Diet-induced iron deficiency anemia and pregnancy outcome in rhesus monkeys[1–3]

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ABSTRACT

Background: Iron deficiency anemia (IDA) is relatively common in the third trimester of pregnancy, but causal associations with low birth weight and compromised neonatal iron status are difficult to establish in human populations.

Objective: The objective was to determine the effects of diet-induced IDA on intrauterine growth and neonatal iron status in an appropriate animal model for third-trimester IDA in women.

Design: Hematologic and iron-status measures, pregnancy outcomes, and fetal and neonatal evaluations were compared between pregnant rhesus monkeys ($n = 14$) fed a diet containing 10 μg Fe/g diet from the time of pregnancy detection (gestation days 28–30) and controls ($n = 24$) fed 100 μg Fe/g diet.

Results: By the third trimester, 79% of the iron-deprived dams and 29% of the control monkeys had a hemoglobin concentration <11 g/dL. There were also significant group differences in hematocrit, mean corpuscular volume, transferrin saturation, serum ferritin, and serum iron. At birth, the newborns of monkeys iron-deprived during pregnancy had significantly lower hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin values and a lower ratio of erythroid to total colony-forming units in bone marrow than did the control newborns. Pregnancy weight gain did not differ significantly between the iron-deprived and control dams, and the fetuses and newborns of the iron-deprived dams were not growth retarded relative to the controls. Gestation length, the number of stillbirths, and neonatal neurobehavioral test scores did not differ significantly by diet group.

Conclusion: These data indicate that an inadequate intake of iron from the diet during pregnancy in rhesus monkeys can lead to compromised hematologic status of the neonate without indications of growth retardation or impaired neurologic function at birth. *Am J Clin Nutr* 2006;83:647–56.

KEY WORDS Iron deficiency, pregnancy, rhesus monkeys, intrauterine growth retardation, anemia

INTRODUCTION

Iron deficiency (ID) and iron deficiency anemia (IDA), as identified from hematologic measures, are relatively common in the third trimester of pregnancy. The Centers for Disease Control and Prevention (CDC) Pregnancy Surveillance System data for 2002 cited a 29.6% nationwide prevalence of third-trimester anemia (hemoglobin < 11 g/dL) (1). Adverse pregnancy outcomes investigated in connection with ID and IDA during pregnancy include intrauterine growth retardation, premature delivery, and infant hematologic compromise (2–4). However, causal relations between ID and IDA, determined from hematologic measures, and adverse pregnancy outcomes have been difficult to establish. Complicating factors are physiologic changes associated with advancing pregnancy, confounding with other nutritional deficiencies, economic and social stressors, and infectious and parasitic diseases. The link between dietary iron intake and IDA in pregnancy is also poorly understood. Resolving these issues is important to effective public health use of iron supplements in pregnancy.

Animal models are capable of providing important information on the causal link between restricted dietary iron intake, induction of gestational IDA, and adverse pregnancy outcome. Gestational IDA has been studied in laboratory rodents (rats and mice). However, rodents do not provide an adequate model for the effects of third-trimester IDA on the fetus. Rodents are precocial, and the pups are born shortly after completion of organogenesis, whereas an extended period of postembryonic intrauterine development occurs in human and nonhuman primates. For example, the rhesus monkey completes embryogenesis in the first trimester (55 d), which is followed by a prolonged period of fetal development in the second and third trimesters (56–165 d gestation). Rodents, the most common laboratory animal model for human developmental experiments, complete embryogenesis at 15–16 d of gestation and are typically born at 18–21 d of gestation. Also, rhesus monkeys have single-offspring pregnancies and are known to display hematologic changes similar to those of humans in late pregnancy. Although the biology of pregnancy is similar in monkeys and humans, the environmental standardization possible in nonhuman primate studies, and the related lack of confounding factors, markedly increases the sensitivity of small-sample experiments.

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In this study, 2 groups of pregnant rhesus monkeys were fed purified diets that differed only in iron content. The iron-deprived diet contained 10 μg Fe/g. The daily intake, based on an average body weight of 7.7 kg during pregnancy, was 0.35 mg Fe/kg body weight. The comparable value in women, based on a weight of 61 kg (5) and scaling to metabolic body weight, would approximate the 25th percentile of iron intake from food in pregnant US women (data from the third National Health and Nutrition Examination Survey) (5).

Hematologic variables were assessed before diet initiation in the first trimester, during the second and third trimesters, and at birth. In a subsample of pregnant animals, fetal hematology was also assessed through ultrasound-guided blood sampling. Dam and fetal hematologic status, dam food intake and weight gain, intrauterine growth, pregnancy outcome, and neonatal status were evaluated.

MATERIALS AND METHODS

Study design

All animal procedures conformed to the requirements of the Animal Welfare Act (6), and the protocols were approved before implementation by the Institutional Animal Care and Use Committee at the University of California, Davis. Normally cycling adult female rhesus monkeys (*Macaca mulatta*), housed indoors at the California National Primate Research Center (CNPRC), were bred and identified as pregnant by ultrasound (7). Monkeys were selected for the project on the basis of parity (multiparous) and a history of uncomplicated prior pregnancy outcomes. Pregnancy in the rhesus monkey is divided into trimesters by 55-d increments, with 0–55 d of gestation representing the first trimester, 56–110 d of gestation representing the second trimester, and 111–165 d of gestation representing the third trimester (term: 165 ± 10 d). At the time pregnancy was identified (28–30 d of gestation), dams were moved to the same cage room, where they were pair-housed with another pregnant monkey for the duration of pregnancy. At this time they were assigned to an experimental group and fed experimental diets until delivery. Cage mates were in the same group. The experiment was conducted in 2 cohorts; the pregnancies of cohort 2 were initiated 2 y after the pregnancies of cohort 1.

The sample consisted of 38 dams assigned to the dietary protocol whose pregnancies resulted in live births (**Table 1**). The dams were assigned so as to balance age, weight, and parity across groups at conception. The control group ($n = 24$) was larger than the prenatal iron group ($n = 14$) because the design called for offspring of the control group to be subsequently divided into subgroups at birth (no postnatal iron deprivation, $n = 12$; postnatal iron deprivation, $n = 12$). All the dams were born and raised at the CNPRC, except for one dam in the control group and one dam in the prenatal deprivation group who were born in Puerto Rico. Four dams in each group were born by cesarean delivery because of postdates or indications of potential dystocia. Additional pregnant monkeys were initially assigned to the protocol but did not complete a pregnancy with a live birth (**Table 2**). Because of concerns about the rate of stillbirth in cohort 1, fetal sampling was discontinued in cohort 2, and the diet was modified to exclude bitartrate (used as a choline salt) in response to reports of toxic properties of some batches of this agent in rats (8).

TABLE 1
Characteristics of the pregnant iron-deprived and control monkeys in the study population[1]

	Iron deprived ($n = 14$)	Control ($n = 24$)
Fetal sex ratio (M:F)	6:8	12:12
Age at conception (y)	7.6 ± 0.5[2]	8.3 ± 0.5
Parity	3.4 ± 0.5	3.8 ± 0.4
Weight at pregnancy detection (kg)	6.9 ± 0.4	6.9 ± 0.2
Method of delivery (*n*)		
Vaginal	10	20
Cesarean	4	4
Gestation length (d)	168 ± 1	169 ± 1
Weight gain on day 150 (kg)	1.94 ± 0.24[3]	2.35 ± 0.25
Cohort		
1	7	11
2	7	13

[1] There were no statistically significant group differences by ANOVA or chi-square test.
[2] $\bar{x}$ ± SEM (all such values).
[3] $n = 13$.

Twenty-four sires contributed to the sample; 9 sires contributed 2 offspring each to the sample, and 2 sires contributed 3 offspring each to the sample. For 5 of the sires with multiple offspring in the sample, the offspring were split between the 2 diet groups. For the other 6 sires, 2 offspring were in the same group.

Animal care

Pregnant dams were pair-housed in double cages (120 × 65 × 79 cm) in a cage room separate from the rest of the colony. They were fed experimental diet twice a day in premeasured amounts, and water was available ad libitum from an automated system. Their care included daily cleaning of drop pans, twice daily feeding, biweekly cage changes to freshly sterilized cages, automatically controlled light cycles (lights on from 0600 to 1800), and temperature control (68–78 °F) and monitoring. Monkeys were observed each morning for health signs and referred to veterinarians for treatment if needed. An individual medical record was maintained for each animal. Ketamine hydrochloride (10 mg/kg intramuscularly) was used as the anesthesia in all procedures.

Diet and feeding

The National Research Council recommends a dietary iron content of 100 mg Fe/kg for macaque monkeys (9). Before initiation of the experimental diet, monkeys were fed Lab Diet

TABLE 2
Pregnancy outcome of all pregnant iron-deprived and control monkeys entered into the experiment

	Iron deprived		Control	
	Cohort 1 ($n = 10$)	Cohort 2 ($n = 9$)	Cohort 1 ($n = 18$)	Cohort 2 ($n = 19$)
No. of early pregnancy losses at < 90 d	0	1	1	2
No. of nonlive term births	3	1	6	2
No. of live births	7	7	11	13

TABLE 3
Content of the purified diets used in the experiment[1]

Component	Amount
	g/kg
Sprayed egg whites	250
Sucrose	513
Microcellulose (100 μm)	70
Guar gum	30
Soybean oil	60
Tetrabutyl hydroquinone	0.012
Salt mix[2]	40
Vitamin mix[3]	20
$FeSO_4$ premix (5 mg Fe/g)	2 or 20
Molybdenum-sucrose premix (0.1 mg Mo/g)	0.4
Vitamin E acetate–sucrose premix (10 IU/g)	1.8
Zinc-sucrose premix (5 mg Zn/g)	7.2
Banana flavoring	2.0
Choline bitartrate[4]	3.6

[1] The diets were prepared in pelleted form by Dyets, Inc, Bethlehem PA.
[2] Components (g/kg): calcium carbonate, 279; monobasic calcium phosphate, 157.8; magnesium sulfate, 60; magnesium oxide, 34.62; sodium chloride, 57.52; potassium phosphate, dibasic, 324.3; potassium iodide, 0.06; manganese sulfate · 1 H_2O, 3.08; sodium selenite, 0.004; sodium fluoride, 0.023; chromium acetate, 0.044; cupric carbonate, 0.435; sucrose, 83.393.
[3] Components (g/kg): taurine, 25; thiamine HCl, 1.5; riboflavin, 0.25; pyridoxine HCl, 0.5; niacinamide, 12.5; calcium pantothenate, 1.25; folic acid, 0.25; biotin, 0.20; vitamin B-12 (0.1%), 3.0; vitamin A palmitate (500 000 U/g), 1.5; vitamin D_3 (400 000 U/g), 0.25; menadione sodium bisulfite, 4.01; inositol, 0.25; *para*-aminobenzoic acid, 0.5; sucrose 944.04.
[4] Replaced by choline chloride on 21 August 2002.

#5047 (PMI Nutrition International, St Louis, Mo)—a high-protein grain and soy-based monkey feed that contained 420 mg Fe/kg diet. Beginning at the time of pregnancy confirmation (28–30 d of gestations) and continuing until pregnancy termination, the dams were fed a semipurified diet (Dyets, Bethlehem, PA) that conformed to the recommendations of the National Research Council (9). The nutrient composition of the diet is shown in **Table 3**. The energy content of the diet was 4 kcal/g. The iron-sufficient (control) diet contained 100 mg Fe/kg iron as iron sulfate, whereas the iron-deprived diet contained 10 mg Fe/kg. The value for the deprived diet was adopted from limited information in the literature (10), whereas the control diet contained the recent National Research Council recommendation for macaque monkeys (9). Vitamin C was provided separately as a supplement (125 mg/d, 5 d/wk) to avoid promotion of iron uptake by the presence of ascorbate in the diet. Adaptation to the purified diet was achieved by initially mixing it with the commercial diet used in the colony. Monkeys not consuming a full ration of purified diet were supplemented with low-iron foods (fruit and vegetables) until a steady daily intake of purified diet was achieved. The amount of purified diet was increased if all of the diet was consumed for 2 consecutive days. The diets were color-coded but were not labeled with the iron content. Feeder boxes were also color-coded to help ensure that monkeys were fed the appropriate diet.

In accord with CNPRC environmental enrichment policies, the diet was supplemented with foods prescreened for low iron content (puffed rice and wheat cereal, popcorn, and low-iron produce).

Dam evaluations

Maternal weights were obtained before group assignment on 28–30 d of gestation, on 90 and 150 d of gestation, and on the day after birth. For weights, dams were transferred to a scale in a preweighed transfer box. After delivery, the dam's crown-rump length, arm circumference, and skinfold thicknesses were measured as described previously (11) when the dams were anesthetized to remove the newborns. A daily food intake measure was obtained every 2 wk by subtracting the amount of food left uneaten from the amount of food provided. The dams were separated from their cage mate for the 24-h intake monitoring period.

Blood samples were obtained from the anesthetized dam at the time of ultrasound examinations at 30, 90, and 150 d of age and portioned for the measurement of complete blood count (CBC), zinc protoporphyrin (ZPP), ferritin, transferrin receptor (TfR), iron, total-iron-binding capacity (TIBC), and transferrin saturation. A blood sample for a clinical chemistry panel was collected at the time of the ultrasound examination at 150 d. Health records (health problems reported by technicians, veterinarian diagnosis, and treatment) were summarized at birth.

Fetal ultrasound and blood sampling

All dams were immobilized with ketamine hydrochloride for ultrasound evaluations during pregnancy. Sonographic measurements of the fetal head (biparietal and occipitofrontal diameters, area, and circumference), abdomen (area and circumference), and limbs (humerus and femur lengths) and gross anatomic evaluations (axial and appendicular skeleton, viscera, membranes, placenta, amniotic fluid) were made at 90 and 150 d of gestation, as previously described, and all measures were compared with normative growth curves for rhesus fetuses (12). Fetal heart rates and position were also documented. Fetal blood samples (≈1–2 mL) were collected under ultrasound guidance by cardiocentesis with the use of standard techniques (13) for CBCs and hematopoietic progenitor assays at 90 and 150 d of gestation. Maternal blood samples (≈4 mL) were collected immediately before fetal sample collection from a peripheral vessel for CBCs, plasma, and serum.

CBCs were performed on fetal blood samples (gestation day 90 and gestation day 150 for cohort 1 only), infant blood samples (postnatal days 1–4), and maternal blood samples (gestation days 30, 90, and 150 and at birth). The samples were placed directly into microtainer tubes with EDTA. Bone marrow smears were also evaluated for the determination of the relative percentages of immature and mature cellular components (myeloid: erythroid ratio) by direct examination. All smears were stained with Wright-Giemsa for morphologic evaluations.

Delivery

Labor was initiated in the rhesus monkeys shortly after the onset of the dark cycle, and the infants were usually born overnight. Animal care staff monitored the cages at 0700 each morning for births. Newly delivered dams were anesthetized, and the infants were removed, weighed, measured, and transferred to the primate nursery for developmental assessments. The dams were released from the project at this time.

Beginning at 165 d of gestation, pregnant monkeys were screened at 5-d intervals for fetal viability, fetal position, and progress of cervical dilation. Pregnancies with indications of fetal compromise were referred to veterinarians for cesarean

delivery. At 175 d of gestation, the monkeys were considered postdates, and the infants were born by cesarean delivery unless the delivery appeared imminent from ultrasound examination.

Newborn evaluations

A neurobehavioral test battery (NBT) patterned on the Brazelton Neonatal Assessment Scale was conducted on the day of birth. Exam items targeted muscle tone, state control, and presence and vigor of age appropriate reflexes (11, 14, 15). The animals delivered vaginally were tested within 2 h of separation from the dam. For the animals born by cesarean delivery, the neurobehavioral assessment was delayed to 4 h after birth. Vaginally delivered infants were not fed before the exam; animals born by cesarean delivery were fed when placed in the nursery with a sucrose solution followed by formula but were deprived of food during the 2 h before the NBT. Subsequently, the infants were held and fed at 2-h intervals with commercial human infant formula (Similac Advance, with or without iron; Ross Product Division, Abbott Laboratories, Columbus, OH). A morphometric exam followed the NBT and included the measurement of crown-rump length, foot length, femur length, head width, length and circumference, and skinfold thicknesses (triceps, thigh, scapula, and iliac region) (14).

On days 1, 2, and 3 after birth, depending on scheduling restrictions, the infants were anesthetized with ketamine and underwent bone marrow sampling and blood sampling for CBC and iron status. (Cohort 1 infants also had a liver biopsy sample taken, but the samples proved too small for analysis.) The infants were then returned to the nursery for follow-up evaluations, which will be reported separately.

For bone marrow sampling, infants were sedated with ketamine hydrochloride (10 mg/kg) after an approximate 4-h fast, and aseptically prepared for a bone marrow aspirate (≈1 mL). Briefly, the area over the right iliac crest was shaved and scrubbed, and 0.05 mL lidocaine was injected. With the use of a sterile technique, a sterile needle (20 g × 1.5 mL) previously flushed with sterile heparin was placed into the iliac crest, a 12-mL sterile syringe was attached, and ≈10 mL negative pressure was applied.

Complete blood counts and clinical chemistry panel

CBCs, including white blood cell differentials and red blood cell distribution width (RDW), were conducted by the Clinical Laboratory of the CNPRC. The clinical chemistry 20 panel was performed by using an automated method at the Veterinary Diagnostic Laboratory, School of Veterinary Medicine.

Expanded iron-status panel

Serum TfR, iron, and TIBC assays

A commercial enzyme-linked immunosorbent assay kit (Ramco, Houston, TX), with appropriate external controls, was used to analyze serum TfR. Internal quality controls were analyzed along with each batch of samples. The within-assay CV was <3% and the between assay CV was <5%. Serum iron and transferrin (TIBC) were measured by using standard colorimetric methods with ferrozine as the dye (16). Transferrin saturation was then calculated as plasma iron/TIBC ×100.

Ferritin assay

A ferritin assay was performed with the use of an automated method (ACS:180; Bayer Healthcare, Tarrytown, NY). The assay used is a 2-site direct sandwich immunoassay that uses chemiluminescence technology. The first antibody is a polyclonal goat anti-ferritin labeled with acridinium ester. The second antibody is a mouse monoclonal anti-ferritin labeled with paramagnetic particles. Reagents are then added to elicit the chemiluminescent reaction. The amount of ferritin present is a direct relation to the amount of relative light units detected by the system. The linearity of the instrument is 0.5-1650 ng/mL. To determine the ability of the assay, which was developed for human ferritin, to detect rhesus monkey ferritin, serial dilutions of purified monkey ferritin were prepared. Spike recovery averaged 10% in the biological range, with a correlation of >0.99 in serially diluted samples. Values below the level of detection were assigned the value of the level of detection (0.5 ng/mL) for purposes of statistical analysis.

Hematopoietic progenitor assays

Progenitor assays (blood, marrow) were established, as previously described (17), by using 0.5×10^5 nucleated blood cells/plate and 0.2×10^5 nucleated bone marrow cells/plate in 1 mL Methocult GF^+ H4435 (StemCell Technologies Inc, Vancouver, Canada) and plated in triplicate. Evaluation after a standard 10-d incubation period included total colony counts and quantitation of individual erythroid and myeloid progenitors [burst-forming unit–erythroid (BFU-E), colony-forming unit–erythroid (CFU-E), CFU–granulocyte macrophage (CFU-GM), and CFU-mix].

ZPP assay

Aliquots of peripheral blood (0.1–0.2 mL) were placed in EDTA-coated tubes and refrigerated for ≤1 wk. Twenty microliters were placed on a glass slide for reading in a ZP Hematofluorometer (model 206D; Aviv Biomedical, Inc, Lakewood, NJ). Two values (μmol/mol heme) were obtained for each sample aliquot. Some assays included previously frozen control samples with a low, medium, or high ZPP content for quality control.

Statistical analysis

An analysis of variance (ANOVA) with the use of linear models and diet group as the major independent variable was the most common approach used for statistical analysis. Cohort, maternal age, maternal parity, sex of fetus, type of delivery (vaginal or cesarean), and gestation length were considered as covariates in all analyses. If associations with a given endpoint were found, the covariate was included in the ANOVA model for diet-group effects. Because relevant covariates varied by time point, each gestational time point was evaluated with a separate ANOVA. For dam characteristics and pregnancy outcome endpoints, Student *t* test and Fisher's exact test were used for continuous and dichotomous variables.

To determine whether the relation between hemoglobin and other hematologic variables was different within the anemic subgroups of the 2 diet groups, we selected a permutation test (18). The permutation test is a powerful statistical method that can directly calculate *P* values for any statistic without the need to invoke arguments concerning the asymptotic behavior of the tails of statistical distributions. For all our tests, 1000 permutations of the sample data were constructed, the appropriate test statistic

TABLE 4
Anemia and iron-status variables in the pregnant iron-deprived and control monkeys[1]

	30 d Gestation		90 d Gestation		150 d Gestation	
	Iron deprived ($n = 14$)	Control ($n = 24$)	Iron deprived ($n = 14$)	Control ($n = 24$)	Iron deprived ($n = 14$)	Control ($n = 24$)
RBC ($\times 10^6/\mu L$)	5.18 ± 0.10	5.14 ± 0.10	5.18 ± 0.08	5.29 ± 0.11	4.59 ± 0.10	4.87 ± 0.08
Hemoglobin (g/dL)	12.3 ± 0.3	12.2 ± 0.2	12.2 ± 0.3	13.0 ± 0.3	10.0 ± 0.3[2]	11.8 ± 0.3
Hematocrit (%)	36.5 ± 0.7	36.7 ± 0.7	36.0 ± 0.6	37.9 ± 0.8	29.9 ± 0.7[2]	34.7 ± 0.7
MCV (fL)	71 ± 1	71 ± 1	70 ± 1[3]	72 ± 1	65 ± 2[4]	71 ± 1
MCH (pg)	23.8 ± 0.2	23.8 ± 0.2	23.6 ± 0.3[3]	24.3 ± 0.3	21.9 ± 0.6[2]	24.2 ± 0.3
MCHC (pg/fL)	33.7 ± 0.2	33.3 ± 0.2	33.9 ± 0.3	33.8 ± 0.2	33.5 ± 0.4	34.0 ± 0.4
RDW (%)[5]	12.8 ± 0.2	13.0 ± 0.2	12.9 ± 0.2	13.2 ± 0.2	12.7 ± 0.3	13.2 ± 0.2
ZPP (μmol/mol heme)[6]	43 ± 3	38 ± 3	94 ± 9	96 ± 10	137 ± 25	111 ± 11
Serum iron (μg/dL)	150 ± 12	167 ± 7	134 ± 10	158 ± 10	117 ± 11[3]	151 ± 9
TIBC (μg/dL)	355 ± 12	354 ± 6	401 ± 12	385 ± 17	427 ± 15	392 ± 11
Tf saturation (%)	42 ± 3	48 ± 2	34 ± 3[3]	41 ± 2	28 ± 3[3]	39 ± 2
Ferritin (μg/dL)[7]	53 ± 12	65 ± 7	26 ± 9	38 ± 6	13 ± 9[3]	36 ± 6
TfR (μg/mL)	0.70 ± 0.11	0.65 ± 0.08	0.75 ± 0.12	0.87 ± 0.13	0.76 ± 0.11	0.75 ± 0.10

[1] All values are $\bar{x}$ ± SEM. RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, RBC distribution width; ZPP, zinc protoporphyrin; TIBC, total-iron-binding capacity; Tf, transferrin; TfR, transferrin receptor. Cohort was included as a covariate in several analyses of hematologic endpoints (day 30: hemoglobin, MCH, and TfR; day 90: RBC, hemoglobin, hematocrit, MCH, and TfR; day 150: RBC, hemoglobin, MCHC, TfR, and serum iron). Fetal sex was a covariate for TfR at gestation days 30 and 100 and for Tf saturation at gestation day 90.

[2–4] Significantly different from control at the same time point (ANOVA with post hoc tests): [2]$P < 0.001$, [3]$P < 0.05$, [4]$P < 0.005$.

[5] $n = 12$ and 20 at 30 d; $n = 10$ and 23 at 90 d; $n = 9$ and 22 at 150 d; $n = 10$ and 22 at birth.

[6] $n = 7$ and 3 at 30 d; $n = 4$ and 12 at 90 d; $n = 4$ and 10 at 150 d; $n = 7$ and 13 at birth (second cohort only).

[7] The antibody used in the ferritin assay detects ≈10% of monkey ferritin.

was calculated, and the relative ranking of the observed test statistic (/1000) was reported as a *P* value.

Recursive partitioning, a method of tree-based classification (19), was used to classify monkey dams into their actual diet group on the basis of the different iron measures. Recursive partitioning is a nonparametric tool that imposes no a priori restrictions on the distribution of the iron measures. At each partitioning step the algorithm iterates over all possible branch points to find the univariate division of the data that produces the greatest increase in diet group classification accuracy. For each potential split, cases must move to the left or right descendant based on the answer to a yes-no question. To find the best iron measures split, the algorithm calculates the splitting index for each possible division. From this, it computes the improvement in diet group classification accuracy resulting from the proposed decision rule. The chosen iron measures split combines the highest reduction in the splitting index with the greatest improvements in the number of correct diet group assignments. The algorithm then continues by taking the subpopulation and repeating the process until no additional partitioning is reasonable. To avoid overly complex trees that are hard to generalize to new data, the algorithm prunes the tree by using the technique of cross-validation.

All statistical tests were 2-sided, and the level of significance (alpha) was set at 0.05. JMP software (SAS Institute, Cary, NC) and SAS software version 9.1 (SAS Institute, Cary NC) were used for the statistical analysis The permutation tests were done with SAS. Recursive partitioning was done in R (20) using RPART package version 3.1–22 (free software available at http://www.r-project.org).

RESULTS

Food and iron intakes

Food intake (g/d) during pregnancy varied widely between individual monkeys but did not differ between the 2 diet groups (control group: 169 ± 4 g/d; iron-deprived group: 165 ± 6 g/d). The iron intake of the control monkeys averaged 16.9 ± 0.09 mg/d during pregnancy; the average iron intake of the iron-deprived group was 1.67 ± 0.40 mg/d.

Maternal weight gain during pregnancy

The 2 diet groups did not differ in maternal weight before initiation of the experimental diets; at 30, 90, or 150 d of gestation; or at delivery. Dams gained an average of 2 kg, about one-third their initial body weight, from 30 to 150 d of gestation and returned to prepregnancy weight at birth. Morphometric measures did not differ between groups when measured at delivery. Parity was a covariate for the weight analyses, with dams of higher parity having lower weights, but did not affect the pregnancy weight gain measure.

Maternal hematologic and iron-status variables

The iron-deprived group began to show differences from controls at the 90-d (midgestation) time point, 2 mo after initiation of the iron-deprived diet (**Table 4**). The measures showing the earliest response to iron deprivation were transferrin saturation, MCV, and MCH. By 150 d of gestation, a full spectrum of variables were affected. Average values for ZPP and TIBC tended to be higher, and MCHC and RDW tended to be lower in

TABLE 5
Fetal morphometric measurements obtained by ultrasound in the pregnant iron-deprived and control monkeys[1]

	Iron deprived (n = 14)	Control (n = 24)
90 d gestation		
Biparietal diameter (mm)	32 ± 0.5	32 ± 0.4
Foot length (mm)	21 ± 0.4	20 ± 0.3
150 d gestation		
Biparietal diameter (mm)	49 ± 0.4	49 ± 0.4
Foot length (mm)	43 ± 0.3	44 ± 0.5

[1] All values are $\bar{x}$ ± SEM. There were no statistically significant group differences by ANOVA. Crown-rump length of the dam was a covariate for both fetal measurements at 150 d gestation; the dam's preconception weight and the fetal sex were also covariates for foot length at 150 d gestation.

the iron-deprived group than in the controls, but the group differences were not significant.

On the basis of the human CDC criterion for third-trimester IDA (hemoglobin < 11 g/dL), 11 of 14 iron-deprived and 7 of 24 control dams had IDA in the third trimester (150 d) (P = 0.004, Fisher's exact test). Of the 7 control dams classified as anemic on the basis of a hemoglobin concentration < 11 g/dL, 1 dam had a hemoglobin value of 10.9 g/dL and 2 others had MCVs > 70 fL. These 3 monkeys would be unlikely to be judged anemic as individuals. The remaining 4 control monkeys were anemic according to 3 criteria (hemoglobin < 11 g/dL, MCV < 70 fL, and ferritin < 1.5 ng/mL). These monkeys were 4 of the 5 control monkeys with the lowest body mass index (weight/trunk length) in the first trimester (30 d of gestation). Thus, development of anemia on the control diet seemed to be related to being underweight at the beginning of pregnancy.

In addition to hematologic and iron-status measures, a clinical chemistry panel was performed on gestational day 150 for all dams. There were no effects of iron deprivation on the clinical chemistry measures (data not shown). Plasma protein increased significantly (P = 0.001, paired t test) from gestation day 30 (6.9 g/dL) and gestation day 90 (6.8 g/dL) to gestation day 150 (7.2 g/dL) in the sample as a whole, which indicated that plasma volume expansion did not occur in the study.

Fetal growth and hematologic measures

Fetal morphometric measurements were available for both cohorts (**Table 5**); there were no significant diet group differences. Fetal blood samples were obtained during the pregnancies of cohort 1 (10 control and 5 iron-deprived monkeys). The small sample size limited power for these endpoints. No diet group effects were seen for the hematologic variables (**Table 6**). None of the infants were outside the range of historical fetal controls in our colony. However, fetal CFU-E numbers were higher (P = 0.01) and fetal MCVs tended to be lower (P = 0.06) in the iron-deprived group than in the controls at 90 d of gestation (Table 6), which suggested an effect on the developing hematopoietic system at that time. Diet did not affect the numbers of colonies formed from CFU-GM cells.

Birth weight and neonatal morphometric variables

The only significant influence of prenatal iron deprivation was on head width (P = 0.03), which was smaller in the iron-deprived group (**Table 7**).

Dam and newborn hematologic measures at birth

At the time of birth, most of the hematologic indexes differed between the iron-deprived dams and the controls (**Table 8**). In neonates, prenatal iron-deprivation significantly affected mean corpuscular volume (MCV) (P = 0.001), mean corpuscular hemoglobin (MCH) (P = 0.005), and hemoglobin (P = 0.029) (Table 8). The size of the deficit in the prenatally iron-deprived newborns was small, ≈5% for MCV and MCH and ≈10% for hemoglobin. Hemoglobin and red blood cell (RBC) variables [MCV, MCH, and MCH concentration (MCHC)] were found to be correlated between dams and infants in the sample as a whole (Table 8). The correlation for MCHC was largely attributable to the very substantial effect of cohort on this endpoint (P < 0.0001). The correlations of mother and infant MCHC within cohorts were not significant.

No criteria were available for judging clinically relevant ID or IDA in newborn monkeys. The normal range of hemoglobin in human neonates is 14–20 g/dL in human neonates (21). One prenatally deprived neonate had a hemoglobin value below this range (12.3 g/dL). At the time of birth, dams were released from

TABLE 6
Hematologic variables for the fetuses of the pregnant iron-deprived and control monkeys from cohort 1[1]

	90 d gestation		150 d gestation	
	Iron deprived (n = 5)	Control (n = 10)	Iron deprived (n = 4)	Control (n = 10)
RBC (× $10^6/\mu$L)	3.40 ± 0.08	3.22 ± 0.13	4.22 ± 0.15	4.30 ± 0.12
Hemoglobin (g/dL)	13.3 ± 0.2	12.8 ± 0.6	14.2 ± 0.5	15.0 ± 0.3
Hematocrit (%)	35.4 ± 0.8	35.1 ± 1.5	39.3 ± 2.1	40.9 ± 0.9
MCV (fL)	104 ± 3	109 ± 1	93 ± 3	95 ± 1
MCH (pg)	39.2 ± 0.9	39.9 ± 0.6	33.7 ± 1.1	35.0 ± 0.7
MCHC (pg/fL)	37.7 ± 0.7	36.6 ± 0.3	36.2 ± 0.7	36.6 ± 0.4
CFU-E[2]	14 ± 4[3]	5 ± 2	0.3 ± 0.3	2 ± 0.5
CFU-GM	58 ± 7	45 ± 6	7 ± 2	16 ± 3
Colonies	72 ± 7	50 ± 8	7 ± 2	17 ± 3

[1] All values are $\bar{x}$ ± SEM. RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; CFU, colony-forming units; E, erythroid; GM granulocyte macrophage.

[2] Sex of the infant was a covariate for CFU-E.

[3] Significantly different from control, P < 0.01 (ANOVA).

TABLE 7
Morphometric measurements in the newborn monkeys of iron-deprived and control dams[1]

	Iron deprived (n = 14)	Control (n = 24)
Weight (g)	527 ± 15	543 ± 15
Crown-rump length (mm)	198 ± 3	199 ± 2
Foot length (mm)	81 ± 1	79 ± 1
Femur length (mm)	66 ± 1	65 ± 1
Head circumference (mm)	193 ± 2	196 ± 2
Head width (mm)	51.6 ± 0.3[2]	52.6 ± 0.3
Head length (mm)	68.7 ± 0.6	68.7 ± 0.4
Subcutaneous fat (mm)[3]	1.52 ± 0.04	1.54 ± 0.05

[1] All values are $\bar{x}$ ± SEM. Gestation length was a covariate for birth weight, crown-rump length, foot length, and femur length. Cesarean delivery was a covariate for birth weight and crown-rump length. Cohort was a covariate for crown-rump length and head circumference. The dam's preconception weight was a covariate for birth weight and foot length. Sex was a covariate for head width.
[2] Significantly different from control, $P = 0.03$ (ANOVA).
[3] Mean of 4 sites.

the experiment. At this time, anemia was diagnosed by veterinarians as part of their clinical surveillance, and iron dextran therapy was applied to 9 of 14 iron-deprived dams and 6 of 24 control dams.

Neonatal iron status was also compared between dams with and without third-trimester IDA (hemoglobin < 11 g/dL), irrespective of diet group. The only variable that differed between groups was neonatal MCH (IDA: 30.5 ± 0.4; no IDA: 31.6 ± 0.4; $P = 0.01$).

An additional measure of erythrocyte precursors in bone marrow was obtained at birth for the neonates (**Table 9**). The ratio of CFU-E to total colonies was lower for the iron-deprived than for the control group ($P = 0.03$) and confirmed a nonsignificant trend in the samples collected at 150 d of gestation from the subset of fetuses. There were no diet group effects on newborn behavioral assessment (**Table 10**). Veterinary diagnosis and treatment for dams were similar in the 2 groups.

TABLE 9
Hematopoietic progenitor cell (bone marrow) measures in the newborns of iron-deprived and control monkeys[1]

	Iron deprived (n = 14)	Control (n = 23)
CFU-E	11 ± 2	13 ± 3
CFU-GM	42 ± 4	30 ± 4
Colonies	52 ± 5	44 ± 6
% CFU-E	21 ± 4[2]	29 ± 4

[1] All values are $\bar{x}$ ± SEM. CFU, colony-forming units; E, erythroid; GM, granulocyte macrophage. There were no covariates for any endpoint.
[2] Significantly different from control, $P < 0.05$ (ANOVA).

Relations between iron-status variables

Because both the control and iron-deprived groups contained subjects who could be considered anemic in the third trimester by World Health Organization criteria (hemoglobin < 11 g/dL), we conducted a statistical analysis to determine whether the relation between hemoglobin and other hematologic variables was different within the anemic subgroups of the 2 diet groups. Randomization tests (1000 permutations) were used to test the hypothesis that the relations between hemoglobin and 6 other variables (serum iron, TIBC, RBC, MCV, MCH, and MCHC) differed according to diet group in the monkeys that were anemic. The performance of separate randomization tests in the 2 groups was considered appropriate because the interaction between the iron-deprived and control groups for the anemic versus nonanemic groups was marginally significant ($P < 0.1$). The analysis showed that the relation between hemoglobin and MCV was different for the animals judged to be anemic within the 2 diet groups. There was a positive correlation between MCV and hemoglobin in the anemic, iron-deprived group but not in the anemic, iron-sufficient group. The relation between hemoglobin and

TABLE 8
Hematologic measures in the dams and in the neonates at birth[1]

	Dam		Neonate		
	Iron deprived (n = 14)	Control (n = 24)	Iron deprived (n = 14)	Control (n = 24)	Dam-neonate correlation (r)
RBC (×10^6/μL)	3.9 ± 0.1[2,3]	4.1 ± 0.1	6.0 ± 0.2	6.1 ± 0.2	−0.21
Hemoglobin (g/dL)	8.0 ± 0.3[4]	9.9 ± 0.3	17.4 ± 0.7[3]	19.4 ± 0.5	0.02
Hematocrit (%)	24.0 ± 0.8[4]	29.1 ± 1.0	50.2 ± 2.4	55.8 ± 1.5	0.07
MCV (fL)	62 ± 2[3]	69 ± 2	87 ± 1[5]	91 ± 1	0.44[6]
MCH (pg)	20.3 ± 1.0[5]	23.9 ± 0.4	30.2 ± 0.4[7]	31.6 ± 0.3	0.46[6]
MCHC (pg/fL)	33.3 ± 0.5	34.1 ± 0.4	34.8 ± 0.5	34.8 ± 0.4	0.81[8]
RDW (%)	13.8 ± 0.5	13.3 ± 0.2	13.7 ± 0.3	13.5 ± 0.2	0.22
Ferritin (ng/mL)[9]	1.6 ± 1.1[3]	4.6 ± 0.7	6.7 ± 0.6	7.3 ± 0.9	−0.16

[1] RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.
[2] $\bar{x}$ ± SEM (all such values).
[3–5,7] Significantly different from control (ANOVA): [3]$P < 0.05$, [4]$P < 0.001$, [5]$P < 0.005$, [7]$P < 0.01$. Age at sampling and sex were covariates for neonatal RBC, hemoglobin, MCV, and MCH. Age was a covariate for MCHC. Cohort was a covariate for maternal hemoglobin and MCHC. Cesarean delivery was a covariate for maternal RBC and MCV. Parity and maternal age were covariates for maternal RBC.
[6] $P < 0.01$ (linear regression).
[8] $P < 0.001$ (linear regression).
[9] n = 13 and 21 dams; n = 12 and 23 neonates.

TABLE 10
Neurobehavioral test battery ratings in the neonates of iron-deprived and control monkeys[1]

	Iron deprived (n = 14)	Control (n = 24)
Observed muscle tone	1.5 ± 0.07	1.5 ± 0.05
Elicited muscle tone	1.4 ± 0.1	1.4 ± 0.1
Righting and grasp reflexes	1.9 ± 0.07	1.8 ± 0.04
Clasp-grasp reflex		
Time to position change (s)	2.8 ± 0.7	2.8 ± 0.6
Time on cylinder (s)	7.3 ± 1.5	8.8 ± 1.8
Prone progression	1.5 ± 0.2	1.4 ± 0.1
Oral reflexes	1.8 ± 0.1	1.6 ± 0.1
Eye reflexes	1.4 ± 0.02	1.3 ± 0
Moro reflex	1.8 ± 0.1	1.9 ± 0.1
Arousal	0.6 ± 0.2	0.5 ± 0.2

[1] All values are $\bar{x}$ ± SEM. There were no significant group effects detected by ANOVA. Cohort and sex were covariates for oral reflexes; gestation length was a covariate for elicited muscle tone.

other variables did not differ significantly by diet or anemia status. This analysis suggested that the microcytic characteristic of anemia in pregnancy is valuable in distinguishing diet-induced IDA from anemia attributable to other causes.

A second analysis with recursive partitioning was conducted to determine which cutoffs for which variables would be most effective in separating monkeys fed iron-deprived diets from the controls. This would simulate the human situation in which populations are screened for hematologic and iron status and implications are drawn as to the adequacy of dietary iron. With the use of 9 variables (hemoglobin, MCV, RDW, ferritin, TfR, transferrin saturation, serum iron, MCH, and MCHC), a ferritin cutoff of 11.8 μg/dL was most effective in separating the groups at the third-trimester time point (19 of 23 controls and 11 of 14 iron-deprived monkeys identified as nonanemic and anemic, respectively). This was very close to the CDC's hemoglobin cutoff of <11 g/dL (17 of 24 controls and 11 of 14 iron-deprived monkeys appropriately identified). A further cutoff of an MCHC > 35.5 pg/fL within the low-ferritin group correctly identified 22 of 23 controls. At the second-trimester time point, a transferrin saturation cutoff of 38.3% successfully identified 16 of 23 controls and 8 of 14 iron-deprived dams. These analyses indicated that early in the deprivation period, before the period required for complete RBC turnover, transferrin saturation was the best indicator of dietary iron insufficiency. After anemia developed, ferritin and hemoglobin were the best indicators. More specialized iron-status variables, such as RDW and TfR, were not more sensitive than were standard variables in reflecting inadequate dietary iron intake.

DISCUSSION

In this experiment, dietary iron deprivation led to progressive maternal iron deficiency. By the third trimester (day 150 and birth), most hematologic and iron-status variables reflected iron deficiency. Most of the variables were 5–15% lower than controls, but ferritin was 64% lower than controls. Sampling was not conducted often enough to determine the order in which these changes occurred as iron deprivation progressed. However, hemoglobin and MCV, the standard measures of hypochromic microcytic anemia, appeared as sensitive as the other indicators at this stage of anemia (22).

No cutoff for identifying IDA in monkeys has been promulgated. Assuming that the CDC's hemoglobin value of <11 g/dL in the third trimester is appropriate, IDA was seen in 47% of the sample as a whole, in 79% of the iron-deprived dams, and in 29% of the control dams. The observation that 29% (7 of 24) of the dams with an iron-adequate diet were anemic by this criterion emphasizes that a single hemoglobin value late in gestation does not necessarily reflect inadequate dietary iron intake during pregnancy. When multiple criteria were used, 4 of 24 controls were classified as anemic. Examination of weight records indicated that these 4 dams were underweight during the first trimester. Other factors, such as inadequate compensatory gut absorption or erythropoietin production, impairment in RBC iron transport, or deficits in RBC progenitor cells (23) could play a role in the development of anemia in the presence of adequate dietary iron.

Growth was not impaired in fetuses and newborns of the iron-deprived dams. Associations between maternal IDA and intrauterine growth retardation have been reported in human populations (4, 24, 25). There are several reasons, besides species differences, why this association might occur in human populations but did not in our experiment. In our study, limited dietary iron was the sole nutritional-environmental factor that varied, whereas in human studies iron deficiency often co-occurs with dietary and socioeconomic factors, which may play a role in low birth weight. In our study, iron deprivation began in the first trimester, whereas preconceptional iron deprivation may also occur in human populations. Many human studies are intervention trials that test the hypothesis that iron supplements can improve birth weight in populations that are known to include iron-deficient women (4, 22). Finally, human studies typically have a larger sample size and a potentially greater power than our study. Rasmussen (4) has estimated that severe IDA (hemoglobin < 8 g/dL) could lead to a birth weight deficit of 200–400 g. Our experiment had the power to detect a 61-g deficit in monkey infants with an average birth weight of 543 g, the equivalent of a 393-g birth weight deficit in a 3500-g human infant. A nonsignificant 24-g difference, equivalent to a 156-g deficit in humans, was found between iron-deprived and iron-replete groups. In addition to the lack of effect on birth weight, IDA did not lead to linear growth restriction or smaller head circumferences, although the head width of iron-deprived neonates was smaller than that of the controls. Our experiment was able to detect a 4–6% deficit in linear growth (crown-rump length, foot length, femur length) and head measures and a 21% decrease in subcutaneous fat.

In addition to the lack of effect on birth weight, other birth-outcome variables (gestation length, stillbirth, and maternal mortality) were not influenced by iron deprivation in this study. However, an increase in these low-frequency events is unlikely to be detected in a small sample.

Gestational iron deprivation led to compromised fetal hematologic status at birth in the present experiment. A variety of studies of human populations worldwide have failed to find an effect of maternal IDA on most newborn hematologic measures (3). Ferritin has been found to differ between newborns of women with and without IDA (26–30), although other studies reported no differences (31–34). In contrast, we found clear and

highly significant group differences in newborn hemoglobin, MCV, and MCH because of dietary iron deprivation. It is likely that the same compensatory mechanisms seen in women, such as enhanced gut absorption of iron (35, 36), are also present in nonhuman primate pregnancy, but this has not been directly studied to date. It is not clear that the 5–10% differences in hemoglobin and RBC indexes seen here would lead to detection of clinical IDA in human newborns or that they would be detected statistically because of the instability of hematologic variables in human newborns. However, the small differences in hemoglobin pools, which represent 80% of newborn iron stores (37), could affect iron status in the postnatal period when, in the absence of dietary sources of iron, infants rely on iron content present at birth. For instance, delayed cord clamping, which produced a slightly increased newborn hemoglobin, was associated with a reduced incidence of iron deficiency later in infancy (38). It is noteworthy that ferritin and hemoglobin were not related in the mother-infant pairs, although RBC variables (MCV, MCH, and MCHC) were significantly correlated.

Some data concerning fetal iron status were available from the first cohort. Hemoglobin, hematocrit, and MCV were comparable in the small samples; however, a possible effect of maternal iron deprivation during fetal hematopoiesis was suggested on day 90 (midpregnancy), when circulating erythrocyte precursors were elevated. Studies in humans have shown elevated erythropoietin in cord blood of neonates born to anemic women (31).

Effects on hematopoiesis in newborns suggest that other fetal tissues, including brain, could also be adversely affected. Fetal hematopoiesis is thought to have priority for available iron at the expense of other tissues (39). Georgieff et al (40) showed depletion of tissue iron (liver and muscle) when augmented hematopoiesis was elicited in fetal lambs by induction of hypoxia or administration of erythropoietin. Limited information on priority for maternal-fetal distribution of iron to fetal brain comes from a study in rhesus monkeys in which ^{59}Fe iron dextran was administered intravenously during the third trimester (41). At the lower of 2 doses used in the study, the percentage of labeled iron to total iron in tissues was 96% for spleen (primarily RBCs), 42% for liver, 10% for muscle, and 34% for brain, which suggests priority distribution of iron for erythropoiesis. Several studies in rodents have shown that iron deprivation during brain development produces permanent deficits in brain iron. In rats, short periods of IDA during pregnancy led to lower brain iron contents in adult offspring (42). Low-iron diets can reduce brain iron concentrations when administered during the postnatal brain growth spurt in rodents (43, 44) or throughout brain development in mice (45). In rhesus monkeys the period of brain growth peaks in the third trimester, whereas in humans it peaks around the time of birth (46). Thus, fetal brain may be a susceptible tissue for iron depletion in third-trimester IDA. Although neurobehavioral examinations at birth did not show abnormalities in the iron-deprived newborn monkeys, behavior will be evaluated postnatally during follow-up of the present infant cohort to examine functional brain effects.

Mechanisms of iron uptake, storage, and distribution in tissues are gradually being elucidated at the cellular level (47). The location of iron importers, such as DMT1, and iron exporters, such as FTP1, as well as the regulation of expression of these proteins under conditions of iron deprivation is being explored in placenta (48–51). However, the ability to detect tissue deficiency and to establish resource allocation and compensation in the complex maternal-placental-fetal system has not been studied sufficiently at this time. The development of a nonhuman primate model for third-trimester IDA may provide an opportunity to further explore these regulatory mechanisms.

In conclusion, this prospective study of maternal iron deficiency and fetal growth and development notes for the first time in a monkey model that modest maternal iron deficiency does not lead to growth failure, despite compromised neonatal hematologic iron status. The functional consequences of maternal third-trimester IDA on cognitive and behavioral development of the infant are currently being investigated. This new model of human maternal iron deficiency allows more refined examinations of the single micronutrient deficit in third-trimester pregnancy than are possible in human studies.

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MSG designed the experiment, analyzed the data, and wrote the manuscript. CEH designed and wrote the experimental protocols and was responsible for the day-to-day conduct of the study. AFT performed the ultrasound exams, collected the fetal blood and neonatal bone marrow samples, and supervised the progenitor cell assays. JLB supervised and helped interpret the iron-status assays. MKG helped interpret the neonatal hematology data. SLG helped plan the study, write the protocols, and design the purified diets. AC conducted the multivariate and recursive partitioning analyses. BL contributed to the study design and statistical analysis. The authors reported no conflicts of interest.

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