SUPPLEMENTARY APPENDIX

The opposing effects of acute inflammation and iron deficiency anemia on serum hepcidin and iron absorption in young women

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Supplemental Material

Subjects

Inclusion criteria for both groups were: age 18-49 years; without inflammation, defined as a CRP <5 mg/L; body-mass index 18·5 to 27·5 kg/m^2; bodyweight <70 kg; no chronic diseases or medications that affect iron metabolism; not pregnant or lactating; non-smoking; and no intake of vitamin and/or mineral supplements for at least two weeks before and during the entire study. Additional inclusion criteria were: for the IDA group: iron-deficient, defined as SF <15 µg/L and anemic, defined as Hb <12·0 g/dL; and for the nonanemic group: Hb >12·0 g/dL.

Test meal preparation

The standardized test meal consisted of two low-extraction wheat-flour-based rolls (30g), butter (10g) and honey (25g). The rolls were halved and an aqueous solution of 6mg labeled ^{57}Fe as NaFeEDTA (Chemgas, Boulogne-Billancourt, France), prepared as previously described,(1) was added to one half. The vial containing the iron solution was then rinsed twice with 0.5ml water, and the rinsing solution was added to a second half. Then, all four halves were covered with butter and honey using a plastic knife. Under direct supervision of the study investigators, subjects first consumed the two halves containing the iron solutions, then the remaining two halves.

Study design

All subjects underwent two stable iron isotope absorption studies, one before and one at 24h after the influenza/DTP vaccination. Subjects were instructed to not eat meat, fish or poultry on the two days preceding and on the day of the absorption studies. On study day 1, an afternoon baseline blood sample (≈6ml) was taken by forearm venipuncture at 4:00 $_{PM}$ ±1h. On study day 2, after an overnight fast, a baseline morning venous blood sample (≈6ml) was taken at 8:00 $_{AM}$ ±1h and we administered a test meal containing 6mg labeled ^{57}Fe as ethylenediaminetetraacetic acid ferric sodium salt (NaFeEDTA), added to a standardized wheat flour-based test meal, given with bottled water. Subjects were instructed to not eat or drink anything for three hours after the test meal. Venous blood

samples were taken in the afternoon on day 2 at 4:00 $_{PM}$ ±1h as well as the next morning (day 3) at 8:00 $_{AM}$ ±1h. After a 19-day isotope incorporation period, on study day 22, a venous blood sample (≈10ml) was taken at 4:00 $_{PM}$ ±1h to measure erythrocyte iron incorporation; this blood sample also served as the new baseline afternoon blood sample for the second absorption study. In the morning of day 23, a morning venous blood sample was taken at 8:00 $_{AM}$ ±1h. Then, all subjects received the trivalent Influenza Virus Vaccine Vaxigrip (Sanofi Pasteur, Lyon, France) and the Diphtheria-Tetanus-Pertussis (DTP) Virus Vaccine Dultavax (Sanofi Pasteur) given intramuscularly in the upper arm. Venous blood samples were taken at 8h, 24h and 36h after vaccination. At 24 h after vaccination, on study day 24 at 8:00 $_{AM}$ ±1h, an identical labeled test meal was administered to all subjects, as described above. The final blood sample (4ml) was taken on day 45 at 8:00 $_{AM}$ ±1h. We assessed iron absorption by measuring the amount of stable isotopic tracers incorporated in red blood cells 19 days after administration of the labeled test meals.

We decided to perform the second test meal 24h after vaccination based on findings from two pilot studies in which we administered the influenza/DTP vaccination to young women (n=12) and monitored serum IL-6 and SHep in venous blood samples taken every 8h for 72h after the vaccine administration, and measured peaks of serum IL-6 and SHep at 12h and 24h, respectively (data not shown).

Iron status and oral iron absorption

Hb was measured on the day of collection by using a Hematology analyzer (Sysmex XT 2000, Kobe, Japan). Blood was centrifuged at 3000 rpm for 10 minutes, and the serum was stored at -20°C until the day of analysis. Serum samples were analyzed for SF, sTfR, CRP and AGP by using a multiplex ELISA.(2) CRP and AGP are complementary measures of systemic inflammation.(2) IDA was defined as Hb < 12 g/dl and SF < 15 µg/L.(3) Inflammation was defined as a CRP > 5 mg/L or AGP >1 g/L. We measured SHep using the DRG Hepcidin 25 (bioactive) HS enzyme immunoassay (DRG Instruments GmbH, Marburg, Germany). EPO was measured using the Quantikine IVD Human Erythropoietin immune assay (R&D Systems, Minneapolis, MN 55413, USA). BIS were calculated from the sTfR/SF

ratio according to Cook et al.(4) BMI was calculated from the body weight [kg]/(height [m])^2 ratio. Blood samples were analyzed in duplicate for their iron isotopic composition as previously described. (5) Erythrocyte iron incorporation (which includes both dietary iron absorption and its utilization by erythrocytes) was calculated as previously described.(6, 7)

Statistical analysis

We performed the statistical analyses using SPSS (IBM SPSS statistics, Version22). Normally distributed data were reported as mean (SD), normally distributed data after log transformation were reported as geometric mean (-SD, +SD), non-normally distributed data after log transformation were reported as median (IQR). Serum iron, TIBC and TSAT values presented and used for analysis were mean values from a morning and afternoon blood sample from the corresponding day. We used linear mixed effect model analysis to assess the effect of the group (anemic vs non-anemic) and treatment (vaccination) on different variables. Group and treatment were defined as fixed effects, participants as random intercept effects using a variance component structure matrix. Erythrocyte iron incorporation, IL-6, SHep, SF, sTfR, EPO, CRP and AGP were the dependent variables for each separate model. Two separate linear regression analyses were done at baseline and at 24h after vaccination with SHep as the dependent variable and sTfR, Hb, EPO and IL-6 as the independent variables. The same regression analyses were also done with FIA and serum iron as dependent variables. For the regression on serum iron, in place of sTfR, SHep was included as an independent variable. For normally distributed data, Pearson's correlation was used, if data were not normally distributed after log transformation, Spearman's correlation was used. For within-group effects on IL-6, SHep, erythrocyte iron incorporation, and other biomarkers, dependent sample t-tests were used. If data were not normally distributed after log transformation, a related samples nonparametric test was used. For between-group effects, independent sample t-tests were used and if data were not normally distributed after log transformation, a non-related samples nonparametric test was used. P values <0·05 were considered as significant.

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