

Influence of iron supplementation frequency on absorption efficiency and mucosal ferritin in anaemic rats

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(Received 28 October 1996 – Revised 27 January 1997 – Accepted 20 February 1997)

It is believed that frequent Fe doses decrease the efficiency of absorption as a consequence of the loading of intestinal mucosal cells with Fe from the previous supplemental dose. We examined this premise in thirty anaemic Sprague-Dawley rats given Fe supplements as FeSO₄ in 1 g preparations of a 50:50 (w/w mixture of low-Fe diet and sucrose under one of the following regimens: one 3 mg Fe dose daily for 3 d, four 0.75 mg doses daily at 6 h intervals for 3 d, and one 9 mg dose on day 1 followed by two placebo (low-Fe diet) doses on days 2 and 3. All groups were fed on two low-Fe meals daily (8.3 mg Fe/kg diet). After an overnight fast rats were dosed with 1 ml of an ⁵⁹Fe-labelled ferric nitrilotriacetic acid solution (37 kBq ⁵⁹Fe, 50 µg Fe) orally and killed 10 h later. Absorption of ⁵⁹Fe was measured as the percentage of the ⁵⁹Fe retained by the carcass without the gastrointestinal tract 10 h after dosing relative to the initial ⁵⁹Fe dose. Haemoglobin-Fe gain, liver non-haem-Fe, and mucosal duodenal ferritin were determined after the 3 d supplementation period. Absorption of the test dose in rats supplemented once 3 d before assessment of Fe absorption was 2.6-fold greater than those supplemented with daily single doses and 1.9-fold greater than those supplemented with daily multiple doses. Our data indicate that both mucosal ferritin and liver Fe levels account for the higher absorption efficiency found in rats supplemented once to simulate intermittent regimens.

Iron: Ferritin: Mucosa

Fe deficiency is one of the most common nutritional disorders worldwide (Yip, 1994). Approximately half the children and women and 25 % of the men in developing countries are Fe deficient. In developed countries, about 7–12 % of the children and women are Fe deficient (DeMaeyer & Adiels-Tegman, 1985; DeMaeyer, 1989). The United Nations University through its International Iron Nutrition Program is supporting research to test the efficacy of weekly Fe supplementation (Viteri, 1996). The rationale for weekly Fe supplementation trials are mainly based on findings from an animal study that showed a higher Fe absorption efficiency when intermittent Fe doses were given at intervals approximating to mucosal cell lifetimes (Viteri *et al.* 1995). The lower absorption efficiency of Fe provided daily compared with Fe provided intermittently is explained by the hypothesis that oral Fe supplements trigger a mucosal block of Fe absorption from Fe supplements taken subsequently. It is assumed that the blocking effect during daily Fe supplementation occurs as a consequence of the loading of the cells with Fe from the previous supplemental dose and that newly-produced mucosal cells of lower Fe status have a higher Fe absorption efficiency. However, the concentration of ferritin in intestinal

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mucosal cells is influenced by the amount of Fe transported into them (Smith *et al.* 1968), and in states of Fe deficiency, when little storage Fe exists in any of the cells of the body, there is not much ferritin or apoferritin in mucosal cells. The transport of Fe from the gastrointestinal tract through the mucosal cells into the body in states of Fe deficiency is fast and efficient.

The objective of the present study was to understand the basis for the idea of dose spacing that inspired intermittent supplementation. We wanted to understand what happens at the mucosal level during Fe therapy with oral treatments of various doses and frequencies. We studied the effect of three different oral supplementation regimens on the Fe status of the mucosal cells as determined by mucosal ferritin and on absorption efficiency as well as the relationship between mucosal ferritin and absorption. We measured absorption by whole-body counting 10 h after radio-Fe dosing.

MATERIALS AND METHODS

Animals and iron supplements

Animal care procedures and experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Thirty male weanling Sprague–Dawley rats with an average body weight of 36 g were purchased (Camm Research Lab Animals, Wayne, NJ, USA) and housed individually in stainless-steel cages with wire-mesh bottoms in a temperature-controlled room (20–22°) on 12 h dark–light cycle. Initially, the rats had free access to a low-Fe diet of 8.3 (SD 0.4, *n* 3) mg Fe/kg diet (Dyets Inc., Bethlehem, PA, USA) for 4 d. The rats were then trained to meal feed by offering a 1 g pre-meal consisting of sucrose and low-Fe diet (50:50, w/w) for 15 min (Viteri *et al.* 1995). Animals that completely consumed the pre-meal within the allotted time were immediately offered the low-Fe diet for 1 h. Those rats that did not finish the pre-meal in 15 min were denied food until the next scheduled feeding, i.e., 12 h later, so that they would be more motivated to consume the pre-meal. Within 2–3 d, all animals learned to finish their pre-meals within 15 min. Two meals were offered daily during this depletion period which lasted 10 d.

Blood samples were taken from the tail vein with a needle (25G 5/8") connected to a Micro-Hematocrit Tube coated with ammonium heparin (catalogue no. 1020; Clay Adams, Division of Becton Dickinson and Company, Parsippany, NJ, USA) and collected in a Microtainer Tube coated with EDTA (catalogue no. 02-669-38; Fisher Scientific, Pittsburgh, PA, USA).

The Fe supplements contained 0.75, 3, or 9 mg Fe as reagent-grade FeSO₄.H₂O (Fortitech Inc., Schenectady, NY, USA). The supplements were prepared by adding the required amount of FeSO₄ to a 50:50 (w/w) mixture of low-iron diet and sucrose.

Iron test dose

The Fe test dose was prepared fresh each day as needed. It consisted of ferric nitrilotriacetic acid (NTA) at 1:2 molar ratio, Fe:NTA in 1 ml isotonic saline (9 g NaCl/l) at pH 2 with 37 kBq ⁵⁹Fe and 50 µg elemental Fe. The solubility of Fe in this preparation was 98%. Solubility was determined by centrifuging at 2575 g for 5 min, measuring Fe in the supernatant fraction with bathophenanthroline disulfonic acid (catalogue no. B1375; Sigma Chemical Company, St. Louis, MO, USA) and hydroxyl amine (catalogue no. H2391, Sigma Chemical Company), and reading absorbances at 533 nm (Miller *et al.* 1994).

The ^{59}Fe was obtained as $^{59}\text{FeCl}_3$ (DuPont, Biotechnology Division, Wilmington, DE, USA) and the stock non-labelled Fe was an atomic absorption standard solution (catalogue no. I-9011; Sigma Chemical Company).

Experimental design

After they were fed on the low-Fe diet for 10 d the rats became anaemic (mean Hb 90.0 g/l) and weighed 93 (SD 6.5) g. The rats were then assigned to groups according to haemoglobin concentration and randomly allotted to one of three treatments as follows: one 3 mg Fe dose daily for 3 d (1 \times daily), four 0.75 mg doses daily for 3 d (4 \times daily), and one 9 mg dose on day 1 followed by two placebo (low-Fe diet) doses on days 2 and 3 (1 \times). During this 3 d supplementation period all groups received two meals daily (06.00 and 18.00 hours) of low-Fe diet available for 1 h. The Fe supplements were offered in 1 g preparations of a 50:50 (w/w) mixture of low-Fe diet and sucrose, either once daily before the morning meal at 06.00 hours (1 \times daily) for 3 d; four times daily at 06.00, 12.00, 18.00, and 24.00 (4 \times daily) for 3 d; or a single dose before the morning meal at 06.00 hours on day 1 followed by two placebo doses also at 06.00 hours on days 2 and 3 (1 \times). After the 3 d supplementation period, animals were deprived of food for 18 h then administered the ^{59}Fe dose.

Experimental procedure

The animals were given 1 ml of the labelled test dose orally and assayed for radioactivity in a whole-body gamma scintillation spectrometer immediately after dosing. No food was given to the animals after dosing but water was always available. After 10 h, the rats were again assayed for ^{59}Fe then killed by CO_2 asphyxiation. The entire gastrointestinal tract, from gastro-oesophageal junction to rectum, was removed and placed in a glass dish on ice. The carcass without the gastrointestinal tract was assayed for 60 s and the liver was removed. Absorption was defined as the percentage of the initial ^{59}Fe radioactivity retained by the carcass without the gastrointestinal tract 10 h after dosing.

The small intestine was flushed with 20 ml cold Hanks' balanced salt solution (HBSS; Hanks & Wallace, 1949) and the contents and rinsings were collected in a counting vial. The intestine was cut into three segments: duodenum (anterior 100 mm) jejunum (midsection less duodenum and ileum), and ileum (posterior 50 mm). Each duodenal segment was kept cold and moist with HBSS. After each segment was assayed for ^{59}Fe , it was slit open longitudinally and deposited in a 50 ml centrifuge tube for mucosal cell isolation.

Mucosal cell suspensions, liver, all three segments of the small intestine, stomach, and large intestine were assayed for ^{59}Fe in the gamma scintillation spectrometer to determine the distribution of ^{59}Fe . Weights of large and small intestines, liver, and stomach were recorded.

Mucosal cell isolation and ferritin determination

The isolation of mucosal cells from the duodenum was done by mechanical vibration (Momtazi & Herbert, 1973; Savin & Cook, 1978; Whittaker *et al.* 1989). The duodenums were transferred to 50 ml screw-cap centrifuge tubes prefilled with 10 ml Ca- and Mg-free HBSS (catalogue no. 14170; Life Technologies, Inc., GIBCO, Grand Island, NY, USA) buffered with 0.005 M-Tris hydrochloride, pH 7.4. This solution was kept on crushed ice

throughout the entire procedure. Tubes were mechanically vibrated with a vortex mixer for 2 min to dislodge mucosal cells. The cell suspensions were pelleted by centrifuging at 1000 g for 5 min. The supernatant fraction was discarded and the pelleted cells were resuspended in 10 ml fresh buffer. The cells were disrupted by sonication (Bransonic® ultrasonic cleaner; Branson Ultrasonics Corporation, Danbury, CT, USA) for two 60 s periods and the debris removed by a final centrifugation at 10 000 g for 10 min. The supernatant fraction was assayed for ^{59}Fe and stored in a freezer at -20° for later ferritin and protein analyses.

Ferritin was measured by an immunoassay procedure (Spectro Rat Ferritin, catalogue no. RF69; Ramco Laboratories, Houston, TX, USA). Protein concentration was measured using a protein assay kit (catalogue no. 500-0112; Bio-Rad Laboratories, Life Science Group, Richmond, CA, USA) with bovine serum albumin as the standard. All measurements were done in duplicate.

^{59}Fe analyses

Carcasses were assayed in a small-animal whole-body gamma scintillation spectrometer (Welch & House, 1980). Standards and blanks were prepared to simulate the geometry of the animal. Livers and other samples were analysed for ^{59}Fe in an automatic gamma counter (Packard Auto-Gamma model 5530; Packard Instruments, Downers Grove, IL, USA). All ^{59}Fe data were corrected for isotopic decay and differences in the counting efficiency of the two counters by expressing the data as percentages of the appropriate standards.

Iron status indices

Liver non-haem-Fe was measured by the method of Torrance & Bothwell (1968) and haemoglobin concentrations were determined by the cyanmethaemoglobin method (National Committee for Clinical Laboratory Standards, 1984).

Haemoglobin-Fe gain was calculated from values for haemoglobin concentration and estimated blood volume. It is assumed that 7% of the body weight (g) is equal to blood volume (ml) and that haemoglobin contains 3.4 mg Fe/g (Mudd & Stranks, 1985).

Statistical analyses

Data were analysed according to a one-way ANOVA using Minitab (1994) software. When the F test was significant ($P < 0.05$), differences among means were determined by Tukey's test (Snedecor & Cochran, 1989). When necessary, data were log transformed to obtain homogeneous variance. Values were considered significantly different at $P < 0.05$. Regression analyses were used to determine the relationship between absorption and two predictors, i.e. mucosal ferritin and liver Fe.

RESULTS

Summation of ^{59}Fe data from the various compartments (carcass, gastrointestinal tract, liver, and rinses) revealed almost complete recovery of the activity in the initial dose.

The entire dose had cleared the stomach by the time animals were killed. Only about 0.4% of the ^{59}Fe was found in the stomach of the daily-supplemented groups (1 \times daily, 4 \times daily) and about 2% in the 1 \times group but the difference among groups was not

significant. No significant differences in the weights of stomachs, small intestines, large intestines, and livers were observed.

Haemoglobin-Fe gain (Fig. 1), as determined by haemoglobin concentration and estimated blood volume at the beginning and end of the 3 d supplementation period, was significantly higher in the group receiving four supplements daily (4 × daily). We found no difference in haemoglobin-Fe gain between the group supplemented daily for 3 d (1 × daily) and those supplemented only once on the first day (1 ×).

Values for Fe absorption from the radio-Fe test dose, mucosal ferritin, and liver Fe of the three different treatment groups are presented in Table 1. Absorption of ^{59}Fe by rats in the 1 × group (69 %) was 2.6-fold greater than that by rats in the 1 × daily group (26 %) and nearly 2-fold greater than that by rats in the 4 × daily group (36 %). No significant difference in absorption was found between the two groups supplemented daily, although the 4 × daily absorbed 1.4 times more than the 1 × daily. Mucosal ferritin concentration of the 1 × daily group was 1.8- and 3.3-fold higher than that of the 4 × daily and 1 × groups respectively, and that for the 4 × daily group was 1.8 times higher than that for the 1 × group.

There was no difference in liver Fe (Table 1) between groups supplemented daily (1 × daily, 4 × daily). Liver Fe of the group receiving a single 9 mg Fe dose (1 ×) was significantly lower than that of those rats supplemented four times for 3 d (4 × daily).

Table 2 shows the percentage of absorbed ^{59}Fe found in the liver. Livers of rats in the single-dose group (1 ×) contained more ^{59}Fe than did livers of rats in the daily-supplemented groups (1 × daily, 4 × daily). Rats in the 1 × daily group had more ^{59}Fe in mucosal cells than did rats in the other groups. There was a negative correlation between ^{59}Fe in the mucosal cells and ^{59}Fe in the carcass without the gastrointestinal tract ($r = 0.520$, $P = 0.009$).

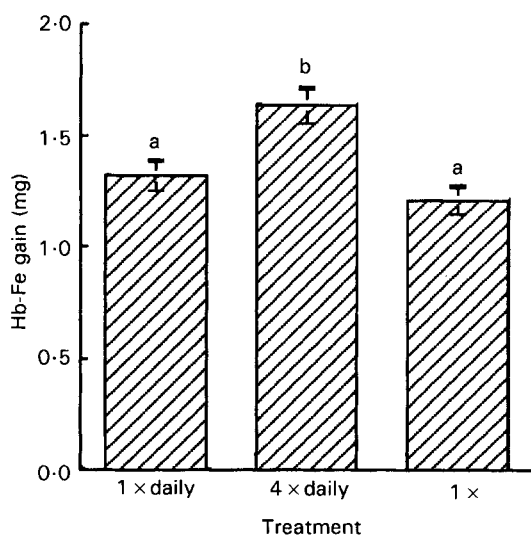


Fig. 1. Haemoglobin (Hb)-iron gain of rats receiving supplements under three different regimens: (1) 1 × daily, 3 mg daily for 3 d; (2) 4 × daily, 0.75 mg four times daily for 3 d; and (3) 1 × daily, 9 mg for 1 d and low-iron doses (8 mg iron/kg diet) for 2 d. Means with unlike superscript letters were significantly different ($P < 0.05$). Values are means with their standard errors represented by vertical bars for ten rats. For details of animals and procedures, see pp. 470–472.

Table 1. Iron absorption as a percentage of administered dose, mucosal ferritin, and liver non-haem-iron concentration in rats provided iron supplements at different frequencies*

(Values are means with their standard errors for eight to ten rats per group)

| Treatment group | Fe absorption† (%) | | Mucosal ferritin (ng/mg protein) | | Liver Fe (µg/g) | |
|-----------------|--------------------|------|----------------------------------|-------|----------------------|-------|
| | Mean | SE | Mean | SE | Mean | SE |
| 1 × daily | 26.22 ^a | 3.51 | 92.40 ^a | 13.60 | 114.22 ^{ab} | 6.41 |
| 4 × daily | 36.42 ^a | 6.64 | 50.33 ^b | 9.08 | 131.80 ^a | 13.50 |
| 1 × | 69.20 ^b | 5.02 | 27.91 ^b | 4.99 | 84.00 ^b | 12.20 |

1 × daily, 3 mg daily for 3 d; 4 × daily, 0.75 mg four times daily for 3 d; 1 ×, 9 mg for 1 d and low-Fe doses (8 mg Fe/kg diet) for 2 d.

^{a,b} Values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* For details of animals and procedures, see pp. 470–472.

† ⁵⁹Fe retained in carcass 10 h after dosing orally with 50 µg ⁵⁹Fe³⁺ nitrilotriacetic acid (NTA) with Fe:NTA molar ratio 1:2.

Mucosal ferritin was the best single predictor of absorption ($r = 0.671$, $P < 0.001$; Fig. 2). A lower but significant correlation between liver non-haem-Fe and absorption ($r = 0.592$, $P = 0.002$) was also observed. However, the model with the best predictive value of Fe absorption was the one that included both mucosal ferritin and liver non-haem-Fe ($r = 0.808$, $P < 0.001$).

DISCUSSION

Fe transfer into the body, after its uptake by the mucosal cells, occurs in two phases: a short phase of rapid transport occurring within 4–6 h and a longer phase of slower transport lasting up to 24 h (Wheby & Crosby, 1963). In our study we wanted enough time to allow the digesta to pass through the duodenum before killing the animals, while minimizing the time during which Fe may be lost by mucosal cell sloughing. We considered 10 h adequate for that purpose.

Results from our short 3 d supplementation period indicate that Fe provided in consecutive daily single doses (1 × daily) is absorbed less efficiently than an equal amount

Table 2. Distribution of ⁵⁹Fe test dose 10 h after dosing* in rats receiving iron supplements at different frequencies†

(Values are means with their standard errors for eight to ten rats per group)

| Treatment group | ⁵⁹ Fe in liver (% of absorbed Fe) | | ⁵⁹ Fe in mucosal cells (pmol/µg protein) | |
|-----------------|--|------|---|------|
| | Mean | SE | Mean | SE |
| 1 × daily | 12.91 ^a | 0.64 | 43.10 ^a | 7.48 |
| 4 × daily | 12.95 ^a | 0.69 | 21.28 ^b | 3.16 |
| 1 × | 16.55 ^b | 0.91 | 16.29 ^b | 1.87 |

1 × daily, 3 mg daily for 3 d; 4 × daily, 0.75 mg four times daily for 3 d; 1 ×, 9 mg for 1 d and low-Fe doses (8 mg Fe/kg diet) for 2 d.

^{a,b} Values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* Test dose administered orally containing 50 µg ⁵⁹Fe³⁺ nitrilotriacetic acid with Fe:NTA molar ratio 1:2.

† For details of animals and procedures, see pp. 470–472.

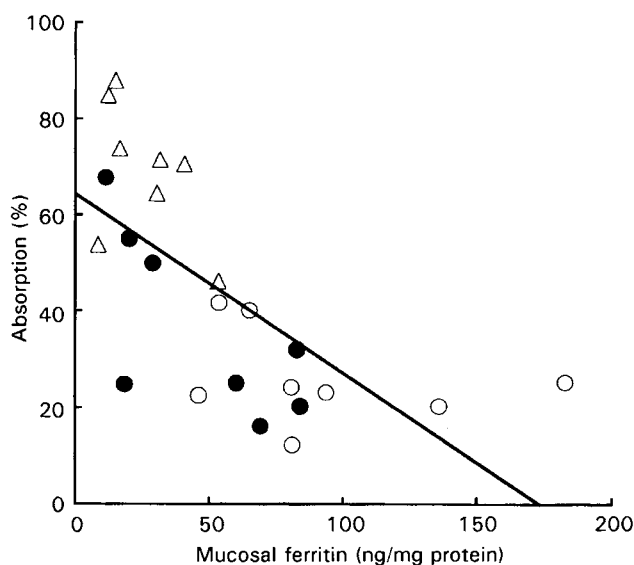


Fig. 2. Relationship between absorption of ^{59}Fe dose and mucosal ferritin in rats receiving iron supplements under three different regimens ($r = 0.671$, $P < 0.001$). (○) 3 mg daily for 3 d (1 \times daily); (●) 0.75 mg four times daily for 3 d (4 \times daily); (△), 9 mg for 1 d and low-iron doses (8 mg iron/kg diet) for 2 d (1 \times). For details of animals and procedures, see pp. 470–472.

of Fe provided daily in multiple smaller portions (4 \times daily) and as efficiently as an intermittent single dose (1 \times). Consecutive large daily doses of Fe (1 \times daily) resulted in more mucosal ferritin than when the same amount of Fe was given in multiple smaller doses (4 \times daily), indicating that mucosal uptake exceeded the capacity to transfer Fe into the plasma and that Fe not transported to the circulation was stored as ferritin in the cells. Haemoglobin regeneration efficiency (haemoglobin-Fe gain/Fe intake) was indeed significantly higher in the 4 \times daily group (18%) than the 1 \times daily group (15%) and the 1 \times group (14%).

The absorption of supplemental Fe is determined by factors such as quantity of dose, type of supplement, and time of ingestion. The greater effectiveness observed in 4 \times daily in improving Fe status reflects the common knowledge from single-dose studies that a greater proportion is absorbed with a smaller dose (Hahn *et al.* 1951). However, we also evaluated the efficiency of mucosal cells to absorb Fe after exposing them to Fe at different frequencies independent of dose quantity. In this respect, a significantly greater absorption efficiency was found in the 1 \times group, in which the test dose was administered 3 d after the single large supplemental dose, as compared with the daily groups. For the daily-supplemented groups, our data indicate a trend of just slightly greater efficiency when mucosal cells are exposed to small but more frequent doses (4 \times daily) *v.* a single large dose (1 \times daily), but the difference was not significant.

Our absorption data are in agreement with other animal studies reporting that intermittent supplementation is as effective as daily supplementation (Wright & Southon, 1990; Viteri *et al.* 1995). In our study we examined the hypothesis that newly-produced mucosal cells of lower Fe status are responsible for the higher absorption efficiency when Fe supplementation is timed to match mucosal cell turnover. Mucosal ferritin has been

shown to be closely related to the state of Fe repletion (Conrad *et al.* 1987). In Fe deficiency there is little Fe in the cells. However, our experiment shows that during the treatment of Fe deficiency the large oral Fe dose presented to the mucosal cells triggers the synthesis of apoferritin, which in turn traps incoming Fe not transported into the circulation. Small intestinal cell renewal in the rat takes place every 2–3 d (Holt *et al.* 1983). When the mucosal cell is sloughed off the Fe is excreted as ferritin.

The role of mucosal ferritin in Fe absorption has been the subject of debate ever since Hahn *et al.* (1943) proposed the mucosal block theory to describe a diminished avidity of the intestinal mucosa for Fe following an orally-administered blocking dose. However, no hypothesis to date has explained in a satisfactory manner the relationship between mucosal ferritin and Fe absorption.

Since it has been stated that oral treatment of Fe deficiency anaemia first normalizes the haemoglobin value then restores storage Fe (Bothwell & Finch, 1962), we designed the present study with a short 3 d supplementation period in order to test the question of whether the Fe status of the mucosal cells alone affects Fe absorption before a significant impact on body Fe stores is observed. We found, however, a significant accumulation of Fe in the liver with only 3 d of administration of supplemental Fe. In fact, a single large supplemental dose in 1 d ($1 \times$) was enough to replenish liver Fe levels. Concentrations of non-haem-Fe in unsupplemented rats in our earlier studies with anaemia levels similar to those in the present study ranged from 9 to 14 $\mu\text{g/g}$ (P. Benito, W. House and D. Miller, unpublished results). We also found that rats in the $1 \times$ group had significantly higher amounts of ^{59}Fe in the liver than did rats provided daily Fe supplements. This may be the result of lower liver non-haem-Fe levels in the $1 \times$ group, suggesting that Fe stores were still rebuilding in that group and that the $1 \times$ daily and $4 \times$ daily groups were more replete. It seems that in mildly-anaemic rats Fe stores start to accumulate early during treatment. Similar findings have been reported by Mahoney & Hendricks (1976) in anaemic rats. Their repletion diets at concentrations of 60–70 mg Fe/kg caused noticeable contribution to the liver before haemoglobin levels reached normal values. One human study has also observed early accumulation of storage Fe in anaemic subjects after only 4 weeks of treatment (Norrby, 1974), but there is little evidence of substantial overlap between these stages in human subjects. The lower liver Fe in the group receiving a single 9 mg dose ($1 \times$) as compared with $4 \times$ daily group reflects the fact that a lower percentage is absorbed from a greater dose (9 mg) and that a higher percentage would be absorbed from a smaller dose such as the 0.75 mg administered to $4 \times$ daily group (Hahn *et al.* 1951).

In summary, small multiple doses of Fe were more effective in increasing haemoglobin gain and liver Fe stores than a single large daily Fe supplement. It is likely that a greater proportion of Fe was absorbed from the smaller doses than the larger dose. However, mucosal cell Fe absorption efficiency did not differ between groups receiving either single or multiple daily Fe supplements. Increased Fe absorption efficiency was observed in rats provided the Fe supplement 3 d before dosing, the time chosen to simulate the mucosal cell turnover cycle. This latter result suggests that Fe consumed on an intermittent or alternate basis may offer absorptive advantages as compared with Fe consumed on a consecutive or daily basis.

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