron (IV) supplement has also been limited by availability and cost, the general belief of anaphylactic reactions and the toxic side effects of IV iron due to the free elemental iron from the supplemental drug[43;44].

Currently, several approaches including either one or a combination of supplementation, food-based approaches such as dietary diversification, mass food fortification or point-of-use food fortification; with other public health control measures such as deworming, health and nutrition education have been recommended as intervention strategies to prevent and treat micronutrient deficiencies (WHO, 2011; Peña-Rosas et al., 2019). As an integral component of the prevention strategies for iron treatment, food-based approaches have been recommended on a global level (WHO, 2001). Various programs and efforts are being made to increase access to iron-rich foods and their consumption. The use of ascorbic acid as an iron absorption enhancer to increase the bioavailability of iron when consumed has been reported in previous studies, while the removal of inhibitors of iron absorption such as calcium, phytates in cereals; tannins in tea and coffee is being recommended[45]. Hence, the enrichment of widely consumed foods with iron and the foods retaining their organoleptic properties and prices have been recommended as an effective public health intervention to improve the iron status of populations[46].

Fortification of widely consumed foods popularly known as staple foods is now renowned for its ability to improve the overall consumption of iron in a population[41]. The WHO has recommended the universal fortification of staple foods including rice, maize flour and cornmeal with iron to prevent ID and IDA in at-risk populations[47;48]. With ID and IDA resulting in serious health and national consequences, the lack of preventative and corrective interventions including developing nutrition-specific and/or nutrition-sensitive strategies to increase individuals' intake and absorption of iron accordingly contribute to the continuation of the poverty cycle and stall progress in such populations/countries.

1.7 Fortification as a Major Nutritional Intervention Strategy

Food fortification is a cost-effective public health intervention strategy capable of correcting or preventing widespread nutrient deficiencies and associated complications, balancing the total nutrient dietary profile or appealing to the consumers' dietary wants[49]. It is described as a deliberate practice of increasing essential micronutrients/trace elements contents in a food solely to improve the nutritional quality of the food supply while providing a public health benefit with the least risk to the population's health[50]. Food fortification has been effective in enhancing nutrient intakes of various populations with demonstrated health, economic and social benefits. It is noteworthy that food fortification programs are part of the sensitive programs conducted in many countries around the world as the recommended long-term strategy to improve certain micronutrients consumption in the general population[51]. They include the fortification of vitamin A in cooking oil, margarine, and sugar; vitamin D in milk and margarine; folic acid in flour; iodine in salt; iron in milk, corn flour, beans, pearl millet, and wheat flour[52]. These programs are however likely to be more successful if mandated by the government with support from the food industry[53].

Several factors determine the most appropriate and effective fortification practices of the different countries, and these include certain micronutrient deficiencies prevalence, most affected populations, dietary compositions, available infrastructure, food processing capacities and production systems, alongside national regulation and governmental leadership[54]. Three fortification approaches are currently in use, and they are the Large-scale/Mass fortification, voluntary/market driven fortification and targeted/focused fortification. The large-scale/mass fortification is usually mandatory, and entails addition of micronutrients to commonly consumed foods to enhance their nutritional value during central processing. Voluntary/market driven fortification of processed products by food industry is guided by the dietary demands of consumers to increase the diverse nutrients available. Targeted fortification involves adding micronutrients to specially designed foods like the infant formula for

infants less than 6 months of age, and others meant for specific population subgroups[55;56]. Selecting an appropriate food or vehicle for fortification and identifying the at-risk micronutrient deficiency populations is therefore an important element of the intervention and may vary among countries due to the obviously diverse patterns of diets.

Iron deficiency is a significant public health challenge in several countries, especially the developing countries and fortifying staple foods with iron have been reported to increase iron consumption in those vulnerable populations[52;57]. Some potentially acceptable staple food or vehicles used in common micronutrient fortification for public health programmes include refined or raw sugar, edible vegetable oils, fats, and cereal grains (rice); wheat flour, maize flour, or corn meals; condiments and seasonings; and powdered or liquid milk[55]. Iron fortification of rice has been recommended by the WHO as a choice vehicle for iron deficiencies in at-risk populations of a rice-consuming region[48]. Rice fortification with iron increased the hemoglobin and serum ferritin levels, and reduced iron deficiency anemia prevalence from 100% to 33% among preschool age children, pregnant women, adolescents, and adults in previous studies[58;59]. Rice is a prominent staple food of about three billion people; its proper fortification with iron therefore has the potential to reduce the prevalence of ID and IDA in rice-consuming countries[60]. Thus, the stability of the iron fortificants in the rice throughout the marketing process, the choice and cost of fortification processing and relative cost of the iron fortificants are important considerations for an efficacious fortification approach.

1.8 Rice Fortification Techniques

With rice consumption as a whole grain and not flour, the successful fortification with micronutrients on a large-scale continues to be a technological challenge compared to wheat or maize flours which have gained successes around the world[61]. Past attempts to fortify rice through dusting and coating were reported to be unsuccessful due to the typical household methods of rinsing and cooking rice in most of the developing countries[62]. Additionally; due to the much greater size difference between rice kernels and micronutrients, the mixture of micronutrient blends with rice kernels ordinarily can readily result in the easy separation

and non-homogeneous distribution of the micronutrients, increased loss of micronutrients during production, transportation, as well as the rice preparation methods employed by various households. This has significantly increased micronutrient losses, with approximately 90% of water-soluble micronutrients being increasingly lost from rice when cooked in excess water[61]. There are currently two major sophisticated techniques developed for the large-scale fortification of rice and counter the problems of micronutrient losses; they are coating and extrusion techniques[63;64]. Both methods typically entail fortifying about 2% of the rice kernels and blending to the other unfortified retail rice.

Coating is a more advanced, relatively inexpensive method involving a combination of ingredients including waxes and gums with the choice fortificant mix to form a liquid mix that is sprayed in several layers onto the rice kernels to produce a fortified rice-premix. These coated rice-permixes are subsequently blended with the unfortified/normal retail rice between a ratio of 1:50 to 1:200 to fortify the rice product[61;65]. Several studies have shown coating to be stable during washing, cooking and to effectively reduce micronutrient losses while washing the grains below 1% for some micronutrients fortified using the coating method[66]. It exerts its effects by the interaction of the waxes and gums which form a waxy layer covering on the rice kernels, making the micronutrients stick to the rice kernel and reducing the loss of these nutrients when the grains are washed before cooking[63]. However, some of the major challenges of coating include the possible coloration of the kernels as the coated micronutrients layer of the kernel makes them highly visible, impact on the taste and loss of micronutrients during washing and cooking and therefore affecting the consumers' preference compared to the extruded counterparts. Coating is considered a lower initial financial investment compared to extrusion, the cost of fortified rice is however relatively comparable per metric ton[64].

Extrusion is a new technological advancement in the food industry that utilizes low-cost broken rice as its raw material for the production of rice kernels. The extruded products are made according to preset specific shape, color, and nutrient requirements in line with the retail rice specification to be blended together for fortification[65]. It is a versatile, continuous process that combines diverse processing steps comprising various constituent mixtures,

degassing, thermal and mechanical heating, forming, and expanding[61]. The technique is primarily used for processing biopolymers, such as carbohydrates and semi-crystalline polymers like starch which have both glass transition and melting temperatures[67;68]. Two types of extrusion technology are mainly utilized in the food industry, and they include the hot and cold extrusion, also known as cooking extrusion and shape-forming respectively. Hot extrusion process involves passing the rice flour dough/premix through a pre-conditioner containing water and steam and thereafter extruded using a single or twin screw extruder at relatively high temperatures (70° to 110°C). The extruded product is cut into rice-shaped structures at the die upon extrusion and dried afterwards, resulting in a consistent and translucent product very similar to the natural rice grain[63]. The cold extrusion process is similar to hot extrusion, it however occurs at temperatures above glass transition but below starch melting temperatures (<70°C). Cold extrusion utilizes a pasta-type extruder which shapes the native or heat-treated rice flour dough containing water, a vitamin/mineral premix, binders, moisture barrier agents, or other additives into rice analogues closely resembling natural rice but a little more opaque[61].

In fortifying rice, extruded rice kernels containing the choice vitamins and minerals are added to intact rice kernels between ratio 1:50 to 1:200 to produce the fortified rice. The extruded rice kernels are similar to the vitamin/mineral–coated rice kernels, but differing in their performance level as the extruded rice kernels are able to retain their micronutrient contents. Both cold and hot extrusion processes produce extruded fortified rice kernels similar to the natural rice, and having the nutrients embedded in them effectively protected during washing and cooking[65]. Hot- and cold-extruded fortified rice kernels containing iron and vitamin B1 have both been reported to retain 100% iron and about 63-83% vitamin B1 when assessed in both products after rinsing the rice kernels severally, soaking in water for 30 minutes, frying some samples prior to cooking, and rigorous preparation and cooking methods[69]. Several studies have shown efficacy and correlation between the consumption of extruded rice grains fortified with zinc, iron, and vitamin A and improvements in zinc status, iron, vitamin A status, reduced iron deficiency and various elevated minerals and vitamins concentration in school children and other adults[70;71]. Additionally, both the hot-

and cold-extruded grains have also been well accepted by adult consumers, schoolchildren and their teachers based on their organoleptic properties[65].

1.9 Iron Fortification Compounds

Three major criteria determine how successful iron fortification strategy would be; they are the baseline nutritional deficiency prevalence, how widely consumed the choice of food/vehicle is, and suitability of the iron compound[72]. The fortification of staple foods including cereal-based products, dairy products, legumes and widely consumed condiments with iron are considered most sustainable and affordable strategies of enhancing iron status and in achieving the daily iron requirements in a population due to their wider consumption[73;74]. Numerous iron fortification compounds have been reported; however, getting suitable iron fortificants with increased absorption level in the human digestive system without modifying the sensory characteristics of the food matrix still remains a challenge in the food industry. Therefore, attention is now being focused on less bioavailable compounds capable of being supplied in larger quantities with no adverse organoleptic impacts on the vehicles[75]. In an order of preference, the WHO has recommended ferrous sulfate, ferrous fumarate, ferric pyrophosphate, and electrolytic iron as preferred iron compounds for most iron fortification processes[9].

Iron fortificants are popularly categorized into readily water soluble, poorly water soluble but soluble in dilute acids, and partially soluble in dilute acid based on their water solubility and properties, which determine their ability to dissolve in gastric juice and their absorption rate[76]. Ferrous sulfate ($FeSO_4$) and ferrous gluconate are common examples of the recommended readily water soluble iron compounds that are better absorbed. They are however highly reactive, and often causing precipitations, color and/or flavor changes to sensitive foods and rancidity during storage of cereals[77]. These readily water soluble iron compounds including $FeSO_4$ have been reported to be limited in rice fortification process as a result of its interaction with the rice matrix. As a result of their water solubility, they are readily lost during the washing and cooking process of rice in excess water and while draining the water after cooking[61]. Nevertheless, several studies have established that these freely water-soluble

iron compounds are better absorbed than their counterparts[78].

Two common examples of the poorly-water-soluble but dilute acids soluble iron compounds are the ferrous fumarate and ferrous succinate. These compounds have fewer or no sensory impacts on sensitive foods with relative bioavailability similar to ferrous sulfate's bioavailability level[78]. This implies that these dilute acids soluble iron compounds are readily dissolved in the gastric juice during digestion and are capable of being used to replace ferrous sulfate in certain cases. They cannot be used for fortifying rice due to their effects on the color and taste of rice, but are popular for fortifying commercial cereal-based complementary foods and as micronutrient powders[61]. Ferric pyrophosphate (FePP) and elemental iron powders are water insoluble and only partially soluble in the gastric juice during digestion. FePP, has a nearly white or off-white color, and has little or no interaction with the other rice components and nutrients as a result of its low solubility at the pH of rice.

The FePP, a partially-soluble-in-dilute-acid iron compound; has a minimal impact on the color of rice when stored and does not cause vitamin A rancidity. FePP has a low bioavailability, with only 13–15% absorption upon consumption when compared to $FeSO_4$[79;80]. The mean particle size of a regular ferric pyrophosphate is approximately 20m, with very minimal interaction with the food matrix, but shown to have the lowest bioavailability of iron among the ferric pyrophosphate compounds. Previous attempts to improve the iron status and absorption of FePP-fortified foods by reducing its particle size and adding FePP to bouillon cubes have however failed to demonstrate practical benefits in the study participants[79]. Although elemental iron is cheap, it is also not recommended for fortifying rice due to its gray discoloration and low absorption when consumed[61]. Additionally, ferric phosphate ($FePO_4$) is a poorly acid-soluble iron compound, having stability in foods and no adverse organoleptic effects on the food matrix. It however has a very poor absorption of about 25% compared to the $FeSO_4$[81;82]. These iron compounds however still remain the most widely used and recommended in fortifying foods due to their minimal impacts on the sensory properties of the fortified food despite their poor availability[76;83].

Sodium iron ethylenediaminetetraacetic acid sodium salt (NaFeEDTA) is an iron compound that utilizes its ethylenediaminetetraacetic acid (EDTA) component to chelate nu-

merous metals, and reduce the percentage of iron compounds bound to inhibitors while increasing absorption to about thrice that of $FeSO_4$ in foods[77]. It is commonly used in cereal fortification especially wheat and maize flours, and is reported to exert superior iron bioavailability in foods containing a high content of phytic acids including legume grains as a result of this property[84]. The affinity of NaFeEDTA to the various metal components may however differ as it is being digested due to numerous factors including the medium's pH, and the molar ratio of EDTA and the metal. NaFeEDTA is also limited in use for rice fortification involving multiple nutrients coating due to its color effect at high concentrations in the fortified kernels[61;77].

1.10 The Enhancers and Inhibitors of Iron Absorption

Various approaches are being considered to enhance the bioavailability of iron in fortified foods and combat iron deficiency. Particle-size reduction is one of such common approaches, and has resulted in more bioavailable iron candidates in fortified products[85]. One mechanism by which particle size reduction enhances iron bioavailability is through the increased surface area, thereby increasing the rate of solubility and absorption rate of iron in the gastric juice[86]. Iron can only be absorbed in the ferrous form (Fe^2+) by humans, and this can be readily enhanced by the activities of reducing agents such as ascorbic acid on ferric iron (Fe^3+). The reducing and chelating properties of ascorbic acid in fruits and vegetables, and partially digested muscle proteins are described as main enhancers of dietary iron absorption in humans.

Ascorbic acid is the only food component typically added to iron-fortified foods to enhance native iron and nearly all other iron fortificants absorption in multiple folds besides iron chelates. It is the most efficient non-heme iron absorption enhancer when stable, and largely suppresses the inhibitory effects of phytic acid, calcium, milk and legume proteins, and phenolic compounds on iron by converting ferric to ferrous iron and weakening its ability to combine with the inhibitory food components[78]. Additionally, the use of ligands such as citric acid (CA), trisodium citrate (TSC) and EDTA in FePP-fortified rice have been

reported to significantly improve the bioavailability of iron in humans and act as in-vitro solubilizing agents in large-scale rice fortification[87;88]. The co-extrusion of CA and TSC with FePP in rice significantly increased iron absorption without affecting the organoleptic properties of the rice[89]. Soluble ferric pyrophosphate (SFP), a product of chelation of ferric iron to citrate and pyrophosphate ligands has been reported to be more bioavailable than the FePP alone[90]. Adding a mixture of CA and TSC to rice flour before extrusion led to the formation of SFP due to the applied pressure, heat and subsequent boiling; resulting in an increased iron bioavailability[87].

The fortification of foods with iron chelates including NaFeEDTA or the ferrous bisglycinate has been successful as alternative strategies for enhancing iron absorption from foods rich in phytic acid or other inhibitors of iron absorption. NaFeEDTA has been recommended by the WHO as the choice iron enhancer in high phytate cereal flours, with iron absorption of around 2-3 times more than that of ferrous sulfate from high phytate meals[91;92]. The NaFeEDTA is commonly used in cereal flours, and its recent combination with ferrous fumarate in low-cost complementary foods is considered an essential alternative due to the instability of ascorbic acid during cooking[78]. Like the NaFeEDTA, ferrous bisglycinate also inhibits phytic acid and other inhibitors of iron. It is generally used for the fortification of liquid milk, and is reported to increase the iron levels in the children[93]. It is however capable of causing rancidity in cereals during storage and modifying the color of food when used in sensitive foods[94].

In addition, the phytate:iron ratio of rice can be improved by fortification with iron and the milling of wheat or polishing of rice[61]. Using phytase in the production of cereal or soy-based infant foods or activating natural phytases in wheat or rye can also degrade phytic acid in these foods[95]. Previous studies have reported the degradation of phytic acid and improved iron absorption of food by the addition of phytase during consumption[96]. The encapsulation of iron can also be used to prevent reaction with other components in the food matrix when stored. It is a preferred strategy when fortifying salt with multiple nutrients[97;98]. Tannins and some animal proteins including milk, eggs, soybean proteins, and albumin have also been thought to inhibit iron absorption even when combined with iron absorption enhancers

or cereal-based fortified foods[72].

1.11 Measurement of Iron outcome

Iron absorption is a function of the host's iron stores, the solubility of the consumed iron compounds, and the presence of inhibitors or enhancers of iron absorption in a food/meal. The absorption of iron is commonly assessed by measuring the rate of iron incorporation into the erythrocytes of study subjects fed meals containing radioisotope-labeled iron. It is measured as relative bioavailability against a control meal containing $FeSO_4$[99]. Hemoglobin concentration, serum ferritin, serum transferrin receptor, erythrocyte zinc protoporhyrin measurements or a combination of these investigations can be used to determine the bioavailability and bioefficacy of iron. Functional bioefficacy has been determined in children by evaluating changes in the frequency of iron-deficient anemia and growth rates[9]. The human intestinal Caco-2 cell line is an in-vitro experiment used extensively for studies involving nutrient transports[100;101]. Increased bioavailability of soluble ferric pyrophosphate (SFP) resulting from the proposed protective effect by the surrounding pyrophosphate and citrate ligands have been reported in Caco-2 cells[90].

Hepatic iron concentration is a preferred iron bioavailability measurement in animal studies due to the liver being a major repository of iron and containing about 20-30% of body iron[102]. There are two common methods for assessing iron bioavailability in animals, and these are the depletion-repletion method and preventative-prophylactic method. The depletion-repletion method measures iron status by maintaining animals on diets low or deficient in iron to reduce the iron stores of the animals, and thereafter feeding iron-rich diets to the animals to correct the iron deficit which is known as the repletion period[103]. Contrarily, the preventative-prophylactic method involves placing the animals on the iron-rich diets immediately after weaning them[104]. The preventative-prophylactic study is however considered a faster approach for assessing iron bioavailability because of its shorter study duration.

Several studies have reported varying outcomes on the bioavailability of different iron

compounds in extruded rice. FePP fortification of extruded rice grains significantly improved the iron status of participants in various feeding trials involving anemic participants[79;105]. Some efficacy studies reported improvements in the iron level of women or children who consumed encapsulated micronized dispersible FePP- (MDFP) and high concentrations of micronized ground FePP- (MGFP) fortified extruded premix rice respectively[78]. Other reports have described the improvement of body iron stores in Indian school children fed micronized ferric pyrophosphate fortified extruded rice kernels[106]. In addition, co-fortifying extruded rice with FePP and citric acid/trisodium citrate was shown to double the bioavailability of iron in the women who participated in the study[87]. The consumption of iron-fortified rice has been shown to decrease the prevalence of iron deficiency and anemia in groups over an extended period[107].

Chapter 2

Ferric Pyrophosphate Fortified Extruded Rice Increased Iron Status in Rats

Abstract

Background:

Iron deficiency is the most common micronutrient deficiency worldwide and one of the five prominent causes of years lived with disability in humans, particularly affecting women and children. Ferric pyrophosphate (FePP) and ferric phosphate ($FePO_4$) are commonly used in fortifying rice due to their minimal impacts on its sensory properties, but have both presented with poor iron bioavailability. Particle-size reduction of FePP and addition of ligands such as citric acid (CA) and trisodium citrate (TSC) to extruded FePP-fortified rice have been associated with improved iron bioavailability in iron-fortified rice.

Objective:

To compare the iron outcomes of extruded rice flour formulated with four different iron fortificants in rats.

Methods:

Using the prophylactic-preventative method, 50 male weanling Sprague-Dawley rats aged 20-23 days were randomly distributed into 5 groups and fed ad libitum for a 28-day period. The control group consumed powdered AIN-93G growth diet, while the other four experimental diets were AIN-93G based extruded rice flour diets fortified with micronized FePP (μFePP), a higher FePP concentration, $FePO_4$ with TSC and CA or FePP with TSC and CA at a molar ratio of 1:0.3:5.5 for iron to TSC/CA. Hematological parameters and hepatic iron concentration were assessed for the iron status. The dual energy x-ray absorptiometry (DEXA) PIXImus scan was used to assess the bone mineral density (BMD) and body composition of the rats.

Results:

The consumption of FePP, μFePP, FePP+TSC+CA fortified rice flour significantly increased ($p<0.05$) hepatic iron concentration compared to the $FePO_4$+ TSC+CA fortified rice flour and AIN-93G groups. However, there were no significant differences between the hepatic iron concentrations of the FePP groups or the hematological and anthropometric assessments between all groups ($p>0.05$).

Conclusion:

Increased concentration of FePP in extruded fortified rice can improve iron status, and suggests that neither micronizing FePP nor extruding it with TSC and CA improved iron

status compared to FePP. The extrusion of $FePO_4$ with TSC and CA did not improve iron status.

2.1 Background

About 25% of the world's population have been reported to be affected by anemia, causing several acute and chronic health challenges in pregnant women and children[10;11]. Anemia often results from several factors, with dietary iron deficiency (ID), inherited blood disorders, malaria, and helminthic infestation as its leading causes[14]. Iron deficiency is the most common micronutrient deficiency worldwide and is implicated as the leading cause of anemia globally, particularly affecting pregnant women, infants and young children[30]. Iron deficiency anaemia (IDA) is one of the five prominent causes of years lived with disability in humans, and is classified as a public health challenge that adversely affects young children, and women of child-bearing age[14;26]. Fortification of staple foods is considered a most sustainable and cost-effective public health intervention to improve the overall consumption of iron and iron status of populations[41;92].

The WHO has recommended the universal fortification of staple foods including rice as a choice vehicle for iron to prevent iron deficiency and IDA in at-risk populations[47;48]. Rice is a prominent staple food of about three billion people globally, and its fortification with iron reduced IDA prevalence among preschool age children, pregnant women, adolescents, and adults in previous studies[59;87]. With rice being commonly consumed as a whole grain and not flour, it is often fortified with iron using the extrusion method following a grain premix approach[70;71;108;109]. However, getting suitable iron fortificants with high absorption in the human digestive system without modifying the sensory characteristics of the food matrix still remains a challenge in the food industry. Therefore, attention is now being focused on enhancing less bioavailable compounds that do not adversely affect the taste and color of the food vehicles to reduce costs, and increase acceptability in rice-eating populations[5;87].

Ferric pyrophosphate (FePP) is a widely used and common choice of iron compound for rice fortification as result of its white color and minimal impact on the sensory properties

of the food matrices[79;80]. Additionally, the fortification of extruded rice grains with ferric phosphate ($FePO_4$) results in acceptable organoleptic characteristics with no adverse effects on the food matrix[76;81;83]. These compounds however have very low iron bioavailability and absorption of about 50% and 25% compared to the ferrous sulfate ($FeSO_4$) respectively[64;80;82]. The use of ligands such as citric acid (CA), and trisodium citrate (TSC) in FePP-fortified rice have been shown to significantly improve iron bioavailability in humans and act as in-vitro solubilizing agents in large-scale rice fortification[87;88]. The co-extrusion of CA and TSC with FePP in rice significantly increased iron absorption without affecting the sensory properties of the rice[89].

Soluble ferric pyrophosphate (SFP), a product of chelation of ferric iron to citrate and pyrophosphate ligands has been reported to be more bioavailable than the FePP alone[90]. Adding a mixture of CA and TSC to rice flour before extrusion led to the formation of SFP due to the applied pressure, heat and subsequent boiling; resulting in an increased bioavailability of iron[87]. Other reports have also described the improvement of body iron stores in Indian school children fed micronized ferric pyrophosphate (μFePP) fortified extruded rice kernels[106]. Reducing the mean particle size of FePP is thought to increase the bioavailablity of iron due to an increase in the surface area, which in turn enhances the rate of solubility and iron absorption of the fortified foods[86;110]. We previously reported lower moisture-adjusted total food intake, weight gain, final weight and bone mineral density compared to the AIN-93G group in a similar study conducted in our lab[111]. The poor intake and growth of the rice diet groups likely resulted from the inadequate nutrients and poor protein quality of the rice diets. This study, therefore investigated the iron outcomes in rats which consumed extruded rice flour formulated with four different iron fortificants.

2.2 Methods

Production of extruded fortified rice diets

Extrusion processing for the unenriched rice flour was done using a pilot-scale twin-screw extruder (TX-52, Wenger Manufacturing, Sabetha, KS) equipped with a differential diameter cylinder preconditioner, and having a volumetric capacity of 0.056m^3 (DDC2, Wenger Manufacturing, Sabetha, KS). The preconditioner shaft speed was 379 rpm with an average residence time of 2.8 minutes, a screw diameter of 52mm and an L/D ratio of 16. The material was fed into the extruder at 80 kg/h, and the screw speed fixed at 300rpm as previously reported[112]. A single-opening circular die of 3.7mm was used, and the product cut upon exit with three hard knife blades rotating at 530 rpm. Extrudates were collected and dried in a dual pass dryer (4800, Wenger Manufacturing, Sabetha, KS) at 115°C for 18 minutes, with a 7 minute cooling step. The extrudates were then ground using the hammermill grinder (Fitzpatrick DKAS012), and frozen until further fortification processes.

The iron fortificants for the study were provided by Wright Enrichment Inc. (Lafayette, LA). Mean particle size of the micronized FePP was 2.4μm, while the regular FePP had a larger particle size. The Ferric pyrophosphate (FePP) and Ferric phosphate ($FePO_4$) were blended with rice flour (RIVLAND Partnership; Houston, Texas), and the trisodium citrate (TSC) and citric acid (CA) [Sigma-Aldrich; St. Louis, MO] added to the FePP and $FePO_4$ respectively at a molar ratio of 1:0.3:5.5 iron to TSC and CA as previously described[109] by adding 0.036g CA and 1.014g TSC per kg diet (Table 2.1). They were then mixed with 200g water for 3 minutes using a bench-top mixer (N-50, The Hobart Mfg. Co., Troy, OH) to make up a total dry premix of 1.2kg material. Thereafter, the hydrated blends were placed in resealable bags and allowed to equilibrate overnight under refrigeration. Each blend was prepared using three mixes involving a minor mix comprising components having smaller concentrations. The intermediate mix was made up of components with slightly higher concentration, and the major mix consisted of components with concentrations higher than the intermediate. All blends were extruded in duplicates.

These equilibrated blends were then extruded with a lab scale twin-screw extruder (the Micro-18, American Leistritz Extruder Corp., Somerville, NJ), having 18mm barrel diameter and L/D ratio of 29. The extrusion die used was a circular cross-section die of 3.1mm diameter (dd), and feed rate was 2.1 ± 0.2 kg/h at the extruder screw speed of 350 rpm for all formulations. The barrel temperature for the extrusion was controlled at 40°C, 50°C, 60°C, 70°C, 80°C and 90°C respectively with steam and water added during the preconditioning[112]. Final products of the extrusion process were pushed out of the rice-shaped openings called die, and extrudates collected in clean aluminium trays, these were manually cut and dried in a convection hot-air oven at 70°C for 2 hours to reach a moisture content of approximately 14%. The dried products were ground to particle sizes below 1 mm using a high-speed multifunctional grinder (Moongiantgo Grain Grinder) at different time intervals for 30 seconds two consecutive times, and the grinder allowed to cool for about 3-15mins between each grinding time. Larger particles from each mix are collated, sifted and further ground for 20 seconds two consecutive times at room temperature and stored frozen in resealable bags until ready for final mixing.

Using the mixer (The Hobart Mfg. Co., Troy, OH), 63.2% unenriched extruded rice flour comprising 5,818.6g of the pilot-scale extruded rice flour and 500g of the lab-scale extruded rice flour for the μFePP and FePP formulated diets, 5,814.18g of the pilot-scale extruded rice flour and 494.12g of the lab-scale extruded rice flour for the $FePO_4$+TSC+CA and FePP+TSC+CA formulated diets. Each formulated diet blends were then mixed with 20% casein, 5% cellulose, 7% soybean oil with TBHQ, 3.5% mineral mix without iron, 1% vitamin mix and 0.3% L-Cystine (Dyets, Inc; Bethlehem, Pennsylvania) in line with the AIN-93G composition respectively (Table 2.2) to sustain the nutritional requirements of the growing rats[113]. The prepared extruded rice blends were kept in polyethylene zip-lock bags and stored frozen until use.

Nutritional analysis

Macronutrient proximate analysis of the formulated ride extrudates were determined by the University of Missouri–Columbia Agricultural Experiment Station Chemical Laboratories (Columbia, MO) as previously reported (Ward and Lindshield, 2019). The protein analysis was conducted by combustion method (LECO; AOAC 990.03, 2006) and the fat analysis by using acid hydrolysis (954.02, 2006), and the carbohydrates were calculated. Iron content was determined via the Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) method (AOAC Official Method 993.14 Trace Elements in Waters and Wastewaters).

Carbohydrate = 100% - % (crude protein + ash + crude fat + fiber + moisture)

Animals and experimental protocol

Fifty male weanling Sprague Dawley rats aged 20-23 days and weighing between 42 and 77.2 g were obtained from Charles River (Wilmington, MA) for this prophylactic-preventative study[104]. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University (IACUC-4741). All animals were assessed for well-being before and throughout the study. They were housed individually in wire-bottom cages, provided with a resting board, tongue depressors as enrichment, and provided water and food ad libitum. Rats were randomized into 5 diet groups of ten animals per group. The control group were fed the standard AIN-93G rat diet (Dyets, Inc; Bethlehem, Pennsylvania), while the other groups received one of the four extruded rice formulations: μFePP, FePP, $FePO_4$ + TSC and CA, FePP + TSC and CA (at a molar ratio of iron to TSC/CA of 1:0.3:5.5). Food intake of each rat was calculated from the food remnants, and fresh diets provided afterwards. The weight of the rats was collected at baseline upon arrival, and subsequently measured every other day and weekly. Rats were euthanized after 28 days.

Sample collection and preparation

The rats were sacrificed on the 28th day under euthanasia with carbon dioxide (CO^2) inhalation; blood was collected by cardiac puncture into tripotassium ethylenediaminetetraacetic acid (K_3EDTA) tubes for the hematological analysis. Liver tissues were harvested, weighed, and flash-frozen in liquid nitrogen and stored at -80°C.

Hematological assay

Hemoglobin concentration, hematocrit, and red cell indices were measured in the fresh whole blood in K_3EDTA tubes by flow cytometry using the Siemens Advia 2120i Hematology Analyzer as previously described[87].

Hepatic Iron Concentration

The glassware for the procedure was prepared with 6% nitric acid solution and the frozen liver samples thawed overnight in a refrigerator prior to analysis. 1 g of liver each covered with 10 mL Trace-metal grade nitric acid solution (Fisher Scientific; Pittsburgh, PA) in a 50 mL beaker and allowed to sit for 1 hour as previously described[111]. Samples were thereafter placed on a hot plate, and allowed to gently reflux for roughly 2-8 hours at low heat until approximately 1 mL of the solution was remaining in the beaker. The boiled samples were then made up to 10 mL with distilled-deionized water and transferred to 15 mL polypropylene tubes in preparation for analysis. Iron concentration was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian 720-ES, Agilent Technologies, Santa Clara, CA) at the Kansas State University Soil Testing Lab (Manhattan, KS). All liver samples were prepared in duplicate, and triplicate analyses performed on samples with variance above 15% between duplicates.

Body composition and Bone mineral density

Body scans were performed on the rats' carcass using the dual energy x-ray absorptiometry (DEXA) PIXImus densitometer according to manufacturer's instructions (GE Lunar Corporation, Madison, WI) for body composition and bone mineral density (BMD) detection.

Statistical analysis

Shapiro-Wilk was used to assess data for normality, and non-normally distributed data were transformed using natural log transformations. Levene's test was used to assess the level of homogeneity at $p<0.05$ among the groups. Group differences were determined using one-way analysis of variance (ANOVA) Least Squares Means (LSM) tests. Data collected were analysed using the SAS Studio (version 3.8, SAS Institute Inc., Cary, NC) and expressed as mean $\pm$ SEM for each group. Differences were considered significant at $p<0.05$.

2.3 Results

Diet composition, Food intake and Anthropometric evaluation

The rice diets contained 22.35g protein, 6.20g fat, 7.36g moisture, 1.12g fiber, 3.44g ash per 100g, and varying amounts of iron as indicated in table 2.3. The iron content of the FePP diet was however higher; roughly 2-folds than the other formulated diets. There were no significant differences in the weekly food intake (Figure 2.1), total food intake (Table 2.4), weight gain, average weekly weights (Figure 2.2), final body weights, lean mass and bone mineral density (Table 2.4) between groups.

Effects of formulated diets on hematological parameters and hepatic iron

There were no significant differences in the hemoglobin concentration, hematocrit, red blood cell count, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume and total white blood cell counts between the groups. However, the hepatic iron concentrations for all the FePP groups (FePP, μFePP, FePP+TSC+CA) were significantly increased compared to both the AIN-93G and $FePO_4$+TSC+CA groups. There was however no dose-response effect in the iron bioavailability of the FePP. No significant differences were also observed in the iron outcomes between the FePP groups. Hepatic iron concentration was also not significantly different in the $FePO_4$+TSC+CA group compared with the AIN-93G control (Table 2.5).

2.4 Discussion

The increased hepatic iron concentration in all the FePP-fed groups compared to the AIN-93G and $FePO_4$+TSC+CA groups suggests that these iron compounds are capable of improving iron status. Iron is stored in both the hepatocytes and Kupffer cells of the liver, where about 20–30% of excess body iron is stored in a bioavailable form as ferritin and as hemosiderin[21]. It is therefore possible that FePP might have resulted in an increased liver iron store in the form of ferritin due to the preventative-prophylactic method used in this study. FePP fortification of extruded rice grains, and co-fortification with CA and TC have been reported to improve the iron status and body iron stores of participants in various feeding trials involving anemic participants[79;105;109]. Although the FePP diet had a higher iron content than the other diet groups, there were however no significant differences between all the FePP groups. Therefore, increasing the amounts of FePP in the rice flour did not result in a significantly higher hepatic iron concentration.

Despite the higher iron content of the the FePP group in this study, the hepatic iron concentration was lower than found in a previous study conducted in our laboratory where

cooked FePP-extruded rice blended with unenriched white rice were fed to rats[111]. Several factors may influence these divergent findings, including the fact that the current study involved grinding the extruded FePP-fortified rice and feeding them directly to the rats as against cooking the extruded FePP-fortified rice in the previous study. Thus suggesting that cooking the extruded FePP-fortified rice may be beneficial to the availability of its iron as reported in other previous studies[87;106]. It is also possible that the higher concentration of the FePP counteracts its acid-driven dissolution in the stomach, thereby resulting in a lower overall solubility of the FePP, and subsequently lower bioavailability.

Ligands such as citric acid (CA), trisodium citrate (TSC) and EDTA are considered one of the best ways of enhancing iron bioavailability in FePP-fortified rice and in-vitro solubilizing agents in rice fortification[87;88]. Co-extrusion of a mixture of TSC and CA to FePP-fortified rice flour resulted in the in-situ formation of soluble ferric pyrophosphate (SFP) in the presence of pressure, heat and boiling; which increased iron bioavailability and absorption in a human study[89]. The ability of FePP+TSC+CA extruded fortified rice flour to form SFP could have also accounted for the increased level of hepatic iron concentration in the FePP+TSC+CA fed rat group. The TSC/CA ratio used in our study was same with that used in a previous study where co-extrusion of CA/TSC with FePP significantly increased iron absorption in Zurich women[109], the lack of significant difference in the FePP+TSC+CA group compared to the other groups was however an interesting and unexpected discovery which we are yet to proffer an explanation for. To our knowledge, this is the first study to investigate iron absorption from extruded rice cofortified with $FePO_4$ and TSC/CA. However, the addition of TSC/CA did not enhance the bioavailability of iron from $FePO_4$ despite the higher molar ratio of iron to TSC and CA used. Similarly, $FePO_4$ was poorly absorbed (25%) compared to $FeSO_4$ in a radiolabeled farina-based meal that were fed to human participants and rice test meals fortified with bulk $FePO_4$[82;103]. There is a remarkable similarity in the poor hepatic iron outcome of $FePO_4$ determined in our present study and that observed in our previous study involving cooked $FePO_4$ fortified extruded rice kernels[111].

Reduction of the particle size of FePP is reported to increase iron bioavailablity of fortified products as a result of its increase in surface area[110]. This enhances the rate of solubility

and iron absorption of the fortified foods[86]. Similar outcomes in the relative bioavailability of iron for emulsified μFePP with mean particle size (MPS) of 0.5 μm and $FeSO_4$ in iron-depleted rats was previously reported, with larger MPSs FePP molecules resulting in lower relative bioavailability than $FeSO_4$[85]. Improvements in iron stores and reduction of iron deficiency have also been shown with regular intake of μFePP-fortified extruded rice kernels among school children in India[106]. In contrast, the fortification of rice flour with μFePP did not significantly increase the absorption of iron in this study compared to FePP alone. It is therefore possible that there was a poor upregulation of the absorption of iron from the small-particle-sized μFePP as previously noted[79]. Additionally, a previous study involving iron-depleted rats reported no significant difference in the bioavailability of regular FePP with MPS of about 21 μm when compared with μFePP of around 2.5 μm MPS[85].

Hemoglobin concentration is one of the reliable indicators of iron absorption and the application of its cut-offs is commonly used in differentiating iron deficiency and iron deficiency anemia[9;114]. We have previously reported higher hemoglobin concentration in the FePP+TSC+CA and FePP groups compared to the AIN-93G group, and they were not significantly different than FePP alone in the study[111]. However, the hemoglobin concentrations in the various groups were not significantly different but trended higher in all the FePP groups than others in the present study. The various iron compounds also did not enhance the hematocrit, red blood cells and red cell indices of the various groups when compared together. There was no significant difference between the total white cell counts of the various groups, indicating that the concentration of the iron compounds and diet components used had no adverse effect on iron apparent absorption and the well-being of the rats.

The rice diets were formulated based on the AIN-93G; a recommended rodent diet by the American Institute of Nutrition, and the extruded rice flour replaced the dyetrose, cornstarch, and sucrose in the formulated diets. In this study, the iron content of the formulated rice diets were higher compared to the AIN-93G's. The use of a higher concentration of the iron compounds for the formulated rice diets is due to the lesser bioavailability of $FePO_4$ and FePP compared to ferric citrate used in fortifying the AIN-93G[115]. Although we had previously reported lower moisture-adjusted total food intake, weight gain, final weight and

BMD compared to the AIN-93G control group in our laboratory[111], there were no significant differences in the weekly food intake, total food intake, weight gain, final weight, average weekly weights, lean mass and BMD between the various groups in the present study. This improvement resulted from the current study's formulated diets' composition being based on the AIN-93G's. Our formulated rice diets were well consumed.

Our study has some limitations; we tested the diet formulations in apparently healthy rats and cannot exclude a different iron absorption rate under a depleted rat model as would a mass fortification program. We also did not cook the extruded fortified rice which may have been beneficial in improving the iron bioavailability of the iron compounds used. In addition, we did not scrutinize the sensory properties of the different formulated rice types.

Conclusions

This study, therefore, suggests that increasing the concentration of ferric pyrophosphate when fortified in extruded rice can improve iron status to mitigate iron deficiency and iron deficiency anemia. Our study also suggests that adding TSC/CA to $FePO_4$ did not enhance the iron status compared to other groups. Further studies that examine the findings of this present study to account for the potential effectiveness of the increased FePP in extruded fortified rice and confirm that the findings in rats can be extrapolated to humans would be welcomed. In addition, the effect of cooking on the iron bioavailability of FePP, μFePP and FePP +TSC/CA fortified extruded rice requires further investigation

Tables

Table 2.1: *Iron compounds and ligands composition per kilogram rice flour*

Components	μFePP	FePP	$FePO_4$ + TSC + CA	FePP + TSC + CA
$FePO_4$	-	-	0.121g	-
Fepp	-	0.14g	-	0.14g
μFePP	0.14g	-	-	-
TSC	-	-	1.014g	1.014g
CA	-	-	0.036g	0.036g

Table 2.2: *Percentage composition of vitamins and minerals of formulated diets*

Constituent	*AIN-93G	μFePP	FePP	$FePO_4$ +TSC+CA	FePP +TSC+CA
Extruded rice flour without iron	-	63.2	63.2	63.2	63.2
Casein	20	20	20	20	20
Cellulose	5	5	5	5	5
†Soybean oil with TBHQ	7	7	7	7	7
Mineral mix without iron	3.5	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3	0.3
Dyetrose	13.2	-	-	-	-
Cornstarch	39.7486	-	-	-	-
Sucrose	10	-	-	-	-
TBHQ	0.14	-	-	-	-
Choline bitartrate	0.25	-	-	-	-

*Percentage composition is based on product label and not analyzed protein content
TBHQ = t-Butylhydroquinone
†Soybean oil used in the rice diet formulations contained TBHQ, while TBHQ was added to the AIN-93G diet separately.

Table 2.3: *Macronutrients and iron content of formulated rice and AIN-93G diets (g/100g)*

Components	*AIN-93G	μFePP	FePP	$FePO_4$+TSC+CA	FePP+TSC+CA
Carbohydrate	59.3	59	59.4	59.3	60.2
Protein	17.9	22.8	22.7	22.2	21.7
Fat	7.0	6.2	6.2	6.3	6.2
Fiber	5.0	1.3	0.8	1.4	1.0
Moisture	6.6	7.3	7.5	7.3	7.4
Ash	4.2	3.4	3.4	3.5	3.5
Iron (mg/100g)	4.5	11.8	20.6	9.3	10.8

* Components are based on product label and not analyzed protein content

Table 2.4: *Food intake and anthropometric measures of study groups*

	AIN-93G	μFePP	FePP	$FePO_4$+TSC+CA	FePP+TSC+CA
Total food intake (g)	559.6 ± 9.5	551.5 ± 13.1	547.0 ± 18.6	552.6 ± 13.4	515.1 ± 21.2
Total weight gain (g)	251.7 ± 5.6	264.0 ± 6.1	253.0 ± 6.2	263.9 ± 6.3	239.7 ± 9.0
Final body weight (g)	310.6 ± 7.9	324.32 ± 8.4	313.2 ± 7.1	322.5 ± 6.7	298.6 ± 11.0
Lean mass (%)	89.7 ± 0.6	91.0 ± 0.3	90.1 ± 0.4	90.5 ± 0.4	90.0 ± 0.4
Bone mineral density (g/cm^2) x 1000	113.8 ± 2.4	112.1 ± 3.3	107.7 ± 1.7	112.9 ± 2.2	108.1 ± 2.9

Data represent Mean ± SEM
No significant difference between groups
Percentage lean mass: Total weight minus fat mass divided by total weight x 100.
Food intake was measured every other day by subtracting food remnants from the previous food given.

Table 2.5: *Hematological and hepatic iron outcomes*

	AIN-93G	μFePP	FePP	$FePO_4$+TSC+CA	FePP+TSC+CA
Hematocrit (%)	49.1 ± 0.7	50.1 ± 0.9	49.5 ± 0.5	49.8 ± 0.7	50.5 ± 0.6
Hemoglobin concentration (g/dl)	13.8 ± 0.2	14.3 ± 0.3	14.2 ± 0.2	14.0 ± 0.20	14.4 ± 0.2
Red blood cell counts (M/μL)	6.9 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	7.1 ± 0.1	7.0 ± 0.1
Mean cell volume (fl)	71.0 ± 1.1	73.1 ± 0.7	72.0 ± 0.6	69.9 ± 1.7	72.4 ± 0.9
Mean cell hemoglobin (pg)	20.0 ± 0.3	20.8 ± 0.2	20.7 ± 0.2	19.6 ± 0.5	20.6 ± 0.3
Mean cell hemoglobin concentration (g/dL)	28.2 ± 0.1	28.5 ± 0.2	28.8 ± 0.2	28.0 ± 0.2	28.5 ± 0.3
Total white blood cell count (K/μL)	12.7 ± 0.8	12.6 ± 0.6	12.3 ± 0.9	11.8 ± 0.9	13.8 ± 1.1
Hepatic iron (μg/g)	7.1^a ± 0.5	11.4^b ± 1.3	11.7^b ± 1.0	8.1^a ± 0.4	11.5^b ± 1.2

Data represent Mean ± SEM
Values in the same row with different superscripts are significantly different at $p<0.05$.

Figures

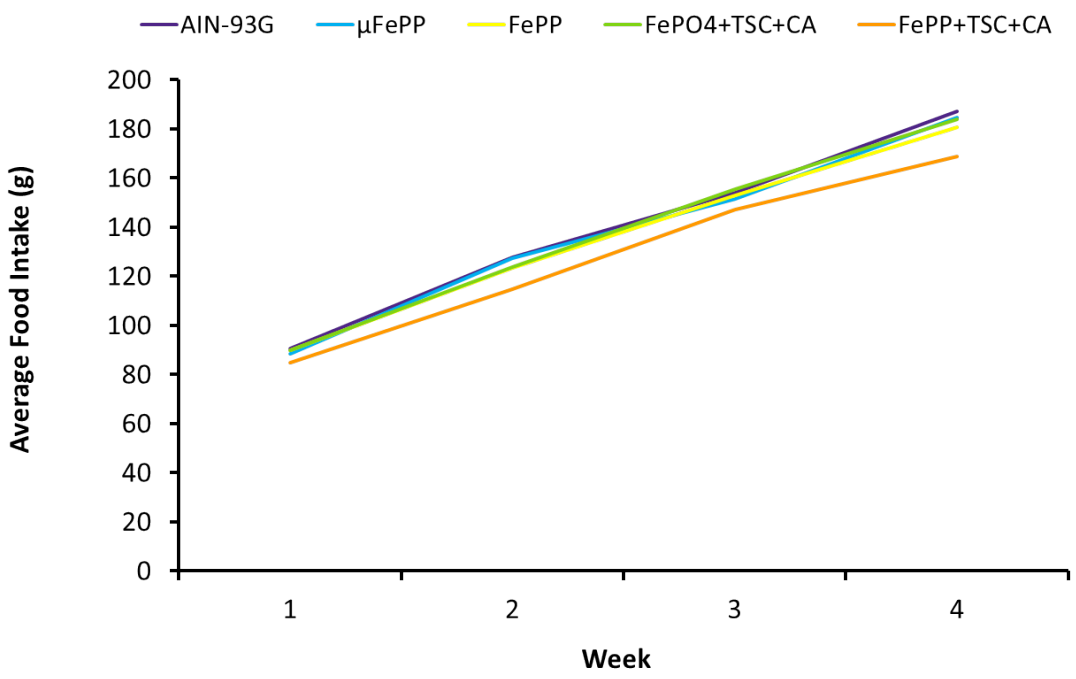

Figure 2.1: *Average weekly food intake per group*

A trend chart showing the average weekly intake of food for the various groups. Each colored line graph represents the respective study groups.
No significant difference in the mean weekly food intake between groups.

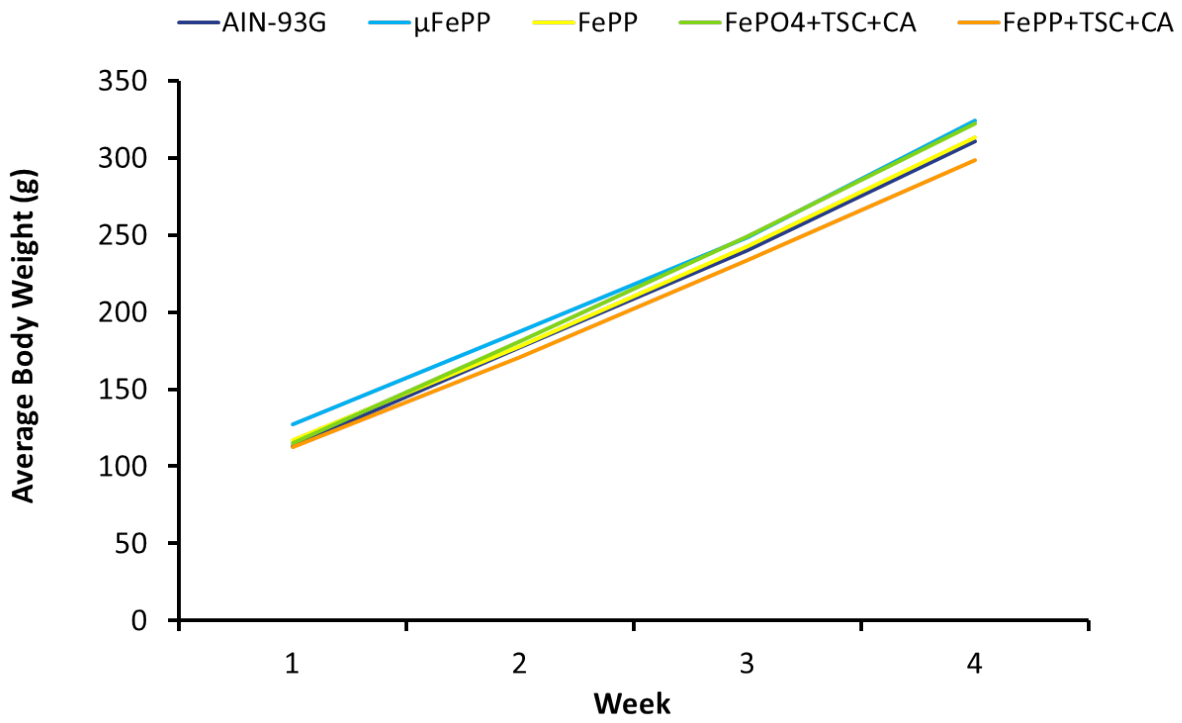

Figure 2.2: *Average body weight of diet groups per week*

A trend chart showing the average weekly body weight of rats for the various groups. Each colored line graph represents the respective study groups.
No significant difference in the mean weekly weights between groups.

Chapter 3

Conclusions

Given the outcomes and limitations revealed in the first similar experiment in our laboratory, we made diets' composition to be based on the AIN-93G where extruded rice flour replaced the dyetrose, cornstarch, and sucrose in the present experiment. We also ensured that all mixes for the the diets were done under the guidance of our collaborator in the Grain Science department; Dr. Sajid Alavi who routinely produces pet and other food products. This helped to ensure standardization of the formulated diets and prevented the loss of any of the micronutrients in our premixes. The findings from this study show that iron absorption from the different FePP-fortified rice groups was higher compared to the $FePO_4$ and AIN-93G groups. FePP is a simple, less expensive iron compound having a low reactivity and better sensory impacts on food matrices, and is generally considered safe. This therefore indicates that fortifying μFePP, FePP+TSC/CA and increased concentration of FePP in extruded rice can improve iron status to mitigate iron deficiency and iron deficiency anemia. Our study also suggests that adding TSC/CA to $FePO_4$ did not enhance the iron status compared to other groups. No significant differences were observed in the food intake, growth, and anthropometric features of the various groups. The formulated rice diets were well consumed by the rats.

We have learned much from this model of iron bioavailability study; although numerous aspects of our approach worked well, there are a few things we could do differently

in the future. Most importantly, I would suggest conducting a similar animal study that would include a depleted rat model group to assess their absorption rate relative to other groups, as well as a prophylactic-preventative group, an $FePO_4$ alone group, alongside a $FePO_4$+TSC+CA group using the dose and time-course model. Although challenging, it would be very informative to conduct the study for an extended duration beyond our current timeline for extensive toxicity testing of $FePO_4$+TSC+CA, FePP+TSC+CA and the increased regular FePP used in this study. A key implication of this research is in advancing our understanding of iron fortificants and iron absorption enhancers for the purpose of improving iron status. Thus, further studies that examine the findings of this present study to account for the potential effectiveness of the increased FePP in extruded fortified rice and confirm that the findings in rats can be extrapolated to humans is highly encouraged. Additionally, the effect of cooking on the iron bioavailability of FePP, uFePP and FePP +TSC/CA fortified extruded rice can also be further investigated.

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Appendix A

List of Abbreviations

AIN	American Institute of Nutrition
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BMD	Bone Mineral Density
CO_2	Carbon Dioxide
CA	Citric Acid
DEXA	Dual Energy X-ray Absorptiometry
EAA	Essential Amino Acid
EDTA	Ethylenediaminetetraacetic Acid
$FePO_4$	Ferric Phosphate/Orthophosphate
FePP	Ferric Pyrophosphate
$FeSO_4$	Ferrous Sulfate
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IACUC	Institutional Animal Care and Use Committee
IDA	Iron Deficiency Anemia
LSM	Least Square Means
MPS	Mean Particle Size
μFePP	Micronized Ferric Pyrophosphate
RBV	Relative Bioavailability
NaFeEDTA	Sodium Iron EDTA
SFP	Soluble Ferric Pyrophosphate
TSC	Trisodium Citrate
WHO	World Health Organization