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Fractional magnesium absorption is significantly lower in human subjects from a meal served with an oxalate-rich vegetable, spinach, as compared with a meal served with kale, a vegetable with a low oxalate content

Torsten Bohn, Lena Davidsson*, Thomas Walczyk and Richard F. Hurrell

Laboratory for Human Nutrition, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, Zurich, Switzerland

The aim of the present study was to evaluate Mg absorption from a test meal served with an oxalate-rich vegetable, spinach, as compared with a test meal served with a vegetable with a low oxalate content, kale. Mg absorption was measured by a stable-isotope technique based on extrinsic labelling of the test meals and faecal monitoring of the excreted isotope labels. Nine healthy adults participated in the study. The test meals were based on 100 g phytate-free white bread, served with 300 g spinach (6·6 mmol oxalate; 0·7 mmol ^{25}Mg label added, 5·0 mmol total Mg) or 300 g kale (0·1 mmol oxalate; 1·2 mmol ^{26}Mg label added, 4·8 mmol total Mg). The test meals were served on days 1 and 3, at breakfast and lunch, using a cross-over design. The results from the present study demonstrated that apparent Mg absorption was significantly lower from the meal served with spinach (26·7 (SD 10·4) %) than the meal served with kale (36·5 (SD 11·8) %) ($P=0·01$). However, the lower fractional apparent Mg absorption from the test meal served with spinach can be assumed to be, at least partly, counterbalanced by the higher native Mg content of spinach as compared with kale. Although based on indirect evidence, i.e. not based on an evaluation of added (or removed) oxalic acid, the difference in Mg absorption observed in the present study is attributed to the difference in oxalic acid content between the two vegetables.

Magnesium absorption: Spinach: Oxalate: Stable isotopes: Faecal monitoring

Oxalic acid and its salts are ubiquitous in plant cells and relatively large amounts are found in leafy vegetables such as spinach (Tabekhia, 1980), and also in fruits, grains, nuts, tea, coffee, and cocoa (Zarembski & Hodgkinson, 1962; Souci *et al.* 1994). Oxalate intakes vary with dietary habits and have, for example, been reported to be in the range of 70–150 mg/d in the UK (Zarembski & Hodgkinson, 1962; Anderson *et al.* 1971; Hodgkinson 1977*a*).

Although oxalic acid forms insoluble complexes at physiological pH with divalent cations such as Ca^{2+}, Zn^{2+}, and Mg^{2+} (Weast, 1989), the influence of oxalate or foods rich in oxalate on mineral and trace element absorption in man has not been evaluated systematically. However, Ca absorption from a vegetable rich in oxalate, spinach, has been reported to be significantly lower than from kale (a vegetable with a low oxalate content) in adults (Heaney *et al.* 1988; Heaney & Weaver, 1990). In addition, Schwartz *et al.* (1984) reported significantly lower net (apparent) Mg absorption from bran muffins with added spinach as compared with added collard greens, a vegetable botanically similar to kale, but no difference as compared with added lettuce or turnip greens. However, these results are difficult to interpret as the quantity of spinach and the content of oxalate in the test meal are not reported and the study only included four subjects. An inhibitory effect of oxalic acid-rich vegetables on Mg and Zn absorption in man is indicated by the observation that spinach added to the diet resulted in negative Mg and Zn balances (Kelsay & Prather, 1983). Furthermore, Mg has been shown to inhibit oxalate absorption in man (Berg *et al.* 1986; Hanson *et al.* 1989), suggesting that Mg forms insoluble, non-absorbable complexes with oxalic acid in the gastrointestinal tract.

The aim of the present study was to evaluate Mg absorption from test meals served with vegetables with high or low oxalate content (spinach or kale) in healthy adults. Mg absorption was measured by a stable-isotope technique based on extrinsic labelling of the meals and faecal monitoring of the excreted isotope label.

Subjects and methods

Subjects

Apparently healthy, free-living, i.e. non-hospitalised, subjects (ten adult men and women) were recruited. Lactating

* **Corresponding author:** Dr Lena Davidsson, fax + 41 1 7045710, email lena.davidsson@ilw.agrl.ethz.ch

and pregnant women were excluded from the study. No medication was allowed during the study except for oral contraceptives. The intake of mineral and vitamin supplements was not allowed from 2 weeks before the start of the study and during the entire study. The participants were asked not to change their dietary habits or lifestyle during the study. Information about the aims and the procedures of the study was given orally and in writing. Written informed consent was obtained from all participants. The study protocol was reviewed and approved by the ethical committee at the Swiss Federal Institute of Technology, Zurich.

Isotopic labels

Highly enriched ^{25}MgO (1·04 (SD 0·01) % ^{24}Mg, 98·73 (SD 0·01) % ^{25}Mg and 0·23 (SD 0·01) % ^{26}Mg) and ^{26}MgO (0·39 (SD 0·01) % ^{24}Mg, 0·11 (SD 0·01) % ^{25}Mg and 99·50 (SD 0·01) % ^{26}Mg) labels were purchased from Chemotrade (Dusseldorf, Germany). The enriched ^{25}Mg label (28 mmol as ^{25}MgO) and ^{26}Mg label (43 mmol as ^{26}MgO) were each dissolved in 4 M-HCl (10 ml) and diluted to 100 ml with water. Solid NaHCO$_3$ (Merck, Darmstadt, Germany) was added to adjust to pH 6. The concentration of ^{25}Mg and ^{26}Mg isotope labels in solution was determined by isotope-dilution MS against a commercial Mg standard of natural isotopic composition (Titrisol; Merck, Darmstadt, Germany). Unless otherwise specified, all chemicals were of analytical grade, and acids were further purified by surface distillation. Only 18 MΩ water (Milli Q water system; Millipore, Zurich, Switzerland) was used for laboratory work and test-meal preparation.

Test meals

Kale and spinach were purchased frozen at a local supermarket, then cooked and puréed to prepare one batch of each vegetable. Individual servings were weighed and frozen (−25°C) immediately after preparation. Weighed samples of the isotope-label solutions were added to the test meals about 15 min before administration to ensure the accurate monitoring of individual doses. Stable-isotope labels were mixed with the puréed vegetables. Higher doses of ^{26}Mg label than of ^{25}Mg label were used in the present study due to differences in measurement precision (see p. 604).

Phytate-free wheat bread was prepared from white wheat flour (Migros, Zurich, Switzerland), water, salt, sugar and dry yeast. The dough was left to ferment for 5 h at room temperature. Bread rolls were baked for 15 min at 200°C, and stored frozen (−25°C) until served.

The rare-earth elements Yb and Eu were used as non-absorbable faecal markers to evaluate the completeness of faecal collections. Both compounds (in chloride form; Aldrich, Buchs, Switzerland) were added to water served as a drink together with the labelled test meals.

Study design

Before the start of the study, a faecal sample was collected to determine the baseline Mg isotope ratios. In addition, brilliant blue (100 mg; Warner Jenkinson Europe, King's Lynn, UK), a dye used as a faecal marker, was administered

in a gelatine capsule to indicate the start of the faecal pooling. After an overnight fast, a venous blood sample (10 ml) was drawn into a heparinised glass tube (Vacutainer Systems, Plymouth, UK) for the determination of Mg concentration in plasma. Plasma was separated by centrifugation (Omnifuge 2.0 RS; Heraeus, Zurich, Switzerland) at 20°C and about 500 *g* (5 min), and stored in acid-washed plastic vials at −25°C until analysed.

The study had a cross-over design, with each subject acting as his or her own control. Test meals A and B were randomly allocated to be served on day 1 or day 3. All test meals were based on 100 g phytate-free wheat bread served with either 300 g spinach (test meal A), to which the ^{25}Mg label was added, or with 300 g kale (test meal B), to which the ^{26}Mg label was added. Spinach and kale were heated in a microwave oven before serving. Water (18 MΩ water; 600 ml) with the added rare-earth elements was served as a drink with test meals A and B. The test meals were divided into two identical portions served at breakfast (07.30–08.30 hours) and at lunch (12.00–13.00 hours).

No food or drink was allowed between breakfast and lunch on days 1 and 3 and for 3 h after lunch. Standardised dinners (pizza and white wheat crisp bread) were provided on days 1 and 3. Drinking water (18 MΩ water; 2 litres) was provided on days 1 and 3. No additional foods or drinks were allowed on days 1 and 3. Diet was unrestricted at all other times.

Pre-weighed polypropylene containers (Semadeni, Ostermundigen, Switzerland) were provided for stool collections. The subjects collected all stools separately, starting immediately after the intake of the first labelled test meal on day 1. On day 8, a brilliant blue capsule was again administered. The collections were continued until excretion of the second brilliant blue marker. Stool samples were stored frozen (−25°C) until processed.

Preparation of faecal pools

Each individual stool sample was freeze-dried using a freeze dryer (Modulyo; Edwards, North Bergen, NJ, USA) and ground to a powder in a mortar. All stools, from the first stool dyed by brilliant blue until, but not including, the stools dyed by the second dose of brilliant blue, were included in the faecal pool. After a drying step (20 h at 65°C) in a drying chamber (Binder, Tuttlingen, Germany) to standardise humidity, followed by cooling at room temperature (4 h), all individual stools were weighed and milled, starting with the first (most enriched) samples. A mill (ZM1; Retsch, Haan, Germany) equipped with a sieve of 1 mm pores was used for this purpose. Each milled stool was transferred back into its original container, dried again for 20 h at 65°C, cooled for 4 h at room temperature and re-weighed to determine losses. All milled stools included in a single pool were combined in a 2 litre polyethylene container (Semadeni, Ostermundigen, Switzerland) and mixed for 90 min using a rotator (UG 70/20; Micro Motor, Basel, Switzerland).

Wet ashing

Portions of the freeze-dried pooled stool samples (1·0–1·6 g), wheat bread (0·25 g), kale and spinach (1 g) as

well as plasma (1 g), were wet ashed in a microwave system (MLS 1200; MLS GmbH, Leutkirch, Germany) in a mixture of 14 M-HNO$_3$ and 8·8 M-H$_2$O$_2$ (Merck, Darmstadt, Germany). All samples were wet ashed in duplicate.

Separation of magnesium

Mg was separated from the wet-ashed stool samples by cation-exchange chromatography using a strongly acidic ion-exchange resin (AG 50W X-8, 200–400 mesh; Bio-Rad, Hercules, CA, USA). Samples containing about 30 μmol Mg were evaporated to dryness, re-dissolved in 0·7 M-HCl (1 ml) and transferred onto the top of the column (10 mm inner diameter; Bio-Rad, Hercules, CA, USA), filled with ion-exchange resin to a height of 70 mm. The column was rinsed with 0·7 M-HCl (56 ml), followed by 0·9 M-HCl (24 ml) to elute Na and K. Mg was eluted with 1·4 M-HCl (12 ml). The solution was evaporated to dryness and re-dissolved in 50 μl water. Mg recovery, evaluated with a diluted Mg standard solution (Titrisol; Merck, Darmstadt, Germany) was found to be 94·8 (SD 1·8) % (n 10). Resins were regenerated with 6 M-HCl (30 ml) and replaced after the fifth run. Only acid-washed Teflon and polyethylene laboratory ware were used for sample processing. Samples of the ^{26}Mg isotope label were processed in parallel with each batch for blank monitoring, starting at the ion-exchange chromatography step. Sample contamination due to natural Mg was found to be 10·8 (SD 7·0) nmol (n 9) for the combined sample preparation and filament loading, which was <0·4‰ of the amount of Mg separated.

Isotopic analysis by thermal ionisation mass spectrometry

About 20 nmol separated Mg from faecal samples was loaded onto the metal surface of the evaporation filament of a double-Re filament ion source. Mg was coated with 5–10 μg silica gel 100, 0·8 μmol boric acid and 30 nmol Al as AlCl$_3$ (all chemicals from Merck, Darmstadt, Germany). Compounds were loaded as aqueous solutions and dried at a current of 0·8 A after each step. Finally, the evaporation filament was heated to dull red heat (a current of 1·6 A) for 30 s. The ionisation filament remained unloaded. Isotope ratios were determined with a single-focusing magnetic sector field instrument (MAT 262; Finnigan MAT, Bremen, Germany), equipped with a Faraday cup multicollector device for simultaneous ion beam detection. The evaporation filament was heated to 1230°C, using a standardised procedure. The ionisation filament was heated gradually to 1250–1350°C until a stable Mg$^+$ ion beam at a current of $1-2 \times 10^{-11}$ A was obtained. Each measurement consisted of thirty consecutive isotope-ratio measurements. Reproducibility (five independent runs) had a relative standard deviation of ±0·2 % for the ^{24}Mg:^{25}Mg isotope ratio and ±0·4 % for the ^{24}Mg:^{26}Mg isotope ratio.

Atomic absorption spectroscopy

Quantitative Mg analysis of the wet-ashed and diluted plasma, bread, faecal, and vegetable samples was performed by flame atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, Australia). Plasma samples were measured by external calibration using a commercial Mg standard (Titrisol; Merck, Darmstadt, Germany). All other samples were measured by an internal calibration technique (standard addition) to minimise matrix effects. In addition, all solutions contained La(NO$_3$)$_3$ at 5000 mg/l to suppress matrix effects. Certified reference materials (Seronorm Trace Elements Serum; Nycomed, Oslo, Norway, and wheat flour 1567 a; National Bureau of Standards, Gaithersburg, MD, USA) were analysed in parallel to monitor the accuracy of analysis. Yb and Eu were measured in the wet-ashed and diluted faecal samples by electrothermal atomic absorption spectroscopy by external calibration, using a standard solution containing Yb and Eu (Titrisol; Merck, Darmstadt, Germany), following standard procedures.

Analyses of oxalate and phytate

Total and soluble oxalate contents were determined enzymically (no. 1856120; Roche Diagnostics, Mannheim, Germany) after the extraction of oxalate in the puréed vegetables with 2 M-HCl (total oxalate) or water (soluble oxalate). Phytic acid was extracted from a 1 g sample of freeze-dried bread with 0·5 M-HCl. The extract was purified by anion-exchange chromatography, evaporated to dryness, and re-dissolved in water before analysis by HPLC reversed-phase chromatography as described by Sandberg & Ahderinne (1986).

Calculations

Molar amounts and ratios of the ^{25}Mg and ^{26}Mg isotope labels in the faecal pools were calculated based on double-isotope dilution principles (Walczyk et al. 1997; Sabatier et al. 2002). Fractional apparent Mg absorption (AA%) was based on the dose (μmol) of Mg stable isotope administered (D$_o$) and the amount of the isotopic label excreted in faeces (F$_o$).

$$AA(\%) = \frac{D_o - F_o}{D_o} \times 100.$$

Recovery of the rare-earth elements Eu and Yb was used to evaluate the completeness of the stool collections.

Statistics

Calculations were made using commercial software (Excel 97; Microsoft, Chicago, IL, USA and SPSS 10.0; SPSS Inc., Chicago, IL, USA). Results are presented as arithmetic means and standard deviations. The normal distribution of absorption values was verified by the Kolmogorov–Smirnoff test. Paired Student's t tests (two-tailed) were used to compare Mg absorption from the two different test meals. P values below 0·05 are referred to as statistically significant.

Results

The mean age and mean BMI of the subjects were 23 (SD 1) years and 22·6 (SD 3·2) kg/m^2 (n 9). The mean plasma Mg

concentration was 0·80 (range 0·73–0·89) mmol/l. Two individuals had slightly lower plasma Mg concentration than the reported normal range of 0·75–0·96 mmol/l (Lowenstein & Stanton, 1986).

The Mg and oxalate contents of the test meals are presented in Table 1. The native Mg content of the bread, spinach, and kale was 0·96 (SD 0·03), 1·12 (SD 0·09), and 0·89 (SD 0·01) mmol/100 g, respectively (*n* 3). Of total oxalates, 87·5 (SD 2·8) % were found to be soluble in spinach (*n* 3) and 51·8 (SD 11·7) % in kale (*n* 3). The phytic acid content was below the detection limit in wheat bread ($<0.5\,\mu$mol/100 g).

Mg absorption from the test meal served with spinach (26·7 (SD 10·4) %) was significantly lower ($P=0.01$) than from the test meal served with kale (36·5 (SD 11·8) %) as shown in Fig.1. The within-subject difference in Mg absorption between the two test meals was 9·8 (SD 7·2) %. Absorption ratios (test meal served with spinach/test meals served with kale) were 0·73 (SD 0·19). One subject

Table 1. Test meal contents of total magnesium, stable-isotope labels (^{25}Mg or ^{26}Mg), oxalic acid (OA), oxalic acid: magnesium molar ratios and non-absorbable faecal markers, ytterbium and europium*

(Mean values and standard deviations)

	Bread plus spinach		Bread plus kale	
	Mean	SD	Mean	SD
Total Mg (mmol)	4·98	0·01	4·82	0·05
Added Mg label				
^{25}Mg (mmol)	0·66	0·01	–	
^{26}Mg (mmol)	–		1·19	0·02
OA (mmol)	6·6	0·2	0·11	0·01
OA:Mg molar ratio	1·33		0·02	
Yb (nmol)	31·38	0·58	–	
Eu (nmol)	–		33·17	0·26

*Test meals consisted of 100 g phytate-free white wheat bread served with 300 g spinach or kale.
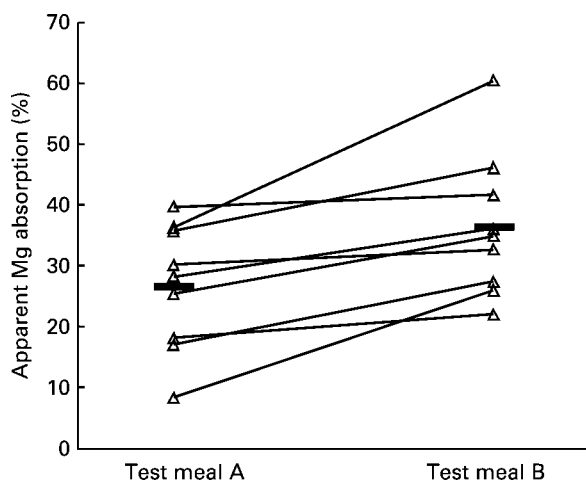

Fig. 1. Apparent magnesium absorption from a meal based on 100 g white wheat bread served with 300 g spinach (6·6 mmol oxalate; test meal A) or 300 g kale (0·11 mmol oxalate; test meal B). (△), Individual data (*n* 9); (━), mean absorption values.

was excluded from the evaluation due to a low recovery of Yb (<85 %). For all other subjects, mean Yb recovery was 98·8 (range 90·6–115·4) % and mean Eu recovery was 95·0 (range 90·0–99·1) %.

Complete faecal collections were made during 8·9 (SD 0·6) d. The mean loss of faecal material during sample preparation, determined by weighing faecal pools before and after milling, was 1·1 (SD 0·6) %. The measured isotopic enrichment of the stool pools was 5·6 (SD 1·8) % (^{24}Mg:^{25}Mg range 2·5–9·3 %) and 8·1 (SD 2·4) % (^{24}Mg:^{26}Mg range 5·3–13·1 %) based on differences of the measured isotope ratios of faecal pools and natural isotope ratios of a standard (Titrisol; Merck, Darmstadt, Germany), divided by the measured isotope ratio of the standard.

Discussion

The present study is, to our knowledge, the first report clearly demonstrating an inhibitory effect of an oxalate-rich vegetable on Mg absorption in human subjects, based on the stable-isotope technique. The mean fractional apparent Mg absorption from the labelled test meal served with spinach was about 35 % lower than from the test meal served with kale in the present study. However, these results should not be interpreted as suggesting that spinach is a poor source of dietary Mg. The observed decrease in fractional apparent Mg absorption can be assumed to be, at least partly, counterbalanced by the approximately 30 % higher Mg content of spinach as compared with kale (Holland *et al.* 1994; Souci *et al.* 1994); native Mg content of spinach was 26 % higher than that of kale in the test meals evaluated in the present study.

In the present study, we evaluated Mg absorption from extrinsically labelled test meals based on phytate-free wheat bread, served with spinach or kale. Limited information is available on the validity of the extrinsic labelling technique for studies of Mg absorption in human subjects although an earlier study reported no significant differences in Mg absorption from intrinsically and extrinsically labelled leafy vegetables, including spinach and collards, in man (Schwartz *et al.* 1984). Similar findings have also been reported in rats (Schwartz *et al.* 1980); however, it should be stressed that the usefulness of animal models in these evaluations is not clear. Contrary to the data (which are rather limited) on the validity of extrinsic labelling of leafy vegetables with Mg isotopes, the extrinsic labelling technique has been demonstrated not to be valid for Ca absorption from spinach (Weaver & Heaney, 1991). Interestingly, the study by Weaver & Heaney (1991) reported Ca absorption from calcium oxalate to be twice as well absorbed as from spinach oxalate in healthy adult women. These data thus indicate different absorption mechanisms for, or different physical and chemical properties of, pure calcium oxalate and spinach Ca and highlight the limited usefulness of evaluating the influence of pure calcium oxalate on Ca absorption from complex food matrices such as spinach.

Oxalates in plants are present as water soluble, bound to Na or K or present as free oxalates, as well as water-insoluble compounds, i.e. calcium oxalate and magnesium

oxalate (Hodgkinson, 1977b; Souci et al. 1994). It is not clear to what extent insoluble oxalates dissolve in the gastric juice and it could be speculated that soluble oxalates would have a more pronounced negative impact on mineral and trace element absorption due to their ability to form complexes with minerals and trace elements in the gastro-intestinal tract. In addition, calcium oxalate has been suggested to be absorbed intact (Hanes et al. 1999) and it could be hypothesised that the more soluble magnesium oxalate could also be absorbed intact. In the present study, a large proportion (88 %) of total oxalates was present as water-soluble oxalates in spinach. However, at the present time, there is no information about the relative importance of soluble and insoluble oxalates on mineral and trace element absorption in man. Published data on the proportion of soluble oxalates relative to total oxalates also differ considerably; for example data for spinach vary from 15–20 % (Toma & Tabekhia, 1979; Souci et al. 1994) up to 93 % (Hodgkinson, 1977b).

Although based on indirect evidence, the observed difference in fractional Mg absorption in the present study is attributed to the differences in oxalate content; 6·6 mmol in the meal served with spinach and 0·1 mmol in the meal served with kale. Based on present knowledge, it is improbable that other plant components present in green leafy vegetables such as fermentable fibre or phenolic compounds would have influenced the results. For example, kale contains about 2 % fibre as pentosans and hexosans, about double the amount found in spinach (Souci et al. 1994). These partly soluble hemicelluloses are non-digestible and could be expected to be fermented by bacteria in the large intestine, similarly to fructo-oligo-saccharides, which were reported to increase fractional Mg absorption in human subjects significantly when consumed in amounts of 10 g/d (Tahiri et al. 2001). Recently, the enhancing effect of polyols (100 g/d) on apparent Mg absorption in man was also demonstrated (Coudray et al. 2003a). However, information about the effect of dietary fibre on Mg absorption is not conclusive, as several human studies have not demonstrated statistically significant differences in Mg absorption after an increased intake of fermentable fibre (for a review, see Coudray et al. 2003b). In addition, a study based on the chemical balance technique reported a significant negative effect on Mg absorption after the intake of a mixture of galactans, pentosans, and hexosans (14 g/d) by adolescent boys (Drews et al. 1979). Furthermore, phenolic compounds have been reported to have a strong negative effect on Fe absorption (Gillooly et al. 1983; Brune et al. 1989). However, although phenolic compounds have not been evaluated for their effect on Mg absorption, the amount of total phenolic compounds has been reported to be similar in spinach and kale (Lucarini et al. 2000).

In conclusion, the results from the present study demonstrate that the mean fractional apparent Mg absorption from a test meal served with spinach was about 35 % lower than from a test meal served with kale. It is suggested that this reduction in Mg absorption is due to the higher oxalate content of spinach. However, the significantly lower fractional apparent Mg absorption from spinach can be assumed to be, at least partly, counterbalanced by the higher native Mg content of spinach as compared with kale.

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