Chapter

Hemolytic Disease of the Fetus and Newborn

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Abstract

Hemolytic disease of the fetus and newborn (HDFN) also called as "erythroblastosis fetalis" is characterized by the increased rate of red blood cells (RBCs) destruction. Hemolysis should always be investigated even if the anemia is mild and apparently trivial. The principle clues which suggest hemolytic anemia includes: increased number of reticulocytes and/or circulating nucleated RBCs, unconjugated hyperbilirubinemia, a positive direct antiglobulin test and characteristic changes in red cells in the blood films. Based on etiology, hemolysis in newborn can be immune or non-immune mediated. The immune-mediated hemolysis due to blood group incompatibility between the mother and the fetus is the main cause of HDFN.

Keywords: hemolysis, blood groups

1. Introduction

Hemolytic disease of the fetus and newborn (HDFN) also called as "erythroblastosis fetalis" is characterized by the increased rate of red blood cell (RBC) destruction. Hemolysis should always be investigated even if anemia is mild and apparently trivial. The principle clues which suggest hemolytic anemia include increased number of reticulocytes and/or circulating nucleated RBCs, unconjugated hyperbilirubinemia, a positive direct antiglobulin test, and characteristic changes in red cells in the blood films [1, 2]. Based on etiology, hemolysis in newborn can be immune or nonimmune mediated. The nonimmune causes include α-thalassemia, RBC membrane, or enzyme defects [1]. The immune-mediated hemolysis due to blood group incompatibility between the mother and the fetus is the main cause of HDFN. Immune-mediated hemolysis of fetal red cells, due to blood group incompatibility, occurs when there is transplacental passage of maternal antibody active against paternal red cell antigen of the infant [2–4]. Both naturally occurring and immune antibodies are implicated leading to a spectrum of clinical sequela, ranging from anemia and hyperbilirubinemia to fetal hydrops, kernicterus, and death [3, 4]. Although more than 60 different RBC antigens are capable of eliciting an antibody response, significant morbidity is associated primarily with D antigen of Rh group [2]. The prevalence of red cell antibodies other than anti-D with the potency to induce HDFN is about 1 in 500 pregnancies [5–7].

2. History

The royal family of England was not spared from the features of HDFN. Henry VIII's first wife, *Katherine of Aragon*, conceived six times, among which five died in

the perinatal period due to features presumed to be of HDFN [8]. The well-documented description of HDFN was made in 1609 by the midwife in the French literature. The case was a twin gestation in which the first fetus was stillborn and the second twin developed jaundice and succumbed soon after birth [9]. In 1940, Rh blood group system was described by Landsteiner and Wiener, and in 1941 Levine et al. determined that D antigen in Rh system is the agent for HDFN [10, 11]. The main cause of sensitization though was stated by Levine in 1940 but was described in detail by Keenan and Pearse in 1963 [12]. In 1961 Liley, for the first time, described intrauterine transfusion into the abdominal cavity of the fetus as a preventive measure for the disease [13]. Exchange transfusion, introduced by Wallerstein, and induced premature delivery are other treatment options employed for the management of HDFN [14, 15]. Until 1960, HDFN due to rhesus blood group system was considered the major contributor to the perinatal mortality rates. In 1961, Finn et al. defined the administration of anti-D Ig in the prevention of Rh sensitization and later in 1967 along with two German scientists Schneider and Preisler proved that anti-D is not useful in already sensitized mothers [16, 17]. This was a major breakthrough in prevention of sensitization with administration of 400 μg of human anti-D globulin within 72 h after delivery. Since 1971, the WHO recommends empirical use of anti-D Ig following any sensitizing event including after delivery of Rh-positive newborn and for abortions.

3. Blood group antigens and antibodies

An antigen is any substance which, when introduced into the body of an immunocompetent individual, stimulates the immune system by production of an antibody by interacting with the immunoglobulin receptor of B cell. Each red cell membrane is a bi-phospholipid layer containing millions of antigen on its surface [18]. The blood group antigens are either protein or carbohydrate structures present on the red cell membrane. An individual's blood group is determined by the antigen expressed on the surface of red cell membrane. The carbohydrate antigens are expressed as it is, while the protein antigens stretch in the bi-phospholipid layer by transmembrane proteins [19].

Antibodies are recognition proteins found in the serum and other body fluids of vertebrates that react specifically with the antigens that induce their formation. Antibodies belong to a family of globular proteins called immunoglobulins. The terms antibody and immunoglobulins are used synonymously. They are produced by the lymphocyte-plasma cell system. Antibodies bind antigen, fix complement, facilitate phagocytosis, and neutralize toxic substances in the circulation [18]. IgG, IgM, and IgA are the most significant from the point of view of transfusion medicine. Most clinically significant antibodies are IgG type, reacting at body temperature (37°C) with the antigens, and cause significant in transfusion reactions as they are a class of antibodies produced in response to nonself-antigens on the blood products. IgM antibodies are mostly naturally occurring antibodies [19].

4. Alloimmunization during pregnancy

Alloimmunization can be caused due to pregnancy, blood transfusion, or tissue/organ transplantation or grafting, due to genetic difference between the individuals [20]. On exposure to a foreign red cell antigen, the immune system is activated which is mediated by lymphocytes. The first step involved is recognition of the antigen by T cell. The recipient's helper T cell interacts with the MHC class II

Antepartum	Intrapartum	Postpartum
Early pregnancy	Caesarean section	Blood transfusion
Abortion (spontaneous or induced)	Manual removal of placenta	
Ectopic pregnancy		
Late pregnancy		
Placetal abruption		
Abodominal trauma		
Obstetric procedures		
Amniocentesis		
Chorionic villus sampling		
External cephalic version		
Fetal blood sampling		

Table 1.
Sensitizing events for feto-maternal haemorrhage [24].

molecule expressed on the donor red cells. Following the initial interaction, T cells trigger a second signal for the B lymphocytes, in order to stimulate humoral immune response [20, 21]. Initially, IgM class of antibody is produced in primary response to an antigen and is formed as early as 4 weeks to 3 months period. However, there is a switch of IgM to IgG class during the secondary response [20]. The secondary response is more rapid, potent, and specific than the primary.

Placenta is a natural barrier present between mother and fetus. Only IgG antibodies can cross the placenta. The transfer is mediated by the neonatal Fc receptors (FcRn) [6]. The immunoglobulin is bound and transported by FcRn of syncytiotrophoblast which also protects IgG molecule from normal serum protein catabolism. In the first trimester, there is relatively less transfer; however, it subsequently increases exponentially in the second and third trimester. Mean concentration in the fetus at the 24th week of pregnancy is 1.8 g/dL. The IgG antibody levels are higher in the fetus than in the mother toward the term [22]. Of the four subclasses of IgG antibody, IgG3 and IgG1 are more efficient in RBC hemolysis than IgG2 and IgG4, though all the four classes are efficiently transferred across the placenta [23].

4.1 Sensitizing events

Transplacental feto-maternal hemorrhage occurs in over 75% of pregnancies. The average volume of fetal blood in the maternal circulation following delivery is less than 1 mL in 96% of pregnancies [24]. As the pregnancy progresses, the possibility of feto-maternal hemorrhage increases, 3% in first trimester, 12% in the second, 45% in the third, and 64% at the time of delivery as shown by Bowman [22]. It has been reported that as little as 0.1 mL of antigen-positive blood is sufficient to cause sensitization in an antigen-negative mother [23]. Feto-maternal hemorrhage can occur due to various antenatal and postnatal events in **Table 1**.

5. Pathophysiology for HDFN

HDFN is the destruction of fetal and newborn red cells by maternal alloantibodies specific for the inherited paternal red cell alloantigens. While IgM is usually detected in the maternal circulation during primary response, IgG is found during secondary response, which appears about 5–15 weeks after feto-maternal hemorrhage. Because exposure to fetal red cells and resulting maternal alloimmunization typically occurs late during pregnancy and at delivery, and IgM does not cross the placenta, the fetus and newborn of the first pregnancy are rarely affected. Re-

exposure to red cell antigen during subsequent pregnancies produces IgG in sufficient concentration [4].

The sensitized fetal red cells by maternal IgG antibody are unable to continue in the circulation, and these red cells are destroyed by the fetal spleen resulting in anemia. Compensatory erythropoiesis is induced by fetal anemia initially [25]. The exception to this rule is antibodies Kell blood group system and MNS system which cause destruction of erythroid progenitor cells, causing early anemia without erythroblastosis [26, 27]. Hyperdynamic circulation tries to compensate anemia in the fetus, which subsequently leads to cardiomegaly, and finally fetal hydrops develops [25]. Due to hemolysis of the fetal cell, there is rise in the bilirubin level in the fetus. In utero, the bilirubin is excreted by the mother, when it is transported across the placenta, so the severity of hyperbilirubinemia is not observed. After delivery, the hemolysis continues, but the comparatively immature liver of the neonate is unable to sufficiently conjugate the excess of bilirubin. This subsequently leads to severe hyperbilirubinemia and, when left untreated, could result in "kernicterus" [4, 25, 28].

6. Clinical relevance of different red cell alloantibody specificities

The risk of developing severe HDFN depends on several factors, including Ig class, specificity of the red cell alloantibodies, and level of expression of the involved blood group antigen on the fetal red cells and other tissues as shown in **Table 2** [25].

6.1 Rh blood group system

Rh system is more complex than the single antigen system. Five principal Rh antigens D, C, c, E, and e are responsible for the majority of clinically significant antibodies, but over 50 different Rh antigens have been described [29].

Antibody specificities	Risk to develop HDFN in antigen-positive children and clinical course of disease
ABO	Low risk for disease, in general mild, incidentally severe
Rh D c E Other Rh antigens	High risk for disease, often (very) severe, otherwise mild High risk for disease, (very) severe or mild Medium risk for disease, sometimes severe, but mostly mild Medium risk for disease, incidentally severe, but mostly mild
Kell K Other Kell antigens	High risk for disease, (very) severe or mild Medium risk mild to severe disease
Duffy Fya/Fyb	Medium risk for disease, mostly mild
Kidd Jka/Jkb	Low risk for disease, only mild
MNS M, N, S, s Other antigens	Low risk for disease, mostly mild disease, very rarely severe Low risk for disease, mostly mild disease, very rarely severe
I, Le, P1, Lu, Yt	No risk, because of very low expression of these antigens by fetal cells
Other antigen systems	Very low risk, very rarely severe disease can develop

Table 2.
Red cell antibody specificities in reference to induce HDFN [25].

D is by far the most immunogenic of all the Rh antigens. Hence, it is common in clinical practice to equate D with Rh and to use the terms Rh-positive and Rh-negative to describe "D-positive" and "D-negative" [30].

Rh antibodies implicated in HDFN are:

- Severe HDFN—anti-D and anti-c [31, 32]
- Mild disease—anti-C, anti-E, and anti-e [33–37]

6.1.1 D antigen and antibodies

The D antigen carried by the RhD proteins is the most immunogenic and most important blood group antigen leading to HDFN. There is no antithetical antigen to D [4]. The first blood group antigen to be associated with HDFN was described by Levine et al. in 1945 [9]. About 15% of Western world and 8% of blacks are D-negative [38, 39]. If a unit of D-positive blood is transfused to a D-negative recipient, the recipient will form anti-D in around 90% of cases, and subsequently D-positive red cells cannot be given safely to these patients [29].

Sensitization to D antigen can occur in reaction to less than 0.1 mL of fetal blood, resulting in formation of anti-D in the maternal circulation [22]. Before 1945, more than 50% of all fetus with HDFN died of kernicterus or hydrops fetalis, and anti-D was the most common associated [9, 40]. With improvement of treatment, in industrialized countries the mortality reduced to 2–3%. But anti-D is still among the most frequently detected antibodies in sensitized pregnancies.

Very early, it was understood that, if the D-negative mother was carrying an ABO-compatible D-positive fetus, her risk of Rh immunization was 16%. If the D-positive fetus was ABO-incompatible, the risk was only 2%. So, the overall risk of Rh immunization is 13.2% [9].

Not only in Rh-negative pregnancies does anti-D causes HDFN, but also case reports have been reported for HDFN due to anti-D in Rh-positive pregnancies [41–43]. These are mostly due to the Rh variants: weak D, Du, and partial D described by molecular analysis [30].

6.1.1.1 Blocked D phenomenon

The blocking of D antigen sites by IgG anti-D in severe cases of HDFN is a rare phenomenon explained by Wiener in 1944 [44]. Only a handful of case reports have been described in the literature [45–48]. The coating of maternal anti-D IgG on the D-positive red blood cells (RBCs) of the newborn gives false-negative D typing, when IgM typing reagent anti-D is used. This phenomenon is not limited to anti-D, but is seen with other blood groups [49]. BCSH describes guideline for resolving such cases [50].

6.1.1.2 Rh immunoglobulin prophylaxis

Rh immunoglobulin (RhIG) prophylaxis for D-negative pregnant women is now the international cornerstone for prevention of maternal alloimmunization to the D antigen and subsequent HDFN [50, 51].

During the mid-1960s, experiments were carried out in various parts of the world for preventing HDFN due to anti-D. Clinical trials showed that, when unimmunized mothers who have delivered D-positive infants, were given RhIG prevented the development of anti-D in the mother. RhIG is obtained from the

human plasma. RhIG has to be given within 72 h after delivery of a D-positive infant. Since 1968, RhIG is licensed for prevention of HDFN [9, 21, 22].

The effectiveness of RhIG in order to prevent isoimmunization is determined by adequate dosage and should be administered before initiation of Rh isoimmunization [50].

There are various mechanisms describing the role of RhIG in preventing HDFN. Though antigenic epitopes are not fully masked by anti-D, they are still available for immune system recognition. But anti-D is be able to destroy RBCs without triggering the adaptive immune response, by inhibition of FcgammaRIIB signaling in B cells which is called as antibody-mediated immune suppression (AMIS) [21, 52, 53]. The T-cell response and memory may still be intact.

Various studies were carried out in the 1970s after systemic implementation of systemic anti-D prophylaxis, which showed reduction in HDFN from 16 to 0.3% [54].

The standard guidelines recommend to administer RhIG as soon after delivery as the infant is determined to be D-positive or latest within 72 hours after delivery or after any antenatal procedure, where the risk of feto-maternal hemorrhage is high as shown in **Table 3** [50]. It has been shown experimentally that at least partial protection is afforded by giving RhIG up to 13 days after exposure to D-positive RBCs. Rh prophylaxis therefore is recommended up to 28 days after delivery, with the understanding, however, that the longer the prophylaxis after delivery is delayed, the less likely it is to be effective [28, 55].

In 2014, Cohen et al. described a case report on severe HDFN caused due to passive transfer of anti-D from maternal RhIG [56].

6.1.2 Other Rh system antibodies other than anti-D

With widespread use of RhD immunoglobulin, the focus has shifted to the non-RhD antibodies causing isoimmunization. Other Rh antigens include C, c, E, and e antigens. DCe is the most common haplotype in Caucasians (42%), Native Americans (44%), and Asians (70%) [57].

6.1.3 Anti-c

Anti-c is usually described as the next most common cause of severe HDFN after anti-D. Various case reports have been reported, stating that anti-c isoimmunization can cause HDFN from mild to severe degree [31, 32, 58, 59]. A titer of more than 1:32 is associated with hydrops fetalis as described by David et al. [58]. BCSH guidelines state that women with anti-c should be retested following the same protocol as for anti-D [50]. Quantification of the antibodies is expressed in terms of IU/mL. Mothers with antibody concentration of less than 7.5 IU/mL are advised to continue the pregnancy, while 7.5–20 IU/mL are at a risk of moderate HDFN and more than 20 IU/mL, severe HDFN. It should be kept in mind that anti-c causes delayed anemia in neonate [50].

6.1.4 Anti-D + anti-C or anti-G

D-positive or C-positive RBCs have G antigen which was first described by Allen and Tippett in 1958 [60]. The G antigen is co-distributed either with C or D antigen which causes anti-G to appear serologically as anti-C plus anti-D [60]. During pregnancy, it is apparently important to distinguish between anti-D, anti-G, and anti-D + C. As the pregnancies without anti-D are candidates for the administration

Recommendations	Strength of recommendation	Quality of evidence
Postpartum prophylaxis	A	I
• Anti-D 120–300 μg within 72 h of delivery	B	II
• Anti-D up to 28 days after delivery	C	Insufficient
• Routine FMH testing after delivery		
Antepartum prophylaxis	A	I
• Anti-D 300 μg at 28 weeks	C	III
• Repeat antibody screening at 28 weeks	C	III
• Routine paternal testing	D	III
• Anti-D for "weak D" (e.g., Du)	D	III
• Repeat anti-D at 40 weeks		
Early pregnancy loss and termination	B	II-3
• Anti-D 120–300 μg after spontaneous/induced abortion	B	III
	B	III
• Antibody screening prior to anti-D after abortion	B	III
• Ectopic pregnancy: 120–300 μg Rh immune globulin		
• Molar pregnancy: 120–300 μg Rh immune globulin		
Invasive fetal procedures	B	II-3
• Amniocentesis: 300 μg Rh immune globulin	B	II
• CVS: 120–300 μg Rh immune globulin	B	II-3
• Cordocentesis: 300 μg Rh immune globulin		
APH, abdominal trauma, ECV, FMH	B	III
• Quantitative FMH testing	B	III
• Anti-D 120–300 μg following placental trauma		
Consent	C	III
• Informed consent prior to administration of anti-D		

Table 3.
Anti-D prophylaxis and quality of evidence available [50, 54, 55].

of RhIG. The administration of RhIG can be avoided if anti-D has already developed. D-negative mothers with anti-G are potential candidates to receive RhIG in order to prevent formation of anti-D. It also avoids the associated social or medicolegal complications [61]. The clinical significance of anti-G alone in causing mild to severe HDFN still remains controversial [62]. The isolation of anti-G by double adsorption and elution is a tedious and relatively complex procedure [63]. The technique to distinguish anti-D + C from anti-G is recently described by Fatima et al. [64].

6.1.5 Anti-C, anti-E, anti-e, and others

Anti-RhC, anti-RhE, and anti-Rhe antibodies are of Rhesus family and usually occur in low titer in conjunction with anti-RhD antibody. Their presence can be additive to the hemolytic effect of the anti-RhD on the fetus [65, 66]. Various reports have been published on pregnancies alloimmunized only to RhE [36, 37].

Hardy and Napier in their review of red blood cell antibodies among Rh-positive women in South and Mid Wales over a 30-year period (1948–1978) described two infants with hemolytic disease caused by anti-C [67].

Anti-e is usually a very rare cause of HDN; the disease is usually mild [68].

In addition to the above antibodies, there are many other antibodies belonging to Rh family which are associated with HDFN [35, 69, 70].

6.2 Kell blood group system

A mnemonic goes "Duffy dies, Kell kills, and Lewy lives" [71]. Kell blood group system is clinically significant in terms of transfusion medicine and perinatology. It relates to the polymorphic nature of the Kell protein. It is also associated with the Kx and Gerbich blood group systems. K is formed in fetuses of 10–11 weeks and k at 6–7 weeks of gestation [72, 73].

Alloimmunization to Kell blood group antigens is due to previous blood transfusion or feto-maternal hemorrhage induced during pregnancy [73]. Kell alloimmunization is the second major cause for fetal hemolytic anemia, with a reported and still increasing incidence in a large US series of 3.2 in 1000 and affecting 1 in 10,000 neonates [74].

6.2.1 Anti-K

Anti-K antibodies differ from the other blood group system antibodies that cause HDFN in suppressing fetal non-hemoglobinized erythropoiesis, causing severe anemia and often death of the fetus. The high bilirubin level is not a characteristic feature as the precursor cells are destroyed. Amniocentesis therefore does not give an indication of the severity of the disease. Successful management of RBC-alloimmunized pregnancies depends on early detection of fetal anemia and timely intervention by intrauterine blood transfusions [75–77].

Perinatal survival in severe Kell alloimmunization was only 58% as recorded after implementation of routine screening nationwide in the Netherlands from 1988 to 2005 [75].

In some countries it is usually practiced to give K-negative red cells for girls and women of childbearing age group [78].

6.3 Detection of feto-maternal hemorrhage (FMH)

6.3.1 KB test

As mentioned earlier, feto-maternal hemorrhage increases as the pregnancy progresses: 3% in the first trimester, 12% in the second, 45% in the third, and 64% at the time of delivery [22]. These fetal cells which have crossed the placenta can be detected by acid elution method of differential staining described by Kleinhauer and Betke in 1960 [79].

6.3.2 α-Fetoprotein (α-FP)

α-FP is an analogue of albumin [6]. Seppala and Ruoslahti in 1972 and Caballero et al. in 1977 used α-FP as an index of transplacental hemorrhage (TPH) [80, 81].

6.3.3 Others

Apart from quantification, the serological methods for detecting transplacental hemorrhage are also available. This includes rosetting test and flow cytometry.

Rosetting test is not sensitive, as it needs at least 15 mL or more of fetal cells to be present in the maternal circulation to give a positive result [19].

Flow cytometry is the most sensitive test technique in detecting the amount of TPH [19, 82].

The other surrogate markers of FMH include enzyme-linked antiglobulin test (ELAT), placental alkaline phosphatase (PLAP), polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH).

6.4 Antibody screening

It had been a common practice to screen the sera of all Rh(D)-negative pregnant women for Rh antibodies. Later, when it was found that Rh(D)-positive women could also have babies with HDFN due to Rh antibodies (other than anti-D) and non-Rh antibodies, it was suggested that sera from all pregnant women should be screened for antibodies [6, 25, 83].

6.4.1 Screening methods

The indirect antiglobulin test (IAT) using reagent red cells suspended in low ionic strength saline (LISS) is the most suitable method for detection of clinically significant red cell antibodies [84]. Column agglutination methods, liquid-phase tube tests, and solid-phase methods have also been found to be suitable [50].

6.4.2 Guidelines for antenatal antibody screening

Various countries have developed guidelines for screening of antenatal cases.

Scientific Section Coordinating Committee of the American Association of Blood Banks (AABB) has issued guidelines (not AABB standards) for serological testing of pregnant women [85]. **Table 4** shows the recommendations for prenatal testing.

The BCSH Task Force has also laid guidelines in 2007 for blood grouping and antibody testing in pregnancy as follows [50]:

Testing and condition	Timing
ABO	Initial visit
First pregnancy	Initial visit
Subsequent pregnancies	For pretransfusion testing
Other	
Rh (test for weak D optional)	Initial visit and at 26–28 weeks' gestation
First pregnancy	Initial visit
Subsequent pregnancies	For pretransfusion testing
Other	
Unexpected antibodies	Initial visit
All pregnancies	Before Rh Ig therapy (optional)
D− pregnancies	Third trimester if transfused or history of unexpected
D+ pregnancies	antibodies
Other	For pretransfusion testing
Antibody identification	Upon initial detection
Unexpected antibodies present	At time of titration
Confirmatory testing	
Antibody titration	Upon initial detection
Rh antibodies	Repeat at 18–20 weeks' gestation
Other potentially significant	Repeat at 2- to 4-week intervals if below critical titer [16–32]
antibodies	As above, with discussion with obstetrician

Table 4.
AABB recommendations for prenatal testing [85].

- "*All pregnant women* should have samples taken early in pregnancy, ideally at 10–16 weeks gestation, for ABO and D typing and for screening for the presence of red cell alloantibodies."

- "All pregnant women, *whether D-positive or -negative* should have a further blood sample taken at 28 weeks gestation for rechecking the ABO and D group and further screening for red cell alloantibodies."

- "No further routine blood grouping or antibody screening is necessary *after 28 weeks of gestation*."

Australian and New Zealand guidelines have been adapted from AABB recommendations for pregnant women [85, 86]. However, repeat testing of RhD-negative women only at 28 weeks, prior to administering RhIG, is becoming the accepted protocol in most Australian centers, eliminating the norm of antibody screening at 34–36 weeks. In New Zealand, in the absence of routine antenatal prophylaxis, the normal practice is to test RhD-negative women at 28 and 36 weeks of gestation [86].

The latest guidelines for alloimmunized pregnancy framed and followed in Japan since 2014 are as follows [87]:

- "Identify the antibody when a screening test, such as the indirect Coombs test, suggests the presence of an atypical antibody against red blood cells."

- "Assess the titer of the antibody if the antibody belongs to IgG class that may cause hemolysis in the fetus."

- "Monitor the fetal well-being, paying special attention to anemia and hydrops, in women with an elevated titer of an IgG antibody that may cause hemolysis in the fetus."

- "Be prepared to administer un-crossmatched packed red blood cells compatible with an ABO blood type if the pregnant woman develops unexpected massive bleeding."

6.5 Methods of red cell antibody detection and identification

Antibody detection plays a critical role in detection and monitoring of antenatal cases who are at risk of delivering neonates with HFDN [88].

Most of the clinically significant antibodies are IgG in nature, which are non-agglutinating or incomplete antibodies, so they can only sensitize red cells but cannot produce agglutination. Coombs et al. in 1945 described "antiglobulin test" for detection of these non-agglutinating antibodies [89].

The presence of red cell antibodies in patient's serum or plasma and an in vitro reaction between red cell detection are demonstrated by indirect antiglobulin test [89]. Indirect antiglobulin test is considered to be the most effective and reliable method for detection of clinically significant antibodies [84]. Several studies have shown that the column agglutination test is better than tube and solid-phase tests.

The gel technique has shown sensitivity as compared with conventional test tube (CTT) methods (93.5–100% for CAT vs. 50% for CTT) [90–93]. The

sensitivity of SPRCA has been found to be superior to CTT and comparable with that of CAT [94].

6.6 Quantification of antibody in the serum

Titration is a semiquantitative method to estimate the strength and concentration of antibodies present in a serum sample [95]. Titers give only rough estimates of the amount of antibody bound to the target RBCs and do not measure the amount of antibody remaining free in solution at the endpoint of agglutination [96]:

- The critical titer for anti-D (the level below which HDFN and hydrops fetalis are considered unlikely is usually 16 or 32 in antihuman globulin (AHG) phase). These titer criteria apply to anti-D, to other Rh antibodies, and generally to other clinically significant antibodies, with anti-K as possible exception.

- A critical titer of 8 is considered for anti-K.

- As long as the titer is 8 or lower (except anti-K), the pregnancy can be followed up every 4–6 weeks until delivery.

- A difference of two dilutions or a score of 10 is considered a significant change [4].

- A score may also be assigned, based on the strength of reactivity.

- Each reaction is given a value, and score is determined by adding up individual values.

- Most hospitals have set up their critical antibody titer, at which amniocentesis is recommended.

There are various methods of performing the titration, conventional tube test (CTT), or by gel microcolumn assay (GMA) [95, 97]. A study by Thakur et al. showed that gel technique is more sensitive for antibody detection. It does not show a linear correlation with tube titers in predicting the outcome in RhD-sensitized women, while Rachel et al. suggested that GMA gives comparable results to the CTT in titrating alloantibodies to Rh and Kell antigens [95, 97].

6.7 Amniotic fluid analysis

Amniocentesis is helpful in determining the overall condition of the fetus and is mostly indicated when the clinically significant maternal antibody titer is 1:32 or greater in the fourth or fifth month of pregnancy [4, 19, 98]. If there is a history of a previous pregnancy complicated by HDN, amniocentesis is indicated regardless of the present maternal serum antibody titer.

In 1961, Liley developed a chart depicting change in amniotic fluid bilirubin levels (delta OD450) with period of gestation, with three zones delineating the severity of rhesus disease [99]. The chart is useful only after the 27th week of gestation. Currently, cordocentesis is the only reliable means of assessing the fetal condition accurately prior to 27 weeks. In 1993, Queenan proposed a chart showing delta OD450 from 14 to 40 weeks, with four zones to guide management [100].

6.8 Other methods for assessing the severity of HDFN

6.8.1 Ultrasound

Some of the pathophysiological changes in the fetus due to anemia could be shown using ultrasound [6]. Real-time sonography accurately predicted the clinical course in 86% of the cases, with no false-positive predictions [101].

With recent advances, Doppler ultrasonography, which measures fetal hemodynamics, has been used, and it gives better results in predicting fetal anemia as early as the 18th week. The Doppler assessment of peak systolic velocity in the middle cerebral artery (MCA-PSV) is done [102]. It is hypothesized that faster rate of blood flow indicates a more severely anemic fetus, with severe anemia being an indicator of fetal hydrops.

6.8.2 Fetoscopy, percutaneous umbilical blood sampling (cordocentesis), and chorionic villus biopsy

Fetoscopy is a technique in which the second-trimester fetus can be visualized directly and fetal blood (or other tissues) can be sampled through an endoscope introduced transabdominally into the amniotic cavity. The technique is only reliably successful at 16 weeks of gestation and later. Fetoscopy carries a mortality rate of 5% as compared to a 1–2% mortality rate after midtrimester amniocentesis. The fetal blood can be tested for blood type, DAT, hemoglobin, and hematocrit [96]. MacKenzie and coworkers suggested that the technique would be of benefit in cases where the father of the baby is known to be heterozygous for the offending blood group antigen in the following situations:

1. Patients with a history of previous babies having severe HDFN.

2. Patients with high-titer anti-D (e.g., in excess of 20 IU/mL) during the first 20 weeks of pregnancy.

6.8.3 Prenatal determination of fetal blood groups

The father's probable genotype is predicted. If the father is thought to be homozygous, the baby is assumed to possess the putative antigen. If the father is heterozygous, there is a 50% chance that the baby is antigen-positive [6, 96]. Antenatal genotyping of the fetus is now in widespread use as an aid to the clinical management in cases where there is a possibility of occurancy of hemolytic disease of the newborn [121]. Molecular genotyping is a major clinical application which has led to the determination basis of blood group antigens expressed, most of which have been defined at the level of the gene. All assays used are dependent on the polymerase chain reaction amplification of fetal DNA derived from.

6.8.4 In vitro predictive tests utilizing functional cellular assays

They are based on the in vivo mechanisms of RBC immune destruction. The interactions with the monocytes are measured by recording RBC adherence/phagocytosis by a monocyte monolayer assay (MMA), antibody-dependent cellular cytotoxicity (ADCC) using 51Cr-labeled RBCs, or a chemiluminescence test (CL) using luminol. These tests are not very accurate in predicting the severity of HDFN [6, 96].

6.9 Antenatal treatment of hemolytic disease

6.9.1 Plasma exchange in mother

A known therapeutic approach for red cell alloimmunization is plasmapheresis in the mother in order to reduce maternal antibody titer [103]. In the current era, plasma exchange treatment appears to be useful in cases of HDFN developed early in pregnancy (before 20 weeks). The American Society for Apheresis in 2013 proposed that plasmapheresis should be considered early in pregnancy (from the 7th to the 20th week) and continued until IUT can be safely administered (approx. 20 weeks of gestation) [104].

6.9.2 Absorption of alloantibodies onto red cells

Plasma containing the antibodies is drawn from the patient, the antibodies are absorbed using appropriate cells, and then plasma is returned to the patient [19]. During the 1980s, this procedure was attempted by Robinson and Yoshida et al. for an Rh-immunized woman [105, 106].

6.9.3 Intravenous immunoglobulin given to the mother

The use of intravenous immunoglobulin (IVIG) to the mother is one of the alternative strategies developed in past 20 years for the management of severely alloimmunized pregnancies [107]. The mechanism by which IVIG might act is saturation of FcRn, thereby inhibiting placental transfer of anti-D to the fetus as shown by Morgan et al. in 1991 [108]. A single course of 2 g/kg over 5 days or repeated weekly injections of 1 g/kg have been tried in conjunction with plasma exchange or with intravascular transfusion of the fetus [109]. There is no other description of the dosing during the antenatal period.

6.9.4 IVIG given to the fetus

IVIG given to the fetus did not show any beneficial effect [19].

6.9.5 Intrauterine transfusion

In 1970, Pontuch stated five strategies as a preventive measure for HDFN, which are valid to this day [110]:

1. "Prevention of leakage of fetal erythrocytes into maternal circulation and antibodies in the opposite direction"

2. "Blockage of antibody production in the maternal circulation"

3. "Prevention of sensitization of maternal erythrocytes with fetal erythrocytes"

4. "Prevention of Rh sensitization by anti-D administration"

5. "Intrauterine transfusion in pregnancy"

One of the oldest methods, introduced by *Sir William Liley*, is intraperitoneal fetal blood transfusion into the abdominal cavity of the fetus under X-ray guidance [111]. The donor cells were absorbed into the fetal circulation via the

subdiaphragmatic lymphatics and thoracic duct, which, in conjunction with the use of amniotic fluid analysis for bilirubin levels, markedly improved the management of Rh-sensitized pregnancies.

From the time of its initiation, IUT has come a long way. Initially, it was intraperitoneal, but in 1981, Rodeck et al. described intravascular transfusion by using the umbilical cord and fetoscope [112].

The formula for calculation of blood volume for IUT is shown below [113]:

$$\boxed{V = \frac{(\text{Desired PCV} - \text{Fetal PCV}) \times \text{Fetoplacental BV}}{(\text{Donor PCV} - \text{Desired PCV})}.}$$

Red cell prerequisites for IUT as described by BCSH guidelines are as follows [113]:

- "Group O (low titre haemolysin) or ABO identical with the fetus (if known) and RhD negative"
- "IAT-cross-match compatible with maternal serum and negative for the relevant antigens determined by maternal antibody status"
- "Less than 5 days old and in citrate phosphate dextrose (CPD) anticoagulant"
- "CMV seronegative and irradiated"
- "Should have a haematocrit (packed cell volume, PCV) of up to but not more than 0.75"
- "Not be transfused straight from 4°C storage"
- "The rate of transfusion should be 5–10 mL/min"

The target of a single IUT is to reach Hct 48–55% in non-hydropic fetuses [103].

6.9.6 Induction of GvHD

Immunomodulatory effect due to transfusion to the fetus by IUT due to the HLA group of the donor causes GvHD [19].

6.9.7 Premature delivery

Premature induction of labor may be considered, as after the birth of the infant, placental transfer of antibody ceases [6, 28].

6.10 Postnatal intervention for hemolytic disease

6.10.1 Exchange transfusion

The main purposes of exchange transfusion in HDFN are as follows [28, 114]:

1. With sedimented red cells, it can raise the hematocrit without increasing the blood volume of a severely affected erythroblastotic newborn infant in the first minutes of life.

2. It can remove antibody-coated cells from the circulation of newborn before they hemolyze and produce bilirubin.

3. It can remove bilirubin in the circulating plasma and some from extravascular areas, so that its concentration can be kept below levels which are generally considered to be toxic to tissues—particularly central nervous system tissues.

The method was introduced by Diamond (1947). Blood was withdrawn and injected, intermittently, through a plastic catheter passed up the umbilical vein [110].

For HDFN double-volume exchange is mostly preferred, where approximately 85% of the blood volume is replaced and will cause an approximate reduction of 50% in pre-exchange bilirubin level [114–116]. The American Academy of Pediatrics (AAP) has published guidelines, recommending not to initiate early ET, such as within the first 12 h of birth. The bilirubin threshold for the start of ET depends on the gestational age at which the baby is born [117].

Components used in exchange transfusion.

Red cells for exchange transfusion should meet the following BCSH criteria [113, 118]:

- "Group O or ABO compatible with maternal and neonatal plasma, RhD negative (or RhD identical with neonate)"
- "Negative for any red cell antigens to which the mother has antibodies"
- "IAT-cross-match compatible with maternal plasma"
- "5 days old or less (to ensure optimal red cell function and low supernatant potassium levels)"
- "Collected into CPD anticoagulant and CMV seronegative"
- "Irradiated and transfused within 24 h of irradiation. Irradiation is essential if the infant has had a previous IUT and is recommended for all ETs"
- "Hematocrit of 0.50–0.60"
- "Not to be transfused straight from 4°C storage. Care should be taken to avoid over-heating of the component"

Fresh frozen plasma (FFP)—The RBCs are suspended in AB plasma in order to provide plasma proteins, coagulation factors, and albumin [113]. Reconstituted whole blood is prepared by adding appropriate amount of FFP into the RBC unit which is preservative-free. The hematocrit of the unit should be 40–45% [119]. Volume of blood for exchange is calculated using an estimate of the neonate's circulating blood volume [113]:

- Term infants: 80–160 mL/kg
- Preterm infants: 100–200 mL/kg

$$\boxed{\text{Volume to be exchange} = 2 \times \text{circulating blood volume}}$$

The volume to be given is calculated below [119]:

$$\text{Total volume (in mL)} = \text{infant's weight in kg} \times 85^{*}\ \text{mL/kg} \times 2$$

$$\text{Absolute volume of RBCs required (in mL)} = \text{total volume} \times 0.45\ \text{(desired hematocrit)}$$

$$\text{Actual volume of RBCs required (in mL)} = \frac{\text{Absolute volume}}{\text{Hematocrit of unit after any manipulation}}$$

$$\text{Necessary volume of FFP} = \text{total volume required} - \text{actual volume of RBCs required}$$

$$*(85\text{–}100\ \text{mL/kg, depending on estimated blood volume})$$

6.10.2 Phototherapy

Phototherapy has been proven to be effective in treatment of hyperbilirubinemia by denaturing the bilirubin at appropriate wavelength [28]. AAP and NICE have laid down guidelines for initiation of treatment, considering gestational age, birth weight, and cause of hyperbilirubinemia [117, 120].

6.10.3 Intravenous immunoglobulin

IVIG blocks the Fc receptor sites on the cells of the reticuloendothelial system, thus preventing the hemolysis of sensitized cells. It is mostly used for ABO HDFN and is not very effective in anti-D-mediated HDFN [25, 28].

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