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# Optimal iron fortification of maternal diet during pregnancy and nursing for investigating and preventing iron deficiency in young rhesus monkeys ☆

Christopher L. Coe [a,*], Gabriele R. Lubach [a], Mark Busbridge [b], Richard S. Chapman [b]

[a] Harlow Center for Biological Psychology, University of Wisconsin, Madison, WI 53715, United States
[b] Department of Clinical Biochemistry, Charing Cross Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom

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ABSTRACT

The realization that pregnant and infant monkeys were challenged by high nutritional needs for iron led vendors to markedly increase iron concentrations in commercial diets. Yet, no systematic research was conducted to investigate the consequences of this important dietary change. Hematology and iron panels were determined for 142 infant rhesus monkeys gestated and reared on 3 different diets varying in iron concentration (180, 225 or 380 mg Fe/kg). Anemia was significantly more prevalent in offspring from females fed the 180 and 225 mg Fe/kg diets (32–41% versus 0 for the 380 mg Fe/kg diet, $P < 0.001$). Higher hepcidin levels were protective against iron overload in infants from the 380 mg Fe/kg condition. These findings indicate a highly fortified diet during pregnancy continues to have postnatal benefits for the growing infant. However, for those interested in iron deficiency, lower iron diets provide a reliable way to generate anemic infant monkeys for research.



## 1. Introduction

Pregnant females and infants of many animal species are challenged by the dietary need to obtain high levels of iron and thus are prone to developing an iron deficiency anemia (IDA), with the risk comparable to that seen in women and young children (Allen, 2005; Bothwell, 2000; Looker et al., 1997). The likelihood of IDA in nonhuman primates, as well as in litter-bearing rodents and some farm animals, led to increased fortification of commercial diets. Since 1972 the recommended Minimal Daily Requirements have been updated repeatedly by advisory panels in the U.S. (National Research Council, 2003). Iron concentrations in most primate diets today are over twice the level from two decades ago, and almost fourfold higher than the amount considered adequate for a non-pregnant adult monkey (i.e., 100 mg Fe/kg). Given the diverse effects of iron, including on oxidative metabolism and the synthesis of neurotransmitters and myelin, these changes in nutriture over time are likely to have impacted the health of monkeys in zoos and laboratories and the findings of many studies (Rao and Georgieff, 2002; Ortiz et al., 2004). Highlighting this type of concern, it was found previously that vitamin A levels had actually been excessively high in primate diets, with toxic hepatic effects evident upon histological exam and from elevated liver enzyme values (Penniston and Tanumihardjo, 2006).

Two hematological surveys documented that the occurrence of anemia in infant monkeys used to be very common in research facilities (Bicknese et al., 1993; Kreite et al., 1995). The 30–40% occurrence of anemia was comparable to the prevalence in American children prior to the fortification of infant formula and cereals, and concurs with the widespread IDA still evident in non-industrialized countries (Zetterstrom, 2004). While lactoferrin, the primary source of iron in breast milk, is readily absorbed, it is also critical that substantial amounts of maternal iron be acquired transplacentally before birth in order to meet the infant's growth needs for iron (Davidson et al., 1990; Golub et al., 2006). Monkeys born with low storage iron, as indexed by serum ferritin, are at increased risk to become anemic by the end of the nursing period at 4–6 months of age. In keeping with the importance of this prenatal iron acquisition, adult female monkeys fed a low iron diet during pregnancy are predisposed to birth infants that will progress to a clinical anemia (Lubach and Coe, 2006). Moreover, primiparous monkeys are less likely to provide sufficient iron than are multiparous dams, a maternal/fetal conflict also seen in adolescent human pregnancies (Iannotti et al., 2005; Meier et al., 2003).

Although the infant's hematology gradually improves with the consumption of solid food, some lingering effects of the iron deficiency may remain evident, including on brain dopamine and

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* Corresponding author. Address: Harlow Center for Biological Psychology, University of Wisconsin, 22 North Charter Street, Madison, WI 53715, United States. Tel.: +1 608 263 3550; fax: +1 608 262 6020.
*E-mail address:* ccoe@wisc.edu (C.L. Coe).

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norepinephrine activity and myelination (Coe et al., 2009; Lozoff et al., 2006; Patton et al., 2012). Iron transport proteins, such as transferrin and divalent metal transporter, also stay up-regulated in cerebrospinal fluid, suggesting that the acquisition of brain iron takes longer to be completely normalized (Geguchadze et al., 2008). Thus, it is important to better understand the maternal factors that influence the occurrence of infant anemia and to evaluate when the protracted effects on hematology and iron biology resolve. A second aim of our study was to assess if there is evidence of iron overload in monkeys consuming a very fortified diet (Tanno and Miller, 2010). Iron absorption should be tightly regulated, particularly by the peptide hepcidin, and from heme feedback signals including transferrin saturation (TSAT) (Ganz, 2011; Kemna et al., 2008). Prior research in rodents had also suggested that low hepcidin in an iron-deficient infant could result in a rebound overshoot of iron absorption during rapid repletion (Hegde et al., 2011).

To address these issues, hematology and iron measures were evaluated in infant monkeys born to females fed 3 different diets: (1) the current diet used by most facilities today, (2) a diet formulation similar to the standard one used prior to 1995, and (3) a customized diet with intermediate iron levels. Serum hepcidin levels were determined because of its known role in the regulation of iron absorption and tissue storage. A final aim was to assess if parity influenced the effects of maternal diet, which was investigated by screening infants of primiparous and multiparous monkeys in each diet condition.

## 2. Methods

### 2.1. Subjects

This research determined the hematology and iron status of 142 young rhesus monkeys (Macaca mulatta) reared and housed under standardized indoor conditions at the Harlow Primate Laboratory and adjacent Wisconsin National Primate Research Center. Light/dark schedules were regulated and constant across the year; room temperature was controlled at 21 °C; and the monkeys lived in stainless steel caging, which was cleaned daily and completely sanitized every 2 weeks. Similar veterinary care and clinical treatments (if needed) were provided to all animals, and all monkeys received annual physical exams to monitor their general health status. The mothers were fed one of 3 diets during pregnancy and nursing (180 mg/kg [$N = 73$], 225 mg/kg, [$N = 29$], 380 mg/kg [$N = 40$]). All infants were healthy, from full term, singleton births and delivered naturally, with primiparous and multiparous dams represented in each diet condition ($N = 51$, and 91, respectively). It was also possible to consider the possible influence of infant gender (78 males, 64 females).

Blood samples were collected twice from infants in the two lower iron diet conditions, at 6–7 months and 12 months of age, because some were iron-deficient and it was important to determine if their anemia resolved. For the 40 infants from the high iron diet condition, half were assessed at each age point, with parity and gender completely balanced. Samples were collected from infants of 10 primiparous and 10 multiparous dams at each time point, with each parity condition comprised of 5 males and 5 females. All procedures were reviewed and approved by the Animal Care and Use Committee at the University of Wisconsin.

### 2.2. Diet

Three different diets varying in iron concentration were assessed (Table 1). The low iron diet (180 mg Fe/kg) was comparable to the standard diet used in the U.S. prior to 1995 (Purina 5L1Q, PMI Nutrition International, St. Louis, MO). The high iron biscuit (380 mg Fe/kg) is the diet commonly used by research facilities and primate centers today (Harlan Teklad 2050, Madison, WI). In addition, with the assistance of the vendor, we specifically created an intermediate level of iron fortification (225 mg Fe/kg), while maintaining other constituents comparable to the low iron diet (Purina 5LFD). Ferrous sulfate was used during manufacture to supplement the natural ingredients up to the appropriate iron level. Each day the monkeys were given a specified number of biscuits. A small amount of fruit was provided in the afternoon as part of an environmental enrichment program required by federal regulations.

**Table 1**
Mineral and vitamin concentrations, as well as ingredient composition, for the 3 diets fed to the rhesus monkeys in this study.[a]

| Fe concentration | 180 | 225 | 380 |
|---|---|---|---|
| Ingredients | | | |
| Protein, % | 15.6 | 15.7 | 20.0 |
| Carbohydrate, % | 68.9 | 68.7 | 40.1 |
| Fat,% | 6.0 | 6.0 | 5.4 |
| Ash, % | 5.2 | 5.3 | 6.1 |
| Fiber (crude), % | 4.6 | 4.5 | 8.1 |
| Minerals | | | |
| Iron[b], mg/kg | 180 | 225 | 380 |
| Zinc, mg/kg | 114 | 110 | 72 |
| Copper, mg/kg | 20 | 21 | 14 |
| Vitamins | | | |
| A, IU/g | 20.0 | 20.0 | 19.5 |
| B12, mg/kg | .022 | .073 | .040 |
| C, mg/kg | 500 | 500 | 910 |

[a] The vender sources were Purina (5L1Q and 5LFD for the 180 and 225 mg Fe/kg biscuits, respectively) and Harlan Teklad (2050) for the 380 mg Fe/kg diet.
[b] Based on typical daily consumption (220 g), each day the adult female monkeys were provided approximately 39.6, 49.5, or 83.6 mg of Fe, respectively, from the 3 diets.

### 2.3. Hematology and iron panel

Blood samples (<4 mL) were obtained via femoral or saphenous venipuncture in order to generate the panel of hematological and iron measures. Results are presented for two red blood cell (RBC) parameters with known clinical cutoffs for IDA in monkeys, mean corpuscular volume (MCV, for IDA < 60 fL) and hemoglobin (Hb, for IDA < 110 g/L). Three measures from the iron panel are also summarized: (1) serum ferritin, (2) iron level in circulation and (3) TSAT (serum iron/total iron binding capacity x 100). In addition, serum hepcidin levels were analyzed at each age point from a representative subset of 21 infants, including 6 from the high iron diet as well as for 15 iron-sufficient (IS) and IDA monkeys from the 225 mg Fe/kg diet condition ($N = 8$ and 7, respectively).

### 2.4. Hepcidin assay

Hepcidin-25 (Hep-25) levels were analyzed with an established competitive radioimmunoassay, which has been described in detail previously (Busbridge et al., 2009). Rabbit anti-Hep-25 polyclonal antibody was generated using synthetic hepcidin (Bachem Ltd, UK), and the displacement of Hep-25 radiolabeled with iodine ($^{125}$I) used to quantify serum titers. Prior comparisons had verified that Hep-25 values determined by this RIA were highly correlated with established SELDI-TOF-Mass Spectrometry methods ($r = 0.96$) (Ashby et al., 2010). The lower limit of detection was 0.22 nmol/L; intra-assay precision averages 7.2%, and the inter-assay coefficient of variation averages 7.6%. The sequence homology of Hep-25 is conserved across species, with the Hep-25 of rhesus monkeys differing by only one amino acid, a threonine for an alanine. From