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A comparison of methods for the *in vitro* determination of the effects of tea on iron availability from foods

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Two *in vitro* techniques were used to estimate the effect of tea on the availability of iron in semi-synthetic rat diet and in rice. Both methods entailed measurement of soluble iron after simulated digestion *in vitro* but one (diffusibility technique) also incorporated a dialysis step so that high molecular weight species (> about 12000) were excluded from the estimated bioavailable fraction. In the case of the semi-synthetic diet a feeding trial was carried out to confirm the negative effect of the tea on the iron status of rats after a prolonged feeding period. The diffusibility technique failed to detect the reduction in iron availability caused by the addition of tea leaves to the rat diet. It was concluded that *in vitro* methods must be used with caution, and the inclusion of a dialysis step in the technique may cause true availability to be seriously underestimated.

INTRODUCTION

Dietary iron availability is dependent upon the chemical form of iron, and its interaction with various dietary components (Hallberg, 1981), and may be a more important determinant of iron nutrition than total iron intake (FAO, 1988). There is, therefore, an urgent need for methods to accurately measure iron availability from foods and diets, both in industrialised countries and in the developing world. However, the measurement of bioavailability from different foods and meals in man is time-consuming, expensive, and statistically complex because of wider inter- and intra-individual variations in iron absorption due to physiological factors (Fairweather-Tait, 1987) and adaptive responses (Cook, 1990). Because of these difficulties there have been many attempts to develop reliable methods for the estimation of iron availability using *in vitro* techniques to model the intraluminal stage of iron absorption.

The present study was undertaken to compare two different methods for estimating iron availability *in vitro*. Both techniques incorporate a simulated digestion procedure, followed by a measurement of soluble iron, which is used as an indicator of iron availability. However in the technique of Miller *et al.* (1981), as modified by Hazell and Johnson (1987), dialysis tubing is used to separate radioactively labelled iron complexes of high and low molecular weight. In the method of Madriaga *et al.* (1984), which is based on that of Narasinga Rao and Prabhavathi (1978), total ionisable iron is determined chemically.

Tea, a known inhibitor of iron availability, was used in this study, and the *in vitro* results obtained were compared with the effects of tea on iron status in the rat. Tea is a rich source of tannins, which form insoluble complexes with iron and render it unavailable for absorption. When taken as a beverage in conjunction with foods containing iron, tea infusions inhibit iron availability in man (Disler *et al.*, 1975; Hallberg & Rossander, 1982). Regular consumption of tea has been associated with iron-deficiency anaemia in infants (Merhar *et al.*, 1985).

MATERIALS AND METHODS

Preparation of test foods

The normal-iron and low-iron semi-synthetic rat diets, were prepared with or without the incorporation of 20 g

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Table 1. Composition of experimental rat diets

	Control diet	Tea leaf diet
Maize starch	309	299
Sucrose	309	299
Casein	200	200
Solka floc	40	40
Maize oil	80	80
Mineral mix[a]	40	40
Vitamin mix[a]	20	20
Methionine	2·5	2·5
Tea leaves	—	20

[a] Micronutrient mixes were formulated to provide normal requirements for growth. The added Fe was in the form of $FeSO_4.7H_2O$. The control diet contained 38 μg Fe/g; the low-iron diet (no added $FeSO_4.7H_2O$) contained 9 μg Fe/g, but was otherwise identical.

tea leaves/kg diet (PG Tips, Brooke Bond, Croydon, Surrey, UK). The composition of the experimental diets is shown in Table 1. Rice (long grain Patna type, Tesco, Cheshunt, Herts, UK) was cooked by boiling in distilled water for 15 min, and draining off the excess liquid. Tea infusion was prepared by mixing 2 g tea leaves (PG Tips) with boiling distilled water (100 ml), and allowing it to stand for 10 min, after which it was stirred and filtered. All the test meals were homogenised with saline (0·9% w/v NaCl) in a food blender to give a creamy dispersion containing approximately 10 g dry matter per 100 g homogenate. Where tea infusion (5–25 ml) was added to the incubation, the volume of saline was appropriately reduced to maintain a constant dry matter to liquid ratio.

Reagents

The pepsin suspension for the diffusible iron technique (method 1) was prepared by stirring pepsin powder (16 g, Sigma Chemical Co., Poole, Dorset, UK; porcine stomach mucosa) into 0·1N HCl, and adjusting to 100 ml final volume. For the extractable iron technique (method 2), pepsin powder (0·5 g, Sigma Chemical Co.) was similarly suspended in 100 ml 0·1N HCl. Pancreatin (4 g, porcine pancreas, Sigma Chemical Co.) and bile extract (25 g, porcine, Sigma Chemical Co.) were dispersed in 0·1M $NaHCO_3$ and made to 1 litre final volume. The chromagen solution used in method 2 was prepared by dissolving α, α,-dipyridyl (0·2 g, Sigma Chemical Co.) in 100 ml acetic acid in water (10% v/v). Radioactive ^{59}Fe (Amersham International, UK), as $FeCl_3$ in 0·1N HCl, was used (110–740 MBq/ mg Fe), each incubation containing 37 kBq of ^{59}Fe.

Diffusible iron – method 1

The release of diffusible iron from the test foods was determined using a modified version of the method of Miller *et al.* (1981) and Hazel and Johnson (1987). After blending, the pH of the homogenates was adjusted to pH 2·0 with 6M HCl, and the homogenates were 'spiked' with 37 kBq of ^{59}Fe. Pepsin suspension (3 ml) was added and the homogenate (100 ml) was incubated at 37°C for 2 h to simulate gastric conditions. Duplicate 20 ml samples of the pepsin digest were then transferred to wide-base conical flasks. Segments of dialysis tubing (molecular cut-off 12000 Da, Medicell International, UK) containing 25 ml distilled water and an amount of $NaHCO_3$ equal to the number of equivalents of NaOH required to titrate the combined pepsin digest pancreatin-bile extract mixture to pH 7·0 was added to each flask. The precise quantity of $NaHCO_3$ was pre-determined by titration of 20 g samples of pepsin digest to which 5 ml of the pancreatin-bile extract mixture has been added.

The flasks were covered with parafilm and incubated in a reciprocating shaking water bath (100 strokes/min) for 30 min at 37°C. Pancreatin-bile extract mixture (5 ml) was then added to each flask, and the incubation continued for a further 2 h. At the end of the incubation period, the dialysis sacs were removed, rinsed and blotted. The weight of the contents of each flask (weight of retentate) was determined, and the volume of liquid in each dialysis sac (volume of diffusate) was measured. The radioactivity in 5 ml samples of the retentate and diffusate was assessed in triplicate, using a Philips PW4750 gamma spectrometer.

The percentage of diffusible (dialysable) iron was determined using the following equation:

$$\frac{(\text{Counts/min } ^{59}\text{Fe/ml diffusate} \times \text{ml diffusate}) \times 100}{(\text{Counts/min } ^{59}\text{Fe/ml diffusate} \times \text{ml diffusate}) + (\text{counts/min } ^{59}\text{Fe/g retentate} \times \text{g retentate})}$$

Extractable iron method 2

The release of extractable iron from the test foods was determined by the method of Madriaga *et al.* (1984). A weighed sample of homogenised food was mixed with pepsin–HCl solution (1:2 w/v), the initial pH was recorded, the suspension was 'spiked' with 37 kBq of ^{59}Fe and incubated in a reciprocating shaking water bath (100 strokes/min) for 2 h at 37°C. At the end of the incubation period, sub-samples (1 ml) were taken to determine initial activity, and the pH of the digest was adjusted to pH 7·0 using 50% (w/v) sodium acetate trihydrate in water. Chloroform (0·5 ml) was added to the remaining digest, to remove fat and coloured compounds. Available iron was then extracted in triplicate from 1 ml sub-samples, using 1 ml of chromagen solution. Similar sub-samples were treated with water instead of chromagen for 'blank' determinations. Anhydrous magnesium carbonate (0·2 g) was added to each tube, mixed, allowed to stand for 15 min, and centrifuged at 3000 rev min for 20 min. The extracted sub-samples were assayed for radioactivity using a Philips PW4750 automatic gamma spectrometer.

The percentage available iron (E) was calculated using the equation:

$$E = B - C$$

$$B = \frac{\text{counts/min } ^{59}\text{Fe/ml chromagen extract} \times 100}{\text{initial count } ^{59}\text{Fe added (cpm/ml)}}$$

$$C = \frac{\text{counts/min } ^{59}\text{/ml water extract} \times 100}{\text{initial count } ^{59}\text{Fe added (cpm/ml)}}$$

In vivo study

Ninety male weanling Wistar strain rats were randomly allocated to six groups of 15 and housed in wire-bottomed, stainless steel and plastic cages. Three groups received a control diet (38 μg Fe/g), and three groups received a low-Fe diet (9 μg Fe/g). The compositions of the diets are given in Table 1 and described in detail elsewhere (Fairweather-Tait *et al.*, 1991). At each dietary Fe level, one group was fed semi-synthetic diet containing tea-leaves and received distilled water *ad libitum*; a second group was given a control diet and tea infusion to drink *ad libitum*; and a third group received a diet and drink that were both free of tea. Food and liquid intakes were measured daily and animals were weighed at regular intervals.

After four weeks the animals were killed with a lethal dose of sodium pentobarbitone (Euthatal, May & Baker Ltd., Dagenham, UK), given by intraperitoneal injection (1·0 ml; 20 mg/ml). Blood was collected by cardiac puncture, the liver was removed, rinsed in saline (9 g NaCl/ml), blotted dry, freeze-dried and homogenised by grinding in a pestle and mortar.

Analysis of Fe status

Samples of freeze-dried liver were heated to 480°C for 48 h in silica crucibles, the ash was dissolved in hot concentrated hydrochloric acid, Analar grade, and the solution was made up to an appropriate volume with distilled water (Analytical grade, Fisons, UK). The Fe content was determined by flame atomic absorption spectroscopy (PU 9000, Philips, Cambridge, UK), using bovine liver as a reference standard (National Bureau of Standards, Gaithersburg, USA).

Haemoglobin concentration was determined from blood samples using the cyanomethaemoglobin method (Crosby & Houchin, 1957). Packed cell volume (PCV) was determined by a microhaematocrit technique.

Statistical analysis

The significance of differences between control values and treatment values was determined using Student's unpaired t-test.

RESULTS

In vitro studies

Estimates of bioavailable iron in the two semi-synthetic diets, with and without tea leaves or tea infusion, are given in Fig. 1. In the case of method 1, the estimated availability was never greater than 2·5% but showed a tendency to rise as the quantity of tea infusion added to the diet was increased. In contrast, iron availability, as estimated with the extractable iron technique, method 2, declined sharply with the addition of 5 ml of tea infusion. Higher levels of tea had a small additional effect. The apparent availability of iron was also reduced to approximately the same extent by the addition of tea leaves. In the absence of tea, the percentage of available iron estimated by method 2 was about four times higher than the value obtained using method 1.

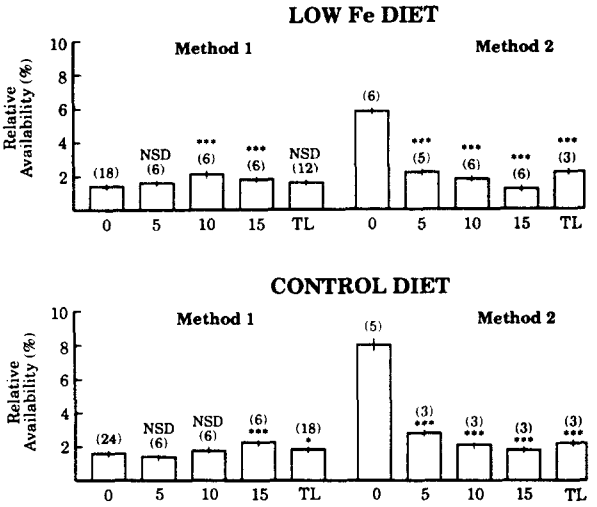

Fig. 1. Relative availability of iron from semi-synthetic rat diets determined *in vitro* by the iron diffusibility method (method 1) or by the total extractable iron technique (method 2). The control samples (0) contained no iron; test samples contained 5, 10 or 15 ml of tea infusion or tea leaves (TL). Each value is the mean, and standard error for the number of analyses given in parentheses. Asterisks indicate the significance of the difference between the control values (0) and the tests (NSD = no significant differences; $*p < 0{\cdot}05$; $**p < 0{\cdot}01$; $***p < 0{\cdot}001$).

A somewhat different pattern emerged when the two techniques were applied to rice (Fig. 2). In contrast to the effects on a semi-synthetic diet, a decline in available iron was observed with the addition of tea infusion in both methods, and the values obtained by the two methods were in reasonable agreement.

In vivo studies

The final body weights of the animals given the low-iron diets were approximately 20% lower than those of the control groups ($p < 0{\cdot}001$), but there was no signifi-

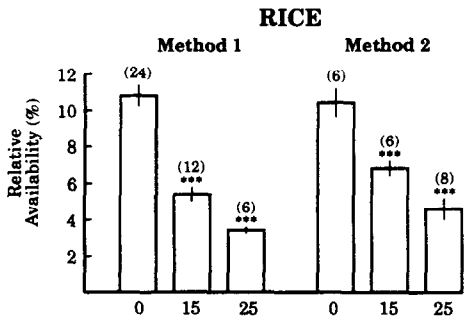

Fig. 2. Relative availability of iron from rice, determined *in vitro* by the iron diffusibility method (method 1) or the total extractable iron technique (method 2). Control samples (0) contained no tea; test samples contained either 15 or 25 ml of tea infusion. Values are means, with standard errors for the number of analyses given in parentheses. Asterisks indicate the significance of the differences between the control values (0) and the tests (NSD = no significant difference; $*p < 0{\cdot}05$; $**p < 0{\cdot}01$; $***p < 0{\cdot}001$).

cant effect of tea on body weight, whether given as an infusion or as tea leaves. The iron concentration in liver tissue, the total iron content of the livers, and the haemoglobin and PCV of rats given the low-Fe diet were all significantly lower than those of control animals (Fig. 3; $p < 0{\cdot}001$). In animals given the control diet, consumption of tea, whether as an infusion or as tea leaves, was associated with relatively low liver iron stores and small reductions in haemoglobin and PCV. The animals given a low-iron diet showed similar trends (Fig. 3).

DISCUSSION

The bioavailability of dietary iron depends upon luminal interactions between iron and other food components, and upon the absorptive capacity of the mucosal cells, which are under a high degree of physiological control. The use of *in vitro* techniques for estimating iron availability is an attempt to measure that proportion of dietary iron which is released during digestion in a form suitable for absorption: this should be a consistent measure that is reproducible between laboratories when carried out under identical conditions. However, problems have been reported with *in vitro* methods (Forbes *et al.*, 1989). *In vitro* methods are based on the principle of measuring soluble iron, but the technique of Miller *et al.* (1981) — a modified version of which has been used in this study — also incorporates a dialysis system to exclude high-molecular-weight soluble complexes. There can be no possibility of simulating the physiological factors that account for major variations in the absorption of iron, both between individuals, and within individuals over time. Nevertheless, *in vitro* techniques should be able to rank individual foods in order of available iron content and to predict correctly the effects of known enhancers and inhibitors *in vivo*. The present study has demonstrated important inconsistencies in the predictive value of two different *in*

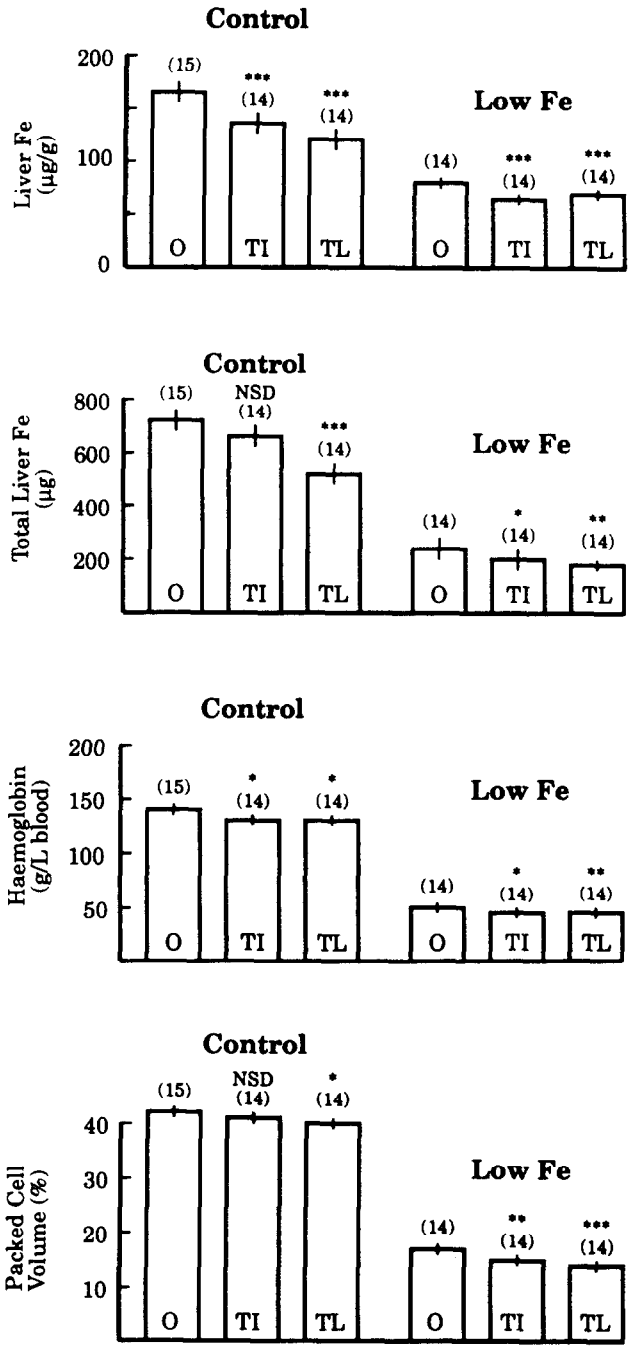

Fig. 3. Indices of iron status in rats drinking tea infusion (TI) or eating a diet containing tea leaves (TL). Values are means, with standard errors of the number of animals given in parentheses. Asterisks indicate the significance of differences between the control values (0) and the tests (NSD = no significant difference; $*p < 0{\cdot}05$; $**p < 0{\cdot}01$; $***p < 0{\cdot}001$).

vitro methods applied to relatively simple food matrices containing a known inhibitor of iron absorption.

As expected, the prolonged consumption of either tea infusion or dried tea leaves led to a reduction in iron status in growing rats. Indeed, liver iron stores were reduced by almost 30% in rats given a high-iron diet containing tea leaves (Fig. 3). There was a surprising divergence, however, in the iron availability measurements obtained by the two *in vitro* methods when

applied to the semi-synthetic diets. The modified Miller technique (method 1) failed to detect any inhibitory effect of tea and, indeed, there was a tendency for availability to rise slightly with the addition of tea infusion (Fig. 1). Method 2 indicated a reduction in available iron with the addition of tea, and there was a trend towards declining apparent availability as the quantity of tea infusion was increased.

Some indication of the quantitative validity of the techniques can be obtained by comparing the effects of tea leaves with those of dietary iron depletion in the rats. The presence of tea leaves in the semi-synthetic diet was associated with a 70% reduction in apparent availability as measured by method 2 (Fig. 1). At the end of the feeding study the hepatic iron stores of rats fed the high-iron, tea-enriched diets were about 30% lower than those of the tea-free control animals. Animals fed the iron-depleted diet consumed over 75% less iron than their control-fed counterparts, and at the end of the feeding period their hepatic iron stores were over 50% lower than those of the controls. These observations indicate that the *in vitro* method 2 correctly predicted the direction of the change in iron availability resulting from the addition of tea to the diet, but tended to overestimate its magnitude, whereas method 1 completely failed to predict the adverse effects of tea. Both methods gave similar results when applied to rice.

Miller and Berner (1989) have suggested that the measurement of iron diffusibility across a semi-permeable membrane may be a more appropriate predictor of biologically available iron, particularly where there is extensive interaction with proteins. Their argument is based on the proposition that iron which is bound to high molecular weight peptides may be unable to cross the mucosal mucus layer in the proximal small bowel, where iron absorption is most efficient. Thus, the inclusion of this fraction in the measurement of total soluble iron would overestimate iron availability. Some biological evidence supports this proposition: for example, the solubility of iron in the intestinal lumen of rats fed a diet rich in egg protein is high, but egg protein inhibits the absorption of iron *in vivo* (Sato *et al.*, 1987).

The inclusion of a dialysis step in method 1 in the present study eliminated soluble iron species with a molecular weight greater than 10000–12000 Da from the estimated bioavailable fraction. This may account for the anomalous results obtained with the semi-synthetic rat diet. The casein component of this material probably provided high molecular weight peptides in quantities large enough to reduce diffusible iron to very low values that could not be reduced any further by the addition of tea. This effect would be absent, however, when the methods were applied to rice, which is relatively low in protein.

This study demonstrates that considerable caution is necessary when applying *in vitro* methods for estimating iron availability to complex food systems. An estimate of iron solubility under conditions similar to those of the gut during digestion is likely to provide some indication of availability *in vivo*, but the experimenter should be aware of the limitations of the technique. Careful thought needs to be given to the kinds of chemical interactions that are likely to be influencing speciation in the system and, if possible, some form of *in vivo* procedure should be used to validate the observation at key stages in the study. The measurement of diffusibility may be a useful refinement for some foods, but the possibility of seriously underestimating the true availability of iron needs to be considered.

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