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Studies on iron availability in man, using stable isotope techniques

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1. Iron absorption from 10 mg Fe (as ferrous sulphate), labelled with 1·3 mg ^{58}Fe, was measured in fasting, non-anaemic adult subjects by the faecal-balance technique. The measurement was performed twice, each subject being given, in random order, either 50 mg Fe or a placebo 18 h before the ^{58}Fe-labelled $FeSO_4$.

2. The 50 mg Fe load significantly reduced Fe absorption the following day ($P < 0·01$), from a mean of 35·4 (SEM 4·6)% to 29·0 (SEM 5·1)%. This points to the importance of strict dietary control during Fe-absorption studies to eliminate bias in results.

3. In a separate study, the feasibility of using ^{58}Fe-enrichment of erythrocytes, measured by neutron activation analysis (NAA), 10 d after a meal labelled with 0·69 mg ^{58}Fe as an index of Fe absorption was examined. The levels of ^{58}Fe in the blood were detectable by NAA. Regression analysis showed a significant relation between ^{58}Fe-enrichment of blood and ^{58}Fe absorption, calculated as the difference between intake and faecal excretion (R 0·59, $P < 0·05$).

Isotopes are generally regarded as the most accurate and versatile method of studying mineral absorption and metabolism. The radioisotope ^{59}Fe was first used in animal studies in 1939 (Hahn *et al.* 1939) and in humans in 1941 (Ross & Chapin, 1941). However, the hazards associated with ionizing radiation preclude its use in most human studies. This has led to the development of methods for utilizing the completely safe alternative of stable isotopes which have been successfully employed in a number of experiments investigating Fe availability.

The traditional approach to such studies is to use the faecal monitoring technique (Janghorbani & Young, 1980). The test substance is labelled extrinsically with, for example, a small quantity of ^{58}Fe and given to subjects after an overnight fast. The quantity of ^{58}Fe which has been absorbed is calculated as the difference between the dose given and the amount excreted (unabsorbed) in the faeces, after allowing for the naturally occurring isotope present. Since it is presumed that complete isotopic exchange has occurred between the ^{58}Fe label and the endogenous Fe in the test foodstuff, the absorption of ^{58}Fe represents absorption of endogenous Fe from the food.

Two points arise from the previous statements. First, it is firmly established in rats that the level of Fe in the diet consumed during the previous 1–3 d profoundly influences Fe absorption from a subsequent test meal (Fairweather-Tait & Wright, 1984; Fairweather-Tait *et al.* 1985). If this is also true in man, then rigorous dietary control is required in human studies. Second, an alternative approach to measuring Fe absorption, which obviates the necessity for faecal collection and analysis, with all the accompanying drawbacks, is that of isotopic incorporation into the erythrocytes.

The following experiments utilize the stable isotope ^{58}Fe, measured by neutron activation analysis (NAA), to test whether in man previous Fe intake affects subsequent absorption, as already demonstrated in the rat (Expt 1), and to investigate the feasibility of using erythrocyte enrichment with ^{58}Fe as an alternative to faecal balance for estimating Fe absorption (Expt 2).

MATERIALS AND METHODS

Expt 1

Ten adults (six females, four males) aged 18–51 years (mean age 29 (SEM 3) years) were selected for the study on the basis of normal Fe status. None was anaemic as judged by blood haemoglobin (Hb) and all were in good health and not undertaking any form of medication. Fe absorption from ^{58}Fe-labelled ferrous sulphate was measured in each subject twice, with an interval of 1 week between the test drinks. The day before the test drink, in the middle of the afternoon, each subject was given, in random order, either a capsule containing 50 mg Fe (as $FeSO_4$) or a placebo. The subjects were on self-selected diets during the experiment but they were asked to duplicate their diets exactly on both days preceding the test drinks.

On the day of the test drink, the subjects presented themselves after an overnight fast and were given 200 ml Coca-cola containing 10 mg Fe (as $FeSO_4$) labelled with 1·3 mg ^{58}Fe (AERE, Harwell). Coca-cola, containing only trace levels of Fe, was used to mask the unpleasant flavour of the Fe and to retain it in the ferrous form. They refrained from eating or drinking for at least 3 h after the test drink. A complete faecal collection was made from the time of taking the drink until all the unabsorbed ^{58}Fe-labelled $FeSO_4$ had been excreted. This was determined by carmine markers: each subject took 500 mg carmine with their evening meal on the day of the test drink and all stools were collected in plastic bags until the carmine had been fully excreted, plus one further collection. Faeces from each subject for each collection period were stored frozen, bulked, autoclaved at 100 kPa (15 psi) for 20 min, freeze dried and homogenized before analysis.

Expt 2

This experiment formed part of a larger study in which adaptation to low-Fe diets was being examined in collaboration with the MRC Dunn Nutrition Unit in The Gambia. Subjects were non-anaemic adult men from Keneba, and the protocol was similar to that previously described except that the men were fed a 3 d rotating menu for 1 week before and during the entire experimental period.

On the day of the test meal, the fasting subjects were given either 3 mg Fe (as $FeSO_4$) in 200 ml Coca-cola or 300 g cooked rice with 130 g durango sauce (groundnuts, tomato paste, chillies and onion); the test meal also contained 3 mg Fe. The drink and the meal were labelled with 0·69 mg ^{58}Fe. At the same time each subject was given three capsules each containing ten radio-opaque pellets (as used by Dr J. H. Cummings, Dunn Clinical Nutrition Unit, Cambridge), to check for completeness of faecal collection. Carmine capsules were taken with the evening meal so that subjects would know when to stop collecting stools. The faecal samples were collected in plastic bags and stored frozen. Faeces from each subject were bulked and autoclaved, and then oven-dried at 90°. The dried material was X-rayed, the number of radio-opaque pellets counted, and any incomplete collections were excluded from the experiment. The dried material was ground to a fine powder and homogenized.

On the 10th day after giving the ^{58}Fe-labelled material, after an overnight fast and before consuming a second test meal, 10 ml of blood were removed from each subject for total Fe and ^{58}Fe analysis.

Total Fe and ^{58}Fe analysis

Subsamples of dried, homogenized faeces and 5 g portions of blood were ashed in silica crucibles at 480° for 48 h. The ash was subdivided and a weighed portion analysed for total Fe by atomic absorption spectroscopy (Fairweather-Tait *et al.* 1985). The remainder of the

ash was analysed by NAA, together with standards from the National Bureau of Standards (Office of Standard Reference Materials, Washington, USA), as described previously (Fairweather-Tait *et al.* 1983).

Statistical analysis

Results from the first experiment were examined by paired *t* test (Snedecor & Cochran, 1973) and Wilcoxon matched-pairs signed-ranks test (Siegel, 1956). The ^{58}Fe absorption, measured by faecal balance, and ^{58}Fe-enrichment of the blood in the second experiment were subjected to regression analysis using the statistical package GENSTAT (Alvey *et al.* 1977).

Ethical considerations

The first experiment was approved by the AFRC Food Research Institute, Norwich (FRIN) Ethical Committee and the second by both FRIN and the Dunn Nutrition Ethical Committees.

RESULTS

The natural abundance of ^{58}Fe is 0·33% (Eagles *et al.* 1985), whereas enriched sources of ^{54}Fe (AERE, Harwell) were used in the studies: Expt 1, 71·5%; Expt 2, 65·8%.

Naturally occurring ^{58}Fe was deducted from total ^{58}Fe in faeces in order to calculate enrichment with ^{58}Fe from the test meal or drink. A further adjustment to the value was necessary to take into account the proportion of ^{58}Fe in the enriched source which would be contributed by naturally abundant ^{58}Fe (i.e. 0·33%). Thus the following formula was used:

$$^{58}Fe_E = \left(\frac{^{58}Fe_T - 0{\cdot}0033\ Fe_T}{^{58}Fe^* - 0{\cdot}33}\right) \times {}^{58}Fe^*,$$

where $^{58}Fe_E$ is ^{58}Fe from enriched source, $^{58}Fe_T$ is total ^{58}Fe in faeces, Fe_T is total Fe in faeces, $^{58}Fe^*$ is abundance of enriched source (71·5/65·8).

Expt 1

The mean Hb concentration was 148 (SEM 5) g/l blood (♀ 138 (SEM 4), ♂ 164 (SEM 5)). The percentage of ^{58}Fe-labelled $FeSO_4$ absorbed by each subject the day after a 50 mg dose of Fe or a placebo was calculated and the results are shown in Fig. 1. Analysis of the results by paired *t* test or Wilcoxon matched-pairs signed-ranks test showed that significantly-less Fe was absorbed by subjects after they had received a 50 mg dose of Fe on the previous day than after they had received a placebo ($P < 0{\cdot}01$). The mean absorption from 10 mg Fe ($FeSO_4$) was 29·0 (SEM 5·1)% after the $FeSO_4$ and 35·4 (SEM 4·6)% after the placebo.

Expt 2

The mean Hb concentration was 139 (SEM 2) g/l blood. Faecal results from Expt 2 were treated as previously described. Mean absorption from $FeSO_4$ was 56·9 (SEM 5·1)% and from durango and rice 44·2 (SEM 8·7)%. The ^{58}Fe excess per g ashed blood was calculated by deducting the naturally occurring ^{58}Fe (0·33% of total Fe by atomic absorption spectroscopy from the total ^{58}Fe, measured by NAA. Enrichment of the blood with ^{58}Fe was expressed as a percentage of the endogenous (naturally occurring) ^{58}Fe. Regression analysis showed a significant relation between these two measurements (R 0·59, $P < 0{\cdot}05$). The results are shown in Fig. 2.

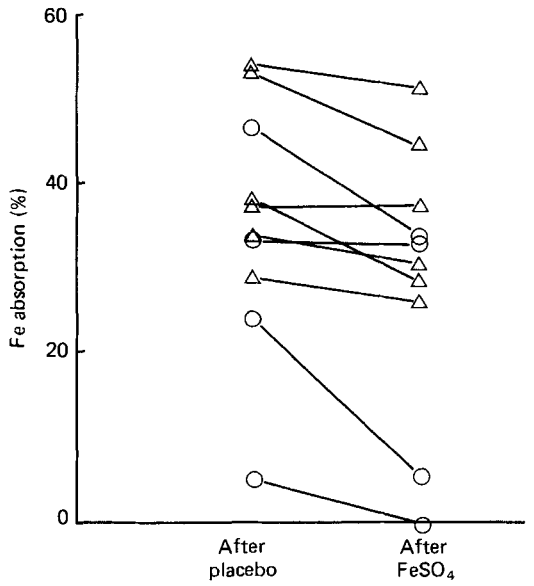

Fig. 1. Iron absorption from 10 mg Fe (as ferrous sulphate) given 18 h after a placebo or 50 mg Fe in four adult male (○) and six adult female (△) subjects ($P < 0{\cdot}01$).

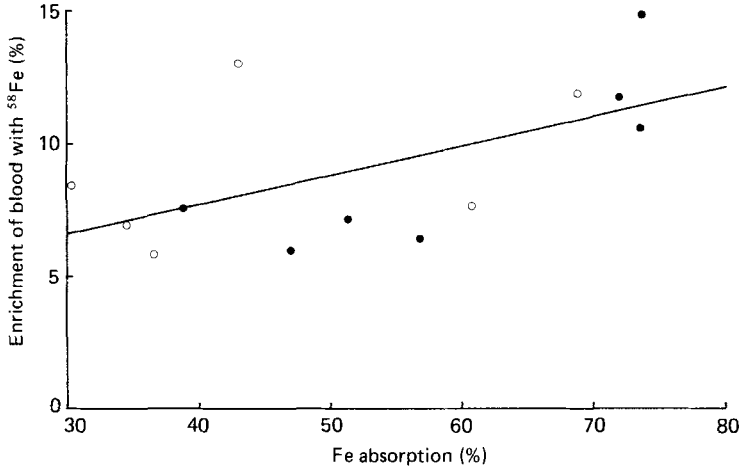

Fig. 2. Percentage Fe absorption (calculated as the difference between ^{58}Fe intake and ^{58}Fe faecal excretion) and percentage ^{58} Fe enrichment of the blood 10 d after giving 3 mg Fe (labelled with 0·69 mg ^{58}Fe) as durango (groundnuts, tomato paste, chillies and onion) and rice (○) or ferrous sulphate (●) in non-anaemic adult Gambian men (R 0·59, $P < 0{\cdot}05$).

DISCUSSION

One of the most striking features of the present work is the large inter-subject variation in Fe absorption. Despite the well-recognized concept that Fe status is one of the major factors determining non-haem Fe absorption (Rossander *et al.* 1979), the differences in these two experiments cannot be explained in terms of differing Fe status, since there is no correlation between Hb concentration and Fe absorption. This finding is not unique, and agrees with previous results (Fairweather-Tait *et al.* 1983) and results reported by other workers on non-anaemic subjects (Martinez-Torres *et al.* 1979; Trinidad *et al.* 1980). Hb concentration is not the ideal method for determining Fe status, but if one accepts that Hb

levels reflect Fe status to some degree, and that Fe status is an important variable influencing Fe absorption, then some sort of relation between Hb concentration and Fe absorption should become apparent. There must obviously be a more important modifier of Fe absorption to explain the well-known inter-subject variations and somewhat less well-documented intra-subject variations in Fe absorption (Cook *et al.* 1969).

The most widely applied method of measuring Fe availability in foods involves measuring Fe absorption from a labelled food (by faecal monitoring, whole-body counting or Hb incorporation) and comparing it with absorption from a well-absorbed Fe salt, such as $FeSO_4$. The availability of the food Fe is then expressed as a percentage of the Fe salt. Subjects are always fasted several hours before and after the test meals because food influences Fe availability, but there is rarely any dietary control in the experimental protocol; subjects are usually allowed to exist on self-selected free-living diets. In view of our previously-published results showing an inverse exponential relation between dietary Fe intake for 1–3 d preceding a test meal and Fe absorption (Fairweather-Tait & Wright, 1984; Fairweather-Tait *et al.* 1985), it may well be necessary to adopt stricter measures of dietary control in Fe availability studies. Results from Expt 1 show that a 50 mg dose of Fe, given as $FeSO_4$, significantly reduced Fe absorption the following day ($P < 0{\cdot}01$), assuming that there was no bias in the intra-subject variation. The mean percentage decrease in absorption was 29 (SEM 13). This was calculated for each subject as ^{58}Fe absorption after the placebo minus ^{58}Fe absorption after 50 mg Fe, divided by ^{58}Fe absorption after the placebo. These results point to the importance of controlling dietary Fe intakes in human studies, as well as in experiments with rats.

Further work is required to measure the effect of altered intakes of dietary Fe (i.e. foods not Fe salts) on Fe absorption, and the importance of timing. We believe that short-term control of Fe absorption is mediated through the mucosal cells of the small intestine and that this mechanism is independent of whole-body Fe status (Fairweather-Tait *et al.* 1985). The influence of dietary Fe intake on subsequent Fe absorption is dependent on the stage of maturity of the affected mucosal cells. Mucosal cell turnover time will also play a role in the duration of the effect. The relative importance of a high-Fe meal shortly before a labelled-Fe test meal and a more long-term dietary change (over several days) needs to be fully investigated with regard to Fe therapy. One could argue that it might be more beneficial to devise an alternate-day dosing regimen whereby the mucosal setting is not constantly depressed because new cells not brought into contact with the high doses of Fe may have a higher absorptive capacity. Indeed Solomons *et al.* (1983) report that consecutive-day administration of therapeutic doses of Fe in preschool children progressively reduced the rise in plasma Fe, following each daily morning administration of $FeSO_4$, whereas alternate-day dosing did not follow this pattern. A minor point is that there could be a potentially beneficial bonus from alternate-day dosing, not directly related to the Fe therapy, in terms of reducing or preventing the chronic constipation often experienced in patients undergoing Fe therapy.

With regard to the methods used to measure Fe availability, the faecal-balance technique is not only time-consuming and unpleasant, but it is fraught with potential errors, including incomplete collections and homogenization problems. These drawbacks have long been recognized and where it is possible to use γ-isotopes, whole-body counting or erythrocyte isotope incorporation are the preferred techniques. As mentioned previously, the use of γ-isotopes in the UK for research purposes is very limited, and certainly not allowed in infants, children, pregnant and lactating women and women of child-bearing age. These groups are particularly important because of their high nutritional requirements for Fe and other nutrients. It is therefore essential to determine the adequacy of their diets in terms of Fe availability using stable isotopes as a safe alternative to radioisotopes.

Expt 2 was a preliminary study to determine the feasibility of measuring erythrocyte enrichment with ^{58}Fe, using the same methodology as ^{59}Fe erythrocyte incorporation. Most absorbed ^{59}Fe is normally incorporated into circulating erythrocytes within 7–10 d of oral administration, with an assumed incorporation rate of 80% (Bothwell *et al.* 1979), and percentage Fe absorption is usually calculated from ^{59}Fe present in whole blood at 14 d. There is excellent agreement between whole-body ^{59}Fe retention, the most accurate method of measuring Fe absorption, and erythrocyte ^{59}Fe incorporation after 2 weeks (Heinrich & Fischer, 1982). The experimental procedure did not allow us to take blood at 14 d but it was thought that the level at 10 d would give a reasonable indication as to whether or not the method was viable, i.e. whether levels of ^{58}Fe in the blood were detectable by NAA and whether they agree with ^{58}Fe absorption calculated from the measurement of ^{58}Fe faecal excretion.

Erythrocyte enrichment with ^{58}Fe was detectable, the range being from 5·96 to 15·00% above natural abundance. No blood volume measurements were made so it was not possible to calculate accurately the actual percentage of the ^{58}Fe dose incorporated into the blood. Blood volume can be estimated from height and weight, but a rather large scatter of values around mean prediction values has been found by all workers, regardless of the methods employed, as discussed by Wennesland *et al.* (1959). A further error lies in the amount of absorbed Fe incorporated into erythrocytes; this is generally taken to be 80% but could be higher in the subjects of low-Fe status (or adapted to a low-Fe diet) and may not have peaked within 10 d. It is clear from Fig. 2 that there is a significant relation between erythrocyte enrichment and ^{58}Fe absorption measured by faecal balance, but the agreement in this preliminary study is not perfect. We feel that the results are promising and, with further refinements in methodology, this technique may offer a valuable alternative to faecal monitoring.

These experiments are an excellent example of the invaluable aid of stable isotopes for furthering studies on Fe availability. The results clearly demonstrate the importance of stricter dietary controls in Fe absorption studies in humans, and point to a feasible alternative to faecal balance, namely ^{58}Fe erythrocyte incorporation, in the assessment of Fe availability.

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