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Title: Hematological outcome in neonatal alloimmune hemolytic disease
Issue Date: 2013-03-07
Chapter 12

Summary and general discussion
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This thesis investigated the management and outcome of neonates with hemolytic disease of the fetus and newborn (HDFN) due to red cell alloimmunization. The focus of this thesis was mainly on the hematological complications and transfusion practices rather than on the management and treatment options of hyperbilirubinemia.

Hematological complications of HDFN include (early and late) anemia, thrombocytopenia, leukocytopenia, coagulation disorders and iron overload. An overview of the literature on these hematological complications and its treatment options in neonates with HDFN due to red cell alloimmunization was given in Chapter 2.

Anemia necessitating top-up transfusions in red cell alloimmune hemolytic disease can be present in the first three months of life. Early onset anemia (within 7 days after birth) is mainly caused by antibody induced hemolysis of red blood cells (RBCs) and characterized by a high bilirubin level and an increased reticulocyte count. Late onset anemia can be subdivided in “late hyporegenerative anemia” characterized by inappropriate erythropoiesis and “late anemia of hemolytic disease” with an active bone marrow and normal or high reticulocyte counts.\(^1\) However, this subdivision is theoretical and in practice these forms can coincide.

Severity and duration of anemia can be difficult to predict in individual cases and as a result frequent monitoring of hemoglobin level and reticulocyte count is required. Factors that are associated with a more severe course of anemia include on one hand a high hemolytic potential of the antibodies (which is determined by the strength (titer) and IgG-subclass) and on the other hand the antigen density of the target antigen on fetal/neonatal red cells or erythrocyte precursor cells. Also treatment with intrauterine transfusion (IUT) by the mechanism of suppression of fetal erythropoiesis contributes to neonatal anemia.\(^2,^3\) In contrast, exchange transfusion (ET) was associated with less occurrence of late anemia in a study of 36 infants with Rh D HDFN, probably due to the elimination of antibodies from the circulation which reduces hemolysis and anemia.\(^1\)

Exchange transfusions

In Chapter 3 we studied the influence of ET practices in a large group of near-term neonates with Rh hemolytic disease. In 2005 a more restrictive ET guideline, based on the American Academy of Pediatrics (AAP) guidelines, was implemented at our center. After implementation of that guideline, ET rate decreased considerably from 66% to 26%. The rate of top-up transfusion however was increased from 68% to 81% after guideline change.
In addition, we found a significant negative correlation between the number of ETs and the number of top-up transfusions in those infants treated with IUT. These results suggest that removal of antibodies and replacing Rh positive cells by Rh negative cells through ET is particularly effective in reducing anemia in the more severely affected neonates with high antibody titers.

In 1925 the first successful ET was performed by Hart for the treatment of severe jaundice in a newborn infant. However, it was until the 1940s that exchange transfusions became common practice in the treatment of neonates with HDFN. ET has more beneficial effects in addition to removing excess bilirubin. ET also removes part of the antibodies but most importantly, similarly as with IUT, the infants’ blood is replaced by red cells that are immunologically compatible with mother and infant, hence not destructed by the action of remaining antibodies. Although ET carries less risk than IUT, ET remains an invasive procedure with a significant risk of side effects. Although reported mortality rates are less than 2%, morbidity rates can be as high as 74% depending on how adverse events are defined and the population in which they are measured. Adverse events include cardio-respiratory events (apneas, cardiac arrest, hypo- and hypertension, cardiac rhythm disorders, pulmonary hemorrhage) catheter-related complications (sepsis, malposition, thrombus), those related to the use of blood products (infection, graft versus host reactions), metabolic derangements (acidosis, disturbances of serum calcium, glucose, potassium and sodium) and other serious complications such as necrotizing enterocolitis, and bowel perforation. We performed a retrospective study to evaluate the type and rate of ET-associated complications in a large series of neonates with HDFN due to red cell alloimmunization exclusively (Chapter 4). Comparison between ET-treated neonates and non-ET-treated neonates showed that ET treatment was independently associated with an increased risk on: clinical and blood-culture positive sepsis (8% versus 1%, OR 8.3, 95% CI 1.7-40.3, p = 0.009), leukocytopenia (88% versus 23%, OR 36.0, 95% CI 17.5-73.8, p = <0.001), severe thrombocytopenia (platelet count <50 × 10^9/L) (63% versus 8%, OR 31.4, 95% CI 14.0-70.4, p = <0.001), hypocalcemia (22% versus 1%, OR 27.4, 95% CI 5.9-126.8) and hypernatremia (8% versus 0%, p = <0.001). Severe symptoms of hypernatremia (seizures) did not occur and neonatal death did also not occur in the group treated with ET. In our center platelet transfusions are given when platelet counts fall below 100 × 10^9/L before planned ET and <50 × 10^9/L after ET. In the ET-treated group only one infant with severe thrombocytopenia showed an intracerebral bleeding (ICB) on cranial ultrasound. In the non-ET-treated group three neonates had signs of ICB on cranial ultrasound and/or MRI of whom one had severe thrombocytopenia for which he received 4 platelet transfusions. We found no difference in mortality and severe morbidity (ICB) between ET- and non-ET-treated neonates.
Intravenous immunoglobulin

Since ET can be associated with significant procedure-related morbidity, alternatives to prevent ET or to optimize the effect of ET have been investigated. These alternatives include (maternal) phenobarbital, albumin, metalloporphyrins, clofibrate and IVIg.\textsuperscript{16} Except for the latter, routine use of these alternatives are not recommended because evidence on the effectiveness is too limited or treatment has been associated with serious adverse effects.\textsuperscript{16-19} Although the exact mechanism of IVIg remains unclear, IVIg might reduce the rate of hemolysis in alloimmune HDFN by blockade of Fc-receptors involved in phagocytosis of antibody coated RBCs. In particular in Rh D antagonism, RBCs are destroyed by an antibody-dependent cellular cytotoxic (ADCC) mechanism mediated by Fc-receptor bearing cells of the neonatal reticulo-endothelial system.\textsuperscript{20} The 2004 guidelines of the AAP recommend the use of 0.5-1.0 g/kg IVIg in neonates with alloimmune HDFN if total serum bilirubin (TSB) is rising despite intensive phototherapy or if TSB level is within 34-51 µmol/L (2-3 mg/dL) of exchange level.\textsuperscript{17} This recommendation was also based on limited evidence. Although results of the Cochrane review in 2002 showed a significant reduction in the need for ET in infants treated with IVIg, the applicability of the results was limited because none of three included studies was of high quality. The Cochrane authors concluded that further well-designed trials were required before routine use of IVIg could be recommended. We therefore performed a double-blind placebo-controlled RCT on the prophylactic use of IVIg in red cell alloimmune HDFN. We demonstrated that IVIg does not reduce the need for and number of ETs and top-up transfusions nor the duration of phototherapy and maximum bilirubin levels (Chapter 5).

Hereafter, we updated the Cochrane review (Chapter 6) and included fourteen (quasi-) RCTs of which only two were of high quality.\textsuperscript{21-35} Meta-analysis of all fourteen trials, comprising 942 infants, showed a reduction in the need for and number of ETs in infants treated with IVIg combined with phototherapy compared to infants treated with phototherapy only. However, analysis of the only two high quality studies showed no reduction in the need for and number of ETs when IVIg was combined with intensive phototherapy. Eight studies measured the effect of IVIg on top-up transfusions for late anemia and found no difference in the need for or number of top-up transfusions. Based on the evidence from high quality trials (without selection bias, performance bias, detection bias, attrition bias and reporting bias) that IVIg is ineffective in preventing ET or top-up transfusion, routine use in alloimmune HDN should be discouraged. However, since there is some evidence that IVIg reduces hemolysis (in laboratory studies)\textsuperscript{36,37}, future high quality studies are needed to determine whether IVIg has limited role in some infants with alloimmune HDN, for example when ET can not be performed promptly or without unusually high risk.
Summary and general discussion

Alloimmunizations other than Rh D

Anti-Kell and anti-c are after anti-D the most important red cell antibodies causing severe HDFN necessitating IUT. In Kell HDFN antibody titer is not well-correlated with disease severity. Kell antibodies are, after anti-D, the most frequent antibodies causing HDFN. Kell HDFN probably has a different manifestation than Rh HDFN. Because the Kell antigen is expressed earlier on cells of the erythropoietic lineage Kell antibodies can cause destruction of progenitor cells (which are not yet hemoglobinized) in addition to hemolysis of erythrocytes. Similar as for Rh HDFN, neonates with Kell hemolytic disease may require top up transfusions up to several months after birth. However, it was not known whether incidence and severity of anemia differs between both types. In Chapter 7 we therefore compared a group of near-term neonates with Kell HDFN with neonates with Rh D HDFN. We found that neonates with Kell HDFN required less phototherapy (2.4 versus 4.1 days) and ETs (6% versus 62%). The need for top-up transfusions was not significantly different between Kell HDFN and Rh D HDFN (62% versus 72%). The median number of days after birth until first top-up transfusion was also not significantly different (16 days versus 17.5 days). Our results show that infants with Kell HDFN require a different management in the early neonatal period (less phototherapy and less ET because of less formation of bilirubin as breakdown product of hemoglobin), but a similar follow up management as infants with Rh D HDFN.

In Chapter 8 we performed a similar study and compared postnatal outcome between infants with Rh c HDFN and infants with Rh D HDFN. In this study we excluded cases with multiple clinical significant antibodies because it is known that disease can be more severe when multiple antibodies are present. We found a trend of a slightly more severe antenatal course of Rh c HDFN reflected by an earlier and higher need for IUT (61% versus 41%). Postnatal course was not significantly different between infants with Rh c and Rh D HDFN in terms of phototherapy duration (mean days 4.8 and 4.5), need for ET (50% versus 46%) and need for top-up transfusions (62% versus 78%). Therefore, also for Rh c HDFN a similar follow up management is justified. In this study we also investigated if antibody titer at birth was correlated with postnatal transfusion requirements. For Rh D HDFN it is known that antibody titer is correlated to postnatal anemia. In our study we also found a positive correlation between anti-D titer at birth and need for and number of top-up transfusions. Whether antibody titer in Rh c HDFN is correlated to postnatal transfusion requirements was not known. We found a positive correlation between antibody titer at birth and need for ET.
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Treatment of anemia

Treatment of anemia mainly consists of top-up transfusion. International guidelines for top-up transfusions in neonates (with HDFN) are lacking. As a consequence, studies on transfusion requirements are difficult to compare due to differences in transfusion thresholds, volumes, products and different outcome measures. In our center we transfuse 15 ml/kg ABO and Rh type specific, leukocyte-depleted RBCs which are negative for the antigen to which maternal antibodies are directed. Irradiated products are used in infants treated with IUT and/or born <32 weeks of gestation and/or birth weight <1500 gram. Transfusion triggers at our center include a hemoglobin level <5 mmol/L (8 g/dL) or <6 mmol/L (9.6 g/dL) when clinical symptoms of anemia are present (need for extra oxygen, poor feeding, tachycardia, tachypnea).

Supplements to support erythropoiesis which are applied in infants with HDFN include folic acid, iron and vitamin E (tocopherol). Folic acid is essential for proliferation of erythroblasts during their differentiation but also for de novo synthesis of the RNA and DNA; hence every growing cell requires folic acid. Gandy and Jacobson et al. demonstrated that 2.5-5 mg/day folic acid supplementation had a beneficial effect on growth, but not on hemoglobin level in infants with alloimmune HDFN. Although evidence is lacking, in our center folic acid is supplemented to infants with alloimmune HDFN in a dose of 0.05 mg/day orally during the first three months of life.

Supplementation of iron is not routine practice in our center and the motivation for this is dealt with later in this chapter.

Vitamin E (tocopherol) is occasionally supplemented in neonates with alloimmune HDFN. This antioxidant reduces oxidative stress to the RBC membrane and vitamin E deficiency can therefore lead to a shorter life span of RBCs. Although infants with alloimmune HDFN have lower vitamin E levels compared to healthy controls, administration of large amount of vitamin E analogue in three neonates with Rh D HDFN showed no beneficial effects. In our center, vitamin E is not supplemented to neonates with alloimmune HDFN.

Recombinant human erythropoietin (rhEPO) is used, often combined with supplements to support erythropoiesis, to treat (late) anemia in HDFN due to red cell alloimmunization. In Table 2 of Chapter 2 an overview is provided of studies/case reports of administration of rhEPO in infants with alloimmune HDFN. Only one small randomized placebo controlled trial has been performed in 20 neonates (≥35 weeks’ gestation) with Rh HDFN showing a significantly lower number of top-up transfusions in the rhEPO treated group.
Associated hematological morbidity

**Thrombocytopenia and coagulation disorders**
Thrombocytopenia was first reported in 1955 in three neonates with alloimmune HDFN.\(^{51}\) In 1970, Nielsen showed that neonates with alloimmune HDFN had a decreasing platelet count and an increasing clot-promoting activity (measured by the thromboplastin activation test in plasma) at birth associated with increase of disease severity.\(^{51}\) In 1979, a report was published by Hey and Jones on coagulation failure in Rh alloimmunization.\(^{52}\) They found very low vitamin-K dependent coagulation factor levels in cord blood of seven infants with severe disease probably caused by liver damage in utero. Five severely diseased infants died of pulmonary or cerebral hemorrhages. In addition, they demonstrated a strong correlation between platelet count and hemoglobin level at birth. Infants with a cord blood hemoglobin level of <10 g/dL (6.2 mmol/L) had a platelet count <150 × 10^9/L.\(^{52}\) The exact etiology of thrombocytopenia in alloimmune HDFN is still unknown, but several mechanisms have been suggested including increased immunological destruction and/or bilirubin toxicity\(^{53}\), sequestration of platelets due to hypersplenism\(^{52}\), dilution due to platelet poor (intrauterine) erythrocyte transfusions\(^{54}\), increased consumption (disseminated intravascular coagulation)\(^{55}\) and decreased production. Koenig et al. suggested a decreased platelet production in favor of increased erythrocyte production.\(^{56}\) In their study group of 20 neonates with Rh HDFN, eleven neonates with hydrops (severe disease) or who were treated with ET (moderate disease) had thrombocytopenia and none of the nine neonates without hydrops or ET had thrombocytopenia. The thrombocytopenic neonates did not have an increased platelet volume. In a severely affected neonate, they also demonstrated that the in vitro maturation of multipotent progenitor cells was altered, with a greater proportion of normoblasts, and fewer neutrophils and megakaryocytes. The significant higher incidence of thrombocytopenia in hydropic (more severely affected) fetuses and the negative correlation between reticulocyte and platelet count that Saade et al. demonstrated, also supports the decreased platelet production theory.\(^{57}\) Because IUT has improved significantly over the years, severe hemolytic disease with hydrops has become less common nowadays. In a recent large study (Chapter 9) we demonstrated that 26% (94/362) and 6% (20/362) of neonates with alloimmune HDFN had thrombocytopenia (platelet count <150 × 10^9/L) and severe thrombocytopenia (platelet count <50 × 10^9/L) at birth, respectively. Risk factors that were independently associated with thrombocytopenia included treatment with IUT, being small for gestational age and a lower gestational age at birth. Although fetal hydrops was not significant at the 5% level,
the relative low p-value (p = 0.083) is suggestive of a possible independent association with thrombocytopenia at birth. Only one neonate had clinical signs of bleeding at birth. This hydropic premature infant with a platelet count of $53 \times 10^9/L$ at birth had an intraventricular hemorrhage (IVH) grade 2 on day one.

In our center a concentrated platelet transfusion in a dose of $20 \times 10^9/kg$ is given if: 1) platelet count is $<20 \times 10^9/L$ in clinically stable neonates; 2) platelet count is $<50 \times 10^9/L$ in neonates with a manifest bleeding, those undergoing a procedure with a risk of bleeding and in clinically unstable neonates weighing $<1500$ grams and of $<32$ weeks of gestation, 3) platelet count is $<100 \times 10^9/L$ before ET (halfway the ET a platelet transfusion is performed). However, the causal relationship between thrombocytopenia and IVH in preterm infants is controversial. Moreover, platelet transfusion are also occasionally associated with adverse effects such as transfusion reactions and transmission of infectious agents.

In addition, the role of platelet transfusions in preventing bleeding is still unclear in neonates.63

**Cholestasis**

Few studies, mostly case-reports, have reported on the occurrence of conjugated hyperbilirubinemia, i.e. cholestasis, in alloimmune HDFN.64-67 Cholestasis in neonates with alloimmune HDFN has been associated with iron overload due to IUTs. Other suggested etiologic mechanisms include overload of pigment causing stasis and blocking of bile canaliculi; liver necrosis caused by hypoxia due to anemia; and pressure by extramedullary hematopoiesis in the liver caused by anemia leading to damage of intrahepatic canaliculi.70 In Chapter 10 we evaluated the incidence, potential risk factors, management and outcome of cholestasis in 313 neonates with alloimmune HDFN due to various anti-RBC antibody specificities. Cholestasis occurred in 13% and was independently associated with IUT treatment and Rh D antibodies. However, 88% of cholestatic neonates had both Rh D HDFN and were treated with IUT, preventing reliable distinction between both risk factors. Extensive tests to rule out other causes of cholestasis were all negative.

However, they were not performed in all neonates with cholestasis. In almost half of the patients cholestasis resolved spontaneously within 1 week to 3 months after birth. In the other half of the patients, measurements of bilirubin and liver enzymes were not monitored until they reached normal values. Median maximum ferritin levels during admission were significantly higher in cholestatic neonates than in the non-cholestatic neonates (1191 (range: 489-73000) µg/L and 657 (range: 86-10195) µg/L, $p = <0.001$). In addition, maximum ferritin level and treatment with IUT were positively correlated ($r = 0.565$, $p = <0.001$). One cholestatic patient with severe hyperferritinemia was treated with iron chelation therapy.
Iron overload
Ferritin reflects total body iron stores and age-appropriate levels are used to determine iron deficiency and iron overload. In healthy term newborns median ferritin levels at birth of 134-141 μg/L have been reported. Mean ferritin levels at birth have been reported to range from 101 μg/L to 183 μg/L. After birth, ferritin levels rise to a mean level of 226-356 μg/L and than decrease progressively over the following months to a mean level around 30 μg/L at the age of 6 months. Although it is known that ferritin levels in the fetal period and at birth can be highly elevated in neonates with alloimmune HDFN, the course of ferritin levels in the following months after birth was not known. We therefore prospectively collected data on the iron status of 35 neonates with alloimmune HDFN treated with or without IUT (Chapter 11). We found that iron overload occurred in 70% (23/33) of neonates with alloimmune HDFN at birth, 50% (10/20) at the age of one month, 42% (5/12) at the age of two months and 18% (2/11) at the age of three months. We found no cases of iron deficiency at birth and only one term infant had iron deficiency at three months of age based on the for this study defined criterion for iron deficiency (<40 μg/L). Whether this infant, who received one IUT and no postnatal transfusions, would have benefitted from iron supplementation is not clear. In our cohort, none of the infants received iron supplementation. We recommend to measure iron status before starting iron supplementation in infants with alloimmune HDFN because iron deficiency as well as iron overload can have detrimental effects. Iron deficiency and overload have both been associated with neurodevelopmental impairment. Iron overload can also lead to damage to the liver, heart and endocrine organs and increase susceptibility to infection. In adults with hereditary hemochromatosis ferritin levels >1000 μg/L have been associated with increased risk for liver cirrhosis. To assess the long term outcome of iron overload and deficiency in infants with alloimmune HDFN more research is warranted.

Leukocytopenia
As shown in Chapter 4, leukocytopenia is a common complication after ET with leukocyte-depleted donor blood. However, Koenig et al. demonstrated that neutropenia was also present before ET in four hydropic neonates with Rh HDFN. They also showed that erythroid progenitors were increased and granulocyte-macrophage progenitors were decreased in two severely affected neonates. Segal et al. described two hydropic neonates with Rh HDFN and neutropenia who were successfully treated with recombinant human granulocyte colony-stimulating factor. These scarce data are insufficient to be conclusive on the incidence and morbidity of neutropenia and its relation with sepsis in alloimmune HDFN.
Conclusion

In conclusion, this thesis focuses on several aspects related to the hematological outcome of infants with HDFN due to RBC alloimmunization, including pathogenesis and management of the disease. The presence of leukocytopenia and thrombocytopenia support the mechanism of suppression of thrombopoiesis and granulopoiesis in favor of the increased erythropoiesis stimulated by anemia and hypoxia. In addition to the problems caused by a shortage of platelets and white and red blood cells, the excess of the RBC metabolites bilirubin and iron also contribute to the morbidity of the disease. Based on our systematic review we can also conclude that there is a lack of high level evidence promoting the use of IVIG. Irrespective of the controversy concerning the use of IVIg, our data show that intensive follow-up is indicated in both Rh D and non-Rh D HDFN and raised awareness about the associated morbidity is of paramount importance. Although a few questions are answered in this thesis, it yielded much more questions to answer. In the next and final chapter, some of these future research questions are discussed.
References


Summary and general discussion


