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ORIGINAL ARTICLE

Oxalic acid does not influence nonhaem iron absorption in humans: a comparison of kale and spinach meals

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Objective: To evaluate the influence of oxalic acid (OA) on nonhaem iron absorption in humans.
Design: Two randomized crossover stable iron isotope absorption studies.
Setting: Zurich, Switzerland.
Subjects: Sixteen apparently healthy women (18–45 years, <60 kg body weight), recruited by poster advertizing from the staff and student populations of the ETH, University and University Hospital of Zurich, Switzerland. Thirteen subjects completed both studies.
Methods: Iron absorption was measured based on erythrocyte incorporation of ^{57}Fe or ^{58}Fe 14 days after the administration of labelled meals. In study I, test meals consisted of two wheat bread rolls (100 g) and either 150 g spinach with a native OA content of 1.27 g (reference meal) or 150 g kale with a native OA content of 0.01 g. In study II, 150 g kale given with a potassium oxalate drink to obtain a total OA content of 1.27 g was compared to the spinach meal.
Results: After normalization for the spinach reference meal absorption, geometric mean iron absorption from wheat bread rolls with kale (10.7%) did not differ significantly from wheat rolls with kale plus 1.26 g OA added as potassium oxalate (11.5%, $P=0.86$). Spinach was significantly higher in calcium and polyphenols than kale and absorption from the spinach meal was 24% lower compared to the kale meal without added OA, but the difference did not reach statistical significance ($P>0.16$).
Conclusion: Potassium oxalate did not influence iron absorption in humans from a kale meal and our findings strongly suggest that OA in fruits and vegetables is of minor relevance in iron nutrition.
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Keywords: iron absorption; oxalic acid; organic acids; stable isotopes

Introduction

Oxalic acid (OA) is a common constituent of plant foods such as green leafy vegetables, rhubarb, parsley, beetroot, carrots, potatoes, cocoa and tea (Zarembski and Hodgkinson, 1962; Chai and Liebman, 2005). Concentrations vary depending on season, variety, age, soil conditions, maturity and part of the plant (Kasidas and Rose, 1980). The largest amounts, up to 1–2 g/100 g wet weight, are found in rhubarb (Honow and Hesse, 2002) and spinach, mangold and purslane (Chai and Liebman, 2005). OA may be present in foods as insoluble calcium or magnesium oxalate crystals or as soluble sodium or potassium oxalate. Relative contributions of these two fractions to total OA seem to vary widely within and between plant species. Zarembski and Hodgkinson analysed OA contents of 80 food items commonly used in English homes and hospitals and calculated the average daily intake to be 118 mg in the household setting and to range between 70 and 150 mg in six hospital diets (Zarembski and Hodgkinson, 1962). This compares to results from a small study including five subjects, in which mean daily OA intake was estimated to be 152 mg (range 44–351 mg) (Holmes and Kennedy, 2000). Additionally, the human body is itself able to synthesize OA, with ascorbic

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acid and glycine being the major precursors (Hodgkinson and Zarembski, 1968).

OA is a well-known inhibitor of calcium absorption in humans (Heaney *et al*., 1988; Heaney and Weaver, 1989), and has been reported to decrease zinc (Kelsay and Prather, 1983) and magnesium (Bohn, 2003) absorption. Formation of insoluble oxalate complexes is the most likely explanation based on the observation that addition of calcium decreased OA absorption from the diet in a dose-dependent fashion (von Unruh *et al*., 2004). However, surprisingly little attention has been paid to the effect of OA on iron bioavailability. Results from human studies are equivocal (Gillooly *et al*., 1983; Ballot *et al*., 1987), whereas rat studies showed a neutral to enhancing effect of adding purified OA to the diet (Van Campen and Welch, 1980; Gordon and Chao, 1984).

To better define the influence of dietary OA on iron bioavailability, we compared the absorption of nonhaem iron from meals containing spinach (high OA), kale (low OA) and kale plus added OA in human volunteers. Both vegetables are reported to contain similar amounts of polyphenols (~100 mg/100 g wet weight) (Ninfali and Bacchiocca, 2003; Chun *et al*., 2004), an important class of inhibitors of iron absorption (Hurrell *et al*., 1999), but may vary in content. Ascorbic acid is a potent enhancer of iron absorption (Cook and Monsen, 1977; Hallberg *et al*., 1986) and kale has been reported to contain 105 mg/100 g compared to 52 mg/100 g in spinach (Souci *et al*., 1994). Iron absorption was measured based on the incorporation of stable iron isotope labels into erythrocytes (Kastenmayer *et al*., 1994; Walczyk *et al*., 1997).

Subjects and methods

Subjects

Sixteen apparently healthy women (aged 19–38 years; maximum body weight 60 kg) were recruited from the staff and student populations of the ETH Zurich, University of Zurich and the University Hospital Zurich, Switzerland. The subjects participated in two studies, which were performed in a randomized crossover design.

Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin or mineral supplements were allowed during the study. Women regularly taking vitamin or mineral supplements discontinued supplementation 2 weeks before the start of the study. No subjects were recruited who had donated blood within 4 months of the beginning of the study or who were planning to donate blood during the study period.

The study protocol was reviewed and approved by the Ethical Committees of the ETH Zurich and of the canton of Zurich. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Test meals

The control meal (meal A) consisted of two wheat bread rolls (100 g) and 150 g spinach. The bread rolls were prepared in batches by mixing 1 kg low extraction wheat flour with high-purity water (18 MΩ, 600 g), salt (10 g), white sugar (32 g) and dry yeast (15 g). After fermentation for 5 h at room temperature, rolls were prepared from the dough, baked for 15 min at 200°C and stored at −25°C. Frozen spinach leaves were purchased at a local supermarket and heated in batches of 1 kg in steel pots until spinach leaves were thawed and started boiling. The spinach was left to boil for another 5 min, samples were pureed with a hand mixer, pooled, and 250 g of butter and 35 g of table salt (no added iodine or fluorine) added to improve taste. Samples of 150 g were weighed into aluminium trays and stored at −25°C until use.

Test meal B (study I) consisted of two wheat bread rolls and 150 g of kale. Fresh kale from a local supermarket was washed under cold tap water, trimmed of the midribs and boiled in tap water for 15 min. After removal of excess water, blanched leaves were pureed with a hand mixer, flavoured with 250 g of butter and 35 g of table salt, and boiled for another 10 min with repeated stirring. After pooling of individually prepared kale batches, samples of 150 g each were weighed into aluminium trays and stored at −25°C until use.

Test meal C (study II) was identical to meal B, except that subjects received an additional OA drink (20 ml, 1.26 g OA) made freshly each day by dissolving an appropriate amount of potassium oxalate monohydrate (Fluka, Buchs, CH) in high-purity water. Total iron content of all kale meals (1.38 mg) was balanced to spinach meal Fe levels (2.68 mg) by adding unlabelled ferrous sulphate (1.30 mg Fe). High-purity water (400 g; 380 g for kale plus OA meals) was served as a drink.

Subjects received a peppermint-flavoured Wrigley's Eclipse® Flash strip after each meal to alleviate any unpleasant aftertaste.

Study design

Two separate studies were made. In study I, iron absorption from kale (meal B, 0.01 g OA) was compared with iron absorption from spinach (meal A, 1.27 g OA). In study 2, iron absorption from kale with 1.26 g added OA served as a drink (meal C, 1.27 g OA) was compared to spinach meal A. Comparisons were made between morning and lunch meals given over three consecutive days. This meal administration was necessary as we wanted the extrinsic tag to contribute as little iron as possible to total meal iron (Fairweather-Tait and Dainty, 2002). Each subject received 12 test meals (study I: 3 × A, 3 × B; study II: 3 × A, 3 × C). One type of meal was administered in the morning and the other at lunch. A few days before the first test meal administration (day 0), a baseline venous blood sample was drawn after an overnight fast for determination of iron status (plasma ferritin, haemoglobin) and subjects' height and weight were recorded. Iron absorption was determined with the use of a

double stable-isotope technique and based on erythrocyte incorporation of ^{57}Fe or ^{58}Fe 14 d after test meal administration. The first pair of test meals (A/B or A/C) was fed on three consecutive days (days 1, 2 and 3) between 0700 and 0900, after the subjects had fasted overnight, and 4 h later under standardized conditions. No food or drink was allowed between breakfast and lunch meals and within 3 h after the lunch meals. Fourteen days later (day 17), a second blood sample was drawn and the second pair of test meals (A/C or A/B) was administered on three consecutive days (days 17, 18 and 19) under the same conditions. Another 14 days later (day 33), a final blood sample was drawn. Meals were compared in a fully randomized crossover design, with each subject acting as her own control. By administering the dose of iron label over the course of 3 days, we achieved a contribution of the extrinsic tag of 31% to the total meal iron in the spinach meal and 61% in the kale meals (due to the lower iron content). The extrinsic tag has been shown to be a valid approach up to a level of 20% for dietary iron absorption studies using complete meals due to rapid isotopic exchange and the formation of a common nonhaem iron pool, with a suggestion that higher levels are also acceptable (Cook *et al*., 1972; Bjorn-Rasmussen *et al*., 1973).

Stable-isotope labels
Isotopically labelled $^{57}FeSO_4$ and $^{58}FeSO_4$ were prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) dissolved in dilute sulphuric acid. The solutions were stored in polytetrafluoroethylene containers flushed with argon to keep the iron in the $2+$ oxidation state.

Quantification of iron isotope labels
Isotope dilution mass spectrometry was used to determine the concentration of the isotopic labels in solution. An accurately known amount of iron of natural isotopic composition was added to aliquots taken from the prepared isotopic labels. The used iron standard was prepared gravimetrically from an isotopic reference material (IRM-014, EU Institute of Reference Materials, Geel, Belgium). Isotopic analysis was performed using negative thermal ionization-mass spectrometry (Walczyk, 1997). Iron concentrations in the isotopic labels were calculated on the basis of the shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels and the natural iron isotopic abundances (Walczyk *et al*., 1997).

Iron status measurements
Venous blood samples (7 ml) were drawn into EDTA-treated vacutainers (368 453, Becton Dickinson, Milian SA, Meyrin, Switzerland) a few days before the first test meal administration and again 14 days after the first and second set of test meals on days 17 and 33 of the study, respectively. Samples were analysed for iron status indexes (plasma ferritin, haemoglobin; day 0) and for the incorporation of ^{57}Fe and ^{58}Fe into erythrocytes (day 17, day 33). Haemoglobin was measured in fresh whole blood using the cyanmethaemoglobin method (D5941; Sigma, Buchs, Switzerland). Plasma was separated and stored at $-25^{\circ}C$ for later ferritin analysis with the use of an enzyme immunoassay (Immulite; DPC Bühlmann GmbH, Salzburg, Austria). Commercial quality control materials for haemoglobin (Digitana AG, Horgen, Switzerland) and ferritin (Immulite) were run with each analysis.

Quantification of iron isotope in the blood
Each isotopically enriched blood sample was analysed in duplicate for its iron isotopic composition as previously described by Walczyk *et al*. (1997). The blood samples were mineralized by using a mixture of nitric acid and hydrogen peroxide and microwave digestion. The iron was separated from the matrix by anion-exchange chromatography and a solvent–solvent extraction step into diethylether. The isotopic analyses were performed by negative thermal ionization-mass spectrometry (Walczyk, 1997).

Calculation of iron absorption
The amounts of ^{57}Fe and ^{58}Fe isotopic labels in blood 14 days after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic (Walczyk *et al*., 1997). Circulating iron was calculated on the basis of blood volume and haemoglobin concentration (Kastenmayer *et al*., 1994). Blood volume calculations were based on height and weight according to Brown *et al*. (1962). For calculations of fractional absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed (Hosain *et al*., 1967).

Food analysis
The iron and calcium contents of the wheat bread rolls, spinach and kale were analysed by graphite tube- and flame-atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, Australia), respectively, after mineralization by microwave digestion (MLS-ETHOS with easyWAVE software version 3.5. 4.0; Egrolyt Laborgeräte, Oberwil, Switzerland) in a mixture of HNO_3 and H_2O_2. Soluble and total oxalic acid were extracted with water and 2 M HCl, respectively, from cooked spinach and kale samples and measured with an enzymic assay (33–591C-1KT Oxalate Kit S, Trinity Biotech, Wicklow, Ireland) according to the manufacturer's instructions. Phytic acid was quantified spectrophotometrically (Makower, 1970) on a MRX microplate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK), with the modification that iron was replaced by cerium in the

precipitation step. Total polyphenol content of spinach and kale was determined using the Folin–Ciocalteu method (Singleton *et al*., 1999) and results are expressed as gallic acid equivalents (GAE). Ascorbic acid in spinach and kale was measured by reversed phase-high performance liquid chromatography (RP-HPLC) (Sapers *et al*., 1990; Nyyssönen *et al*., 1992).

Statistics

Student's paired *t*-test was used to evaluate data within each study. Values were logarithmically transformed before statistical analysis (EXCEL 2002 SP3, Microsoft Corporation, Redmond, WA, USA). Results are presented as geometric means (−1 s.d., +1 s.d.). *Post hoc* power calculations for paired *t*-tests were performed using GraphPad StatMate for Windows, version 2.00 (GraphPad Software, San Diego, CA, USA). Student's unpaired *t*-test was used for inter-study analysis and comparison of nutrient composition between the different meals. Normalization was achieved by dividing the individual absorption values from meals B and C by the corresponding absorption from meal A followed by multiplication with the calculated absorption mean from meal A of all subjects in study I and study II. All results were considered significant at $P<0.05$.

Results

Test meals

The contents of iron, calcium, ascorbic acid, phytic acid, oxalic acid and polyphenols in the different test meals are shown in Table 1. The OA content of cooked spinach was 848 mg/100 g, of which 271 mg were soluble oxalates. Cooked kale OA content amounted to 8 mg/100 g, with 3 mg being soluble. Iron and calcium concentrations were found to be 1.3 mg/100 g and 189 mg/100 g cooked spinach, respectively. In comparison, cooked kale contained 0.4 mg Fe/100 g and 14 mg Ca/100 g ($P<0.0001$ for difference between spinach and kale calcium). The measured content of polyphenols in cooked spinach was 95 mg GAE/100 g, whereas cooked kale contained 48 mg GAE/100 g ($P<0.0001$ for difference). Ascorbic acid content was 10 mg/100 g cooked spinach and 3 mg/100 g cooked kale, the difference between the vegetables being statistically significant ($P=0.0012$). Phytic acid was below the limit of detection (<0.0035%) in freeze-dried samples of cooked spinach, cooked kale and bread rolls.

Subjects

Thirteen of the sixteen subjects initially recruited completed the two studies. The reason for dropout was taste aversion. Mean body weight was 55.8±5.4 kg and mean age was 25.1±4.7 years. One of the subjects displayed borderline haemoglobin with low iron stores (Hb, 119 g/l; plasma ferritin, 12 μg/l) and one had depleted iron stores (plasma ferritin, <12 μg/l).

Iron absorption

The iron absorption data are shown in Tables 2 and 3. Geometric mean iron absorption in study I was 8.4% from spinach and 11.0% from kale (absorption ratio A/B: 0.76, $P=0.19$). Geometric mean iron absorption in study II was 8.0% from spinach and 11.2% from kale plus OA (absorption ratio A/C: 0.72, $P=0.16$). The average serum ferritin of the subjects in studies I and II was similar at 28 and 30 μg/L,

Table 1 Test meal composition

	Bread with spinach (A)		*Bread with kale (B)*		*Bread with kale + OA (C)*	
	Mean	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*
Native Fe (mg)	2.68	0.13	1.38	0.04	1.38	0.04
added Fe[a] (mg)			1.3		1.3	
Calcium[b] (mg)	284	0.9	25	0.5	25	0.5
Ascorbic acid[b] (mg)	15.4	0.5	4.3	0.1	4.3	0.1
OA (mg)						
Total	1272	5	12	6	12	6
Soluble	407	5	5	2	5	2
OA added[c] (mg)					1260	
Phytic acid (%)	<0.0035		<0.0035		<0.0035	
Polyphenols[b] (mg)	142	4	72	1	72	1

[a]Added as ferrous sulphate heptahydrate (2.6 mg Fe/ml) in 0.01 M HCl.
[b]Significantly different between kale and spinach meals, $P < 0.002$ (Student's *t*-test).
[c]Served as an additional drink made from potassium oxalate monohydrate and containing 1.26 g/20 g OA, oxalic acid.

Table 2 Iron absorption from wheat roll meals with spinach or kale[a]

Subjects	*Serum ferritin (μg/l)*	*Meal iron absorption (% of dose)*		*Absorption ratio*
		Spinach (A)	*Kale (B)*	*A:B*
Study I (13F)				
1	54	7.2	9.6	0.75
2	13	4.3	13.1	0.33
3	93	4.5	12.6	0.36
4	26	5.6	2.9	1.90
5	36	9.2	13.0	0.71
6	27	15.4	33.2	0.46
7	12	15.8	37.6	0.42
8	24	6.4	5.3	1.21
9	40	10.6	16.3	0.65
10	49	7.2	4.7	1.53
11	53	6.9	2.5	2.80
12	10	18.5	33.9	0.55
13	15	8.9	13.3	0.67
Mean	28	8.4	11.0	0.76[b]
(−1 s.d.; +1 s.d.)	(5; 51)	(5.2; 13.3)	(4.5; 26.8)	(0.39; 1.48)

[a]Geometric mean (−1 s.d.; +1 s.d.). Subjects aged 19–38 years. F, female.
[b]$P=0.16$ (Student's paired *t*-test).

Table 3 Iron absorption from wheat roll meals with spinach or kale plus added OA[a]

Subjects	*Serum ferritin (μg/l)*	*Meal iron absorption (% of dose)*		*Absorption ratio A:C*
		Spinach (A)	*Kale + OA (C)*	
Study II (13F)				
1	54	10.6	14.7	0.72
2	13	2.7	8.4	0.32
3	93	6.9	9.4	0.74
4	26	2.9	8.6	0.34
5	36	7.6	14.1	0.54
6	27	11.4	11.5	1.00
7	12	18.5	40.4	0.46
8	40	18.5	15.6	1.19
9	49	2.1	9.5	0.22
10	53	8.2	2.0	4.18
11	63	8.2	2.2	3.64
12	10	23.8	42.5	0.56
13	15	9.7	24.0	0.40
mean	30	8.0	11.2	0.72[b]
(−1 s.d.; +1 s.d.)	(6; 55)	(3.8; 17.0)	(4.5; 27.9)	(0.30; 1.73)

[a]Geometric mean (−1 s.d.; +1 s.d.). Subjects aged 19–38 years. F, female; OA, oxalic acid; added OA (1.26 g) was included in the meal as a potassium oxalate solution.
[b]$P = 0.19$ (Student's paired *t*-test).

respectively. Thus when compared to kale, spinach had a 24% lower absorption which failed to reach statistical significance. The addition of 1.26 g OA to kale did not change the absorption ratio, and again a 28% decrease in iron absorption was not significant. Two subjects in each study absorbed much less iron from kale than from spinach.

Absorption from kale plus OA (meal C, 11.2%) was similar to kale alone (meal B, 11.0%, $P = 0.59$) and when these absorption values were normalized for the small differences in the respective spinach meal (meal A), the absorption values (11.5 vs 10.7%) were still not significant ($P = 0.86$). The added OA (1.26 g) therefore did not influence iron absorption.

Discussion

The present study clearly shows that OA added as 1.26 g soluble potassium oxalate to a kale meal does not influence iron absorption in humans. It would be expected therefore that soluble or insoluble oxalates in plant foods are of minor relevance in iron nutrition. This is in line with the report of no relationship between human iron absorption and the OA content of the three OA-rich vegetables, spinach, beetroot greens and beetroot (Gillooly *et al*., 1983). The parallel finding by the same authors that 1 g of calcium oxalate added to a cabbage soup meal decreased iron absorption significantly from 32.0 to 19.5% (Gillooly *et al*., 1983) could be explained by the inhibitory nature of the added 300 mg calcium (Hallberg *et al*., 1991). Similarly, the slightly

Table 4 Stability constants and solubilities of some OA salts (modified from Hodgkinson and Zarembski, 1968)

Oxalate salt	*Stability constant*[a] *(log K_S)*	*Solubility (mg/100 ml)*
Calcium oxalate	3	0.67 (13°C)
Magnesium oxalate	2.76 (20°C)	70.0 (16°C)
Zinc oxalate	4.9	0.79 (18°C)
Ferrous oxalate	>4.7	22
Ferric oxalate	9.4	Very soluble

[a]$T = 25$°C, $I = 0.1$ unless stated otherwise, OA, oxalic acid.

enhancing effect of 100 g rhubarb (537 mg OA) on a rice meal (200 g) (Ballot *et al*., 1987) can be explained by the malic acid (1.7 g/100 g) and citric acid (410 mg/100 g) it contains, as both these compounds at high levels can increase iron absorption (Gillooly *et al*., 1983).

The reason why OA has no influence on iron absorption but decreases absorption of calcium (Heaney *et al*., 1988; Heaney and Weaver, 1989), magnesium (Bohn, 2003) and zinc (Kelsay and Prather, 1983) is presumably related to the respective solubility and complex stability constants (Table 4). The poor water solubility of calcium and zinc oxalate (<1 mg/100 ml) could explain the inhibitory effect of oxalate on zinc and calcium absorption. Magnesium oxalate is more soluble but the solubility is still only 70 mg/100 ml. Both ferrous and ferric iron form stable oxalate complexes ($K_S > 4.7$); however, ferrous oxalate is rather insoluble (22 mg/100 ml) compared to ferric oxalate which is described as very soluble (Hodgkinson and Zarembski, 1968). Presumably, most of the meal iron in the present study was in the ferric form in the gastric and duodenal phases of digestion. It is possible that when iron is in the ferrous form, as in meals high in ascorbic acid, OA may inhibit iron absorption by forming the more insoluble ferrous oxalate. It could also be speculated that in the present study, meal iron in the gastrointestinal tract was equally in the ferrous and ferric form and whereas OA increased the absorption of ferric iron, it reduced the absorption of ferrous iron, so the net effect would be no influence on iron absorption. Some support to this last theory is given by Caco-2 cell experiments, which have reported a fivefold enhancing effect of OA on the uptake of ferric iron but a 20% decrease in the uptake of ferrous iron, both given as the pure iron salts (Salovaara *et al*., 2002).

In our study, there was a 24% lower iron absorption from spinach than from kale which failed to reach statistical significance. It would be expected from the literature that spinach inhibits iron absorption (Gillooly *et al*., 1983; Brune *et al*., 1989), most likely owing to the high polyphenol (Brune *et al*., 1989) and calcium (Hallberg *et al*., 1991) contents, which were twofold and tenfold higher, respectively, in the spinach meals compared to the kale meals used in the present study. The lack of statistical significance can be explained by the study design which aimed to detect a 30% difference at 80% power. We would like to emphasize

that although most subjects had lower iron absorption from spinach than from kale, two subjects in each study had much lower iron absorption from kale than from spinach. Owing to the higher variability in iron absorption values than we observe normally in comparable studies with comparable subject dropout numbers, we would have needed a 42–49% reduction in iron absorption from spinach compared to kale meals to reach statistical significance.

In conclusion, our results strongly suggest that OA in plant foods does not inhibit iron absorption, and that OA does not contribute to the reported inhibitory effect of spinach on iron absorption. However, the possibility of a differential effect of OA on ferrous and ferric iron absorption merits further study.

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