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Chapter 11

Iron status in infants with alloimmune hemolytic disease in the first three months of life

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Submitted

Abstract

Background and Objectives

Ferritin levels are often highly elevated at birth in neonates with alloimmune hemolytic disease of the fetus and newborn (HDFN). Data on ferritin levels in these infants in the first three months of life are lacking. Objective of this study was to examine the course of iron status and incidence of iron deficiency and overload in neonates with alloimmune HDFN up to three months of age. Secondary objective was to analyze bilirubin levels, liver enzymes and red blood cell indices in the same time period and the association with intrauterine transfusion (IUT).

Materials and Methods

Observational study of neonates with alloimmune HDFN admitted to our center between November 2010 and March 2012. Data on iron status, bilirubin levels, liver enzymes and red blood cell indices up to three months of age were collected prospectively and compared between neonates treated with and without IUT.

Results

Thirty-five infants with alloimmune HDFN were included. Iron overload occurred in 70% of neonates at birth and in 50% and 18% at the age of one and three months, respectively. No cases of iron deficiency at birth and only one case of iron deficiency at three months of age were found. No infants received iron therapy. Infants who received IUT had a significantly lower hemoglobin level and reticulocyte count and higher ferritin level at birth.

Conclusion

The vast majority of neonates with alloimmune HDFN have iron overload at birth. Incidence of iron overload gradually decreases within the first three months without iron supplementation.

Introduction

Iron supplementation therapy is occasionally given to support erythropoiesis in neonates with hemolytic disease of the fetus and newborn (HDFN) due to red cell alloimmunization.¹ However, iron stores in neonates with alloimmune HDFN are often not depleted at birth. On the contrary, ferritin levels in fetal and cord blood are often highly elevated.²⁻⁴ Increased fetal and neonatal iron stores are most probably caused by the increased rate of fetal hemolysis.^{4,5} Moreover, intrauterine red blood cell transfusions (IUTs) and/or postnatal red blood cell transfusions (top-up transfusions) can further increase iron overload.^{6,7} Iron overload can cause damage to the liver, heart and endocrine organs, alter immune response and increase susceptibility to infection.⁸ Nevertheless, iron is also crucial for early brain development and function. Iron deficiency as well as iron overload have been associated with neurodevelopmental impairment.⁸

Iron overload is most accurately diagnosed by measuring tissue iron using liver (and cardiac) biopsies.⁹ However, these procedures are invasive, expensive and difficult to perform in neonates. Magnetic resonance imaging (MRI) and magnetic susceptibility are noninvasive methods to measure tissue iron stores, but are also expensive and complex.¹⁰ Serum ferritin, which reflects total body iron capacity, is the most convenient and cost-effective technique to diagnose iron overload. An important limitation of serum ferritin levels as the sole marker for iron overload is that they can be influenced by inflammation, infection, liver damage, hemolysis, ineffective erythropoiesis and ascorbate deficiency independently of changes in body iron.^{9,10}

Although it is known that ferritin levels at birth are frequently elevated, the course of iron status after birth in infants with HDFN is not clear. Primary aim of this study was to examine the course of iron status in infants with alloimmune HDFN. Secondary aim was to measure the course of bilirubin levels, liver enzymes, red blood cell indices, the incidence of iron overload and cholestatic disease and the association with IUT.

Materials and Methods

All term and preterm neonates with HDFN due to maternal red cell alloimmunization admitted to our center between November 2010 and March 2012 were eligible for this prospective observational study. The Leiden University Medical Center (LUMC) is the national referral center for the management and intrauterine treatment of HDFN. Informed consent was obtained to collect data from outpatient clinics. We recorded the following obstetric and neonatal data: type of red cell alloimmunization, number of IUTs, gestational

age at birth, birth weight, small for gestational age (SGA, defined as below the 10th percentile according to Kloosterman et al.¹¹), presence of hydrops at birth, hemoglobin (Hb) level, reticulocyte count, iron status (serum iron, ferritin, transferrin and total iron binding capacity (TIBC)), liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γGT) and lactate dehydrogenase (LDH)) and total serum bilirubin (TSB) and conjugated bilirubin levels at birth. We also recorded duration of phototherapy, number of exchange transfusions (ETs), number of top-up transfusions and days after birth until the last top-up transfusion was given. In addition, we recorded whether infants had sepsis during admission. We prospectively collected data on iron status, bilirubin levels, liver enzymes (measured monthly) and Hb level and reticulocytes (measured weekly) up to three months of age. We collected these data and the number of top-up transfusions received during the first 3 months of life and Hb levels prior to the top-up transfusions through correspondence with the clinical chemistry and/or transfusion laboratories of the outpatient clinics. A uniform transfusion trigger according to our transfusion guidelines was followed. After initial discharge from the LUMC, top-up transfusions were performed when Hb levels fall below 8.0 g/dL or at higher levels (<9.8 g/dL) if clinical symptoms of anemia (lethargy, feeding difficulties or failure to thrive) were present. Iron supplementation was not used; however folic acid (50 µg/day) was administered orally in all infants during the first three months of life.

At birth, iron overload is defined as a serum ferritin level above the 95th percentile and iron deficiency as a serum ferritin level below the 5th percentile according to a recent study of Siddappa et al.⁸ In their study, the 95th percentile for term infants (≥37 weeks of gestation) was 309 µg/L and for preterm infants (<37 weeks of gestation) 267 µg/L. The 5th percentile was 40 µg/L for term infants and 35 µg/L for preterm infants.⁸ From the first day of life until the age of three months, iron overload and iron deficiency were defined as a ferritin concentration above the 97.5th percentile (775 µg/L) and below the 2.5th percentile (40 µg/L) respectively, according to a study by Soldin et al.¹²

To enhance specificity of high ferritin levels in diagnosing iron overload, transferrin saturation (TSAT) is also calculated (serum iron divided by TIBC and multiplied by 100) in infants with iron overload based on the abovementioned definitions. Iron overload is suggested by a TSAT level of >60% based on the 95th percentile measured in premature newborns of 35-36 weeks of gestation by Lackmann et al.¹³

Cholestasis is defined as a conjugated serum bilirubin concentration above 1.0 mg/dL if the total serum bilirubin level is less than 5 mg/dL, or a value of conjugated bilirubin that represents more than 20% of the total bilirubin if the total bilirubin is greater than 5 mg/dL.¹⁴

Data are reported as means and standard deviations (SD) or as medians and interquartile ranges (IQR). Statistical analysis was performed using Student-t test and Mann-Whitney test for continuous variables and Chi-square and Fisher's-exact test for categorical variables. A p-value <0.05 was considered to indicate statistical significance. The associations between iron status parameters and top-up transfusions were examined by Spearman correlation coefficients. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

Results

During the study period, 35 neonates with HDFN due to red cell alloimmunization were admitted to our nursery. Characteristics of included patients are summarized in Table 1. Antibody specificities of included patients were D (n=19), Kell (n=5), E (n=4), c (n=1), D,C (n=1), C,D (n=1), C,E (n=1), D,W^ra (n=1), Kell,c (n=1) and D,C,E (n=1). Six percent (2/35) of infants were SGA, of whom one was treated with IUT. Eighty percent (28/35) of infants received one or more top-up transfusions and 71% (20/28) received their first top-up transfusion within one month after birth.

Table 1. Characteristics of study patients

	n = 35
Neonates treated with IUT, n (%)	22 (63)
Number of IUTs ^a	2 (1-3,25, 1-5)
Gestational age at first IUT, weeks ^a	29.8 (23.8-31.2, 18.29-34.29)
Gestational age at birth, weeks ^a	36 (35-37,33-38)
SGA, n (%)	2 (6)
Birth weight, kg ^b	2.88 ± 0.53
Male, n (%)	20 (57)
Neonates treated with phototherapy, n (%)	35 (100%)
Days of phototherapy per infants ^b	4.49 ± 1.93
Neonates treated with ET, n (%)	9 (25.7)
Number of ETs ^a	1 (1-1.5,1-2)
Neonates treated with top-up transfusions, n (%)	28 (80)
Number of top-up transfusions ^a	2 (1-3,1-6)
Days after birth until first top-up transfusion ^a	17 (3-31, 0-42)
Days after birth until last top-up transfusion ^a	34.5 (25.5-60.5, 0-79)

^a Value given as median (IQR, range)

^b Value given as mean ± SD

Table 2 provides an overview of the course of iron status, bilirubin levels (total and conjugated) and liver enzymes measured at birth, at one month, at two months and three months after birth. Evaluation of all parameters at each time point was often incomplete. Median ferritin level was 442 µg/L at birth (T=0), 763 µg/L at one month (T=1), 630 µg/L at two months (T=2) and 417 µg/L at three months of age (T=3). Minimum and maximum ferritin levels were 103 µg/L and 1211 µg/L at T=0, 154 µg/L and 2700 µg/L at T=1, 70 µg/L and 1504 µg/L at T=2 and 33 µg/L and 1100 µg/L at T=3.

Table 2. Course of iron status, bilirubin, liver enzymes and red blood cell indices in infants with alloimmune HDFN

	T=0 (at birth)	T= 1 (at one month)	T=2 (at two months)	T=3 (at three months)
Ferritin level, µg/L ^a	442 (242-710)	763 (413-936)	630 (311-935)	417 (184-704)
Iron level, µmol/L ^b	34.0 ± 7.5	20.4 ± 7.0	14.2 ± 7.7	12.7 ± 6.1
TIBC, µmol/L ^c	33 (29.3-37)	36 (30-40)	48 (40-53)	n=2: 44 and 49
Transferrin, g/L ^d	1.61 (1.45-1.86)	1.50 (1.30-1.60)	1.70 (1.42-1.94)	2 (1.70-2.24)
TSB level, µmol/L ^e	77 (62-117)	28 (13-52)	12 (7-16)	8 (5.3-10)
Conjugated bilirubin level, µmol/L ^f	10 (8-12)	8 (4.3-16)	6 (1-12)	2 (0.5-12.5)
AST, U/L ^g	34 (26-48)	33 (22-43)	39 (21-51)	39 (26-56.5)
ALT, U/L ^h	12 (8.5-15)	24 (17-28.5)	33 (25-66)	39.5 (24-56)
γGT, U/L ⁱ	126 (73.5-212)	80 (51-116)	51.5 (38.5-83.3)	29 (21.5-40.0)
LDH, U/L ^j	434 (343.5-635.5)	253 (233.5-309.5)	257.5 (206.5-291)	236 (196-290)
Hb level, g/dL ^k	12.9 (11.0-15.8)	8.5 (7.9-9.5)	8.9 (8.2-10.0)	9.8 (9.3-10.8)
Reticulocyte count, % ^l	57 (16-101)	4.5 (2-22.8)	27 (13.3-41.1)	22.8 (20.2-35.7)

Values are given as mean ± SD or median (IQR). Hb = hemoglobin.

^a Measured in 33, 20, 12 and 11 infants at T = 0, 1, 2, and 3, respectively. ^b Measured in 31, 18, 12 and 10 infants at T = 0, 1, 2, and 3, respectively. ^c Measured in 32, 7, 5 and 2 infants at T = 0, 1, 2, and 3, respectively. ^d Measured in 32, 19, 11 and 9 infants at T = 0, 1, 2, and 3, respectively. ^e Measured in 35, 23, 11 and 9 infants at T = 0, 1, 2, and 3, respectively. ^f Measured in 31, 19, 5 and 5 infants at T = 0, 1, 2, and 3, respectively. ^g Measured in 33, 19, 12 and 9 infants at T = 0, 1, 2, and 3, respectively. ^h Measured in 33, 21, 11 and 10 infants at T = 0, 1, 2, and 3, respectively. ⁱ Measured in 34, 23, 10 and 10 infants at T = 0, 1, 2, and 3, respectively. ^j Measured in 29, 17, 12 and 9 infants at T = 0, 1, 2, and 3, respectively. ^k Measured in 35, 29, 22 and 15 infants at T = 0, 1, 2, and 3, respectively. ^l Measured in 35, 22, 18 and 14 infants at T = 0, 1, 2, and 3, respectively.

Table 3. Influence of intrauterine transfusions on iron status, liver enzymes and red cell indices at birth and at one month after birth

	T=0 (at birth)			T= 1 (at one month)		
	IUT	No IUT	P- value	IUT	No IUT	P-value
Ferritin level, µg/L ^a	598 ± 249	270 ± 111	< 0.001	815 (720-982)	434 (315-863)	0.181
Iron level, µmol/L ^b	33 (30.5-37)	29 (26-41.5)	0.367	22.1 ± 5.7	17.8 ± 8.4	0.216
Transferrin, g/L ^c	1.65 ± 0.33	1.71 ± 0.29	0.606	1.46 ± 0.38	1.55 ± 0.14	0.577
TSB, µmol/L ^d	101.5 (60-135.5)	73 (65-110)	0.389	29 (19.5-82)	20 (12-47)	0.280
Conjugated bilirubin, µmol/L ^e	10 (8-13.25)	8 (7.5-9.5)	0.070	8 (4.075-25)	8 (7-11)	0.902
LDH, U/L ^f	434 (332-719)	450 (344-522)	0.830	238 (234-264)	306 (223-379)	0.481
AST, U/L ^g	27.5 (24.8-48.8)	39 (32-50)	0.233	32.5 (22.3-41.5)	35 (18-43)	0.711
ALT, U/L ^h	11.5 (8-14.3)	14 (9-18)	0.355	24 (17-27.5)	25 (12.8-35.0)	0.547
γGT, U/L ⁱ	134.5 (73.5-207.5)	97.5 (71.5-238.8)	0.958	80 (52-116)	82.5 (36.8-180.3)	0.776
Hb, g/dL ^j	12.0 (10.5-13.5)	14.5 (12.6-16.9)	0.026	8.5 ± 1.4	8.9 ± 1.3	0.469
Reticulocyte count, % ^k	24 (2-142)	73 (59.5-124)	0.004	4 (2-20.5)	15.9 (2.75-34)	0.231

Values are given as mean ± SD or median (IQR). Hb = hemoglobin.

^a Measured in 21 (IUT group) and 12 infants (no IUT group) at birth and in 12 (IUT group) and 8 (no IUT group) infants at one month.

^b Measured in 21 (IUT group) and 10 infants (no IUT group) at birth and in 11 (IUT group) and 7 (no IUT group) infants at one month.

^c Measured in 21 (IUT group) and 11 infants (no IUT group) at birth and in 11 (IUT group) and 8 (no IUT group) infants at one month.

^d Measured in 22 (IUT group) and 13 infants (no IUT group) at birth and in 12 (IUT group) and 11 (no IUT group) infants at one month.

^e Measured in 22 (IUT group) and 9 infants (no IUT group) at birth and in 12 (IUT group) and 7 (no IUT group) infants at one month.

^f Measured in 21 (IUT group) and 8 infants (no IUT group) at birth and in 9 (IUT group) and 8 (no IUT group) infants at one month.

^g Measured in 22 (IUT group) and 11 infants (no IUT group) at birth and in 12 (IUT group) and 7 (no IUT group) infants at one month.

^h Measured in 22 (IUT group) and 11 infants (no IUT group) at birth and in 13 (IUT group) and 8 (no IUT group) infants at one month.

ⁱ Measured in 22 (IUT group) and 12 infants (no IUT group) at birth and in 15 (IUT group) and 8 (no IUT group) infants at one month.

^j Measured in 22 (IUT group) and 13 infants (no IUT group) at birth and in 19 (IUT group) and 10 (no IUT group) infants at one month.

^k Measured in 22 (IUT group) and 13 infants (no IUT group) at birth and in 16 (IUT group) and 6 (no IUT group) infants at one month.

Table 3 shows the comparison of parameters measured at birth and at one month after birth between infants treated with or without IUT. Ferritin level at birth was significantly higher in infants treated with IUT than in infants not treated with IUT (mean $\pm$ SD: 598 ± 249 and 270 ± 111 , respectively, $p < 0.001$). Hb level and reticulocyte count at birth were significantly lower in infants treated with IUT (median (IQR): 7.45 (6.5-8.4) and 9 (7.8-10.5), $p = 0.026$ and 24 (2-142) and 73 (59.5-124), $p = 0.004$, respectively). At one month of age, no significant differences were found between the groups treated with or without IUT.

Seventy percent of neonates (23/33) had iron overload at birth and 83% (19/23) of these infants had been treated with IUT. Twenty percent of neonates (2/10) without iron overload at birth had been treated with IUT. Iron overload was present in 10/20 (50%) infants at one month, in 5/12 (42%) infants at two months and 2/11 (18%) infants at three months of age. In all neonates with iron overload at birth TSAT was $>60\%$ (mean of 86%), except one neonate with a TSAT of 44%. The TSAT was measured in only two of ten neonates with iron overload at one month of age (54% and 73%), in only one of five neonates with iron overload at the age of two months (24%) and in none of the neonates with iron overload at the age of three months.

None of the infants had iron deficiency at birth. However, one term infant gradually developed iron deficiency after birth (ferritin level at birth: 145 $\mu\text{g/L}$, at T=1: 154 $\mu\text{g/L}$, at T=2: 70 $\mu\text{g/L}$, at T=3: 33 $\mu\text{g/L}$). This male infant with Rh D alloimmune HDFN had received one IUT and no top-up transfusions. Serum iron level at 3 months was 9 $\mu\text{mol/L}$ and serum transferrin level 2.40 g/L.

Five of 35 neonates (14%) had cholestasis during admission and those five neonates all had iron overload at birth with ferritin levels varying from 322 $\mu\text{g/L}$ to 1211 $\mu\text{g/L}$. Four of five cholestatic neonates had Rh D HDFN, one had Kell HDFN, and four of these five had been treated with IUT.

Only one infant (treated with ET) had signs of neonatal sepsis during admission with a positive blood culture for *Staphylococcus Aureus*. After exclusion of this infant, ferritin level at birth remained significantly higher in infants treated with IUT than those not treated with IUT (598 ± 249 and 269 ± 116 respectively, $p < 0.001$).

There were no significant correlations between the number of top-up transfusions and ferritin level, serum iron, TIBC and transferrin measured at one month after birth (data not shown).

In infants who did not receive top-up transfusions, ferritin levels at one month, two months and three months after birth varied between 154-715 ($n=4$), 70-435 ($n=3$) and 33-122 ($n=2$), respectively.

Discussion

This study shows the course of iron status after birth until the age of three months in infants with alloimmune HDFN. Based on our definitions, we found that 70% had iron overload and none had iron deficiency at birth. After birth, the incidence of iron overload gradually decreased from 50% at the age of one month to 18% at the age of three months.

The high ferritin levels at birth in our study group are consistent with a previous small study by Berger et al. They found a mean ferritin level of 372 µg/L at birth in 14 neonates with Rh D hemolytic disease of whom 9 (64%) were treated with IUT. In addition, the mean serum iron level at birth and the number of infants treated with IUT in our study are also comparable with their study.³ IUTs are associated with increased fetal ferritin levels, but ferritin levels were also increased in fetuses with Rh alloimmune hemolytic disease before the first IUT in a study by Nasrat et al.⁴ The significantly higher ferritin levels at birth in IUT treated neonates in our study are consistent with the findings of Nasrat et al. In a study by Aygun et al. cord blood ferritin levels in infants with Rh D HDFN were also significantly higher than levels in a control group. However, they found no difference in ferritin levels between neonates with Rh D HDFN treated with or without IUT.²

There is a number of case reports published on the risk of severe iron overload, diagnosed by liver biopsies, following IUTs for Rh HDFN.^{6,7,15,16} These infants were all born at 33 or 34 weeks of gestation and received 2-5 IUTs and several postnatal transfusions. Their serum ferritin levels ranged from 2479 to 28800 µg/L.^{6,7,15,16}

In addition to transfusions for alloimmune HDFN, the hemolysis itself can also contribute to iron overload in alloimmune HDFN. Abbas et al. measured maternal and fetal serum ferritin concentrations at 18-38 weeks of gestation in 40 red alloimmunized pregnancies and compared them to those of normal pregnancies. They found that serum ferritin concentrations are increased in the fetus and decreased in the mother. Their explanation for these findings was enhanced transplacental iron transfer despite fetal iron overload due to the extravascular hemolysis similar to increased iron absorption from the gastrointestinal tract in certain types of postnatal hemolytic anemia.⁵

Follow up ferritin levels are described in several studies in term and preterm neonates without alloimmune HDFN.¹⁷⁻¹⁹ However, no studies report ferritin levels for neonates with alloimmune HDFN after birth. In a study by Mukhopadhyay et al. the median ferritin level in 50 term (≥37 weeks of gestation) appropriate for gestational age neonates at birth was 141 µg/L and mean level at one month of age was 226 µg/L.¹⁹ In our study group, ferritin levels at one month after birth were higher than in their study group of neonates without alloimmune HDFN. However, in their study none of the infants received a red blood cell

transfusion in contrast to our study in which 80% of infants were transfused postnatally of whom 71% were transfused within one month after birth.¹⁹ Another difference between both studies was the gestational age of participants. In our study also premature infants were included. Because most of fetal iron is transported from mother to fetus in the third trimester of pregnancy, prematurely born infants have lower iron stores at birth.⁸ Schiza et al. showed that serum ferritin levels decreased from the age of 2 weeks to 6 months and were stable thereafter in late premature infants (34-36 weeks of gestation). At the chronological age of three months they found a mean ferritin level of 70 ($\pm$ 68) μ g/L. Soldin et al. summarized serum ferritin levels in 800 in- and outpatient participants. Reference intervals for ferritin levels in males and females of 0-90 days of age were 40-775 and 79-501 μ g/L, respectively.¹²

In recent literature, a ferritin level <12 μ g/L is used for the definition of iron deficiency during the first year of life.^{17,20} Based on that definition, no cases of iron deficiency were present until three months of age in our study group. Based on the lower reference range (40 μ g/L) in the study of Soldin et al., only one neonate met the definition of iron deficiency. This male term infant with Rh D HDFN received one IUT but no postnatal top-up transfusions. Whether this neonate may have benefited from iron supplementation is not clear.

The incidence of cholestasis in this study is similar as reported in a previous study from our group.²¹ In the previous report IUT and Rh D type of alloimmune HDFN were identified as risk factors for cholestasis. In the present study, the majority of cholestatic neonates was also treated with IUT and had Rh D type of alloimmunization. In addition, all five cholestatic neonates had iron overload at birth. Although the numbers are small, these results are consistent with the previously published hypothesis that iron overload can cause cholestasis in neonates with alloimmune HDFN.²¹

This is the first study describing follow up data on iron status in neonates with alloimmune hemolytic disease exclusively. However, conclusions are limited by the relative small numbers of participants. In addition, iron overload is difficult to diagnose in neonates and ferritin levels could have been influenced by infection, liver damage, ineffective erythropoiesis, and ascorbate deficiency.^{9,10} Follow up studies should also take into account factors that can influence neonatal iron status such as maternal diabetes, pregnancy induced hypertension, maternal smoking, severe maternal iron deficiency, intrauterine growth restriction and type of feeding of the infants (formula or breastfeeding).⁸

In conclusion, iron deficiency in the first three months of life is very rare among infants with alloimmune HDFN and does not occur in infants who received top-up transfusions postnatally. On the contrary, iron overload occurs in 70% of neonates with alloimmune

HDFN at birth, 50% at the age of one month and 18% at the age of three months. Therefore, we advise to measure iron status and we discourage the use of iron supplementation in the first three months of life in neonates with alloimmune HDFN. Hemolysis and intrauterine and postnatal transfusions probably both contribute to the high incidence of iron overload in alloimmune HDFN. Further studies are required to confirm our findings and to study the long term effects of iron overload in neonates with alloimmune HDFN.

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