The American Journal of Clinical Nutrition
Official publication of the American Society for Nutrition

The American Journal of Clinical Nutrition
AJCN/2017/161364
Version 3

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Trial registered: ClinicalTrials.gov Reg No. NCT02365103

A one-hour time interval between a meal containing iron, and consumption of tea, attenuates the inhibitory effects on iron absorption: a controlled trial in a cohort of healthy UK women using a stable iron isotope. (1-5)

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Names for PubMed indexing: Ahmad Fuzi, Koller, Bruggraber, Pereira, Dainty, Mushtaq

Sources of Support: The study was funded by the University of Chester and the UK Medical Research Council (Grant number U105960399) provided support with the iron isotopic analyses.

Short running head: Inhibitory effect of tea on iron absorption

Abbreviation list: NDNS, National Diet and Nutrition Survey; RNI, reference nutrient intake, $^{57}FeSO_4$, ferrous sulfate; EDTA, Ethylenediaminetetraacetic acid; FBC, full blood count; ELISA, enzyme-linked immunosorbent assay; CRP, C-reactive protein; ICP-MS/MS, inductively-coupled plasma-mass spectrometry; COMA, Committee on the Medical Aspects of Food Policy; ANOVA, One-way repeated measures Analysis of Variance; BMI, body mass index; IDA, iron deficiency anemia; TM, test meal

Clinical Trial Registry number: NCT02365103, obtained from www.clinicaltrials.gov

ABSTRACT

Background: Tea has been shown to be a potent inhibitor of non-heme iron absorption but it remains unclear whether the timing of tea consumption, relative to a meal, influences iron bioavailability.

Objective: To investigate the effect of a one-hour time interval of tea consumption on non-heme iron absorption in an iron-containing meal, in a cohort of iron-replete, non-anemic female subjects, using a stable isotope (^{57}Fe).

Design: Twelve women (mean age (± SD): 24.8 ± 6.9 years) were administered a standardized porridge meal extrinsically labelled with 4 mg of ^{57}Fe as $FeSO_4$, on 3 separate occasions, with a 14-day time interval between each test meal (TM). The test meal was administered with: water (TMI); tea administered simultaneously (TMII); and tea administered 1 hour post-meal (TMIII). Fasted venous blood samples were collected for iron isotopic analysis and measurement of iron status biomarkers. Fractional iron absorption was estimated by the erythrocyte iron incorporation method.

Results: Iron absorption was 5.7 ± 8.5% (TM I), 3.6 ± 4.2% (TM II) and 5.7 ± 5.4% (TM III). Mean fractional iron absorption was found to be significantly higher (2.2 %) when tea was administered 1 hour post-meal (TM III) compared to when tea was administered simultaneously with meal (TM II, $p = 0.046$). A 2-fold reduction in the inhibitory effect of tea (relative to water) was observed, from 37.2% (TM II) to 18.1% (TM III).

Conclusions: This study demonstrates that tea consumed simultaneously with an iron-containing porridge meal leads to decreased non-heme iron absorption, and that a one-hour time interval between a meal and tea consumption attenuates the inhibitory effect, resulting in increased non-heme iron absorption. These findings are not only important in relation to the management of iron deficiency, but should also inform dietary advice, especially that given to those at risk of deficiency.

Keywords: iron bioavailability, iron deficiency, hepcidin, polyphenols, tea

INTRODUCTION

Iron deficiency is a condition defined as a lack of iron stores which leads to an enduring negative iron balance (1), commonly affecting women of childbearing age and children worldwide (2). Iron remains a marginal nutrient in the dietary intake of the UK population (3), leading to concerns of an increased risk of iron deficiency. The UK National Diet and Nutrition Survey (NDNS) shows that women aged 19-64 years do not meet the recommended level of dietary iron intake with a mean (±SD) daily intake of 9.6 ± 3 mg which equates to 78 % of the reference nutrient intake (RNI) (4). In countries where iron deficiency is not commonly attributable to iron metabolism-related diseases, it is suggested that insufficiency in absorbable iron to meet the iron requirement is one of the factors that may contribute to iron deficiency (5). The presence of inhibitors and enhancers in the diet will affect iron bioavailability more than the chemical forms of iron itself, and polyphenols have been demonstrated to be potent inhibitors of iron absorption (6). Polyphenols can be found in plant-based sources, including tea, wine, and coffee (7). British adults (aged 19-64) consume an average of 1,114 g of tea, coffee, and water daily, and this amount is higher in women (1,132 g/day) compared to men (1,097 g/day) based on the recent NDNS data (4).

To the best of our knowledge, few human studies to date have investigated the inhibitory effect of tea consumption on iron absorption using an isotope-labelled test meal. Hurrell, et al. (8) found that the inhibitory effect of black tea on iron absorption ranged between 79-94 % in a study carried out using a simple bread roll test meal and different types of phenolic-containing beverages. Significant reductions in iron absorption were also observed ($p<0.001$) when the test meal was given with tea compared to water in a study by Thankachan, et al. (9), which used a rice-based test meal.

A study by Disler, et al. (10) carried out in 10 male Sprague rats, reported that the minimum time interval for the inhibitory effect to be established was 30 minutes, with at least

a 1.8 fold higher iron absorption compared to a when there was no time interval (27.6 to 50.7 %, $p<0.005$).

To date, it is apparent that limited data are available, especially from controlled intervention studies investigating the inhibitory effect on iron absorption of tea components, and no studies in humans addressing iron absorption, particularly time interval, have been carried out in the UK. As tea is not only widely consumed in the UK (11), but extensively worldwide, the relevance of this study has global impact. Therefore, we aimed to assess the effect of time interval of tea consumption relative to a meal, on non-heme iron absorption from a typical western breakfast, using a stable iron isotope (^{57}Fe) in non-pregnant women and assessing the potential link between hepcidin concentration and iron absorption.

SUBJECTS AND METHODS

Subjects

Twelve pre-menopausal women aged 19 - 40 years were enrolled in the study using posters and emails as a recruitment medium. All subjects were healthy, without any known history of gastrointestinal or metabolic disorders, non-pregnant and non-lactating. The subjects were excluded if they had donated blood in the past 6 months, and regularly consumed nutritional supplements. Subjects were provided with a participant information sheet and were briefed on the study protocol before providing written consent prior to partaking in the study.

Sample size:

Sample size was estimated using iron absorption data from a study by Derman, et al. (12) carried out in Indian women aged between 21-71 years using a maize meal labelled with radioisotope ^{55}Fe or ^{59}Fe. The study reported mean (± SD) iron absorption of 6.7 ± 6.2 % when the test meals were administered with tea and 34.0 ± 23.0 % when the test meals were administered without tea. With a Cohen's effect size (d) of 1.32, the total sample size required in the present study was estimated to be 10 subjects (power = 0.95, α error probability = 0.05). Incorporating a potential 20% drop out rate, the total sample size required to demonstrate a significant difference in iron absorption between consumption of meal with and without tea was estimated to be 12. The sample size was estimated using G-Power software, version 3.1.7 (13).

Study design

A non-randomized, controlled intervention study was carried out between November 2014 to March 2015 to investigate the effect of tea consumption on non-heme iron absorption

using ^{57}Fe as a stable isotope (ClinicalTrials.gov registration : NCT02365103). Each subject attended the clinic on five separate occasions over a period of 56 days during which they were administered three standardized test meals and reference iron dose in the following order: (I) test meal administered with water; (II) test meal administered simultaneously with tea; (III) test meal administered with tea 1 hour post-meal; (IV) reference iron dose without a test meal. The study protocol was approved by the National Research Ethics Service Committee North West-Greater Manchester East, United Kingdom (REC reference: 14/NW/0310, IRAS Project ID 154775). All procedures were carried out in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Study Protocol:

Eligible volunteers provided informed consent at the beginning of the study. All subjects were required to fast for approximately 10 hours the night prior to each test dose of iron. The scheduled clinic visits at the clinical research laboratories in the Department of Clinical Sciences & Nutrition, University of Chester were between 8 am and 10 am. All subjects were asked to attend a total of 5 clinic sessions (day 0, 14, 28, 42 and 56) with a 14-day interval between clinics to allow the incorporation of the isotope into the erythrocytes. A standardized test meal of porridge, extrinsically labelled with $^{57}FeSO_4$ was administered to the subjects with water or tea depending on the clinic, under supervision of the investigator. The subjects were not permitted to consume food or drink for 3 - 4 hours after the administration of the test meal. All the containers used to administer the test meals were washed with ultrapure water to ensure complete consumption of both test meals and isotope label. The study protocol is summarised in **Figure 1** and details of each clinic sessions are as follows.

Clinic 1 (Day 0)

The subjects' height and weight were measured and a 30 ml fasted blood sample was collected in a trace element-free EDTA blood collection tube for isotopic analysis and iron status measurements. The whole-blood sample collected at every clinic was used for iron isotopic analysis and the plasma sample was used for iron status measurements. The investigator conducted a 24-hour food recall interview and subjects were given a 3-day food diary to record their dietary intake at home. Subjects were then administered test meal I, with water to serve as a control.

Clinic 2 (Day 14)

The subjects' weight was measured and a 30 ml fasted blood sample was collected in trace element-free EDTA blood collection tubes. The investigator conducted a 24-hour food recall interview and subjects were then administered test meal II, simultaneously with tea.

Clinic 3 (Day 28)

The same procedure as in Clinic 2, but subjects were administered with test meal III, with tea administered 1 hour post-meal.

Clinic 4 (Day 42)

The same procedure as in Clinic 2, but subjects were administered a reference iron dose (3 mg ^{57}Fe as $FeSO_4$ and 35 mg ascorbic acid) without administration of any test meal, and were given a further 3-day food diary to record their dietary intakes.

Clinic 5 (Day 56)

The same procedure as in Clinic 2, with the collection of a final whole-blood sample without the administration of a test meal.

Stable isotope labels

A single isotope technique was employed in the present study using a stable iron isotope (^{57}Fe, 95.93% enriched, Trace Sciences, Ontario, Canada) in metal form (300 mg) which was converted into ferrous sulphate solution ($^{57}FeSO_4$), sterilized and tested for endotoxin (Anazao Health Corporation, Florida, USA) and declared safe for human consumption. The enriched stable iron isotopes were transferred and kept in individual vials, flushed with nitrogen before being sealed and stored at -20 ºC until test meal administration. The specified dosages for test meals and the reference iron dose used in the study were 4 mg and 3 mg, respectively. The reference iron dose of 3 mg ^{57}Fe with 35 mg ascorbic acid administered during a fasted condition was used as a measure of iron absorption under optimal state (14).

Preparation of labelled isotope $^{57}FeSO_4$:

The dissolution process to produce a clear $^{57}FeSO_4$ solution which was added extrinsically to the test meals was carried out by the Anazao Health Corporation based on company standard procedure. Following complete dissolution of the elemental ^{57}Fe with sulphuric acid, which resulted in a clear and colorless solution, the solution was filtered over 0.5 µm membrane. The prepared $^{57}FeSO_4$ solution was shipped to the clinical research laboratory at the University of Chester in one vial containing a 75 ml solution, which was then transferred into individual sterile borosilicate glass clear sample vials with fitted caps (Fisher Scientific UK Ltd), labelled with dosage and concentration. The labelled vials were flushed with nitrogen gas, sealed and stored at -20 ^{o}C until the day of test meal administration. A total volume of 75 ml $^{57}FeSO_4$ will give 4 mg of ^{57}Fe/ml.

Test meals

Test meal preparation and composition:

The test meal used in the present study was instant oat porridge (Sainsbury's UK Ltd). Test meals were freshly prepared on the day of each clinic by adding 200 ml of boiling ultrapure water (TraceSELECT Ultra, Fluka Analytical, Sigma-Aldrich UK Ltd) to 55 g of porridge. This test meal was used at all clinics using the same standardized method of preparation. The nutritional content of the porridge is summarized in **Table 1**. To each test meal, a 4 mg dosage of ^{57}Fe extrinsically was added before consumption. The reference iron dose was extrinsically labelled with a 3 mg dosage of ^{57}Fe with 35 mg of ascorbic acid and administered in ultrapure water without any test meals (Clinic 5).

Preparation of tea:

Black tea (Yorkshire Tea, Bettys and Taylors Group Ltd, UK) was prepared using a standardized method at each clinic, by adding 200 ml boiling ultrapure water (TraceSELECT Ultra, Fluka Analytical, Sigma-Aldrich, UK Ltd) to one tea bag (3 g) and the mixture was steeped and infused for 3 minutes before straining for consumption. In each cup of tea, 12 ml of homogenized semi skim milk (Lakeland Dairies Co-op Society UK Ltd) was added and subjects were offered white granulated sugar (Fairtrade International, Bonn, Germany) with the tea to their preference. The tea was prepared on the day of the clinic and kept warm in a flask before consumption. The tea was administered at specific times either simultaneously with the porridge meal, or 1 hour post-meal.

Anthropometric measurements

Both height (cm) and weight (kg) were measured using electronic scales (Model 875 SECA, Hamburg, Germany) and a wall mounted digital stadiometer (Model 264 SECA, Hamburg, Germany) at baseline before any test meals were administered. The instruments used were calibrated before every measurement. Body mass index (BMI) was then calculated

using the equation: BMI= weight (kg)/height (m^2) and categorized using cut-off values from WHO (15).

Blood handling

Both whole blood and plasma obtained from venepuncture were used in the study for analysis. At each clinic, a 30 ml venous blood sample was collected using a trace element-free blood collection tube containing EDTA. A total of 6 ml of whole blood was aliquoted into micro centrifuge tubes and stored at -80 ºC prior to isotopic iron analyses, and also used to measure full blood count indices on the day of each clinic. The remaining blood samples were centrifuged at 1600 × g at 4 ºC for 10 minutes using a benchtop centrifuge to obtain plasma samples which were aliquoted into micro centrifuge tubes and stored at -80 ºC until the analyses of iron status biomarkers. Whole blood and plasma samples were used for the analysis of iron status biomarkers, including full blood counts (red blood cells, hemoglobin concentration, hematocrit %, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), plasma ferritin, plasma C - reactive protein, and plasma hepcidin concentrations.

Measurement of iron status biomarkers

Whole blood samples were used to measure full blood counts indices (FBC) using an automated Ac.T diff Hematology Analyzer (Beckman Coulter, Inc., California, USA). Plasma samples were used for the analysis of ferritin concentration using automated immunoanalyzer Mini VIDAS (Biomeriuex, Marcy-I'Etoile, France), C-reactive protein concentrations were measured using commercially available human ELISA kits (Quantikine Human CRP Immunoassay ELISA kit, R&D Systems Inc., Minneapolis, USA) and hepcidin

concentrations were measured using a commercially available human ELISA kits (Human Hepcidin ELISA kit, Sincere Biotech Co. Ltd., Beijing, China).

Isotopic analysis of blood samples

Isotope ratio measurements were carried out using an Agilent 8800 triple quadrupole ICP-MS/MS instrument (ICP-QQQ/Agilent Technologies, Japan). Samples and standards were introduced into the ICP with a MicroMist nebulizer and a Peltier-cooled (2 ºC) Scott-type spray chamber. The Agilent 8800 contains an octopole-based collision/reaction cell, which is located in-between two quadrupole analyzers. A mixture of 10 % Ammonia in Helium was introduced into the octopole cell as a reaction gas to remove the interferences on iron isotopes (Supplemental data) and to attenuate the signal at the appropriate level for precise isotope ratio analysis. Iron isotopes were measured as Fe ammonia cluster, $^{m}Fe(NH_3)_2$ at masses 88, 90, 91 and 92. Mass bias correction was performed by external standard correction using Iron (Fe) Pure Single-Element Standard, 1,000 µg/mL, 2 % HNO3 (Perkin Elmer, USA) as a baseline reference.

Sample preparation:

Frozen whole blood samples were allowed to reach room temperature and then vortex mixed. 100 µL of the sample was mixed with 9.9 mL of 0.005 % TMAH (v/v) (BioXtra grade, Sigma-Aldrich, USA).

Estimation of fractional iron absorption

Fractional iron absorption (%) was estimated by the erythrocyte iron incorporation method, 14 days post-dosing of the isotope, which involved the use of published equations to establish the percentage of iron absorption of each subject. Blood volume was used to

236 calculate the circulating iron body pool (16) using a published equation specific for females
237 (17). Based on the estimation of blood volume and circulating body iron pool for each
238 subject, iron incorporation into red blood cells was calculated using another equation which
239 assumed 80% of the absorbed iron was incorporated into erythrocytes (18, 19). The final
240 fractional iron absorption was derived from mole fraction calculations for each iron source
241 ('endogenous' iron and iron from the labelled oral dose) in the blood samples. This required
242 knowledge (from ICP-MS/MS measurements) of the abundance of each iron isotope (^{56}Fe,
243 ^{57}Fe, ^{58}Fe) in the previous and present blood sample and the abundance of the iron isotopes in
244 the oral isotope dosage (mainly ^{57}Fe). The erythrocytes became progressively more enriched
245 with ^{57}Fe as the subjects consumed more test meals since iron is not lost from erythrocytes
246 until they break down after approximately 120 days. This extra enrichment (above natural
247 abundance) was taken into account when fractional iron absorption was estimated after each
248 test meal by using the previous blood sample's iron isotope abundances as the 'endogenous'
249 value. The calculated fractional iron absorption (%) was then normalized to a fixed reference
250 value of 40% using the reference iron dose (14) to take into account inter-subject variability
251 (20).
252
253 **Assessment of dietary intake**
254 Two dietary assessment methods were utilized in the present study. Subjects were
255 required to complete a 3-day food diary within the week after the baseline clinic and within
256 the week before the post intervention clinic (to include 2 weekdays and 1 weekend day) to
257 estimate their habitual dietary intake. To ensure subjects had not consumed unusually high
258 levels of iron-rich foods immediately before each clinic, a 24-hour recall interview was
259 completed at each clinic to assess dietary intake, a day prior to the test meal administration
260 during which subjects were asked to recall food consumption, including drinks for the past 24

hours in detail to the investigator. All the dietary records were analyzed for nutritional content using computerized dietary analysis software (Microdiet for Windows software, Version 2.8.8, Downlee Systems UK Ltd). The food items used for the analysis were derived from McCance & Widdowson's The Composition of Foods 6th Summary Edition 2002 (21). Dietary reference values from the Committee on the Medical Aspects of Food Policy (COMA) 1991 were used for comparison with the intake of energy, macronutrients and micronutrients of the subjects.

Statistical analysis

All statistical analyses were performed with IBM SPSS Statistic Data Editor Software (Version 21). The Shapiro-Wilks test was used to ascertain the normal/non-normal distribution for each parameter. The non-normally distributed data, including the fractional iron absorption (%) and total iron absorbed (mg) were log transformed for the purpose of performing statistical analyses, and results were re-transformed to be reported as mean and standard deviation in the results section. One-way repeated measures Analysis of Variance (ANOVA) statistical tests were performed to compare the iron absorption from each pair of meals in comparison to control test meal (water). Pearson's correlation coefficient tests were performed to investigate the association between different iron status biomarkers and iron absorption (fractional absorption and total absorbed in mg). Differences were considered significant with a p-value ≤ 0.05.

RESULTS

Baseline physical characteristics, iron status and dietary intake of subjects

All 12 women who commenced the study completed all phases of study, with no reports of adverse events. The subjects were healthy women, with a mean (±SD) age of 24.8 ± 6.9 years and body mass index (BMI) of 22.6 ± 2.7 kg/m^2 which is in the normal range (**Table 2**). At baseline, all subjects had plasma ferritin concentration within the normal range for healthy women, and no elevated plasma ferritin concentration due to acute phase reactions, indicated by the normal plasma CRP concentrations. Seven of the 12 subjects were not iron deficient based on plasma ferritin concentration of > 15 µg/l, and only 2 subjects were anemic (hemoglobin concentrations of 9.0 and 10.0 g/dl) at baseline. Mean (±SD) plasma hepcidin concentration was 96.0 ± 119.6 ng/ml, which was within the expected normal range, apart from 3 subjects who had plasma hepcidin concentrations of more than 150 ng/ml, which accounts for the large standard deviation. There are no universal threshold values for normal hepcidin concentration, therefore, a reference value of between 17-286 ng/ml from a study which reported that the 5-95 % normal range for healthy women, sampled in the United States and Italy (22), was used to compare the concentrations found in the present study.

Following dietary analysis of the food diaries, estimated mean (±SD) daily energy intake was 6.75 ± 1.91 MJ which is consistent with the UK population data of 6.78 ± 1.9 MJ for women aged between 19-64 years (4). Mean (±SD) daily iron intake was 6.7 ± 1.5 mg, nearly 50 % of the RNI, and was lower than the UK population mean for women aged 19-64 years of 9.6 ± 3.0 mg/day. Habitual iron intake (based on the 3-day food diary), however, was found to be higher (8.4 ± 1.8 mg) at Day 56 compared to baseline (6.7 ± 1.5 mg) (p=0.035). Mean (±SD) daily iron intake estimated from the 24-hour recall was 9.7 ± 5.5 mg, and the

dietary analysis from each clinic revealed no unusually high intakes of iron, calcium or vitamin C, which may have confounded subjects' iron absorption.

There were also no changes observed in subjects' hemoglobin and plasma hepcidin concentrations between baseline and post-intervention, except for plasma ferritin which was significantly lower at post-intervention (29.4 ± 33.4 µg/l) compared to baseline (38.8 ± 42.7 µg/l) (p=0.026). Despite the lower plasma ferritin concentration at post-intervention, it was within the range of replete iron stores (> 15 µg/l). The absorption observed from the reference iron dose of 25.4 %, suggests that the absorption was not affected by the changes observed in subjects' iron stores.

Iron absorption from test meals

The present study utilized three standardized test meals administered successively at 14-day intervals with either water (TM I), simultaneously with tea (TM II) and tea administered 1 hour after the test meal (TM III). Each test meal did not contain any non-heme iron and was labelled extrinsically with 4 mg of ^{57}Fe as $FeSO_4$, except for the reference iron dose which was labelled with 3 mg of ^{57}Fe as $^{57}FeSO_4$ and 35 mg of ascorbic acid (to enhance iron absorption of the reference dose). TM I serves as a reference meal and was used as the basis for comparison and to calculate iron absorption ratio and tea inhibitory effect. Absorption data for the reference iron dose was used to correct the inter-variability between subjects' iron status background , normalized to a fixed reference value of 40 % (23) based on a published equation (20). **Table 3** shows the iron absorption (%), iron absorption ratio to the control test meal (water) and total iron absorbed (mg). Each mean value for iron absorption was comprised of 12 values; one for each subject. For TM I, three of the subjects had absorption values of '0%' because their enriched isotope ratios ($^{57}Fe/^{58}Fe$) were below the limit of quantification (LOQ) in comparison to the unenriched sample. In other words, the

instrument (ICP-MS/MS) was unable to confidently distinguish a 'signal' from the background 'noise' in these samples. This was also the case for three subjects at TM II and two subjects at TM III.

The mean iron absorption (%) was higher in TM I (meal + water) and TM III (meal + tea + 1 hour) compared to TM II (meal + tea) (data is shown in Table 3), as anticipated. A ~ 35 % reduction in fractional iron absorption was observed when the test meal was administered simultaneously with tea (TM II), compared to the test meal administered with water (TM I), but this difference was not significant (p=0.398). The mean fractional iron absorption was approximately 2.2 % higher, in absolute terms, when tea was administered 1 hour (TM III) after the subjects were administered their test meal (p=0.046), compared to when the test meal was administered simultaneously with tea (TM II). This was similar to the fractional iron absorption of the meal administered only with water (5.73 % versus 5.69 %). Consistent with the percentage iron absorbed, the mean total iron absorbed (mg) was also significantly higher by 0.05 mg in TM III (meal + tea + 1 hour) compared to TM II (meal + tea, p=0.049). There was no statistically significant difference between TM I (meal + water) and TM II (meal + tea) with regards to the total iron absorbed (p=0.530). There were substantial variations in fractional iron absorption between subjects. There was a wide range of percentage absorption, varying from undetectable to 31.1 % (Supplemental data). However, the fractional iron absorption reported in the present study has been normalized using a reference iron dose to account for inter-variability between subjects, and enabled the findings to be compared against the previously published literature.

The inhibition effect was reduced approximately 2-fold, from 37.2 % in TM II (meal + tea) to 18.1 % in TM III (meal + tea + 1 hour), indicating that allowing a 1-hour time interval between a meal and tea consumption will lead to increased iron absorption.

No significant association was observed between both principal iron status biomarkers (plasma ferritin and plasma ferritin concentration) and iron absorption. However, positive associations were observed between plasma ferritin and plasma hepcidin concentrations at the 3 time points the test meal administered with (a) water (r=0.918, p=0.0001) **(Figure 2A)**, (b) tea simultaneously (r=0.882, p=0.0001) **(Figure 2B)**, and (c) tea 1 hour post-meal (r=0.841, p=0.001) **(Figure 2C),** which indicates a very strong association between these two key iron biomarkers that play a substantial role in the regulation of iron absorption.

DISCUSSION

The study investigated the effect of tea consumption on non-heme iron absorption from a porridge meal using ^{57}Fe iron isotope, as well as ascertaining the impact of time interval of the potential inhibitory effect of consuming tea relative to consumption of an iron isotope-labelled porridge meal. In agreement with the previous studies (8, 9, 12, 24-28), the present study shows that tea reduces non-heme iron absorption, by at least 37 % when compared to water used as a control beverage. The study also demonstrates that a 1-hour time interval between tea consumption and a meal has a substantial impact in counteracting this inhibition effect, by at least 1.6-fold.

In the present study, the overall mean (±SD) iron absorption from porridge, administered with either water (TM I) or tea (TM II & TM III) was no higher than 6 %. Depending on the conditions and chemical forms of iron itself (heme/non-heme), the fractional iron absorbed by individuals is reported to be classically low, ranging from as low as 5 % to as high as 35 % (29). It can be presumed that the nature of the cereal-based meal is the main contributory factor leading to the low iron absorption, as the test meal utilized in the present study had a potentially low amount of iron with presence of phytate as an iron inhibitor, which is consistent with findings from previous studies that have used a cereal-based test meal in their study (25, 26). These studies reported a wide range of fractional iron absorption values, between 0.56 – 18.8 % (30, 31).

Consistent with findings reported from previous studies, despite the difference in test meal used, iron absorption was reduced when the tea was administered with a test meal, as opposed to when administered with water. **Table 4** compares iron absorption reported in previous studies where test meals were administered with either water (as a control) or tea, to illustrate the inhibition effect. The magnitude of the inhibitory effect shown in previous studies ranged between 26 – 99 % in comparison to the present study which is approximately

388 37 %. Variability in tea brewing time, brands and the total amount of tea used, which may
389 influence phenolic concentration are probably amongst the factors that contribute to
390 discrepancies in findings (32). The iron status of the subjects may also dictate iron absorption
391 and this has been demonstrated in previous studies. A potential explanation as to why a lower
392 inhibition effect of tea was observed in the present study could be due to the majority of the
393 subjects not being anemic nor iron deficient. This is supported by the low mean (±SD)
394 absorption of the reference iron dose (25.4 ± 18.3 %) compared to previous studies that
395 reported a higher absorption of iron from both test meals and reference doses (Table 4).
396 Thankachan, et al. (9) compared iron absorption from a rice meal between iron deficiency
397 anemia (IDA) subjects and normal subjects and demonstrated that iron absorption was higher
398 in the IDA group (7.1 %) compared to the control group (3.5 %) suggesting that iron status
399 has a significant impact on the level of iron absorption and is based on physiological
400 requirements.

401 The finding that plasma ferritin and plasma hepcidin concentrations were positively
402 associated in the present study is consistent with a limited number of previous studies that
403 have measured iron absorption concurrently with hepcidin and ferritin concentration, despite
404 the different test meals used (33-35). Hepcidin, a systemic iron regulator, directly binds
405 ferroportin to cause its internalization and degradation to hinder iron efflux (36).

406 The inhibition of iron absorption by tea, observed in this study could be due to the
407 formation of insoluble iron-tannin complexes in the lumen of the gut (26). Supporting the
408 findings from previous studies that tea is a prominent inhibitor of iron absorption, the present
409 study demonstrated that a 1-hour time interval has a substantial effect in reducing the
410 inhibition effect by increasing the absorption by at least 37 %. There is limited evidence to
411 support this finding on the time interval effect, specifically in human studies, as previous
412 studies were carried out in rats or using other polyphenol-containing beverages. Disler, et al.

(10) demonstrated that the time interval between administration of tea and an iron-containing meal, substantially reduced the inhibition effect. However, tea was administered at several time-points up to 3 hours before iron solutions were administered, instead of after the test meal, as in the present study. A linear relationship between the time interval and iron absorption was observed, and the absorption was approximately 2-fold higher if the tea was administered 1 hour before a meal, which is similar to the 1.6-fold magnitude (TM II versus TM III) in our study.

It should be noted that the iron absorption levels reported in the present study are relative to a specific meal. However, gastric emptying of solid foods can vary between individuals and can also be affected by variations in meal components and consistency (37) which has been reported to range between 45-108 minutes in several studies in healthy subjects fed test meals of different compositions (38-40).

In conclusion, the present study demonstrates that tea can be distinctly regarded as a potent inhibitor of non-heme iron absorption from a cereal-based breakfast. It is also evident from this study that not consuming tea simultaneously with a meal will have an impact on attenuating the inhibition effect of tea on iron absorption. In addition to attenuating the effect of inhibition of iron absorption, it is pertinent to incorporate dietary advice that would increase iron absorption, such as incorporating ascorbate-containing fruit juice to a meal to enhance iron absorption. The findings of the present study may have implications in the management of iron deficiency, especially in at-risk groups, predisposed to iron deficiency. Translating the findings of the present study into dietary advice at a healthcare level, would help maximize dietary iron absorption as tea is not only widely consumed in the UK (11), but is extensively consumed worldwide.

ACKNOWLEDGEMENTS

The authors would like to thank the subjects for their contribution in partaking in this study. We also would like to acknowledge the UK Medical Research Council (Grant number U105960399) for their support with the iron isotopic analyses. SM designed research; SFAF conducted research; SFAF analyzed data; SM, SFAF, DK, SB, DIAP and JRD wrote the paper; SM had primary responsibility for final content. All authors read and approved the final manuscript. There was no conflict of interest.

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Table 1. Nutritional composition of porridge test meal

Nutrient	Per serving (1 pot = 55g)
Energy (MJ)	8.63
Total fat (g)	3.4
SFA (g)	0.6
MUFA (g)	1.3
PUFA (g)	1.3
Carbohydrate (g)	32.8
Sugars (g)	8.7
Starch (g)	24.0
Fibre (g)	3.9
Protein (g)	8.7
Salt (g)	0.14

Table 2. Baseline subject characteristics, iron status, and nutritional intake (n=12)

Variable		Mean ± SD
Age (years)		24.8 ± 6.9
Physical characteristics		
	Height (m)	166.6 ± 6.5
	Weight (kg)	63.0 ± 10.8
	BMI (kg/m^2)	22.6 ± 2.7
Iron status biomarkers		
	Hemoglobin (g/dl)	12.33 ± 1.52
	Hematocrit (%)	35.97 ± 4.49
	RBC (x 10^{12}/l)	3.93 ± 0.55
	Mean cell volume (fl)	91.84 ± 6.93
	Plasma ferritin (µg/l)	38.8 ± 42.7
	Plasma C-reactive protein (mg/l)	0.62 ± 0.77
	Plasma hepcidin (ng/ml)	96.0 ± 119.6
Mean Daily Nutritional intakes		
	Energy (MJ)	6.75 ± 1.91
	Protein (grams)	66.1 ± 23
	Iron (mg)	6.7 ± 1.5
	Tea intake/day	2 cups (average infusion)

Table 3. Fractional iron absorption (%) from test meals with specified beverages and iron absorption ratio (tea administration relative to water)

	Test meals		
	Test meal I (Administered with water)	Test meal II (Administered with tea simultaneously)	Test meal III (Tea administered 1 hour post-meal)
Fractional iron absorption (%)	5.7 ± 8.5	3.6 ± 4.2	*5.7 ± 5.4
Iron absorption ratio	1	0.65 ± 0.67	1.18 ± 1.19
Total iron absorbed (mg)	0.23 ± 0.51	0.14 ± 0.26	$^{\Psi}$0.19 ± 0.28

* Significantly different from test meal II (p = 0.046)

$^{\Psi}$ Significantly different from test meal II (p = 0.049)

A one-way, repeated measures analysis of variance (ANOVA) test was performed to compare assess the differences in mean iron absorption between each test meal

Table 4. Inhibition effect (%) of tea consumption on iron absorption (%) in comparison to water as a control beverage

Study	Population / Mean age (±SD) or range (years)	Test meals	Absorption (%)			
			Meal (water)	Meal (tea)	Inhibition %	Reference iron dose
Present	Healthy women (n=12) / 24.8 ± 6.9	Porridge + 200 ml tea	5.7 ± 8.5	3.6 ± 4.2	37	25.4 ± 18.3
[I]	Healthy women (n=8) / 26-60	Bread + 200 ml black tea	10.4 ± 4.4	3.3 ± 3.0	68	35.8 ± 25.0
		Rice & soup + 200 ml black tea	10.8 ± 4.1	2.5 ± 1.6	77	34.7 ± 24.7
[II]	Healthy women (n=22) / 21-71	Maize porridge + 150 ml black tea	3.8 ± 4.0	2.1 ± 2.5	45	50.5 ± 31.5
[III]	Thalassemia patients (n=5) / 11-23	Hamburgers + 240 ml tea	15.6 ± 12.8	5.2 ± 5.5	67	NA
[IV]	Healthy adults (n=37) / 18-50	Beef hamburger + 200 ml tea	3.71 (2.94,4.68)	1.32 (1.01,1.71)	64	15.73 (13.22,18.71)
[V]	Hemochromatosis patients (n=18) / 47.4 ± 16.1	Homogenized rice & beef + 200 ml tea	22.1 ± 3.4	6.9 ± 1.4	69	NA
[VI]	Healthy adults (n=77) / 19-40	Bread roll + 275 ml tea				
		275 ml tea (n=9)	12.9 (10.7,15.6)	0.74 (0.57,0.95)	94	NA
		275 ml tea (n=10)	5.63 (4.64,6.84)	0.89 (0.68,1.16)	84	
		275 ml tea (n=10)	4.46 (3.47,5.72)	0.92 (0.72,1.18)	79	
		275 ml tea (n=10)	8.64 (6.00, 12.4)	0.83 (0.56,1.23)	90	

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		100% strength (n=9)	6.58 (4.76,9.10)	0.59 (0.45,0.96)	91	
		50% strength		1.05 (0.72,1.53)	84	
		25% strength		0.66 (0.45,0.96)	99	
		25% strength (n=9)	4.33 (3.31,5.67)	1.18 (0.84,1.66)	73	
		10% strength		1.48 (0.99,2.21)	66	
		5% strength		1.47 (1.03,2.12)	66	
[VII]	Healthy women (n=10) / 26 ± 4	Pasta + bread + 4 ml green tea extract	12.1 ± 4.5	8.9 ± 5.2	26	38.6 ± 18.1
[VIII]	IDA and healthy women (n=20)/ 18-35	Tomato rice + tea (different amount)				NA
	Iron deficient anemia group (22.6 ± 3.5)	150 ml tea	18.2 (12.6,26.4)	7.1 (4.3,11.7)	61	
		300 ml tea	19.7 (13.5,29.0)	5.6 (3.1,10.1)	72	
	Iron replete group (24.3 ± 2.9)	150 ml tea	7.5 (4.0,14.2)	3.5 (2.1,5.9)	53	
		300 ml tea	5.2 (2.6,10.8)	1.6 (0.7,3.8)	69	

*[I] Disler et al., 1975, India (24); [II] Derman et al.,1977, India (12); [III] De Alarcon., 1979, Italy (25), [IV] Morck et al., 1983, USA (26), [V] Kaltwasser et al., 1998, Germany (27), [VI] Hurrell et al., 1999, USA (8), [VII] Samman et al., 2001, Australia (28), [VIII] Thankachan et., al 2008, India (9).

*Inhibition effect (%) = $\frac{\text{[meal (water) – meal (tea)]}}{\text{meal (water)}} \times 100$

*All meals were extrinsically labelled with either stable or radio isotopes except the second sub-study of [1]

*[1 & 9]: stable iron isotopes; [2-8]: radio iron isotopes

Figure Legends

Figure 1. Flow chart of study protocol (5 clinics with 14 day intervals).

Figure 2. Associations between $\log_{10}$: (A) plasma ferritin concentration and plasma hepcidin concentration (Day 14), (B) plasma ferritin concentration and plasma hepcidin concentration (Day 28) and (C) plasma ferritin concentration and plasma hepcidin concentration (Day 42) (n=12).

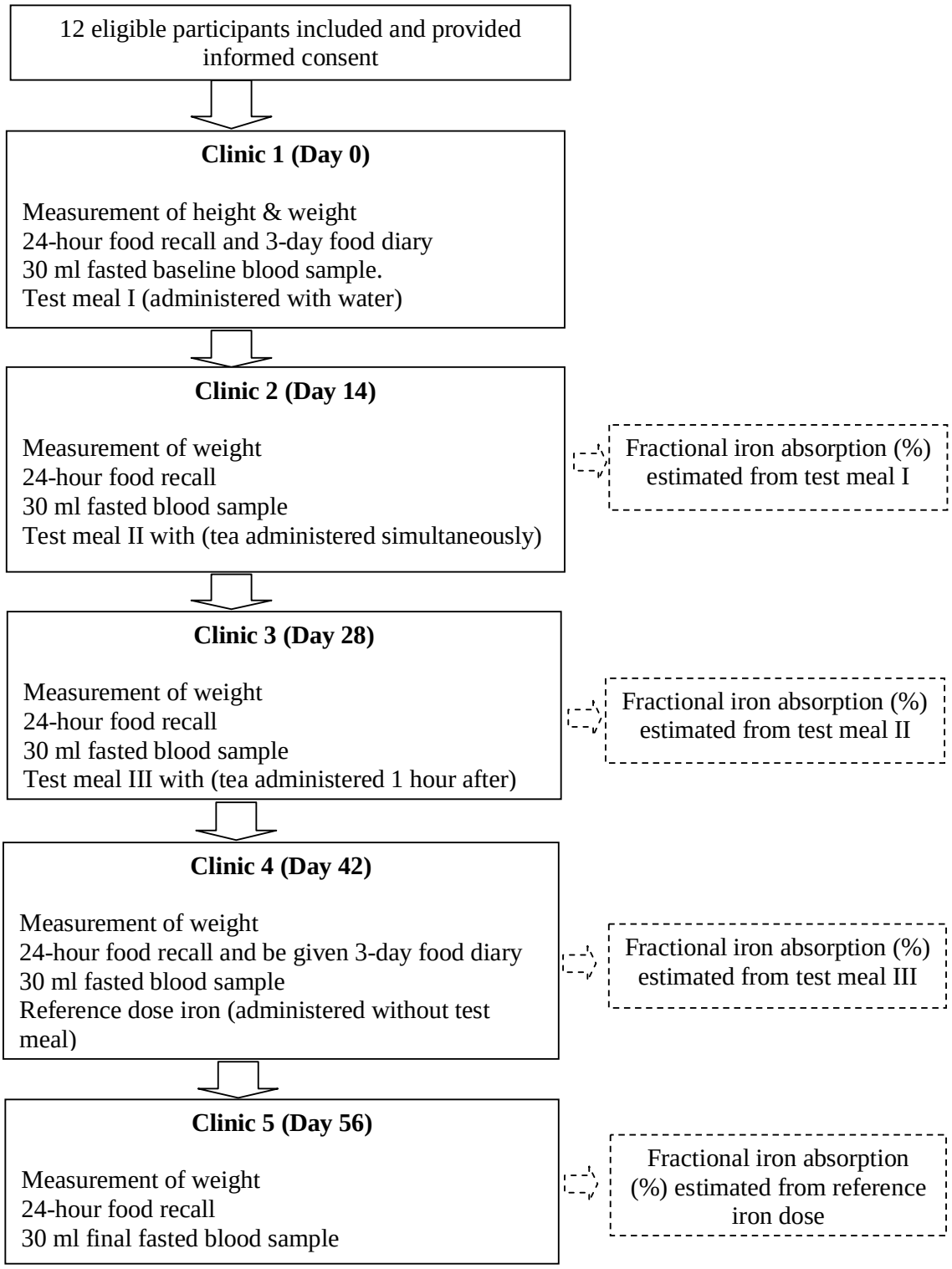
12 eligible participants included and provided informed consent
Clinic 1 (Day 0)
Measurement of height & weight
24-hour food recall and 3-day food diary
30 ml fasted baseline blood sample.
Test meal I (administered with water)
Clinic 2 (Day 14)
Measurement of weight
24-hour food recall
30 ml fasted blood sample
Test meal II with (tea administered simultaneously)
Fractional iron absorption (%) estimated from test meal I
Clinic 3 (Day 28)
Measurement of weight
24-hour food recall
30 ml fasted blood sample
Test meal III with (tea administered 1 hour after)
Fractional iron absorption (%) estimated from test meal II
Clinic 4 (Day 42)
Measurement of weight
24-hour food recall and be given 3-day food diary
30 ml fasted blood sample
Reference dose iron (administered without test meal)
Fractional iron absorption (%) estimated from test meal III
Clinic 5 (Day 56)
Measurement of weight
24-hour food recall
30 ml final fasted blood sample
Fractional iron absorption (%) estimated from reference iron dose

Figure 1

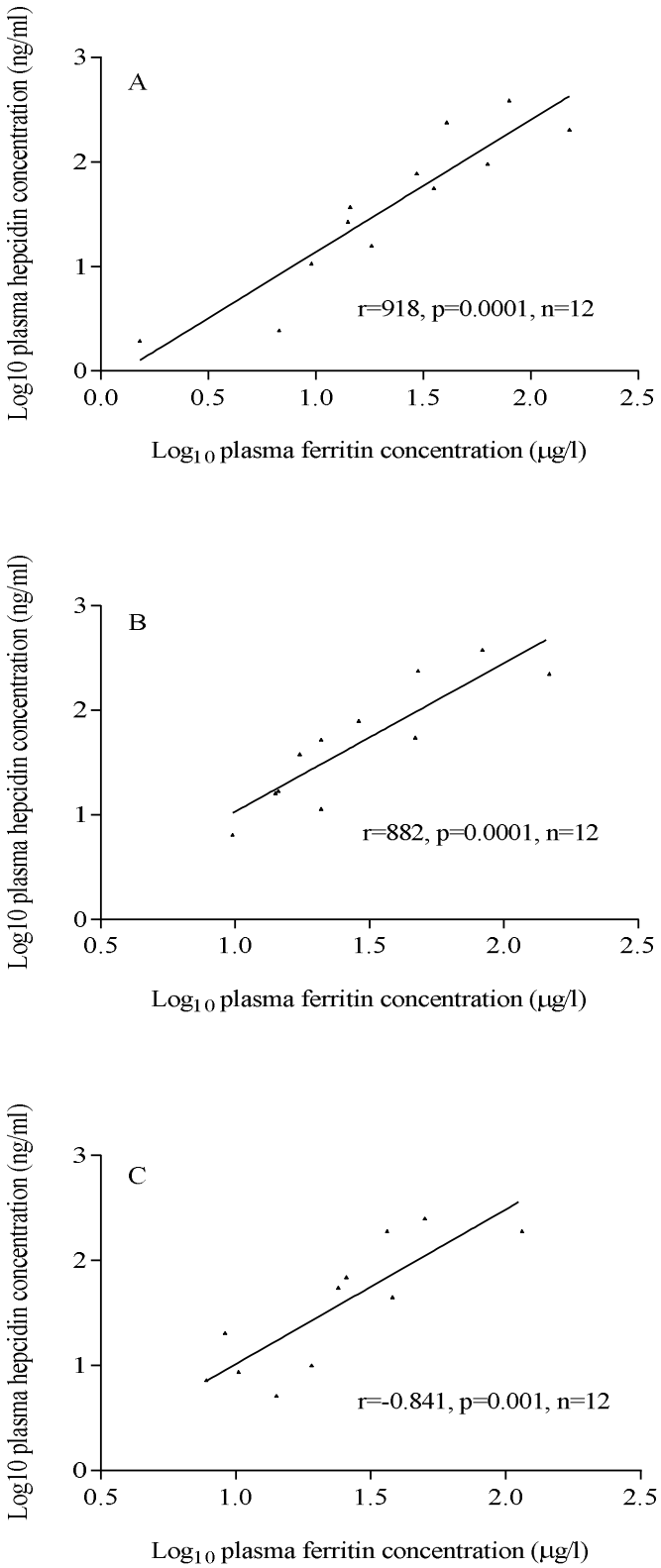
A
Log10 plasma hepcidin concentration (ng/ml)
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
r=918, p=0.0001, n=12
Log10 plasma ferritin concentration (µg/l)
B
Log10 plasma hepcidin concentration (ng/ml)
3
2
1
0
0.5
1.0
1.5
2.0
2.5
r=882, p=0.0001, n=12
Log10 plasma ferritin concentration (µg/l)
C
Log10 plasma hepcidin concentration (ng/ml)
3
2
1
0
0.5
1.0
1.5
2.0
2.5
r=-0.841, p=0.001, n=12
Log10 plasma ferritin concentration (µg/l)

Figure 2