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Hematological Characterization of Neonatal Hyperbilirubinemia during Hemolytic Disease of Newborn (HDN): An Approach to Management of Hyperbilirubinemia

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Abstract: Hemolytic disease of newborn (HDN) consequences lead to considerable morbidity and mortality, and so immediate diagnosis and treatment to prevent serious repercussions are crucial. Since investigative and preventative measures of hemolytic disease of the fetus and newborn (HDFN) in Sudan lack adherence to standard international protocols (due to a lack of adequate antenatal care), this study may be useful in improving quality of life. The present study aimed to determine different hematological parameters (Hb, HCT, RBC, and erythroblast values) and maternal–fetal ABO blood type incompatibility among Sudanese jaundiced neonates with HDN. A descriptive cross-sectional study was carried out on 305 mothers and their fetuses admitted to Omdurman Maternity Hospital, Sudan. ABO and rhesus (RhD) blood groups, direct antiglobulin test (DAT), CBC, and a comment on blood smear were determined for the jaundiced newborns and used for data analysis. The history of total bilirubin, direct bilirubin level, mother's age, gestational age, weight, gender, phototherapy, blood exchange, and presence of fever of neonates were recorded during the study. Statistical data analysis was performed using SPSS Software Version 16. The overall frequency of HDN with positive DAT was 57 (18.9%). RhD, ABO, and other blood group system incompatibility in HDN were 4 (1.3%), 18 (5.9%), and 35 (11.4%), respectively. There was statistical significance in total and direct bilirubin, gestational age, transfusion history, and phototherapy. There was a highly significant association between a positive Coomb's test and the presence of abnormal peripheral cells (spherocyte, NRBCs, polychromasia, and target). This study revealed that the prevalence of HDFN with hyperbilirubinemia was 57 (18.9%), which remains a challenge due to the lack of standard protocols in Sudan. RhD remains the most significant antigen contributing to HDFN, although prophylactic anti-D was used. There was a significant association between DAT and abnormal blood cells.

Keywords: hemolytic disease of newborn, hyperbilirubinemia**,** ABO, blood group.

新生儿溶血病(高清网络)期间新生儿高胆红素血症的血液学特征：一种管理高胆红素血症的方法

摘要：新生儿溶血病(高清网络)后果导致相当大的发病率和死亡率，因此立即诊断和治疗以防止严重后果至关重要。由于苏丹胎儿和新生儿溶血病(高密度宽带网络)的调查和预防措施缺乏

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对标准国际协议的遵守（由于缺乏足够的产前护理），因此本研究可能有助于提高生活质量。本研究旨在确定患有高清网络的苏丹黄疸新生儿的不同血液学参数（血红蛋白、HCT、红细胞和成红细胞值）和母胎ABO血型不相容。对苏丹乌姆杜尔曼妇产医院收治的305名母亲及其胎儿进行了描述性横断面研究。确定黄疸新生儿的ABO和恒河猴(博士)血型、直接抗球蛋白试验(数据)、加拿大广播公司和血涂片评论，并用于数据分析。研究期间记录总胆红素、直接胆红素水平、母亲年龄、胎龄、体重、性别、光疗、血液交换和新生儿发热情况。使用SPSS软件版本16进行统计数据分析。具有阳性数据的高清网络的总体频率为57(18.9%)。高清网络中博士、ABO和其他血型系统不相容分别为4例（1.3%）、18例（5.9%）和35例（11.4%）。总胆红素和直接胆红素、胎龄、输血史和光疗有统计学意义。库姆试验阳性与异常外周细胞（球形红细胞、NRBC、多染性和靶细胞）之间存在高度显着的关联。这项研究表明，高胆红素血症的高密度宽带网络患病率为57人（18.9%），由于苏丹缺乏标准方案，这仍然是一个挑战。尽管使用了预防性抗D，但博士仍然是导致高密度宽带网络的最重要抗原。数据与异常血细胞之间存在显着关联。

关键词: 新生儿溶血病、高胆红素血症、ABO、血型。

1. Introduction

Hemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloimmunization to blood group antigens that are expressed by fetal red blood cells, which induces fetal anemia with severe neonatal hyperbilirubinemia, kernicterus, and increased risks of fetal death in severe cases [1–3]. Most severe cases of HDFN are attributed to rhesus (RhD) incompatibility between an RhD–negative woman and her RhD–positive fetus, with RhD alloimmunization having occurred during a previous pregnancy [3, 4].

RhD incompatibility refers to the discordance between maternal and fetal RhD types, leading to the development of maternal RhD sensitization and hemolytic disease of the newborn (HDN). If their erythrocytes have expressed the RhD antigen, an individual can be classified as RhD-positive; an individual without the RhD antigen is classified as RhD-negative. This phenomenon becomes clinically significant if a mother that is RhD-negative becomes sensitized to the RhD antigen and subsequently produces anti-D antibodies (i.e., alloimmunization) that can bind to and potentially lead to the destruction of RhD–positive erythrocytes. This can result in consequences along the spectrum of HDN, ranging from self-limited hemolytic anemia to severe hydrops fetalis [5].

On the other hand, ABO hemolytic disease occurs almost exclusively in infants with A or B blood groups born to O blood group mothers because the antibodies IgG anti-A and anti-B occur more commonly in blood group O than blood group A or B individuals. However, in blood group A2 mothers with high titer anti-B, rare cases of ABO incompatibility have been reported. DAT-positive tests have a higher prevalence in Asian and Black ethnicities than Caucasians [6]. The lower expression of A and B antigens on fetal red cells minimizes the feto-maternal incompatibility, and ABO hemolytic disease is generally a mild occurrence. However, descriptions of unusually severe disease necessitating active intervention and exchange blood transfusions have been documented in the literature and have also been reported by us [7]. The prevalence of non-RhD alloantibodies in pregnancy has been found to range from 0.15% to 1.1% [8]. About one-third of the new cases in a study from Norway on immunization to the Rh system were found in RhD–positive women and included anti-C and anti-E antibodies [9]. A common disorder during the neonatal period is hyperbilirubinemia, which is associated with a variety of physiologic and pathologic conditions [10]. Isoimmune hemolytic disease has been identified as a pathologic cause of neonatal hyperbilirubinemia attributed to blood group incompatibility. Blood group A and blood group B newborns of blood group O mothers are defined as having ABO incompatibility. ABO hemolytic disease is the major cause of neonatal hyperbilirubinemia [11]. Hence, the current study aimed to determine different hematological parameters (Hb, HCT, RBC, and erythroblast values) and maternal–fetal ABO blood type incompatibility among Sudanese jaundiced neonates with HDN.

2. Materials and Methods

2.1. Design of the Study and Participants

This prospective cross-sectional hospital-based study was carried out at Omdurman Maternity Hospital, Sudan. The study was conducted between March and June 2021. A total of 305 individuals (305 mothers and their 305 jaundiced newborns) were enrolled in the study. Ethical permission was provided by the Ethical Research Committee, Faculty of Medical Laboratory Sciences, Alzim Alazhari University, Sudan. Informed consent was acquired from the selected subjects before participation in the study. The primary data were gathered using a self-administered questionnaire designed to obtain data that would support the study.

2.2. Sample Collection

Venous blood samples (2.5 mL) were collected from mothers and their newborns using sterile disposable plastic syringes after cleaning the venipuncture area with 70% ethanol. The blood was added to anticoagulant at a ratio of 1.5 mg of EDTA to 2.5 mL of blood.

2.3. Methods

Measurement of ABO and RhD blood groups, direct Coomb's test, and complete blood counts were performed, and stained thin blood films were also taken to confirm the presence of abnormal peripheral cells that indicate types of hemolytic disease. The Sysmex KX-21N hematology analyzer was used to determine 17 hematological parameters.

Also, the history of total bilirubin, direct bilirubin level, mother's age, gestational age, weight, gender, phototherapy, blood exchange, and fever of neonates were recorded during the study.

2.4. Statistical Data Analysis

Statistical analysis was performed by Statistical Package for Social Sciences (SPSS Software Version 16) for windows. P-values equal to or less than 0.05 were considered statistically significant.

3. Results

Three hundred five individuals (mothers with their jaundiced newborns) were admitted to Omdurman maternity hospital. The frequency of hemolytic disease in the fetus and newborn was 57(18.9%); 4 (1.3%) and 18 (5.9%) had positive DAT with RhD incompatibility and ABO incompatibility, respectively, 35 (11.4%) with RhD/ABO compatibility. 40 (13.1%) with ABO HDN had blood group O:A, and 28 (9.1%) had blood group O:B incompatibility (mother's:child's, respectively). There were no cases of HDN due to the sub-blood group of A (A2:[A1, B, and A2B] [mother: child, respectively]). Regarding the frequency of ABO compatibility, of the selected 305 patients, 237 (77.7%) were found ABO-compatible, and 68 (22.3 %) were ABO-incompatible. Mother–fetus RhD compatibility was also observed in all the groups' different mating types. So, within the RhD-compatible group, mother_fetus was highest in 294 (96.4%) and lowest in 11 (3.6%) with mother fetus RhD incompatibility (Table 1).

Table 1 Baseline data of study subjects

	Frequency (n = 302)	**Percentage %**
Gestational age		
Term	149	49.3%
Preterm	156	51.7%
Blood group types		
ABO HDN	18	5.9%
RhD HDN	4	1.3%
Other blood group system	35	11.4%
Total	57	18.9%
ABO compatibility		
Compatible fetal mother of ABO group	273	77.7%
Incompatible fetal mother of ABO group	68	22.3%
Rh compatibility		
RhD-compatible	294	96.4%
RhD-incompatible	11	3.6%
History of blood transfusion		
Yes	20	5.6%
No	285	94.4%
Babies under phototherapy		
Yes	74	24.5%
No	231	76.5%

Table 2 shows mean levels of bilirubin, RBCs, Hb, and HCT between gestational ages. Mean direct bilirubin, RBCs, HB, and HCT levels were close approximately for the term and preterm neonates, and there was no significant difference between both. However, there was a significant difference in the mean level for total bilirubin between term (10.77 ± 6.52) and preterm neonates (9.423.51) (P-value = 0.02). In addition, there was a significant difference in the mean level of direct bilirubin (0.96 ± 0.78) between the genders (P-value ≤ 0.03) (Table 3). According to Table 4, there was a highly significant difference between total (18.68 ± 9.99) and direct bilirubin (± for the study participants) (P-value = 0.001). Table 5 shows that mean RBCs, HB, and HCT levels were close approximately for phototherapy neonates, and there was no significant difference between both. However, there was a highly significant difference between total and direct bilirubin for both (P-value = 0.001).

Table 2 Mean levels of bilirubin, RBCs, Hb, and HCT between gestational ages

	Gestational age	N	Mean	Std. Deviation	P-value
Total bilirubin mg/dl	Term	149	10.77	6.52	0.02
	Preterm	156	9.42	3.51	
Direct bilirubin mg/dl	Term	149	1.42	2.38	0.08
	Preterm	156	1.05	1.21	
RBCs/Microliter	Term	149	3.81	0.86	0.5
	Preterm	156	3.74	0.85	
g/dL	Term	149	13.39	3.07	0.8
	Preterm	156	13.32	3.30	
HCT%	Term	149	36.98	9.05	0.9
	Preterm	156	36.96	10.23	

Table 3 Comparison of bilirubin, RBCs, Hb, and HCT means and newborn gender

	Gender	N	Mean	Std. Deviation	P-value
Total bilirubin mg/dl	Female	130	9.56	5.19	0.1
	Male	175	10.46	5.25	
Direct bilirubin mg/dl	Female	130	0.96	0.78	0.03
	Male	175	1.43	2.38	
RBCs/microliter	Female	130	3.73	0.82	0.4
	Male	175	3.80	0.88	
g/dL	Female	130	13.22	3.06	0.5
	Male	175	13.45	3.28	
HCT%	Female	130	36.97	9.05	0.9
	Male	175	36.97	10.11	

Table 4 Comparison of bilirubin, RBCs, Hb, and HCT means and history of blood transfusion

	Baby blood transfusion	N	Mean	Std. Deviation	P-value
Total bilirubin mg/dl	Yes	20	18.68	9.99	0.001
	No	285	9.47	4.13	
Direct bilirubin mg/dl	Yes	20	2.53	4.22	0.001
	No	285	1.14	1.57	
RBCs/microliter	Yes	20	3.75	0.74	0.9
	No	285	3.78	0.86	
g/dl	Yes	20	12.68	1.92	0.3
	No	285	13.40	3.25	
HCT%	Yes	20	35.26	7.08	0.4
	No	285	37.09	9.81	

Table 5 Comparison of bilirubin, RBCs, Hb, and HCT means and phototherapy

	Babies under phototherapy	N	Mean	Std. Deviation	P-value
Total bilirubin mg/dl	Yes	74	14.96	5.75	0.0001
	No	231	8.51	3.95	
Direct bilirubin mg/dl	Yes	74	1.86	2.87	0.0001
	No	231	1.03	1.38	
RBCs/microlitre	Yes	74	3.89	0.89	0.1
	No	231	3.74	0.84	
g/dL	Yes	74	13.55	3.16	0.5
	No	231	13.29	3.20	
HCT%	Yes	74	37.29	10.06	0.7
	No	231	36.87	9.55	

Table 5 shows that mean total bilirubin, direct bilirubin, and RBCs levels were close approximately for RhD-incompatible and RhD-compatible neonates; mean HB and HCT were lower, and there was no significant difference between both. Table 7 shows that mean total bilirubin, direct bilirubin, RBCs, HB, and HCT levels were close approximately for ABO incompatibility and ABO compatibility neonates. There was no significant difference between both. Table 8 illustrates the correlation of blood smears with abnormal peripheral cells in RhD incompatibility, ABO incompatibility, and the Coombs test. There was a highly statistically significant association between blood smears and abnormal cells in all cases. In the gestational age group, NRBCs were found to be significantly associated with abnormal cells and gestational age.

Table 6 Comparison of bilirubin, RBCs, Hb, and HCT means and RhD compatibility

	Rh compatibility	N	Mean	Std. Deviation	p-value
Total bilirubin mg/dl	Rh-incompatible	11	9.22	4.11	0.4
	Rh-compatible	294	10.15	5.31	
Direct bilirubin mg/dl	Rh-incompatible	11	1.10	1.06	0.7
	Rh-compatible	294	1.24	1.93	
RBCs/microliter	Rh-incompatible	11	3.67	1.10	0.5
	Rh-compatible	294	3.78	0.83	
g/dL	Rh-incompatible	11	12.70	3.77	0.3
	Rh-compatible	294	13.40	3.14	
HCT%	Rh-incompatible	11	34.82	10.44	0.2
	Rh-compatible	294	37.15	9.59	

Table 7 Comparison of bilirubin, RBCs, Hb, and HCT means and blood ABO

	ABO compatibility	N	Mean	Std. Deviation	P-value
Total bilirubin mg/dl	ABO incompatibility	68	10.63	6.40	0.1
	ABO compatibility	237	9.71	4.25	
Direct bilirubin mg/dl	ABO incompatibility	68	1.46	2.31	0.06
	ABO compatibility	237	1.07	1.51	
RBCs/microliter	ABO incompatibility	68	3.79	0.74	0.7
	ABO compatibility	237	3.76	0.91	
g/dL	ABO incompatibility	68	13.36	2.95	0.9
	ABO compatibility	237	13.34	3.35	
HCT%	ABO incompatibility	68	37.09	9.11	0.8
	ABO compatibility	237	36.89	10.04	

Table 8 Types of cells in peripheral blood film

Types of cells	Chi P-value			
	RhD-Incompatible	ABO Incompatibility	Positive Coomb's test	Gestational age group
NRBCs	0.002	0.0001	0.005	-
Spherocytes	0.0001	0.0001	0.0001	-
Target cells	-	0.0001	0.0001	-
Polychromasia	-	0.0001	0.0001	0.02

4. Discussion

HDFN with positive DAT was detected in 57 (18.9%) cases; 4 (1.3%) and 18 (5.9%) cases had a positive DAT with RhD incompatibility. ABO incompatibility with RhD/ABO compatibility was detected in 35 (11.4%) cases, so this may be due to antibodies against other blood group systems. However, this frequency will be higher than 18.9% due to the presence of cases with negative DAT**.** Thus, this frequency (18.9%) indicates confirmed cases of ABO and RhD HDN incompatibility. Our findings agree with [12], who found that 25.7% of newborns were DCT positive and 74.3% of newborns were DCT negative; ABO and RhD incompatibility was found in 94.4% and 5% of newborns, respectively. The same result was obtained in [13]. The process by which newborns with blood group incompatibility become vulnerable to brain trauma is still unknown. Methemoglobin and other hemolysis products can be toxic to the brain or worsen bilirubin encephalopathy. Therefore, the antibody screening test for mothers should be implemented to monitor infants born with ABO and RhD-incompatible pregnancies.

This study revealed that there was a statistically significant difference in gestational age regarding total serum bilirubin. This study is in agreement with [14] and [15], who noted that the direct bilirubin levels were significantly higher in female infants than in male infants during the neonatal period.

On the other hand, our study showed a significant difference in total and direct serum bilirubin (mean 14.96 ± 5.75 and 1.86 ± 2.87) between the fetuses during the exchange blood transfusion (P-value = 0.0001). This study agrees with [16-20]. Exchange transfusion is a relatively secure therapeutic option for NNH because it induces fast removal of serum bilirubin, decreasing the risk of kernicterus in such patients.

Our findings revealed a highly significant difference in total and direct serum bilirubin between newborns under phototherapy. Therefore, this study is in concordance with [21-23].

There was no significant difference in red cell indices and total serum bilirubin at RhD incompatibility and compatibility. This study disagreed with [22], who found that hyperbilirubinemia was more severe among babies with RhD incompatibility; neonates had a hemoglobin value < 13 g/dL.

Moreover, current results demonstrated no significant difference in red cell indices (RBCs, HB and HCT) between ABO incompatibility and compatibility. This study agrees with [23, 24], who found no statistically significant difference in hematological parameters, and

disagrees with [25], who found red blood cell (RBC), hemoglobin (Hb), and hematocrit (Hct) values significantly lower and erythroblast values higher for the ABO-incompatible than compatible [25]. The severity of the ABO HDN in the neonates may relate in part to the level of IgG Anti-A or anti-B in the mothers and the IgG subclass. There are a small number of fully developed A or B antigen sites on fetal red blood cells. Antibodies of IgG Anti-A and anti-B are absorbed onto other tissues bearing these surface antigens. This study revealed no significant difference in total serum bilirubin between ABO incompatibility and compatibility. This study agrees with [26]. Nevertheless, it disagreed with an Indian study conducted by Shetty A and Kumar BS in 2014; they concluded that ABO blood group incompatibility was the most common cause of hyperbilirubinemia [25]. The same result was documented by several other studies as well [28-30]. A highly statistically significant association was found between (NRBC) cells with ABO and/or RhD-incompatible cells. This study agreed with Kahvecioğlu D (2021), who noted that upon comparing NRBCs/100 WBCs, NRBCs were statistically higher in their study subjects, and certainly higher with positive direct Coomb's test than in patients with negative Coomb's test ($p < 0.05$) [27]. Our result revealed that there was a highly significant association between (spherocyte, polychromasia, and target) cells in ABO incompatibility and non-compatible fetal-mother ABO; this is in agreement with [23, 29].

Our findings showed that there was a highly significant association between positive Coomb's test with presence of abnormal peripheral cells (spherocyte, NRBCs, polychromasia, and target) rather than negative Coomb's test, which indicates the presence of cases of HDN [29, 30].

Finally, hyperbilirubinemia is known as a common pathologic cause of isoimmune hemolytic disease in newborns, which can lead to serious consequences. Accordingly, both parents and healthcare professionals should take preventive measures to appropriately diagnose and treat the disease. Health authorities should manage neonatal jaundice conferences, seminars, and trainings for mothers. Doctors and researchers should seek additional therapies and protection strategies that have no adverse effects and can enable babies to recover faster. Before marriage, partners should have their ABO blood groups and Rh factor checked. Marriage between relatives should be precluded.

Disagreement and conflict between our results and other studies may be due to different related factors, such as the lack of antibody screening of mothers, difference in ethnic groups and laboratory techniques used, as well as sample size of study participants. In Sudan, there is a general need for routine screening and investigation of blood group incompatibility, such as incompatibility of RhD, ABO, and other blood groups, that causes HDN. In particular, ABO and RhD incompatibilities should be evaluated as soon as possible for all neonates born to O positive and RhD negative mothers, respectively, to detect high risk of developing hyperbilirubinemia and/or hemolytic disease among neonates with blood group incompatibilities.

5. Conclusion

This study revealed that the prevalence of HDN with hyperbilirubinemia was 57 (18.9%), which remains a persistent problem due to the lack of standard testing protocols in Sudan. Despite the use of prophylactic anti-D, RhD remains the most prominent antigen contributing to HDN, having a significant association with abnormal blood cells as shown by DATs.

Investigation and prevention of HDN in Sudan have lacked the application of standard international protocols and proper antenatal care. Therefore, considering that there are few studies published regarding these issues in the Sudanese population, this study may be useful in the development of better diagnostic and treatment methods for improvement of the quality of life of the affected individuals. One of the causes of neonatal hyperbilirubinemia is maternal–fetal ABO incompatibility. We hypothesize that in cord blood units, the hemoglobin, hematocrit, and RBC values decrease and the erythroblast values significantly increase for maternal–fetal ABO incompatible blood groups as compared to those for compatible blood groups. Additionally, blood group incompatible newborns have been linked to a higher risk of considerable hyperbilirubinemia and associated morbidities, such as the development of kernicterus.

6. Limitations of the Study and Future Prospective

The current study had the following limitations: First, the study had a small sample size as all participants were recruited from one hospital; this was not a sufficiently representative sample of the entire Sudanese population. Second, the study did not account for the mode of delivery and its correlation to the rate of complications. Third, the study did not include the screening and identification of antibodies to determine various types of HDN. Lastly, other causes of neonatal hyperbilirubinemia were not screened for.

Prospective points of view: In Sudan, there is a general need for routine screening and investigation of blood group incompatibility, such as incompatibility of RhD, ABO, and other blood groups, that causes HDN. In

particular, ABO and RhD incompatibilities should be evaluated as soon as possible for all neonates born to O positive and RhD negative mothers, respectively, to detect high risk of developing hyperbilirubinemia and/or hemolytic disease among neonates with blood group incompatibilities. More studies for investigations, especially those based on antibody screening and identification, to determine various types of HDN need to be conducted. Future studies with large sample sizes need to focus on the association between cord blood laboratory values and clinical outcome in order to clarify the clinical role of the cord blood RBC indices in the diagnosis of hyperbilirubinemia and/or hemolytic disease due to blood group incompatibilities. Best practices for detecting neonatal jaundice need to be implemented. If severe neonatal jaundice occurs in a setting of blood group incompatibility, treatment with intravenous immunoglobulin, which usually avoids the invasive procedure of an exchange transfusion, should be considered.

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