Response of Various Indices of Iron Status to Acute Iron Depletion Produced in Menstruating Women by Low Iron Intake and Phlebotomy

David B. Milne, Sandra K. Gallagher, and Forrest H. Nielsen

We investigated response sensitivities of indices of iron status to controlled iron depletion and repletion in 11 premenopausal women. The women were depleted of storage iron (as reflected by serum ferritin) through a combination of a low-iron diet and phlebotomy. They then consumed a diet containing 13.7 mg of iron per 2000 kcal, supplemented with either ascorbic acid or placebo (for 5½ weeks) and a daily 50-mg iron supplement (for the subsequent 17 days). The relative sensitivities of different indices for detecting iron depletion were as follows: ferritin > % transferrin saturation > plasma iron > hemoglobin > hematocrit > zinc protoporphyrin (ZnPP) and erythrocyte protoporphyrin (EP). Ascorbic acid treatment during repletion, before iron supplementation, significantly (P < 0.05) affected changes in hemoglobin, ZnPP, ZnPP/heme, and EP/heme. Changes in heme synthesis evidently do not occur until iron stores are depleted and, conversely, during iron repletion hematopoiesis must be satisfied before iron stores, as reflected by ferritin, increase. Thus, the use of only one index of iron status is of limited value for detecting iron depletion.

Additional Keyphrases: ferritin • transferrin saturation • zinc protoporphyrin • erythrocyte protoporphyrin

Iron deficiency is the most commonly recognized nutritional deficiency in the United States and is the major cause of anemia. However, iron stores must be depleted before overt symptoms such as anemia are observed (1). Diagnosis of pre-anemic latent iron deficiency is important, both for identification of individuals at risk of developing anemia and because evidence from both humans and experimental animals indicates a relation between deficiencies of essential iron compounds in tissue and abnormalities in behavior, immune function, thermogenesis, and work performance (2).

Ferritin and iron in serum, transferrin saturation, erythrocyte protoporphyrin (EP), and zinc protoporphyrin (ZnPP) in whole blood are commonly used measures of iron stores and of subclinical iron deficiency. Values for these indices have been determined by comparative surveys of iron-deficient, normal, and blood-donor populations (2, 3). Serum ferritin concentration has been directly related to iron stores (4, 5), but primarily the quantitative relationship has been described for men undergoing repetitive phlebotomy (4, 6, 7).

Recently, the value of serum ferritin in diagnosis of iron deficiency has been questioned, because it is not always possible to clearly identify latent iron deficiency on the basis of data for serum ferritin alone (8–11). The usefulness of some of the other indicators of iron status in assessing various states of iron deficiency is also uncertain, because their responses to changes in status have not been rigorously tested under controlled conditions. Thus, we performed a study to determine the sensitivities of these indices of iron status to controlled iron depletion and repletion in menstruating young women, because they are most at risk for becoming iron-deficient. We report here the results of that study, in which all losses or gains in iron stores were strictly quantified.

Subjects and Methods

Eleven women, ages 22–33 years, participated in the study. They were selected on the basis of having low to moderate iron stores, as indicated by serum ferritin concentrations (26, SD 16 μg/L). The subjects entered the study after being informed about the nature of the research, and after medical, psychological, and nutritional evaluations had established that they had no underlying disease and that they were emotionally suited for this project. The protocol was approved by the institutional review board of the University of North Dakota and the United States Department of Agriculture, and followed the guidelines of the Department of Health and Human Services and the Helsinki Doctrine regarding the use of human subjects.

The subjects lived in a metabolic unit during the entire study. During the first phase of the study, they were fed a diet that provided 5.0 mg of iron (actually measured) and 63 mg of ascorbic acid (calculated) per 2000 kcal. Energy intakes were adjusted individually to maintain body weights within 2% of initial values by proportionally adjusting the amounts of all foods. Additional details about the diet may be found elsewhere (12).

To enhance the depletion of iron stores, an average of 85 mL of blood was taken weekly from each subject for the first 40 days of depletion. An average of 33 mL of blood per week then was taken for the remainder of the iron-depletion phase, which lasted 67 to 88 days, depending upon the response of the individual subject. Iron depletion was terminated when a subject's ferritin value declined to 5.0 ± 0.5 μg/L.

Iron was repleted by feeding, for 66 days, a diet (12) that supplied 13.7 mg of iron (measured) per 2000 kcal; the additional iron was primarily from vegetable sources. For the last 17 days of the study, an additional supplement of 50 mg of iron was fed daily in the form of ferrous sulfate "time-release" capsules.

Three subjects experienced gastric distress when ferrous sulfate supplements were fed, so the iron supplement for these women was given as ferrous gluconate during the last...
eight to 15 days of the iron-supplementation period. Their results did not differ markedly from the others.

During iron depletion, we administered 800 mg of supplemental calcium daily to half of the subjects, to test the hypothesis (13) that calcium interferes with iron absorption and utilization. During iron repletion, we supplemented the diets with either placebo or ascorbic acid, 500 mg three times daily (i.e., 1500 mg total), taken with major meals, to examine the effect of ascorbic acid on iron absorption (12).

We measured indices of iron status in blood collected after an overnight fast at weekly intervals throughout the study. We measured hemoglobin, hematocrit, erythrocyte count, and mean corpuscular volume with a Coulter Model S+4 (Coulter Electronics, Hialeah, FL 33030). Ferritin was measured in serum with a competitive binding radioimmunoassay kit (Clinical Assays, Dade-Baxter Travenol Diagnostics, Cambridge, MA 02139). Serum iron and iron-binding capacity were determined colorimetrically after precipitation of protein with trichloroacetic acid (14). Transferrin saturation was calculated from the ratio of serum iron to iron-binding capacity. Zinc protoporphyrin (ZnPP) was determined with a hematofluorometer (ESA, Bedford, MA 01730) that expresses measurements in micrograms per deciliter. EP was determined by a previously described method (15), after total extraction.

Excreta and diets were collected in a manner to avoid contamination with trace-metals. Six-day composites of diets and feces were prepared by homogenization in a 4-L stainless-steel blender. The feces were frozen, then lyophilized, before blending. Aliquots of the diet and feces were digested with concentrated nitric and 70% perchloric acids by method IIA of the Analytical Methods Committee (16). The iron concentrations in the digested materials were determined by inductively coupled plasma-emission spectroscopy with use of aqueous calibration standards. Precision and accuracy were evaluated by concurrent analysis of National Institute of Standards and Technology bovine liver standards (Standard Reference Material no. 1577), pooled samples, and replicated samples containing added iron. We found 189 (SD 16) μg of Fe per gram, which compared well with the certified value of 194 (SD 20) μg/g. Batch-to-batch coefficients of variation were 8.7% and 0.6% for pooled specimens of feces and of diet, respectively. Urinary iron was measured by atomic absorption spectrophotometry (17). Iron balance was calculated by the difference between dietary intake and fecal and urinary losses.

We quantified menstrual loss of iron as follows: Pads or tampons, and gauze wipes, which had been previously selected for low trace-metal contamination, were collected daily in plastic bags each menstrual period. Care was taken to avoid contamination with trace metals. The pads were extracted with dilute (0.12 mol/L HCl). Iron was determined in aliquots of the extract by plasma emission spectroscopy after digestion with nitric and perchloric acids. Analytical recoveries of added iron averaged 102%. Unused pads, tampons, and gauze were carried through the entire procedure to correct for background amount of iron. Iron removed by phlebotomy was calculated as follows: Fe loss (mg) = ([hemoglobin] (3.4) + plasma Fe (mg/dL) (1 - hematocrit)/100 x volume. We estimated total iron loss or gain for each dietary period by subtracting the menstrual and phlebotomy losses from the balance.

The significance of changes in iron indices was deter-

<table>
<thead>
<tr>
<th>Table 1. Menstrual Losses during Iron Depletion and Repletion</th>
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<tr>
<td>Fe loss, mg per menstrual period</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>First menstrual period of study</td>
</tr>
<tr>
<td>Last menstrual period of iron-depletion diet</td>
</tr>
<tr>
<td>Last menstrual period of iron-repletion diet</td>
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<td>n = 11 each.</td>
</tr>
</tbody>
</table>

Results
Menstrual losses of iron were between 1.9 and 28.7 mg per menstrual period (Table 1). Most of the variation was interindividual rather than intra-individual. Menstrual loss tended to decrease as iron stores were depleted. Both the means and ranges for menstrual iron losses tended to be lower towards the end of iron depletion than at the beginning or end of the study (P = 0.07).

Iron balance (dietary minus fecal and urinary iron) was generally positive throughout the study, and it became more positive during iron repletion (Table 2). However, because of iron loss via menses and phlebotomy, there were net losses of iron during both phases of iron depletion. There were net gains in iron during iron repletion and supplementation (Table 2). Each subject lost, on the average, an estimated 156 mg of iron during the first 40 days of depletion, and 68 mg was lost during the remainder of depletion. During iron repletion, there was a net gain of 153 mg of iron and an apparent gain of 373 mg during the iron-supplementation period. This latter amount probably does not accurately reflect changes in iron stores, because an estimated 150 mg of unabsorbed iron would still be in transit in the gastrointestinal tract. Calcium supplements did not affect iron balance or indicators of iron status; thus we ignored this treatment when evaluating the effects of iron depletion.

Most of the measured indices of iron status were affected to varying degrees by the iron depletion and repletion. Hemoglobin concentrations decreased from 134 to 120 g/L during depletion (Table 3), then slowly increased to 127 g/L during repletion and iron supplementation (Table 4). Hematocrit and erythrocyte counts followed a similar pattern, whereas mean cell volume and reticulocyte percentage (data not shown) did not change significantly throughout the study. Reportedly (21), reticulocyte percentage may remain normal during chronic blood loss, as seen in this study.
The ferritin concentration in serum declined rapidly to about 38% of the initial value during the first 40 days of depletion (Table 4). This was followed by a more gradual decline to about 20% of the initial value during the remainder of the iron-depletion period, when the amount of blood taken by phlebotomy was decreased. There was an insignificant increase in serum ferritin during repletion, and a further increase to 9.4 \( \mu g/L \) when dietary iron was supplemented. Serum iron and transferrin saturation followed similar patterns during iron depletion, although the changes were not as great percentage-wise as those for ferritin. However, plasma iron, and consequently transferrin saturation, continued to decline during repletion and did not increase significantly until iron supplements were given. There was no significant change in total iron-binding capacity during either iron depletion or repletion. EP and ZnPP seemed to be relatively insensitive to changes in iron status under the conditions of this study (Table 4). These indices continued to increase during repletion and tended to decrease slightly when additional iron was fed (Table 5).

Figure 1 illustrates the relative sensitivities of different iron status indices to iron depletion, at least under the conditions of this study. If ferritin were used as the sole criterion for iron depletion, iron depletion would be diagnosed with greater than 80% probability within 40 days. Conversely, iron depletion would never be detected if ZnPP were used as the sole indicator of iron status.

Ascorbic acid treatment (Table 5) during repletion, before iron supplementation, significantly improved changes in hemoglobin (\( P < 0.05 \)), ZnPP (\( P < 0.03 \)), ZnPP/heme (\( P < 0.02 \)), and EP/heme (\( P < 0.02 \)). Hemoglobin concentration increased, and ZnPP, ZnPP/heme, and EP/heme decreased when ascorbic acid was fed, whereas there was a decline in hemoglobin and increases in ZnPP, ZnPP/heme, and EP/heme in the placebo group. This effect disappeared...
when iron supplements were fed. All other measured indices were not significantly affected by ascorbic acid supplements when compared with placebo (P > 0.05).

Discussion

Our data indicate that, at least under the conditions of our study, the relative sensitivities of different indices in detecting iron depletion are, in decreasing order, ferritin > transferrin saturation > plasma iron > hemoglobin > hematocrit > ZnPP and EP (Figure 1). This pattern represents the expected course of iron depletion where iron stores, as reflected by serum ferritin, must be depleted before hematopoiesis, as reflected by hemoglobin and hematocrit, is significantly affected (1, 2).

The precipitous drop in serum ferritin concentration seen in this study during the early stages of iron depletion by phlebotomy and low dietary iron confirms the opinions of others that ferritin is a sensitive indicator of iron stores. The ratio of changes in iron stores in relation to changes in ferritin of 9.6 to 14.8 during iron depletion (Table 6) agrees with others (6, 7, 22), who found this ratio to be between 8 and 21 in men undergoing repetitive phlebotomy. During iron repletion, the slower than anticipated recovery of serum ferritin and apparent lack of a good relation between ferritin and increase in body iron (Table 5) is consistent with observations of Jacob et al. (6). They reported gradual recoveries of hemoglobin and transferrin saturation, but little recovery of serum ferritin during several months in three men who had blood repeatedly removed by phlebotomy. When iron is repleted, hematopoiesis apparently needs to be satisfied before iron stores and consequently ferritin increase. If corrections for estimated unabsorbed iron remaining in the gastrointestinal tract (about 150 mg) and the iron incorporated into newly formed hemoglobin (about 136 mg) are made, the gain in storage iron would be 86.6 mg and the estimated ratio for change in iron to change in ferritin would be approximately 26.

The large initial drop in the ferritin concentration in serum and the wide range in estimates of the relationship between iron stores and ferritin seem to preclude a linear relationship. A logarithmic relationship between ferritin and iron stores has been proposed (6, 23) because ferritin values follow a logarithmic distribution on a logarithmic scale. Bezwoda et al. (24) found a close correlation between serum ferritin and leg marrow non-heme iron concentration over a wide range of concentrations. However, upon close inspection of their data, there did not seem to be any relationship between these two indices at concentrations below 50 μg of ferritin per liter. Prieto et al. (25) found that serum ferritin was exponentially correlated with the amount of storage iron as measured by quantitative phlebotomy in patients with iron overload. As storage values approached normal, the rate of fall accelerated relative to storage iron. They suggested that circulating ferritin may be in equilibrium with two functionally distinct ferritin pools. More recently, it has been hypothesized that ferritin represents a rapidly mobilized iron pool and, as the ferritin pool becomes depleted, iron is mobilized from other pools, such as hemosiderin (26). Our data are consistent with both of these hypotheses.

Transferrin saturation, the second most responsive index of iron status seen in this study, is important in the diagnosis of iron depletion (2) and is primarily a gauge of iron supply to tissues (27). Studies have shown that when transferrin saturation is <15%, hemoglobin synthesis is impaired (28). The disadvantage of the transferrin test is the large biological variation that occurs in serum iron concentrations (2), including a pronounced diurnal variation in serum iron, usually with high values in the morning and low values at night.

The relative lack of response of EP and ZnPP to iron depletion and repletion in this study was somewhat surprising. Even when corrected for heme concentration, there were only small but significant increases that remained within the "normal" reference interval during iron depletion (Table 4). Both EP and ZnPP increase in response to impaired heme synthesis (29). In iron deficiency, porphyrins accumulate in erythrocytes because the lack of iron decreases the rate of heme synthesis (30). In lead poisoning, porphyrins accumulate because lead interferes with several enzymes involved in heme biosynthesis (28). Several investigators have found that the EP, ZnPP, or the ratios of EP or ZnPP to heme can be used to detect iron deficiency in infants or young children (31-34) and in young men (35).

Because iron stores must be depleted before heme synthesis is affected and because of the relatively slow turnover of erythrocytes, it may be expected that these indices would not change as rapidly as more-dynamic serum indices such as iron, percent transferrin saturation, or ferritin. Zanella et al. (36) recently showed that serum ferritin was a more sensitive predictor of iron deficiency than was EP in a study of 272 subjects with uncomplicated iron deficiency, both with or without anemia, who responded to iron therapy. The sensitivities for both tests varied as a function of the hemoglobin concentration. Both EP and EP/heme were poor predictors, 25% to 60%, of iron depletion at hemoglo-

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Table 6. Relationship between Iron Loss or Gain and Serum Ferritin Concentration

<table>
<thead>
<tr>
<th>Period</th>
<th>Change in Fe stores, mg</th>
<th>Ferritin Change in ferritin</th>
<th>Change in Fe/Change in ferritin</th>
</tr>
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<tbody>
<tr>
<td>Initial</td>
<td>—</td>
<td>26.3</td>
<td>—</td>
</tr>
<tr>
<td>&lt;40 d depletion</td>
<td>−156.3</td>
<td>10.1</td>
<td>−16.2</td>
</tr>
<tr>
<td>&gt;40 d depletion</td>
<td>−68.1</td>
<td>5.5</td>
<td>−4.6</td>
</tr>
<tr>
<td>Repletion</td>
<td>152.6</td>
<td>6.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Fe supplement</td>
<td>372.6</td>
<td>9.4</td>
<td>3.3</td>
</tr>
</tbody>
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bin concentrations >110 g/L. At hemoglobin concentrations >120 g/L, the predictive value for serum ferritin ranged from 40% to 80%.

Ascorbic acid supplements significantly affect apparent iron retention during iron repelition (12). However, of the measured indices of iron status, only hemoglobin and ZnPP seemed to be affected by ascorbic acid supplements before large iron supplements were given. The lack of an effect of ascorbic acid supplements on storage iron as reflected by ferritin, despite enhanced iron retention, is consistent with the observation of Cook et al. (37). They also found a lack of effect of large doses of ascorbic acid on ferritin in iron-deficient subjects and in two subjects who were initially iron depleted, even when there was an increase of apparent iron retention. That only hemoglobin and ZnPP are affected is further indication that heme synthesis needs to be satisfied before changes in indices reflecting storage iron are seen.

In conclusion, our findings indicate that one index of iron status is of limited value for detecting iron depletion. Each index is sensitive at different stages of iron depletion and several are affected by conditions other than iron status (2). During iron repelition, hematopoiesis must be satisfied before iron stores, as reflected by ferritin, are replenished. Thus, use of two or more abnormal indices would better predict iron depletion.

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