Green tea or rosemary extract added to foods reduces nonheme-iron absorption[1–3]

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ABSTRACT

Background: Phenolic compounds act as food antioxidants. One of the postulated mechanisms of action is chelation of prooxidant metals, such as iron. Although the antioxidative effect is desirable, this mechanism may impair the utilization of dietary iron.

Objective: We sought to determine the effect of phenolic-rich extracts obtained from green tea or rosemary on nonheme-iron absorption.

Design: Young women aged 19–39 y consumed test meals on 4 separate occasions. The meals were identical except for the absence (meal A) or presence (meal B) of a phenolic-rich extract from green tea (study 1; $n = 10$) or rosemary (study 2; $n = 14$). The extracts (0.1 mmol) were added to the meat component of the test meals. The meals were extrinsically labeled with either ^{55}Fe or ^{59}Fe and were consumed on 4 consecutive days in the order ABBA or BAAB. Iron absorption was determined by measuring whole-body retention of ^{59}Fe and the ratio of ^{55}Fe to ^{59}Fe activity in blood samples.

Results: The presence of the phenolic-rich extracts resulted in decreased nonheme-iron absorption. Mean ($\pm$SD) iron absorption decreased from $12.1 \pm 4.5\%$ to $8.9 \pm 5.2\%$ ($P < 0.01$) in the presence of green tea extract and from $7.5 \pm 4.0\%$ to $6.4 \pm 4.7\%$ ($P < 0.05$) in the presence of rosemary extract.

Conclusion: Phenolic-rich extracts used as antioxidants in foods reduce the utilization of dietary iron.   *Am J Clin Nutr* 2001; 73:607–12.

INTRODUCTION

Polyphenolic compounds are diverse in chemical structure and characteristics. They occur in foods such as fruit, vegetables, tea, wine, and spices. Polyphenolic compounds reportedly have a wide range of effects in vivo and in vitro, including antioxidant and metal-chelating activities (1).

The use of extracts from rosemary (*Rosmarinus officinalis*) as food preservatives is well established (2). Phenolic compounds obtained from rosemary were shown to act as antioxi-dants in vitro and to reduce the extent of oxidation of dietary lipids (3) in a dose-dependant manner (4). The constituents of rosemary that accounted for most of its antioxidant activity are rosmarinic acid, carnosol, and carnosic acid (5). The effective-ness of rosemary extracts as antioxidants has resulted in their commercial exploitation (6).

Tea (*Camellia sinensis*) and its phenolic constituents are reported to have antioxidant activity in vitro that minimizes the oxidizability of fatty acids and α-tocopherol (7) and has poten-tial for use in several food products (8). Although the test system and food matrix may influence the response, there appears to be a hierarchy of antioxidant activity of tea constituents, with epi-catechin gallate > epigallocatechin gallate > epigallocatechin > gallic acid > epicatechin = catechin (9, 10).

Extracts derived from green tea and rosemary have the potential to reduce the oxidation of food products and extend their shelf life. The antioxidant action was shown to be dependent on the ability of their constituent phenolic compounds to scavenge free radicals and to chelate metals (11, 12). Tea (13) and other beverages rich in phe-nolic compounds (14), including coffee (15) and wine (16), were shown to inhibit the absorption of nonheme iron; a limited number of studies suggest that the effect is dose dependent. Although the antioxidant effect is beneficial because of the reduced risk of spoilage, the inhibition of nonheme-iron absorption constitutes a potential adverse effect (13).

Because iron deficiency is widespread and the mode of action of some antioxidants includes chelation of metals such as iron, it is prudent to examine the effect on iron absorption of adding these extracts to foods. The aim of this study was to test the

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TABLE 1
Polyphenol contents of the green tea and rosemary extracts[1]

	Content in extract	Content in each meal
	% by wt	*mg (mmol)*
Green tea extract[2]		
Catechin	ND	ND (ND)
Epicatechin	0.84	3.4 (0.012)
Epicatechin gallate	1.39	5.5 (0.014)
Epigallo catechin	2.57	10.3 (0.034)
Epigallo catechin gallate	4.52	18.1 (0.039)
Total	9.32	37.3 (0.099)
Rosemary extract[3]		
Carnosic acid	2.16	8.6 (0.026)
Carnosol	3.45	13.8 (0.042)
Rosmarinic acid	2.57	10.3 (0.029)
Total	8.18	32.7 (0.097)

[1] ND, not detected.

[2] The total phenolic content of the green tea extract was 117.3 mg gallic acid equivalents/g, as determined with the Folin-Ciocalteu method.

[3] Herbor rosemary extract P31 commercially available from FIS SA, Chatel-St-Denis, Switzerland.

effect of clearly defined extracts from green tea or rosemary on nonheme-iron absorption in young women.

SUBJECTS AND METHODS

Subjects

Two studies were carried out with a total of 27 women who ranged in age from 19 to 39 y. The volunteers met the following inclusion criteria: hemoglobin concentration >7.0 mmol/L, no medications (other than oral contraceptive agents) or nutritional supplements during the trial or within the previous 2 mo, not pregnant or breast-feeding, and no participation in other studies or treatments involving radioisotopes or donated blood during the study or within the previous 2 mo. All procedures were in accordance with the Helsinki Declaration II and were approved by the Municipal Ethics Committee of Copenhagen and Frederiksberg (Protocol KF 01-100/97) and by the National Institute of Radiation Hygiene. The participants were given oral and written information about the study and written consent was obtained from all subjects before their participation in the study.

Study design

Iron absorption from single meals was estimated by using the dual-label extrinsic-tag method (17). Each subject consumed a control test meal (meal A) and consumed the same meal with extract added (meal B) on either the preceding or following day. To minimize the potential effects of day-to-day variation in iron absorption, subjects consumed each meal twice; the 4 meals were consumed on 4 consecutive mornings in the order ABBA or BAAB. Subjects were asked to consume the meals within 30 min. They ate the meals with ultrapure water and bread, which was used to wipe residual sauce from the plate. Subjects were asked not to consume any food or beverage for 4 h after the meals. All test meals were provided under supervision between 0800 and 1000, when all subjects had fasted for 12–16 h. The meals were labeled extrinsically with radioiron (either ^{55}Fe or ^{59}Fe, in random order) and absorption was estimated from whole-body retention and isotope activity measured in the blood

2 wk after the test meals. Also at 2 wk after the test meals, a reference dose of iron (3 mg Fe labeled with ^{59}Fe and 30 mg ascorbic acid) was given to each subject; whole-body retention was measured after 2 more weeks.

Composition of the test meals and phenolic-rich extracts

The test meals were extrinsically labeled by drop-wise addition of 1 mL radioisotope solution (FeCl$_3$ in 0.01 mol HCl/L) to the meal ≈16 h before it was served. The solution contained either ^{55}Fe (62.5 and 55.3 kBq in studies 1 and 2, respectively) or ^{59}Fe (74.9 and 73.6 kBq in studies 1 and 2, respectively) (Amersham, Buckinghamshire, United Kingdom). The specific activities of the isotopes were >185 GBq/g Fe for ^{55}Fe and 111–740 GBq/g Fe for ^{59}Fe. The iron from the isotope solution represented <0.2% of the total iron content in each of the test meals.

The test meals were prepared in 2 batches, 1 for each study. The foods that made up the test meals were identical and consisted of pasta (70 g dry weight), a meat sauce [90 g minced pork (15–18% fat), 28 g cream (38% fat), 20 g carrots, bouillon, water, salt, pepper, and sauce thickener], and white bread (20 g). The meals provided 2740 kJ and 27.3 g protein (Danish Tables of Food Composition, DANKOST 2000, version 1.20). The subjects consumed the test meals in the absence (meal A) or presence (meal B) of a flavonoid-rich extract.

The Chinese green tea extract (Licosa-P/The Chinois; Nestec, Lausanne, Switzerland) was obtained by pressing green tea leaves on a hydraulic press with propylene glycol as a carrier as described by R Aeschbach and P Rossi (US Patent US5795609). The rosemary extract was commercially available (Herbor; FIS SA, Chatel-St-Denis, Switzerland). The phenolic content of both extracts (**Table 1**) was determined by HPLC under the conditions reported previously (18). Each extract was diluted (10% wt:vol) by using an ethanol:water solution (2:1 vol:vol); 4 mL of the diluted extract, corresponding to 0.1 mmoles, was added to the meat sauce after preparation. The contents of phenolic compounds in the extracts are shown in Table 1.

The total iron content of the meal was determined by atomic absorption spectrometry (Spectra AA-200; Varian, Mulgrave, Victoria, Australia). Before analysis, the test meals were freeze-dried, homogenized, and then acid digested (MES 1000 Microwave Extraction System; CEM, Matthews, NC). Lyophilized bovine muscle (certified iron value: 79 ± 2 μg, BCR 184-752; Institute for Reference Materials and Measurements, Brussels) was used as a reference material and the analyzed value was 77.3 ± 2.0 μg Fe (*n* = 4). The nonheme iron content was determined spectrophotometrically (19). The heme iron content of the meals was calculated as the difference between the total and nonheme iron contents. The heme iron contents represented 12% and 21% of the total iron in the test meals used in studies 1 and 2, respectively.

Determination of iron status

Subjects were instructed to fast for 12–16 h and to rest in a supine position for 5–10 min before blood sampling. For the analysis of hemoglobin concentration, venous blood (4.5 mL) was collected from the antecubital vein into evacuated tubes containing disodium EDTA (0.01%). Another blood sample (3.0 mL) was collected into plain tubes for determination of serum ferritin concentration. Serum ferritin was determined by using a ferritin kit adapted for the Delfia system (kit A069-101; Wallac Oy, Turku, Finland) and appropriate control sera (World Health Organization NIBSC-ferritin; Blanche Lane, South Mimms,

TABLE 2
Charateristics of the subjects at study entry

	Study 1 ($n = 10$)	Study 2 ($n = 14$)
Age (y)	26 ± 4^{1}	25 ± 5
Weight (kg)	63 ± 8	66 ± 8
BMI (kg/m^2)	22.1 ± 2.3	23.4 ± 2.8
Hemoglobin (mmol/L)	7.9 ± 0.5	$7.9 \pm 0.5\ ^{2}$
Serum ferritin (μg/L)	20.7	23.8
	$(11.9, 36.6)^{3}$	$(16.3, 35.2)^{2}$

[1] $\overline{x} \pm$ SD.

[2] Data shown for $n = 13$.

[3] Geometric mean; 95% CI in parentheses.

United Kingdom). For serum ferritin, analytic intraassay and interassay variations were 5.8% ($n = 18$) and 5.0% ($n = 33$), respectively. Hemoglobin was analyzed in whole blood within 3–4 h after collection of the sample by using an automated procedure derived from the Drapkins method (Cobas Minos, ABX; Roche, Montpellier, France) with appropriate controls (Hematology Reference Control, Monotrol; Roche). For hemoglobin, analytic intraassay and interassay variations were 0.04% ($n = 10$) and 2.4% ($n = 18$), respectively.

Determination of iron absorption

Iron absorption was estimated by using the dual-label extrinsic-tag method as described by Hallberg (17). Before each subject consumed the first test meal, her background radioactivity was determined by whole-body counting. The whole-body counter consisted of a low activity lead-lined steel chamber with 4 plastic scintillation blocks (Nuclear Enterprises Limited, Edinburgh, United Kingdom). The counting efficiency and window settings were established by taking measurements of water-filled phantoms with outlines and weights approximately equal to those of humans. The counting efficiency for a 67-kg phantom was 271 cps/kBq ^{59}Fe, corresponding to an absolute detection efficiency of 27%. To minimize contamination from the environment, subjects were asked to shower, wash their hair, and put on hospital gowns before each measurement. Each subject underwent whole-body counting for 10 min. The results were corrected for chamber background radiation and physical isotope decay.

Two weeks after consumption of the radiolabeled test meals, all subjects underwent whole-body counting to determine the extent of ^{59}Fe retention. Fasting blood samples were also obtained for measurement of ^{55}Fe and ^{59}Fe (17). To minimize quenching, the samples were extracted by using a simplified procedure (20). Briefly, aliquots of whole blood (2×20 mL) were dry ashed at a temperature that increased gradually to a maximum of 550°C. The samples were cooled and reconstituted in 37% HCl (10 mL) and then the volume was reduced to 5 mL by heating in a water bath (100°C). Ammonium hydroxide (16.5 mol/L) was then added drop-wise with intermittent mixing until the pH was ≈10. The samples were then centrifuged at $2600 \times g$ for 15 min at 18°C, the supernate was discarded, and the precipitate was washed with distilled water and dissolved in concentrated phosphoric acid (0.5 mL). The resultant solution was transferred to a scintillation vial with 2 washes with water (2.5 mL) to ensure maximal transfer. Scintillation gel (10 mL Instagel Plus; Packard Instruments, Meriden, CT) was added and the solution was mixed thoroughly and kept in the dark overnight. The samples were then counted by dual-isotope counting with automatic quench correc-

tion and window settings from 0 to 6 keV and from 6 to 500 keV for ^{55}Fe and ^{59}Fe, respectively.

There is marked variability in iron absorption by a single subject when studied on different occasions. To correct for interindividual differences in iron absorption, which is known to be related mainly to iron status, we also measured iron absorption from a standardized reference dose of iron salt (17). After the whole-body counting and blood collection on day 14, all subjects consumed a reference dose of iron on 2 consecutive days. The 10-mL solution contained 3.0 mg elemental Fe as ferrosulfate (FeSO$_4$; Merck, Darmstadt, Germany) and 30 mg L-ascorbic acid (Sigma-Aldrich Co Ltd, St Louis) in 0.01 mol HCl/L. The reference doses were labeled with 70.8 kBq ^{59}Fe (Amersham, Buckinghamshire, United Kingdom) (study 1) and 76.2 kBq ^{59}Fe (study 2). The subjects were fasting on both occasions and were asked not to consume any food or beverage for 4 h after the reference dose. Whole-body counting was carried out 2 wk later.

Iron absorption was expressed as the whole-body retention of ^{59}Fe, which was measured directly, and as the whole-body retention of ^{55}Fe, which was calculated by using the equation shown below. These calculations involve the assumption that the ratio of whole-body activity to blood activity was equal for the 2 isotopes (17).

$$\text{Percentage (\%) } ^{55}\text{Fe absorption} = (^{55}\text{Fe blood activity}/$$
$$^{59}\text{Fe blood activity})$$
$$\times\ ^{59}\text{Fe whole-body retention} \quad (1)$$

Statistical analysis

The results are expressed as means $\pm$ SDs and were analyzed with the paired Student's t test. Differences were considered significant at $P < 0.05$. Analyses were conducted with use of EXCEL (version 8.0; Microsoft, Redmond, WA).

RESULTS

Three of the 13 subjects withdrew from study 1 (green tea extract); therefore, data are presented for the 10 subjects who consumed all 4 test meals. Two of the subjects withdrew for reasons unrelated to the study and the third subject had reconsidered her participation in an experiment involving radioisotopes. In study 2, all 14 participants completed the procedures; therefore, we present data for $n = 14$. Subjects were not asked about the taste acceptability of the meals in either of the studies, but they indicated that they detected the flavor of rosemary but not that of green tea.

The subjects' characteristics and iron status at study entry are shown in **Table 2**. All subjects had hemoglobin concentrations within the normal range, however, 6 subjects had serum ferritin concentrations $<15\ \mu$g/L. Hemoglobin and serum ferritin concentrations at study entry (Table 2) and those measured 2 wk after the test meals were similar (data not shown).

The total iron contents of the meals that were used to test the effects of the tea and rosemary extracts were 2.20 and 2.05 mg/meal, respectively (**Table 3**). The presence of phenolic-rich extracts in the test meals resulted in molar ratios of dietary iron to total phenolic content of 0.39 and 0.36 for the green tea and rosemary extracts, respectively.

Data on nonheme-iron absorption from the test meals and absorption ratios are shown in **Table 4**. In comparison with the control meal (test meal A), the meal containing green tea extract [0.1 mmol (37.3 mg) of a mixture of catechins] (test meal B)

TABLE 3
Iron contents of the test meals[1]

	Study 1	Study 2
	mg Fe/meal	
Meat sauce		
Total iron	0.91 ± 0.03	0.92 ± 0.03
Nonheme iron	0.64 ± 0.12	0.49 ± 0.06
Pasta	1.06 ± 0.04	0.92 ± 0.01
Bread	0.23 ± 0.03	0.21 ± 0.00

[1] $\overline{x}$ ± SD.

inhibited nonheme-iron absorption in 9 of the 10 subjects (mean absorption: 12.1 ± 4.5% and 8.9 ± 5.2% in meals A and B, respectively; $P < 0.01$). Similarly, in comparison with the control meal (test meal A), the meal containing rosemary extract [equivalent to 0.1 mmol (32.7 mg) of phenolic substances] (test meal B) inhibited nonheme-iron absorption in 9 of the 14 subjects (mean absorption: 7.5 ± 4.0% and 6.4 ± 4.7% in meals A and B, respectively; $P < 0.05$).

By measuring each subject's absorption of iron from both the test meals and the reference dose, we could express the results as ratios that represent the relative bioavailability of iron from the test meals (17). Because iron absorption by an individual with depleted iron stores is ≈40%, it is recommended to correct for a 40% absorption from the reference dose (17). This allows comparisons between studies. The ratios we calculated confirmed that the phenolic extracts reduced the bioavailability of iron (Table 4).

DISCUSSION

In this study, we showed that extracts of Chinese green tea and rosemary with well-defined chemical compositions significantly inhibited the absorption of nonheme iron. The extracts were added to the meat component of the meal to reach final concentrations that would be optimal if these extracts were used as food antioxidants.

The observed values for absorption of nonheme iron from the control test meals are consistent with previous data in which similar meals were used (21, 22). The addition of the green tea and rosemary extracts decreased nonheme-iron absorption by 28% and 21%, respectively. Tea is a rich source of flavonoids (23) and black tea inhibits iron absorption because it contains hydrolyzable tannins (tannic acid). The phenolic monomers, polyphenols, and tannins that are found in tea are thought to interfere with iron absorption by forming insoluble complexes in the gastrointestinal lumen, thereby lowering the bioavailability of iron (13). In an attempt to identify the component of tea that is responsible for

chelating iron, Brune et al (24) tested the effects of tannin and catechin on nonheme-iron absorption. The authors concluded that the inhibitory effect is more pronounced when there are 3 hydroxyl groups (galloyl) on the phenolic structure than when there are 2 hydroxyl groups (catechol). These findings are in contrast with those observed in vitro, where it was shown that aromatic rings bearing either catechol or galloyl groups on adjacent carbons have similar iron binding properties (25). A possible explanation for the discrepancy between the in vitro and in vivo findings is the chemical definition of the added phenolic compounds.

The 2 extracts used in this study differed in the degree and pattern of polyhydroxylation; the specific composition was verified by HPLC. The green tea extract was composed of epicatechin, epicatechin gallate, epigallo catechin, and epigallo catechin gallate (Table 1), which consist primarily of galloyl groups. In contrast, the rosemary extract (composed of carnosic acid, carnosol, and rosmarinic acid) consisted primarily of catechol groups. Despite the difference in hydroxylation, these extracts had a similar capacity to inhibit the absorption of nonheme iron.

The test meals used in this study were low in vitamin C. It is possible, on the basis of the Monsen equation (26) and more recent studies (27), that a higher vitamin C content would reduce the inhibitory effect of green tea and rosemary extracts. Increasing the ratio of Fe^{2+} to Fe^{3+}, as would occur in the presence of vitamin C, would decrease chelation by catechol groups (28) and enhance the absorption of nonheme iron.

Hurrell at al (14) proposed that the relation between intake of polyphenolic compounds and absorption of nonheme iron is described by the following equation: relative iron absorption = $2 \times$ (mg polyphenol/serving)$^{-0.5}$. Our results are consistent with this relation despite some methodologic differences between the 2 studies. The meal used in our studies had high iron bioavailability, whereas Hurrell et al (14) used a meal consisting of bread with no enhancing factors. However, the total iron content was comparable in the 2 studies. Our studies used extracts that were clearly defined, whereas Hurrell et al (14) tested whole beverages. It is conceivable that the results of further studies using more precisely defined polyphenol contents will alter the reported relation between polyphenols and iron absorption.

In protocols in which whole-body counting is not possible, iron absorption is calculated on the basis of measurement of radioiron in blood samples, with the assumption that the incorporation of absorbed iron into red blood cells, k, is 0.8 (29). On the basis of whole-body counting and blood activity of the 2 isotopes, the mean (±SD) value of k was determined to be 0.73 ± 0.10 ($n = 24$) in the present study. Although k was not used to calculate absorption in this study, we report its value to indicate the variation in this commonly used factor. The factor 0.8 (range: 0.78–0.83) was derived from a study in 5 men with

TABLE 4
Iron absorption from meals containing green tea or rosemary extracts[1]

	Nonheme-iron absorption			Absorption ratios[2]		
	Control meal (A)	Control meal + extract (B)	Reference dose (C)	B:A	[A:C] × 40%	[B:C] × 40%
	%					
Green tea ($n = 10$)	12.1 ± 4.5[a]	8.9 ± 5.2[a]	38.6 ± 18.1	0.7 ± 0.2	15.6 ± 10.8[b]	11.2 ± 8.8[b]
Rosemary ($n = 14$)	7.5 ± 4.0[b]	6.4 ± 4.7[b]	29.3 ± 15.7	0.8 ± 0.3	11.2 ± 4.4[a]	8.8 ± 2.8[a]

[1] $\overline{x}$ ± SD. Values in the same row with the same superscript letter are significantly different (paired Student's t test): [a] $P < 0.01$, [b] $P < 0.05$.
[2] Mean individual absorption ratios were multiplied by 40 to obtain the percentage absorption of iron corresponding to a 40% absorption of the reference dose.

adequate iron stores (29). The present study, like many other studies involving iron absorption, was carried out in premenopausal women and it is not surprising that the incorporation factor obtained from these 24 subjects is different from that observed in a small number of men.

Some, but not all, ecological studies support the theory that consumption of 30 mg flavonoids/d from tea and other sources reduces the risk of coronary heart disease (CHD) (23, 30–34). In a cross-country comparison, CHD mortality was lowest in Japan, where the average flavonoid intake is 61 mg/d and is mainly derived from green tea (33). The hydroxyl patterns of tea flavonoids fulfill the requirements for antioxidant activity; this effect was suggested as a mechanism for the relation between flavonoid intake and CHD (1). Another possible mechanism is the inhibition of iron absorption because it was reported that high iron status (or iron overload) is positively correlated with CHD risk (35), particularly in the presence of multiple risk factors (36). Increased intake of flavonoids from sources such as tea or wine may maintain a relatively low iron status and may therefore reduce the risk of iron overload. The intake of flavonoids shown to have a favorable effect (ie, ≈30 mg/d) is equivalent to the amount of phenolic compounds in our test meals (Table 1) and is similar to the doses recommended for commercial applications of herb extracts such as rosemary (37). The amounts of antioxidants used in the present study were chosen on the basis of their effectiveness for preventing oxidative changes in processed foods (LR Nissen, G Bertelsen, and L Skibsted, unpublished observations, 1997).

The reduction of nonheme-iron absorption in the presence of green tea and rosemary extracts suggests that chelation of iron may be one of the mechanisms of antioxidant action in vivo. However, in view of the widespread nature of iron deficiency, the efficacy of these and other phenolic-rich extracts should be assessed in terms of not only their antioxidant action in vitro but also their potential to interfere with iron absorption. ✿

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