BASIC SCIENCE ARTICLE

Reticulocyte hemoglobin content as an early predictive biomarker of brain iron deficiency

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BACKGROUND: Fetal and neonatal brain iron content is compromised at the time of anemia, suggesting that screening for iron deficiency by measuring hemoglobin is inadequate to protect the brain. Reticulocyte hemoglobin (Ret-He) reflects iron-deficient (ID) erythropoiesis prior to anemia.

METHODS: At postnatal day (P), 10 and 20 iron-sufficient rat pups were fostered to ID dams to produce a postnatal ID (PNID) group, which was compared to 20 iron-sufficient (IS) pups fostered by IS dams. Pups were assessed from P13 to P15 for hemoglobin, hematocrit, reticulocyte count, and Ret-He. Hippocampal iron status was assessed by transferrin receptor-1 (Tfrc-1) and divalent metal transporter-1 (Slc11a2) mRNA expression.

RESULTS: At P13, brain iron status was similar between groups; only Ret-He was lower in the PNID group. At P14, the PNID group had lower Ret-He, hematocrit, mean corpuscular volume (MCV), and reticulocyte percentage (RET%). Tfrc-1 expression was increased, consistent with brain iron deficiency. Both Ret-He and MCV correlated with brain iron status at P14 and P15.

CONCLUSIONS: Ret-He was the only red cell marker affected prior to the onset of brain ID. The clinical practice of using anemia as the preferred biomarker for diagnosis of iron deficiency may need reconsidering.

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INTRODUCTION

Iron deficiency before the age of 3 years leads to long-term neurobehavioral deficits despite iron treatment.¹ Iron deficiency is the most common single nutrient deficiency in the world, affecting two billion people, most of whom are women of child-bearing age and their children.² Over 50 studies have demonstrated the negative effect of early-life iron deficiency on neurobehavioral development.³ These effects are wide-ranging and include motor and cognitive deficits while the child is iron deficient (ID).⁴ As demonstrated by preclinical models, the neurobehavioral effects are due to brain tissue iron deficiency and not solely due to anemia.⁵ Of greater concern than the effects on brain function during the period of deficiency is the finding that early-life iron deficiency results in long-term neurobehavioral alterations that persist in spite of iron repletion.^{1,4,5} The hippocampus is particularly vulnerable to gestational and early postnatal iron deficiency (PNID), resulting in compromised learning and memory behavior.⁶

Because of these long-term effects, a public health strategy that aims to prevent brain iron deficiency is indicated. Yet, there are no biomarkers that index impending low brain iron status. The current screening and treatment strategy for iron deficiency by detecting and treating anemia does not ensure neuroprotection for three reasons. First, anemia is the end-stage state of iron deficiency. The developing brain is already ID by the time anemia is diagnosed due to prioritization of available iron to the red blood cells (RBC) over all other tissues during negative iron balance.^{7–9} Studies in multiple species including humans indicate that the brain becomes deficient prior to the onset of anemia.^{7–9} Second, brain iron deficiency independent of anemia causes neurological deficits.⁵ Studies in human infants and preclinical models

demonstrate that treatment after the appearance of anemia does not reliably prevent long-term neurological deficits.^{3,10–12} Third, conventional hematological markers of iron deficiency are based on population statistical cut-offs. They are not bioindicators of brain iron deficiency or brain dysfunction.¹² Given the global prevalence of iron deficiency and the societal cost of developmental disabilities across a human lifespan,^{2,13,14} a peripherally measurable biomarker that changes before the brain becomes ID is needed since it would lead to earlier iron repletion.

Reticulocyte hemoglobin content (reticulocyte hemoglobin equivalent: Ret-He) reflects the incorporation of iron into reticulocytes and thus gives insight into functional iron availability. It is easily obtained from whole blood and thus can be read out from instruments that are used to assess hemoglobin as part of routine screening of children. Studies in adults and children have shown that it is a potential marker of pre-anemic iron deficiency,^{14–16} but its relationship to the onset of brain iron deficiency is unknown and is not possible to assess in clinical studies.

The objectives of this study were to determine when Ret-He changes relative to the onset of anemia in a rat pup model of postnatal dietary iron deficiency and to determine whether Ret-He decreases prior to evidence of brain iron deficiency. The hippocampus is a brain region that is exquisitely sensitive to negative iron balance in the neonatal period and thus was utilized for assessing brain iron deficiency.^{17,18}

METHODS

The protocol was approved by the Institutional Animal Care Utilization Committee of the University of Minnesota.

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Study design

Sixteen timed-pregnant Sprague-Dawley rat dams were randomized to an iron sufficient (IS) (298 ppm elemental iron) or ID (3–6 ppm elemental iron) diet on gestational day 2. Dams delivered spontaneously. Pups from the eight IS dams were culled post delivery to an n of 8 per dam and reared by IS dams until postnatal day P10. At P10, these IS pups were randomized to either a dam on the IS diet or a dam on the ID diet. Randomized pups were selected from all litters to avoid litter effects. The intent of the study design was to model postnatal dietary iron deficiency in the human. Thus, P10 was chosen because it represents the rodent hippocampus development equivalent to a term newborn infant. A total of 48 pups were fostered across four IS and four ID dams to generate 24 ID and 24 IS pups.

Eight pups per dietary group had blood drawn and hippocampus tissue harvested at P13, P14, and P15 for assessment of hematologic parameters and brain gene expression, respectively. mRNA levels of genes indexing iron status and iron-dependent brain function were utilized to assess the brain at each timepoint.

Hematology assessment

Hemoglobin concentration, hematocrit, mean corpuscular volume, reticulocyte percentage (Ret%), and Ret-He were assessed using the Sysmex XT2000iV™ instrument. Blood was collected in EDTA vacutainer tubes and 85 μ L of whole blood was assayed in duplicate.

Brain mRNA assessment

mRNA levels were measured by quantitative polymerase chain reaction (MX3000P; Agilent, Santa Clara, CA), run in duplicate, and normalized to S18 as described before.⁵ Values from the IS group were standardized to 1 and the relative expression of the ID group was expressed as a ratio to that standard. Brain iron status was assessed by expression of the two neuronal iron transporters, transferrin receptor-1 (*Tfrc-1*) and divalent metal transporter-1 (*Slc11a2*). Previous studies have shown that *Tfrc-1* and *Slc11a2* gene expression are sensitive to physiologically relevant changes in brain iron status in neonatal rodents.^{5,18} Hippocampal mRNA expression of myelin basic protein (*Mbp*) and calcium calmodulin kinase 2- α (*Camk2-a*) were measured to assess the effects on two iron-dependent neurological processes, myelination and synaptic plasticity, respectively.^{19,20}

Statistical analysis

Mean $\pm$ SEM was calculated for each parameter on each day from both the ID and IS groups. Group means were compared by t test with an alpha set at 0.05. The relationship of brain iron status as assessed by *Tfrc-1* levels to Ret-He, MCV, and hemoglobin concentration on each day was assessed by regression analysis.

RESULTS

PNID reduced Ret-He in the rat pups on P13 prior to all other hematologic indices (Fig. 1). The mean $\pm$ SEM Ret-He was lower in the ID group than in the IS control at P13 (ID: 21.1 ± 0.8 pg vs IS: 24.3 ± 0.5 pg; $p = 0.007$) and remained so at P14 (ID: 15.5 ± 0.2 pg vs IS: 21.1 ± 0.5 pg; $p < 0.001$) and P15 (ID: 16.0 ± 0.4 pg vs IS: 21.8 ± 0.2 pg; $p < 0.001$). In contrast, hemoglobin concentration, hematocrit, MCV, and reticulocyte counts were not different between groups at P13. At P14, hematocrit, MCV, and reticulocyte count were lower in the ID group although the hemoglobin concentration remained similar between groups. At P15, all hematologic measurements were lower in the ID group.

Iron deficiency decreased hippocampal iron status starting at P14 (Fig. 2). At P13, neither *Tfrc-1* nor *Slc11a2* mRNA levels were elevated in the hippocampus of the ID group. *Tfrc-1* was 35% greater in the ID group at P14 compared with IS controls. The expression of both transporters was elevated at P15. This degree

of *Tfrc-1* elevation is consistent with a 20% reduction in brain iron concentration and changes to iron-sensitive synaptic plasticity genes.^{19–21} mRNA expression of *Mbp* and *Camk2-a* in the ID group were similar to control at P13, but both were reduced in the ID group at P14. *Mbp* mRNA expression remained low and declined further at P15 (Table 1).

At P13, there was no relationship between Ret-He and *Tfrc-1* levels (Fig. 3). At that time point, no Ret-He concentration was < 18 pg. At P14, a trend ($p = 0.066$) toward lower Ret-He concentrations associated with higher *Tfrc-1* levels was noted. This relationship was statistically significant at P15 ($p = 0.009$), with 42% of the variability of *Tfrc-1* levels accounted for by Ret-He. At P15, six of seven ID pups had Ret-He < 18 pg, whereas all eight pups in the IS group had Ret-He > 20.8 pg. MCV at P13 had no relationship to brain *Tfrc-1* levels (Fig. 3). Like Ret-He, lower MCV was correlated with higher *Tfrc-1* expression levels at P14 and at P15. All animals with a MCV value < 70 fL had evidence of brain iron deficiency. In contrast, hemoglobin concentration bore no relationship to *Tfrc-1* expression levels at P13, 14, and 15.

DISCUSSION

Iron deficiency is common in children below 3 years of age and leads to long-term motor and cognitive deficits.^{1,3,10} Thus, early detection and treatment of brain dysfunction should be the primary goal of screening for early-life ID. The American Academy of Pediatrics and World Health Organization currently endorse an anemia-based screening approach for iron deficiency by measuring the hemoglobin at 9–12 months of age in order to detect iron deficiency anemia.^{22,23} Yet, anemia is the end-stage state of iron deficiency due to interorgan prioritization of iron to the RBCs over other tissues, including the brain as demonstrated in multiple preclinical models.^{8,9,24,25} Screening for iron deficiency by measuring hemoglobin also fails to detect non-anemic iron deficiency, which is threefold more common than IDA even in the United States²⁶ and causes neurobehavioral abnormalities.^{27–29}

Prior reports in preclinical models of dietary iron deficiency result in both anemia and brain iron deficiency. Thus, it remained unclear whether the brain and red cells are compromised sequentially or concurrently.^{4,11,19–21} The current study utilized a classic neonatal rat pup model of postnatal dietary iron deficiency^{19–21} to demonstrate that brain iron status is compromised prior to the appearance of anemia. The study models the effects of low dietary iron intake beginning at term birth in the human. The P10 rat hippocampus is at a similar point in maturational development as the term-born human. Specifically, brain *Tfrc1* expression, a sensitive marker of iron deficiency^{5,18–20} was increased at P14 in the ID group, a day prior to the detection of a significant reduction of hemoglobin concentration in that group at P15. The brain development of the P15 rat is analogous to human brain development in the first 9 postnatal months.³⁰ Similar results indicating that brain iron deficiency occurs before anemia have been documented in young monkeys at 4 and 6 months of age.⁹ Extrapolating from these cross-species data, there may be value in screening human infants by measuring Ret-He as early as 6 months of age.

MCV has been previously used in combination with other non-hematologic markers such as serum ferritin, serum transferrin receptor, and percent of total iron-binding capacity saturation as a set of pre-anemic biomarkers in ID children with neurobehavioral deficits.^{29,31} In the current study, MCV was not altered until the brain was ID and thus was not an effective predictive marker of impending brain iron deficiency. However, MCV was an effective peripherally measured biomarker to reflect the degree of brain ID once that state was present. All animals with an MCV < 70 fL had evidence of brain iron deficiency and a significant linear relationship existed between MCV and the degree of brain iron deficiency. If a similar relationship exists in children, MCV may be an effective

Fig. 1 Hemoglobin concentration, MCV, reticulocyte count (%), and Ret-He in IS (white) and ID groups (black) at P13, 14, and 15. Values are mean $\pm$ SEM. $N = 6-8$ per group; * $p < 0.05$

Fig. 2 *Tfr-1* and *Slc11a2* expression in the hippocampus of IS (white) and ID (black) rat pups at P13, 14, and 15. Values are mean $\pm$ SEM. $N = 6-8$ per group; * $p < 0.05$

Table 1. Hippocampus mRNA expression of *Tfrc-1*, *Mbp*, and *Camk2-a* at P13, 14, and 15 in the ID and IS groups

Genes	P13		P14		P15	
	IS	ID	IS	ID	IS	ID
<i>Tfrc-1</i>	1.0 ± 0.06	1.09 ± 0.08	1.0 ± 0.09	1.35 ± 0.06 ^a	1.0 ± 0.05	1.24 ± 0.04 ^a
<i>Mbp</i>	1.0 ± 0.17	0.95 ± 0.09	1.0 ± 0.08	0.78 ± 0.05 ^a	1.0 ± 0.17	0.54 ± 0.09 ^a
<i>Camk2-a</i>	1.0 ± 0.03	0.99 ± 0.08	1.0 ± 0.03	0.72 ± 0.04 ^b	1.0 ± 0.11	0.86 ± 0.06

Values are mean ± SEM, $n = 6-8$. Data are shown as the ratio of IS group at each age

^aDifferent from IS at a given age at $p < 0.05$

^bDifferent from IS at a given age at $p < 0.001$

Fig. 3 Relationship of brain iron status as indexed by hippocampus *Tfrc-1* expression as a function of Ret-He, MCV, and hemoglobin concentration at P13, 14, and 15. ID group pups are designated by (x) and IS pups by (.). For Ret-He, no relationship was present at P13 ($r^2 = 0.02$; $p = 0.63$), but an increasingly significant negative relationship was found with advancing age; P14 ($r^2 = 0.30$; $p = 0.066$) and P15 ($r^2 = 0.42$; $p = 0.009$). For MCV, no relationship was present at P13 ($r^2 = 0.02$; $p = 0.65$), but significant negative relationships were present at P14 ($r^2 = 0.39$; $p = 0.03$) and P15 ($r^2 = 0.63$; $p < 0.001$). No significant relationship was present for hemoglobin at P13 ($r^2 = 0.00$; $p = 0.87$), P14 ($r^2 = 0.10$; $p = 0.33$), or P15 ($r^2 = 0.20$; $p = 0.09$)

way of judging brain iron status in the context of non-anemic iron deficiency. Non-anemic iron deficiency is far more common than iron deficiency anemia and is associated with behavioral deficits.²⁷⁻²⁹

An important value of a biomarker resides in its ability to predict the risk of disease prior to disease onset.¹² Ret-He was the only hematologic marker that changed prior to the onset of brain iron deficiency and the reduction of markers of iron-dependent brain function. The lack of a relationship at P13 between Ret-He, which

had already decreased in the ID group and brain iron status, which was preserved, is consistent with that premise. Once brain iron deficiency was present at P14 and P15, a strong correlation was found between the degree of brain iron deficiency and the peripherally measured Ret-He value. At P15, Ret-He values < 20 pg were found in all of the animals with brain iron deficiency, while none of the IS animals had such low values, suggesting that measurement of Ret-He could distinguish brain iron status. The absolute values for Ret-He and MCV found in rat pups in this study

cannot be directly translated to values in human preterm or term infants because of the interspecies differences in normal values.^{15,16} Nevertheless, the data in our preclinical model point to the principle of identifying a peripherally measured biomarker “action point” for initiating iron treatment in order to prevent brain iron deficiency. The specific action point value of Ret-He in humans of various ages remains to be determined.

Another important attribute of a biomarker is dose-responsiveness.³² Ret-He, as well as MCV, demonstrated this characteristic in that abnormalities in both markers correlated with the degree of brain iron deficiency once that deficiency was present at P14 and P15. Both also demonstrated an increasingly stronger relationship with brain iron status as the iron deficiency progressed.

The sensitivity of Ret-He to compromised iron status likely stems from its ability to index iron availability for incorporation into hemoglobin specifically in a dynamic population of developing red cells. Iron begins to be incorporated into developing red cells within 24 h following an erythropoietic stimulus with the appearance of reticulocytes at 48–96 h.²⁴ A significant increase in hemoglobin status, however, is not seen for up to a week or 10 days.²⁴ Measurement of Ret-He thus gives an earlier insight into iron-dependent hemoglobinization and thus may be more sensitive to changes in iron availability. This sensitivity was evident in the current study and demonstrated that both red cells and the brain are compromised by iron deficiency and that red cell compromise was evident and detectable prior to brain compromise.

It is unclear from this study when Ret-He is affected relative to other non-red cell-based indices such as serum ferritin, serum transferrin receptor, or percent of total iron-binding capacity saturation. Similarly, it is not known whether starting iron treatment at the onset of the Ret-He decline, e.g., P13, would prevent brain iron deficiency. Future studies need to address these questions, but a major practical advantage of Ret-He over serum-based assessments is that it can be performed on the same sample as hemoglobin concentration or MCV.

Overall, this study shows that Ret-He identifies individuals at risk for brain iron deficiency in the pre-anemic stage. In addition, both Ret-He and MCV index the degree of brain iron deficiency once that deficiency is present. In contrast, hemoglobin concentration indexed neither. If similar relationships between standard hematologic and brain iron assessments exist in children as we have documented in the rat, the current recommendation of using hemoglobin at 9–12 months of age as the biomarker for diagnosis and treatment of ID in children needs to be reconsidered.^{20,21} Ret-He may be more sensitive than hemoglobin and MCV for detecting impending brain iron deficiency.

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ADDITIONAL INFORMATION

Competing interests: The authors declare that they have no competing interests.

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