SUPPLEMENTARY APPENDIX

Iron absorption from supplements is greater with alternate day than with consecutive day dosing in iron-deficient anemic women

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Supplemental Material

Subjects

Inclusion criteria were: age 18-45 years; iron-deficient, defined as serum ferritin (SF) <20 µg/L; mildly anemic, defined as hemoglobin (Hb) >8 g/dL and <12.5 g/dL; without inflammation, defined as a C-reactive protein (CRP) <5 mg/L; body-mass index (BMI) 18.5 to 29.0 kg/m^2; body weight <85 kg; no chronic diseases or medications that could influence iron metabolism; not pregnant or lactating; non-smoking; no planned intake of mineral and vitamin supplements during the entire study. Women who met these inclusion criteria but had an Hb ≤13.5 g/dl before blood donation were also invited to participate in the study, as we assumed that women with a body weight of 60-65 kg and a blood volume of ≈65 ml/kg body weight would be anemic after the donation of ≈500 ml of blood. In these subjects, Hb was re-measured at least 24 h after blood donation, and if Hb was <12.5 g/dl after donation, we included them in the study.

Iron supplement and label administration

We provided one or two tablets (Eisensulfat Lomapharm) of 100 mg elemental Fe as anhydrous $FeSO_4$ (Lomapharm Gmbh, Emmerthal, Germany) administered with 100 ml of deionized high-purity water containing 4 mg of labeled Fe as $FeSO_4$ in form of (^{57}Fe)-$FeSO_4$, (^{58}Fe)-$FeSO_4$, or (^{54}Fe)-$FeSO_4$ (Chemgas, Boulogne-Billancourt, France), prepared as previously described (1). At administration, we rinsed the plastic cup with an additional 100 ml of water divided in 10 ml and 90 ml portions to ensure complete isotope administration.

Study design

All subjects went through two study cycles of 6 days each, with 16 days in between. To all subjects, we administered oral iron doses in the morning on two consecutive days (days 2 and 3) and a third dose 48 h later (day 5), each dose was labeled with ^{57}Fe, ^{58}Fe or ^{54}Fe. Subjects were randomly assigned to first receive either three doses of 100 mg or three doses of 200 mg. Group assignment was not masked. Then, after the 16-day incorporation period, they were crossed over to the other dose on days 23, 24 and 26 (days 2, 3 and 5) for the second supplementation period (Figure 1). Thus participants acted as their own controls throughout. They were given the iron dose after an overnight fast, followed by an additional 3 h fast and received a standardized lunch on days 1-5 of each supplementation period. The lunch consisted of an apple, a pear and a cheese sandwich made with white bread of low extraction wheat flour, thus containing negligible iron. On day 1, before iron supplementation, baseline venipuncture blood samples (≈6 ml each) were taken at 8:00 $_{AM}$ and at 4:00 $_{PM}$. Iron was administered at 8:00 $_{AM}$ on days 2, 3 and 5. Blood samples were taken at 8:00 $_{AM}$ (before dosing) and at 4:00 $_{PM}$ on days 2, 3 and 5. Additional blood samples were taken on day 4 and day 6 at 8:00 $_{AM}$. Using a questionnaire, subjects were asked whether they had gastrointestinal side effects during the clinic visits on days 2 to 5. The SHep increase was assessed after oral intake of 100 and 200 mg elemental Fe given as $FeSO_4$ on consecutive or alternate days. We assessed iron absorption by measuring the amount of isotopic tracers incorporated in red blood cells 16 days after administration of the third dose in both supplementation periods.

Iron status and oral iron absorption

Hb was measured on the day of collection with a Hematology analyzer (Sysmex XN350, Suisse AG). Blood was centrifuged at 3000 rpm for 10 minutes, serum was stored at -20°C. Serum was analyzed for SF, soluble transferrin receptor (sTfR), CRP, and alpha glycoprotein (AGP) by using a multiplex ELISA (2). CRP and AGP are complementary measures of systemic inflammation (2). IDA was defined as Hb < 12.5 g/dl and SF < 20 µg/L (3). Inflammation was defined as a CRP > 5 mg/L or AGP >1 g/L. Serum was analyzed for serum iron (SFe) and total iron binding capacity (TIBC) by using colorimetry, and transferrin saturation (%TSAT) was calculated using the formula (SFe/TIBC) x 100. SHep was measured using the DRG Hepcidin 25 (bioactive) HS enzyme immunoassay (DRG Instruments GmbH, Marburg, Germany) (4). Body iron stores (BIS) were calculated from the sTfR/PF ratio according to Cook et al. (5). BMI was calculated from the body weight [kg]/(height [m])^2 ratio. Blood samples were analyzed in duplicate for their iron isotopic composition by multi collector inductively coupled plasma mass spectrometry (ICP-MS) (Neptune, Thermo-Finnigan, Bremen, Germany) as previously described (6). FIA was calculated from isotopic ratios measured in blood samples as previously described (7). Total iron absorption (TIA) was then calculated by multiplying the dose of iron administered by the FIA (7).

Statistical analysis

We performed the statistical analyses using SPSS (IBM SPSS statistics, Version22). To assess the effect of consecutive vs alternate day dosing with 100 and 200 mg on FIA, SHep, iron status (PF, sTfR, SFe, TIBC, TSAT), inflammation (CRP, AGP) and erythropoietin (EPO) we fitted linear mixed models. We used the Shapiro-Wilk test to assess normality, and data that were not normally distributed after log transformation were reported as median (IQR); median and IQR are reported from the original data. All data were log-transformed before

inclusion into the statistical models. In the linear mixed model summary statistics, normally distributed data after log transformation were reported as geometric mean (-SD, +SD). Dose and time (reflecting the supplement administration day) were defined as fixed effects, participants as random effects (intercept) using a variance component structure matrix. We estimated parameters using the maximum likelihood method. Different levels within main effects (days and doses) were compared with paired comparisons with the estimated marginal means command using Bonferroni adjustment for multiple comparisons. For the models shown in Table 2 as well as in Figures 2 and 3 we included measures from the time points when iron was administered, as the parameters measured at that time point may affect iron absorption. For the models shown in Figure 4 we included the parameters measured at all the 10 blood sampling time points in each of the two study cycles. For normally distributed data, within-group and between-group differences between baseline parameters on days 1 and 22 were compared using dependent sample t-tests and independent sample t-tests, respectively; if these data were not normally distributed after log transformation, we used Wilcoxon matched-pair signed-rank tests and Mann-Whitney U test, respectively. Incidences of side effects were compared using Chi-square. P values <0.05 were considered statistically significant.

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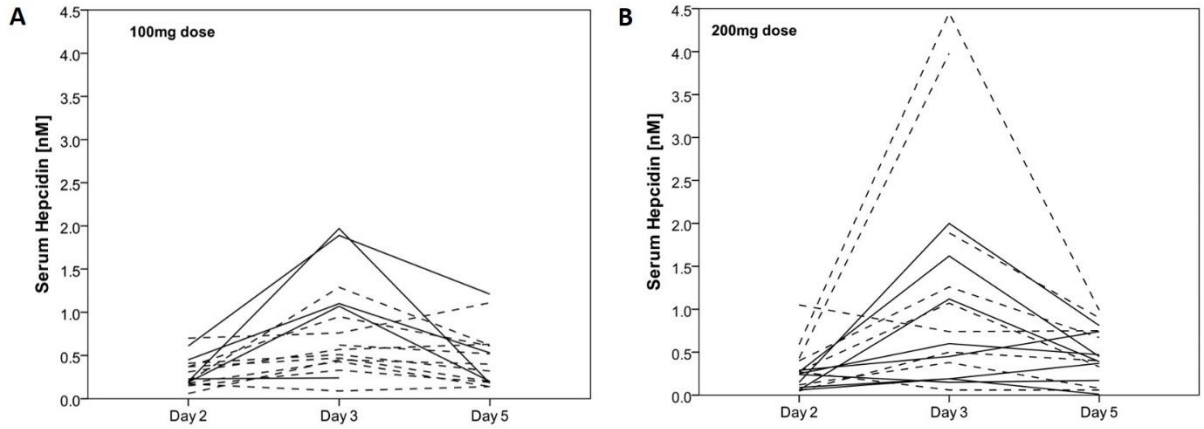
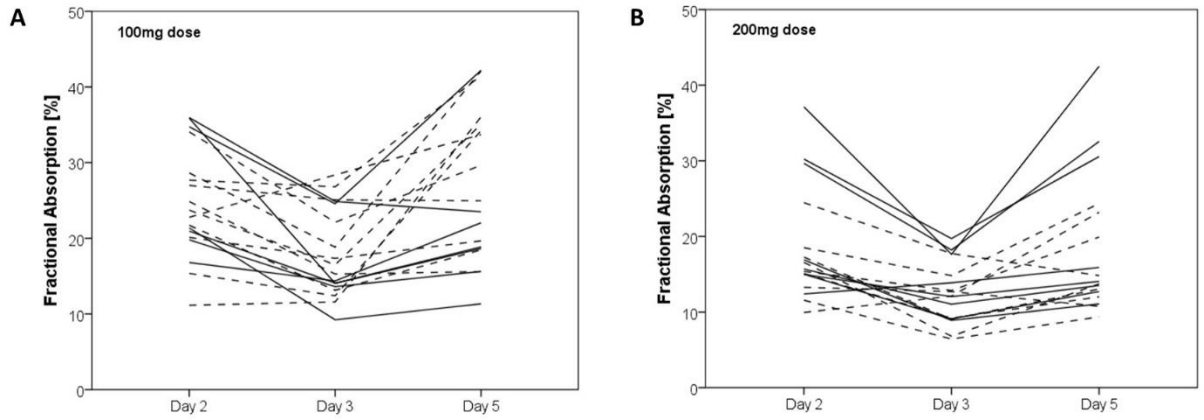


Supplemental Figure 1. Individual serum hepcidin profiles after the intake of oral iron supplements on consecutive (day 3) and on alternate days (day 5). A) For 100 mg iron dose. One subject with a SHep value of 7.85 nM on day 3 is not included in the figure; B) For 200 mg iron dose. Dashed lines: participants received 100 mg iron dosing first; continuous lines: participants received 200 mg iron dosing first.



Supplemental Figure 2. Individual fractional iron absorption from oral iron supplements taken on consecutive (day 3) and on alternate days (day 5). A) For 100 mg iron dose: B) For 200 mg iron dose. Dashed lines: participants received 100 mg iron dosing first; continuous lines: participants received 200 mg iron dosing first.