Intravenous iron supplementation effect on tissue iron and hemoproteins in chronically phlebotomized lambs


Intravenous iron supplementation effect on tissue iron and hemoproteins in chronically phlebotomized lambs. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R2124–R2131, 1997.—Chronic phlebotomy is an important mechanism of iron loss in premature infants. We studied inter- and intraorgan iron allocation in 10 twin lamb pairs undergoing an acute 40–50% reduction in red cell volume followed by smaller intermittent phlebotomies over an 11-day period. One twin received no supplemental iron sucrose, while the other received an average daily intravenous dose of iron sucrose of either 1 (n = 3), 2 (n = 3), 5 (n = 3), or 15 (n = 1) mg·kg⁻¹·day⁻¹. The total iron content of the red blood cells, liver, skeletal muscle, heart, and brain was directly related to iron dose up to 2 mg·kg⁻¹·day⁻¹. Tissue iron concentrations remained stable until liver iron was <200 μg/g dry wt, after which iron was preferentially directed to red blood cells over skeletal muscle, heart, and brain. Hemoprotein concentrations decreased proportionately to tissue iron, except myocardial cytochrome c, which remained preserved. Any available iron in phlebotomized, rapidly growing lambs is preferentially directed to red blood cells, and lambs require iron supplementation to maintain tissue iron and hemoprotein concentrations. A decrease in nonheme tissue iron results in the high prioritization of iron among iron-containing proteins.

Iron deficiency; brain; myoglobin; cytochrome c; red blood cells; heart; sheep.

Premature infants are at risk for significant negative iron balance due to repeated phlebotomy during the acute phase of their illness. Before the onset of enteral nutrition, daily blood losses can be significant, approaching 3–4 ml/kg body wt, at a time when their only source of iron is red cell transfusions (15, 23). Because premature infants are being transfused with red blood cells less frequently (34), negative iron balance can ensue. Without iron replacement, these infants are at risk for developing tissue iron depletion. The use of exogenous erythropoietin as a substitute for red cell transfusion will place additional demands on iron stores and on nonhematopoietic iron-containing tissues as available iron is diverted into red blood cell production (18). We have previously demonstrated in both the fetal (9) and neonatal (26) ovine model that iron status at the initiation of therapy is a primary determinant of the response to exogenously administered erythropoietin.

The purpose of this study was to determine the effect of phlebotomy followed by varying levels of iron repletion on the distribution of tissue iron among storage (hepatic), nonstorage (skeletal muscle, cardiac muscle, and brain), and erythropoietic (red blood cell) pools in the neonatal lamb. An additional objective was to determine the allocation of iron-containing hemoproteins (myoglobin and cytochrome c) within specific tissues. On the basis of previous studies of iron prioritization in the fetal lamb (9), we hypothesized that the level of iron repletion would affect the distribution of iron among various tissues, with red blood cells taking higher priority in an iron-limited environment than brain, heart, skeletal muscle, and liver iron. We further hypothesized that the intraorgan distribution of iron in cardiac and skeletal muscle tissues would favor myoglobin production to facilitate cellular oxygenation at the expense of cytochrome c (30).

Materials and Methods

The study received approval by the Animal Care and Use Review Committee at the University of Iowa. Newborn lambs obtained from a local supplier were housed with their mothers in a temperature- and light-controlled environment. Except for brief periods when procedures were performed, lambs had free and continuous access to their mothers as a source of nourishment.

Experimental protocol. Ten twin pairs of 2- to 4-day-old healthy lambs were randomized by coin flip to intravenous iron sucrose-treated and nontreated groups. After randomization, a percutaneously placed jugular venous catheter was sutured to the skin and protected in a Coban (3M Health Care, St. Paul, MN) neck wrap. After a 1- to 2-day recovery period, lambs underwent an exchange transfusion with saline until hemoglobin levels fell to 5–6 g/dl (a ~40–50% reduction in the red cell mass). Immediately after the initial exchange transfusion, the treated twin received an intravenous bolus injection of 2.3, 4.7, 11.7, or 35 mg of iron sucrose/kg administered within a 3-min period. Subsequent intravenous iron doses were administered every 2–3 days immediately after the lambs underwent phlebotomy (6–9 ml/kg) without saline replacement. When extrapolated to a mean daily iron dose, these doses are equivalent to 1, 2, 5, and 15 mg of elemental iron/kg body wt. One lamb in each of three twin pairs was randomized to the 1, 2, and 5 mg/kg groups and one lamb in the remaining pair was assigned to the 15 mg/kg group. Because of the death of an animal in the 15 mg/kg group coincident with rapid intravenous iron injection, no further animals were studied in this group. The control twin of each of the 10 treated animals received no supplemental iron.

Blood samples for hemoglobin, reticulocyte count, plasma iron, and total iron-binding capacity were drawn before the initial saline exchange transfusion and daily thereafter for the first 5 days. Subsequent sampling was done every 2–3 days until death on day 11 or 12. Body weights were recorded on days of blood sampling and at death. Death was accom-
plished with an overdose of pentobarbital sodium, after which the liver, heart, and brain were removed and weighed. Samples from these tissues and from skeletal muscle were harvested, rinsed in normal saline, and frozen at −70°C until analysis.

Biochemical measurements. Plasma and milk iron concentrations were determined in duplicate using an electrochemical method on 25 µl volumes with a Ferrochem II analyzer (ESA, Bedford, MA). Plasma total iron-binding capacity was determined in duplicate on 100 µl volumes using the same instrument.

Tissue iron was measured by atomic absorption spectroscopy as previously described (9, 16). In doing so, thawed tissue samples were thoroughly rinsed in normal saline, lyophilized for 72 h, weighed, and digested in 10 ml of a 4:1 ratio of 70% nitric acid:70% perchloric acid. Values were compared with iron standards (Sigma Chemical, St. Louis, MO) with results expressed as micrograms elemental iron per gram dry tissue weight.

Cardiac and skeletal muscle tissues were analyzed for myoglobin as previously described (11). Briefly, the tissues were lyophilized, manually crushed, sonically disrupted, centrifuged at 10,000 g for 30 min at 4°C, and filtered through a 0.45 µm low-protein binding filter (4184 Gelman, Ann Arbor, MI). Myoglobin was separated by high-performance liquid chromatography using a 300 × 7.8 mm gel filtration SEC 125 column (Bio-Rad Laboratories, Richmond, CA) and eluted using a phosphate buffer (0.05 M NaH2PO4 · H2O, 0.05 M Na2HPO4, 0.15 M NaCl, and 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. Quantification was done by measuring ultraviolet absorbance at 396 nm (System 3 HRLC; Bio-Rad Laboratories, Richmond, CA) to determine peak heights for comparison with those generated from stock standards of sheep skeletal muscle myoglobin (Sigma Chemical, St. Louis, MO). Hemoglobin peaks, which are also detected at 396 nm, were negligible, suggesting minimal contamination of tissues with hemoglobin.

Cytochrome c was assayed by high-performance liquid chromatography as previously described (9). Briefly, the tissues were lyophilized, manually crushed, sonically disrupted, centrifuged at 10,000 g for 30 min at 4°C, and filtered through a 0.45 µm low-protein binding filter (4184 Gelman, Ann Arbor, MI). Cytochrome c was expressed as intravenous iron in minus hemoglobin iron out, expected to decrease over time) or below (if the data parameter was expected to increase over time) the pretreatment baseline concentration at

<table>
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<th>Parameter</th>
<th>Units</th>
<th>Mean ± SE</th>
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<td>Plasma iron</td>
<td>µg/dl</td>
<td>X ± SE</td>
<td>&lt;0.05</td>
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<tr>
<td>Milk iron</td>
<td>µg/dl</td>
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<td>Myoglobin</td>
<td>µg/g tissue</td>
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<td>Hemoglobin</td>
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<td>Cytochrome c</td>
<td>µg/g tissue</td>
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<td>Reticulocyte count</td>
<td>10,000 erythrocytes</td>
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The statistical analyses were designed to assess the effect of iron dosing on the plasma and tissue laboratory variables. ANOVA was used to demonstrate an effect across dosage groups, whereas regression analysis was used to assess relations among variables. Because plasma Epo levels did not exhibit a normal distribution, logarithmic data transformation was performed for all statistical analyses. Significance for all statistical comparisons was set at P < 0.05. Values are presented as means ± SE.
RESULTS

Hemoglobin and plasma iron concentrations demonstrated a direct dose response to iron treatment (Fig. 1A and B). Similarly, corrected reticulocyte counts were highest in the group with the highest iron dose (Fig. 1C). All groups experienced a rise in plasma Epo concentrations after the initial phlebotomy, with the 10 animals that received no intravenous iron demonstrating persistently increased plasma Epo levels throughout the experimental period (Fig. 1D). Despite the significant anemia induced by repeated phlebotomies, the animals sustained a 60.5 ± 3.5% increase in body weight during the 11-day study period. This was not different among groups. The mean weight gain among all animals during the study period was 2.95 ± 0.20 kg.

Increasing intravenous iron dosage had a significant positive effect on the iron concentrations of all tissues studied (Fig. 2A). Tissue iron content increased with iron dosage in the red blood cells and the liver and was greater with the three higher doses compared with the two lower doses in skeletal muscle and heart (Fig. 2B). There was no significant effect of iron dosage on brain iron content. Net iron balance was negative in the groups that received 0 or 1 mg·kg⁻¹·day⁻¹ of iron, was marginally positive for the 2 mg·kg⁻¹·day⁻¹ group, and was markedly positive for the 5 and 15 mg·kg⁻¹·day⁻¹ groups (Fig. 3). The total measured body iron content was significantly correlated with iron balance in a direct manner (r = 0.92; P < 0.001). Milk samples from 9 of the 10 ewes were centrifuged, and iron concentra-
tion was assayed in the aqueous phase. The iron concentration of the ewes’ milk was 1.5 ± 3.6 µg/dl. The insignificant amount of iron found was not included in iron balance calculations.

The percentage of iron allocated to each of the organs was assessed by study group as a function of iron dose administered (Fig. 4). The red blood cells accounted for 58–93% of the total measured body iron content, and the liver accounted for an additional 0.5–39%. All 13 animals in the 0 and 1 mg·kg⁻¹·day⁻¹ groups had 5% of their total measured iron in the liver (2.8 ± 0.4%) and had liver iron concentrations <200 µg/g dry tissue wt. In contrast, the seven animals in the 2, 5, and 15 mg·kg⁻¹·day⁻¹ groups had mean liver iron percentages of 19.3 ± 3.9% (P < 0.001) and liver iron concentrations >390 µg/g dry tissue wt. The relative proportion of iron decreased in red blood cells and increased in the liver as a direct function of the iron dose administered. Heart iron concentrations were logarithmically correlated with liver iron concentration (Fig. 5) and abruptly decreased at liver iron concentrations <200 µg/g dry wt. A relationship similar to liver iron concentration was present with skeletal muscle (r = 0.67; P < 0.001) and brain (r = 0.55; P = 0.01).

To examine the interorgan allocation of iron between nonheme tissues and red blood cells once hepatic iron stores showed evidence of depletion, the 13 animals in negative iron balance with liver iron concentrations <200 µg/g dry wt and with <5% of total measured iron content in the liver were examined. The percentage of total measured iron found in skeletal muscle (r = -0.90; P < 0.001), heart (r = -0.83; P < 0.001), and brain (r = -0.82; P < 0.001) of these 13 animals decreased proportionately as the percentage of iron measured in red blood cells increased. These findings contrast with those of the entire group (Fig. 4) and with those of the seven animals with >200 µg/g of liver iron remaining.

Intraorgan prioritization of protein-bound iron between the iron-containing hemoproteins myoglobin and cytochrome c was assessed as a function of tissue iron concentration in skeletal muscle and heart. In skeletal muscle, cytochrome c concentration decreased directly with reductions in skeletal muscle iron concentration, whereas myoglobin concentration showed a trend toward the same relationship (Fig. 6, A and B). In heart, muscle myoglobin concentration decreased in parallel with heart iron concentration (Fig. 6C), whereas heart cytochrome c concentration did not (Fig. 6D). In brain, cytochrome c concentration decreased directly with brain iron concentration (r = 0.45; y = 0.07x + 32.71; P = 0.05).

**DISCUSSION**

This study demonstrates three principles of iron metabolism in phlebotomized newborn sheep. First, interorgan iron distribution in newborn lambs made iron deficient by phlebotomy follows the same pattern seen in models of fetal and postnatal dietary iron
deficiency. Specifically, red blood cells take priority over all other organ systems. Although nonheme tissues such as brain, heart, and skeletal muscle maintain adequate iron concentrations until a critical low value of storage (i.e., liver) iron is breached, ultimately iron concentrations of these tissues are sacrificed at the expense of neonatal red cell iron incorporation. The compromise of brain iron during the period of rapid growth and development is of particular concern. Second, the loss of organ iron results in selective reductions of intracellular iron-containing proteins in patterns different from those previously reported in the fetus and older animal. These selective losses would be expected to have specific physiological effects that would affect neonatal organ development and performance. The loss of brain cytochrome c in direct proportion to iron is of particular concern. Finally, iron balance and tissue iron concentrations in phlebotomized lambs can be maintained by intravenous iron sucrose given in dosages that closely approximate the effective iron dose prescribed to phlebotomized preterm infants. This suggests that the neonatal lamb model closely mimics the clinical condition of phlebotomized premature neonates and allows for its use in further studies of neonatal iron metabolism, including the response of neonates to administration of exogenous erythropoietin.

Iron serves an important role in multiple physiological processes in the newborn animal including tissue oxygenation, oxidative energy production, cell cycle regulation, and organ growth and development (17, 19, 32). Disruption of iron balance at any age forces inter- and intraorgan prioritization of the remaining available iron with potential physiological consequences (3, 5, 6, 9, 10, 12, 13, 17, 19, 22, 28, 32, 36). The allocation of body iron during negative iron balance is determined by multiple factors including the species, age, and growth rate of the animal, as well as the rapidity with which negative iron balance is induced (2, 3, 6, 12–14, 22, 28). Depending on the iron dose administered and the extent of phlebotomy experienced, supplementation of phlebotomized animals with intravenous iron can either reduce or prevent the loss of tissue iron.

Clinical phlebotomy loss during the early weeks of life continues to be the primary source of iron loss in critically ill infants in the neonatal intensive care unit (31). Because premature infants are born with low iron stores (25) and are typically given no enteral or parenteral iron (except for red cell transfusions) during the first weeks of postnatal life, the risk of significant late anemia in association with the eventual development of negative iron balance is present. Indeed, unless transfused with red blood cells, hemoglobin concentrations of infants in the neonatal intensive care unit typically decrease rapidly from 150 g/l at birth to <100 g/l within days to weeks. Many centers now use a hemoglobin concentration of 70 g/l as the criteria for red cell transfusion of thriving premature infants not being treated with supplemental oxygen who are growing well and feeding enterally (29, 34). These reductions in red cell hemoglobin and iron are proportional to those produced in our model, although, clinically, the reduction may occur over a period of days in the sick infant to weeks in the stable infant.

The lamb model used in the current study provided a means for studying the inter- and intraorgan distribution of iron during the chronic phlebotomy-induced anemia frequently encountered in the neonatal period. In contrast to the present study, previous studies of iron deficiency have been performed in more developmentally mature, slower-growing animals, typically postweaning rats (2, 3, 6, 12–14, 22, 28, 30), and were designed to examine the effects of long-term dietary iron deficiency without significant hemoglobin iron loss. In contrast, our study investigated the effect of a more rapid shift in iron status in a newborn animal due
cells, the total red cell iron was significantly lower in the two groups in negative iron balance; i.e., 0 and 1 μg·kg−1·day−1. Moreover, their erythropoietic response to phlebotomy was clearly blunted in comparison to lambs receiving higher intravenous iron doses. In addition, the serum Epo concentrations in the two low-dose groups remained elevated throughout the study, suggesting that erythropoiesis was ineffective. These findings are consistent with previous studies in fetal and neonatal sheep, demonstrating that iron sufficiency is essential for mounting an adequate response to endogenously or exogenously administered erythropoietin (9, 26).

Once hepatic iron stores were depleted, iron was preferentially directed to the red blood cells at the expense of skeletal muscle, heart, and brain. These findings are similar to our previous investigations in fetal lambs (9) and in humans fetuses (27), but differ from studies in postweanling rats with dietary iron deficiency (2, 3, 6, 12–14, 22, 28, 30). In the fetal lamb, chronic hypoxia or exogenous Epo administration increased iron demand for erythropoiesis, resulting in loss of heart and skeletal muscle iron and cytochrome c. In the newborn human, depletion of fetal hepatic iron stores was associated with low concentrations of heart and brain tissue iron (27), a finding remarkably similar to the logarithmic relationship observed between nonstorage tissue iron and storage iron in the current study.

Studies of prioritization of interorgan iron in postweanling iron-deficient rats have yielded varied results, depending primarily on the age of the animal, the method and rapidity with which the animal became iron deficient, and the iron-containing tissues examined (3, 13, 14, 22). Joseph (14) demonstrated that the total nonhemoglobin iron, including liver iron stores, decreased before hemoglobin iron in progressive dietary iron deficiency. He did not, however, partition storage and nonstorage iron. Because hepatic iron stores normally comprise a greater percentage of total body iron than nonstorage tissue iron, his conclusions are most likely based primarily on loss of storage iron. McKay et al. (22) showed profound reductions in skeletal muscle and heart iron during severe dietary anemia, although the sequence of the individual organ reductions was contrary to the loss of hemoglobin iron was not studied (22). Dallman et al. (3) demonstrated in adult rats that the peripheral blood hematocrit decreased before reductions in brain iron, suggesting that in older animals brain iron is spared at the expense of the erythron (3). Huebers (13) summarized the findings of these and other postnatal studies recently, concluding that nonstorage tissue iron declines at the same time as erythron iron. His model, based primarily on data from older animals, indicates that there is little to no prioritization between red cell and nonstorage tissue iron (13). The results of our current newborn lamb study and previous studies in the ovine fetus (9, 26) suggest that iron is preferentially directed toward supporting red blood cell production to a greater extent during the fetal and neonatal period than later in life. This prioritization potentially places the rapidly growing and developing heart and brain at risk for the consequences of tissue iron deficiency.

The mechanisms by which this interorgan iron prioritization is regulated have not been fully elucidated. The greater avidity of some tissues over others for iron raises the possibility that the transmembrane transferrin receptor, which regulates iron uptake and release by cells (33), is differentially regulated across various organs. This could be achieved by either greater expression of the receptor on the cell surface or by a higher association constant for diferric transferrin in certain organs. For example, the propensity of the red blood cells to accumulate iron in preference to other tissues in the human may be due to 80–90% of the body's transferrin receptors normally being located on erythroid cells (1) or potentially to a higher avidity of those red cell transferrin receptors for diferric transferrin. Transferrin receptors isolated from different individuals and tissues show variations in transferrin receptor N-glycosylation, which in turn may affect transferrin structure and function (8, 24). The avidity of the cell membrane receptor for diferric transferrin is closely linked to the structure of its asparagine-linked oligosaccharides (35). Although it is known that transferrin receptors isolated from different tissues within the same species can have different affinity constants, it is not known if these differences could account for preferential tissue accretion of iron.

Once organ iron was depleted in our study animals, a pattern of intra-organ prioritization was defined that was different in the skeletal muscle and in the heart. In skeletal muscle, cytochrome c and myoglobin concentrations both decreased proportionally in a manner equivalent to reductions in iron concentrations. This suggests that both oxygen delivery and energy production are compromised by neonatal iron depletion and both are necessary to maintain muscle contractility (28). Similar results have been obtained in iron-deficient postweanling rats (2, 6). In contrast, as myocardial iron concentration decreased, cytochrome c concentration was relatively preserved while myoglobin decreased in proportion to the degree of iron deficiency. Thus it
appears that cellular energetics may be favored over oxygen delivery in the newborn animal.

Our findings differ from those previously described for the skeletal muscle and myocardium in hypoxic iron-deficient fetal sheep (9), in hypoxic iron-deficient fetal guinea pigs (10), and in iron-deficient postweaning rats (2, 6, 12). In the fetal sheep, reductions in heart and skeletal muscle iron concentrations resulted in lower organ-specific cytochrome c concentrations (9). No preservation of heart cytochrome c concentration was seen in that study. Moreover, in the guinea pig, when fetal hypoxia and iron deficiency occurred simultaneously, the opposite pattern of prioritization was seen, i.e., myoglobin concentration was preserved at the expense of cytochrome c (10). Mixed results have also been found in the iron-deficient postweaning rat (2, 6, 12, 30). For example, Hagler et al. (12) demonstrated that myoglobin in the heart was preserved, but cytochrome c in the skeletal muscle and heart was not. Siimes et al. (30) also demonstrated preservation of abdominal muscle myoglobin with concomitant loss of hemoglobin and thigh muscle cytochrome c. These two studies indicate a higher prioritization of iron for myoglobin over cytochrome c in the iron-deficient state and are in direct contrast to the results of our current study of newborn lambs. Others have shown preferential loss of myocardial myoglobin in comparison to cytochrome c (2), a pattern more compatible with the results of our study. We speculate that multiple factors, including organ iron concentration, tissue oxygenation, and age and species of the animal, are involved in the regulation of intracellular iron allocation.

The study lambs did not show any protection of brain iron or cytochrome c with progressive iron deficiency. This is in direct contrast to studies in postweanling rats made iron deficient by diet (2, 3). Diversion of iron from vital, rapidly developing organs such as the brain is of concern because of the multiple iron-containing proteins critical to normal growth and development of the newborn brain. Perinatal iron deficiency results in central nervous system (CNS) hypomyelination (17), brain growth retardation (17), and significant lasting effects on cognitive behavior in rats (5, 36). Biochemically, the effect of CNS iron deficiency includes reductions in 8-9 desaturase and cytochrome b, both of which are involved in fatty acid synthesis for myelination (17), ribonucleotide reductase, which exerts controls on cell growth (32), and cytochrome c, which is involved in neuronal oxidative phosphorylation.

The findings of the current study may have implications for treatment of human infants. As demonstrated in the lambs, the potential for significant negative iron balance after a phlebotomy-induced 40–50% reduction in hemoglobin concentration depends on the strategy for iron repletion. Although red cell transfusion can restore iron lost by phlebotomy, the current trend in the neonatal intensive care unit to administer fewer transfusions will result in an exacerbation of negative iron balance (34). In human infant erythropoietin clinical trials and in studies of lambs treated with erythropoietin, the robustness of the response to an erythropoietic stimulus (either anemia stimulating endogenous erythropoietin secretion or exogenous erythropoietin administration) depends on the availability of plasma iron for immediate incorporation into hemoglobin (20, 26, 29). Iron sucrose delivered intravenously appears to be an effective means of supporting endogenous erythropoiesis. The current study illustrates the extent of negative iron balance that can be induced over a relatively brief period of time by chronic phlebotomy at rates similar to those clinically observed among preterm humans (15, 23).

Nevertheless, differences exist between our term lamb model and the preterm human infant in the neonatal intensive care unit. Prematurity and neonatal illness may have important, but as yet unknown, effects on erythropoietic capability. The term newborn lamb grows at a faster rate than even the healthy preterm newborn and thus expands its blood volume more rapidly. Because our study shows that iron is preferentially directed to red blood cells over all other organs, a greater rate of blood volume expansion in the lamb may put proportionally greater pressure on non-erythropoietic organs during iron-deficient states. Despite these potential limitations, the lamb has been a good model for fetal and perinatal erythropoietic experiments (9, 26).

The findings of our study indicate that allocation of iron among and within organs is different in the phlebotomized neonatal animal than in the animal made iron deficient by long-term dietary manipulation. The allocation of iron among organs is more similar to that observed in the fetal animal than in the adult, suggesting persistence of some or all of the regulatory mechanisms present before birth. In contrast, the prioritization of hemoprotein iron within heart and skeletal muscle appears to more closely resemble the pattern seen in postnatal animals. This suggests that newborn animals are in a transitional phase of iron regulation. The implications of these shifts in iron use with respect to the physiological function of the newborn may be considerable, as significant tissue iron deficiency affects organ growth and development. Because the phlebotomy protocol we used was not dissimilar to that which is commonly used in neonatal intensive care units, the effects of iron depletion on heart and brain iron concentrations are of particular concern.

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