Original Research

Oily Fish Increases Iron Bioavailability of a Phytate Rich Meal in Young Iron Deficient Women

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Background: Iron deficiency is a major health problem worldwide, and is associated with diets of low iron bioavailability. Non-heme iron absorption is modulated by dietary constituents, one of which is the so-called "meat factor", present in meat, fish (oily and lean) and poultry, which is an important enhancer of iron absorption in humans. Food processing also affects iron bioavailability.

Objective: To evaluate the effect of consuming *sous vide* cooked salmon fish on non-heme iron bioavailability from a bean meal, rich in phytate, in iron-deficient women.

Design: Randomized crossover trial in 21 young women with low iron stores (ferritin < 30 μg/L). Two test meals were extrinsically labelled with stable isotopes of iron (Fe-57 or Fe-58). Iron bioavailability was measured as the incorporation of stable isotopes into erythrocytes 14 d after meals consumption.

Results: The addition of fish to the bean meal significantly increased ($p < 0.001$) iron absorption. Serum ferritin concentration and iron absorption were inversely correlated for both the bean meal ($R^2 = 0.294$, $p = 0.011$) and the fish and bean meal ($R^2 = 0.401$, $p = 0.002$).

Conclusion: *Sous vide* cooked salmon fish increases iron absorption from a high phytate bean meal in humans.

INTRODUCTION

Recent estimates of the prevalence of iron deficiency are between 8 and 33% of young women in Europe [1], and 10 to 16% in the United States [2], while in developing countries it affects almost half the population of women of child-bearing age (42.3%) [3]. Iron supplements are considered to be the most effective way of treating iron deficiency anemia, however their role in preventing iron deficiency is less clear, due to problems with poor compliance, long-term acceptability, cost effectiveness, risk of iron overload, and adverse side effects. Preventing iron deficiency through diet is one of the main targets in the World Health Organisation [4].

In patients with iron deficiency, medical advice often consists of increasing the intake of meat. Animal flesh is the most readily available source of iron because it contains heme iron, which is efficiently absorbed, being relatively immune to dietary components that reduce non-heme iron absorption. Animal tissue, such as fish, beef, chicken, pork and lamb contain an active substance, or substances, collectively known as "meat factor", which enhances dietary non-heme iron absorption [5], although the precise "meat factor" mechanism on iron bioavailability is not fully understood. Several studies have demonstrated the muscle tissue enhancing effect of beef [6,7] and pork [8] but few have focused on fish. Hallberg et al [9] showed that iron absorption from 5mg iron doubled with the addition of 60g of lean fish to a simple meal comprised of rice, boiled vegetables and curry. To our knowledge, there are no studies that have assessed the influence of oily fish on iron bioavailability. Much effort has been given to identify the nature of the "meat factor" but no candidate proteins or peptides have been verified. Recent

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studies using an *in vitro* model (Caco-2 cells) has attributed the promoting effect to sulfated glycoaminoglycan carbohydrates isolated from fish muscle tissue [10].

Phytate is a well-known inhibitor of iron absorption [11]. Numerous studies have investigated the effect of phytate on iron bioavailability in various models and matrices, and it is quite likely that the "meat factor" may counteract the inhibitory effect of phytates which predominate in vegetable foods, the most important of which are in cereal grains and legumes [12]. Recent studies on haddock fish using the *in vitro* Caco-2 cell model have determined the levels of "meat factor" needed to enhance iron absorption from phytic acid-iron complexes [13].

Food processing can also influence non-heme iron absorption, due to changes in components that enhance or inhibit iron bioavailability. Phytates lose phosphate groups during fermentation and cooking [14,15]. Cooking pork meat for 60 minutes at 120°C did not impair non-heme iron absorption compared to cooking at 70°C for the same time [16], however sterilization of fish at 105°C for 110 minutes reduced its enhancing influence on iron absorption [17]. The emerging *sous vide* (under vacuum) processing technique offers advantages compared with traditional cooking techniques due to the low heat treatment applied and the absence of an oxygen atmosphere, resulting in a better preservation of vitamin content and lower lipid oxidation [18]. The influence of *sous vide* cooked oily fish as part of a meal on iron bioavailability in humans has never been studied. In the present study cooked red-kidney beans were used because they are rich in phytate.

The objective was to compare non-heme iron bioavailability from a bean meal with or without *sous vide* cooked salmon fish.

SUBJECTS AND METHODS

This study was approved by the Ethics Committee of Hospital Clinica Puerta de Hierro and Spanish Council for Scientific Research, in Madrid (Spain).

Subjects

Volunteer recruitment was carried out through advertisements in the Complutense University campus and by giving short talks about the study between lectures. One hundred and sixty-two healthy, 18–45 year-old, non-smoking, non-pregnant, non-anemic (hemoglobin >110g/L), menstruating women with low iron stores (serum ferritin < 30 μg/L), who had suffered from iron deficiency anemia or had a family history of anemia underwent a pre-study screening, which included a blood test and health questionnaire. None had taken iron supplements in the 12 months before the start of the study, were blood donors or taking any medication that could influence their iron metabolism. Twenty-five women agreed to take part in the nutritional trial, but four of them were excluded for presenting ferritin values higher than 30 μg/L.

Test Meals and Food Labeling

Red-kidney beans (*Phaseolus vulgaris* from Luengo Company, grown in León, Spain) were cooked using a high pressure rapid cooker (WMF, Germany) after standarizing conditions. Salmon fish (*Salmo Salar,* from El Corte Inglés Stores, grown in Norway) was processed by the *sous vide* technique following García-Linares et al procedure [19]. Components of the test meals are presented in Table 1. The total iron content in the bean and bean fish meal was determined by atomic absorption spectrometry. Heme iron was calculated according to Hallberg and Hulthen [20]. Protein and fat contents of the meals were analysed using a protein analyser (FP 2000, LECO, Michigan, USA) and a gas chromatograph with FID detector (Perkin Elmer 8500, Boston, USA) respectively, and total inositol phosphates, inositol hexaphosphate (IP6), and inositol pentaphosphate (IP5) content by HPLC [21].

Non-heme iron in the meals was extrinsically labelled by simultaneously consuming isotopically enriched iron in 100 mL of an isotonic drink (Powerade, Coca-Cola Company, Madrid) with the meal. The drink contained citric acid as a preservative and antioxidant and its pH was 3.56. The stable isotope solution was added to the drink just before consumption. Labelled ferric chloride solutions were prepared by weighing 0.4980 g and 0.1609 g of elemental Fe-57 and Fe-58, respectively, (Chemgas, Boulogne, France) and adding 8.9 mL and 9.5 mL of 6M HCl, respectively, and heating the mixture slowly to dryness in a silica crucible. The resulting powder was dissolved in 5 mL of 0.01M HCl and left overnight. Labelled ferric chloride

Table 1. Test Meal Components

	Total iron	Non-heme iron	Moisture	Protein	Fat	Phytate (mg/portion)			
						IP4	IP5	IP6	Total IP
	mg/portion	mg/portion		%[2]					
Sous vide salmon	0.46 ± 0.04	0.36[1]	58.3 ± 0.2	20.7 ± 0.4	19.8 ± 0.1	ND	ND	ND	ND
Cooked beans	4.1 ± 0.1	4.1	75.5 ± 1.0	5.4 ± 0.1	1.8 ± 0.1	36.1 ± 1.4	50.6 ± 2.9	140.3 ± 1.9	226.8 ± 1.4

All values are mean ± standard deviation of four determinations.

ND: Below limits of detection.

[1] Estimated that fish supplied < 0.1 mg/100g of heme iron [20].

[2] Carbohydrates calculated by difference were 1.2 % and 17.3 %, in *sous vide* salmon and cooked beans respectively.

solutions were used to reproduce the chloride form of iron frequently present in food. The next day, the solution was transferred to a 100 mL volumetric acid-washed flask and the solution was made up to 100 mL with Milli-Q water obtaining a final concentration of 5 mg/mL of Fe-57 and 1.67 mg/mL of Fe-58. The solutions were filtered through a 0.22 μm filter (Osmonics, Lancashire, United Kingdom) and sub-sampled into 1 mL individual doses which were precisely weighed (0.9925 $\pm$ 0.02 for Fe-57 and 1.058 $\pm$ 0.005 for Fe-58). In order to adjust the total iron content of meals to 9.56 mg of Fe, three solutions of unlabelled ferric chloride solutions (3.79 mg/mL, 3.33 mg/mL and 0.46 mg/mL) were prepared from ultrapure iron powder (Sigma-Aldrich, Steinheim, Germany) and, when required, the appropriate amount was added to the isotonic drink before use. The solutions were filtered through a 0.22 μm filter into acid-washed glass bottles, and iron concentrations were confirmed by atomic absorption spectrometry.

Procedures and Analytical Methods

On day 1, a baseline blood sample was taken in Fundación Jimenez-Diaz Unilabs and volunteers attended Instituto del Frio (Madrid) for test meals. Iron isotope content and iron-status indexes were measured. The study was a randomized double crossover trial. After an overnight fast, on days 1, 3 and 5 subjects consumed the cooked beans (196 g cooked weight corresponding to 50 g raw weight) and on days 2, 4 and 6 a meal containing the beans and 100 g (cooked weight) of *sous vide* cooked salmon fish. Meals were extrinsically labelled with a total amount of 15mg (5 mg per meal) and 5 mg (1,67 mg per meal) of Fe-57 and Fe-58, respectively. These different amounts were due to differences in the detection limits of the two iron isotopes. In order to avoid any isotope-related effects, half of the volunteers were randomly assigned to consume the bean meal with isotope Fe-57 and the bean and fish meal with Fe-58, while the other half consumed the bean meal with isotope Fe-58 and the bean and fish meal with Fe-57. All volunteers took the food and drink within 15–20 min and at least one member of the research team was with them during the consumption. No food or drink was allowed 2 h after finishing the meals.

Fourteen days after the last labelled meal was consumed, a 30 mL fasting blood sample was taken for measuring hematological indices and iron isotope enrichment in erythrocytes. Previous reports show that this 14 d period is enough to detect stable iron isotopes in blood [22].

Blood samples were collected by venipuncture into EDTA tubes. Hematological parameters were determined following standard laboratory techniques and using the Symex NE 9100 automated hematology (Symex, Kobe, Japan) and the Modular Analytics Serum Work Area (Roche, Basel, Switzerland) analyzers. Measurements of hemoglobin concentration, serum ferritin, hematocrit, mean corpuscular volume, serum transferrin (TRF) and serum iron were carried out and total iron binding capacity (TIBC (μmol/L) = 25.1 $\times$ TRF (g/L)) and transferrin saturation (serum Fe/TIBC $\times$ 100) were calculated.

Iron absorption from the test meals was determined by measuring the enrichment of Fe-57 and Fe-58 in red blood cells 14d after taking the last meal, the total iron concentration in whole blood and an estimate of blood volume [23]:

$$\text{Blood volume (L)} = [0.3669 \times \text{height (m)}^3] + [0.03219 \times \text{weight (kg)}] + 0.6041$$

In order to determine iron isotope enrichment in whole blood, samples were processed following the method of Roe et al [22]. Isotope ratio analysis was carried out on a focusing Multi-Collector Mass Spectrometer (MC-ICP-MS) (Isoprobe; Micromass, Manchester, United Kingdom) with a desolvating sample introduction system with a microconcentric nebulizer (Aridus and T1H; both from Cetac, Omaha, NE). All samples were run in triplicate and were calibrated against IRMM014 which was measured before and after each sample. The iron content of whole blood was calculated from the hemoglobin concentration as follows:

$$\text{Iron content of blood } (\mu\text{g/mL}) = \text{hemoglobin (g/dL)} \times 34.7$$

Concentrations of the Fe-57 and Fe-58 doses in the sample were calculated from the mole fractions of each isotope measured by ICP-MS, the natural abundance of each isotope, and the mole fractions of each isotope in the doses. The quantities of doses circulating in the blood were calculated by multiplying the measured concentration by the total iron content of the blood. Iron absorption was calculated as the percentage of dose found in red blood cells after 14 d.

Statistical Analysis

Absorption data corresponding to the bean, and bean and fish meal were analyzed by using analysis of variance (ANOVA) with repeated measures, with serum ferritin concentration as a covariant. The relationship between % iron absorption and log serum ferritin concentration values was examined using Pearson's correlation test, and the comparison between regression lines was analysed by ANOVA of the regression coefficients. Values of $p < 0.05$ were considered significant. The SPSS statistical package (version 13.0.1) was used.

RESULTS

The total amount of iron (unlabeled and labeled) ingested per meal was 9.56 mg. Total labeled iron ingested by volunteers was 15 mg of Fe-57 (5 mg/meal, three meals) and 5 mg of Fe-58 (1.67 mg/meal, three meals).

Anthropometric and hematological characteristics of the 21 volunteers are presented in Table 2. Iron absorption in the bean meal was 4.52 $\pm$ 3.0%, and in the fish and bean meal was

Table 2. Anthropometric and Hematological Characteristics of the Subjects Who Participated in the Trial[1]

Age (y)	24.5 ± 4.7
Height (cm)	166 ± 6
Weight (kg)	61.99 ± 6.7
Body Mass Index (kg/m^2)	22.5 ± 2.2
Hemoglobin (g/dL)	13.1 ± 0.9
Serum ferritin (μg/L)*	12.7 (11.5–17.2)
Hematocrit (%)	39.6 ± 2.4
Mean corpuscular volume (fl)	86.4 ± 4.8
Total-iron binding capacity (μmol/L)	393 ± 63
Serum iron (μmol/L)	86 ± 33
Transferrin saturation (%)	22.0 ± 8.1

[1] All values are mean ± standard deviation.
* Geometric mean (range).

6.68 ± 4.1% (mean ± standard deviation). Analysis of variance with repeated measures showed that the presence of fish significantly increased iron absorption ($p < 0.001$, Fig. 1).

There were significant negative correlations between log serum ferritin concentration and iron absorption from both the bean ($R^2 = 0.425$, $p = 0.001$) and the fish and bean meal ($R^2 = 0.530$, $p < 0.001$) and no significant differences between regression lines (Fig. 2). Other correlations between iron absorption and transferrin, mean corpuscular volume, hemoglobin or serum iron were not significant.

DISCUSSION

This study clearly demonstrated for the first time the positive effect of oily fish on iron absorption from a phytate-rich meal in humans. The results show that adding salmon fish to a bean meal increased iron absorption in young women with low iron stores and hence at risk of iron deficiency anemia. The quantities of beans and fish used in the present study represent typical portion sizes in the diet [24]. The phytate content of the beans used in this study (5.36 ± 0.52 and 4.75 ± 0.03 mg/g dry matter of raw and cooked beans respectively, being IP6 the main inositol phosphate (78.7 and 61.9% of the total content respectively), are in agreement with Muzquiz et al [21] who analysed raw *Phaseolus vulgaris* from different regions of Spain. Inositol phosphates IP5 and IP6 are well recognized inhibitors of iron absorption [25–27], the effect being dose-dependent [11]. Taking into account the amount of phytate after cooking, the phytate:iron molar ratio in the present study was 2.6. Molar ratios higher than 1 reduce iron absorption in humans [11]. Ascorbic acid, which is the most powerful enhancer of iron absorption, negated phytate inhibition of non-heme iron absorption in a maize-bran meal with a phytate/iron molar ratio of 1 [28]. Another effective way of overcoming the inhibitory effect of phytate is the inclusion of meat in a phytate-rich meal. Baech et al [8] showed that the addition of 50 and 75 g of pork meat to a meal containing 220 mg of phytic acid (molar ratio phytic acid to iron of 8.7), significantly enhanced non-heme iron absorption by 44% and 57%, respectively, and the cooking temperature of meat did not affect non-heme iron absorption [16]. Engle-Stone et al [13] have recently demonstrated the counteracting effect of lean fish over phytates with regard to iron uptake by Caco-2 cells, which is supported by our present findings in human volunteers.

The effect of different sources of animal proteins on non-heme iron absorption has been compared in a number of studies. Reports indicate that beef, lean fish, chicken, and calf thymus increased iron absorption to about the same extent [29], and these animal tissues increased iron absorption from a

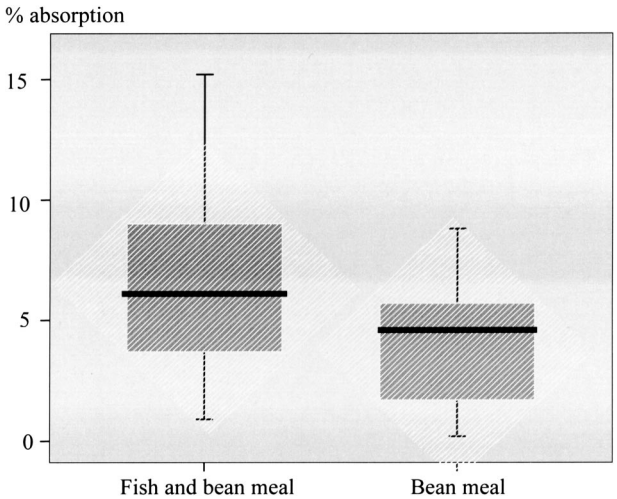

Fig. 1. Non-heme iron absorption (%) from the red-kidney bean and red-kidney bean and salmon diets. Horizontal bold lines indicate median values, boxes represent 5–95% confidence interval, and the bars show the minimum and maximum values. The effect of consuming fish on iron absorption was significant (ANOVA with repeated measures, $p < 0.001$).

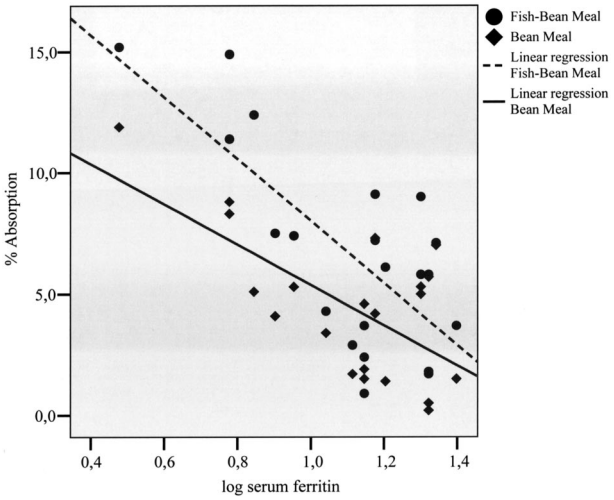

Fig. 2. Relation between serum ferritin and non-heme iron absorption. Fish and bean meal ($R^2 = 0.530$, $p < 0.001$). Bean meal ($R^2 = 0.425$, $p = 0.001$).

semi-synthetic meal of low iron availability by 2 to 4-fold [5]. Engelmann et al [7] observed that 25g of meat (lean beef) enhanced non-heme iron absorption from 80g of vegetable pureé (15% vs. 9.9%, geometric mean) in infants using stable isotopes. The present results, where the addition of 100 g of *sous vide* salmon fish has increased iron absorption by 49% (from 4.5 to 6.7%), are in the same range as previous observations using small amounts (25–75 g) of pork meat [8].

Fish has been shown to contain substances that enhance iron uptake by Caco-2 cells [10,13]. Compared to meat, it has a relatively low total iron concentration e.g. *sous vide* salmon contains only 0.46 mg Fe/100g edible portion, with minimal levels of heme iron (below 0.1 mg/100 g edible portion) [20] compared with grilled beef steak which contains 3.6mg Fe/100g. The nature of the iron enhancer in meat has not yet been identified, but candidates include peptides rich in cysteine residues from muscle tissue [30] and carbohydrate fractions in fish [10]. Further evidence for the enhancing effect of fish is the observation that pre-menopausal women who habitually consumed a poultry/fish rich diet had higher iron stores, measured as serum ferritin, than red meat consumers [31]. However, there is no information concerning the influence of oily fish on iron absorption separated from other animal products. Oily fish may be beneficial in individuals with iron deficiency, because a slightly higher iron absorption from the fish and bean meal is observed in women with lower iron stores (Fig. 2). This is consistent with the general observation that subjects with low iron stores show highest benefit from interventions. However, other components of the food matrix may also interact with the iron-phytate complex and alter the bioavailability of iron.

The present study shows that the intake of salmon containing 20 g of fish oil does not impact negatively on iron absorption, in contrast to other studies indicating that consumption of diets with high levels of polyunsaturated fatty acids decreases iron bioavailability in humans [32] and rats [33–35]. In their study, Lukaski et al [32] used diets containing 50% of daily energy intake from polyunsaturated fat rich in linoleic acid compared to other energy sources during 28 days, and found that apparent iron absorption was reduced and there was a tendency to decrease serum ferritin. Adverse effects of excessive n3 intake have also been reported when fish oil was the only dietary fat source given to rats in comparison to saturated fat or olive oil. These have not been associated with digestive but with metabolic [33–35] effects. An habitual diet that contains a moderately high content of fish may not have the same effect, and the type of fish consumed, oily or lean, should be taken into account.

CONCLUSION

Sous vide cooked salmon fish increases iron absorption from a high phytate bean meal in iron deficient young women. However, further long-term studies are required to confirm the beneficial effects of oily fish observed in the present investigation.

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