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Efficacy of Postnatal Anti-D Immunoprophylaxis in RhD-Negative Pregnant Women in Benin

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Abstract

Hemolytic Disease of the Fetus and Newborn (HDFN) results from fetal-maternal blood group incompatibility, particularly involving the RhD antigen. Despite preventive strategies, HDFN remains a significant cause of perinatal morbidity, especially in low-income regions. This study evaluates the efficacy of postnatal anti-D immunoprophylaxis in RhD-negative women, considering the potential benefits of earlier administration. A longitudinal follow-up study was conducted from June 2023 to September 2024. Data were collected through structured questionnaires and laboratory analyses, including ABO and RhD blood typing using the Beth-Vincent and Simonin methods and irregular antibody screening via the indirect Coombs test. Statistical analyses were performed using Fisher's exact test with contingency tables and Microsoft Excel. Among 174 women, 50.57% were aged 25 - 35, and 56.32% were in their third trimester at the time of enrollment. Over half (53.45%) did not know their spouse's blood type. Of the 131 RhD-negative women with RhD-positive partners, 5.71% developed anti-D antibodies despite postnatal prophylaxis. No significant association was found between immunization and parity, previous transfusions, or miscarriage history. Postnatal anti-D immunoprophylaxis does not fully prevent alloimmunization. These findings highlight the need for earlier immunoprophylaxis, including routine fetal RHD genotyping and targeted antenatal anti-D administration, to improve maternal and neonatal outcomes.

Keywords

Hemolytic Disease of the Fetus and Newborn (HDFN), Irregular Antibodies, Alloimmunization, Pregnant Women, Benin

1. Introduction

Hemolytic Disease of the Fetus and Newborn (HDFN) is a condition caused by fetal-maternal blood group incompatibility, particularly involving the RhD antigen (RH1) of the rhesus system [1]. It was first reported by a French midwife in 1909 [2] and results from the mother's immune response against fetal red blood cells carrying the RhD antigen inherited from the father [3] [4]. The anti-D antibody is responsible for approximately one-third of HDFN cases, whereas HDFN associated with other antibodies is rarely fatal [5]. According to the work of Mirelen Moura de Oliveira Rodrigues *et al*., it turned out that the anti-D antibody is cited in most cases of HDFN [6]. Prior to the 1970s, maternal-fetal antigen-antibody incompatibility was the leading cause of mortality in fetuses beyond 22 weeks of gestation and neonates up to seven days old [7]. The disease remains a major concern in low-income countries, particularly in Asia and sub-Saharan Africa, where it accounts for over 50,000 deaths annually [8]. Despite being largely preventable, Rh(D)-associated HDFN continues to contribute significantly to neonatal morbidity and mortality, particularly in low- and middle-income countries, due to gaps in screening and prophylaxis implementation strategies [9]. Clinically, HDFN manifests as fetal anemia, hyperbilirubinemia, kernicterus, and, in severe cases, death [9].

Maternal alloimmunization occurs when fetal red blood cells from an RhD-positive fetus cross into the maternal circulation of an RhD-negative mother, triggering an immune response. The mother then produces antibodies against the fetal D antigen [10]. This exposure can result from several pregnancy-related events, including spontaneous or induced abortion (with uterine evacuation for hydatidiform mole between 12 and 20 weeks of gestation), ectopic pregnancy, chorionic villus sampling, amniocentesis, fetal death during the second or third trimester, vaginal bleeding before labor, and abdominal trauma [11]. Wilken (1988) suggested that maternal immunization can occur even without a known sensitizing event [12].

To prevent HDFN, the introduction of anti-D immunoglobulin therapy has proven highly effective in reducing the incidence of the disease, particularly in high-income countries [13] [14]. Initially, immunoprophylaxis was introduced prenatally in 1969 [15]. Over time, the practice has evolved to consider the potential circumstances of fetal-maternal blood exchange that could lead to maternal alloimmunization. Postnatal administration of anti-D immunoprophylaxis may be insufficient, as Hollán (1969) demonstrated that prophylaxis achieves greater effectiveness when administered earlier in pregnancy [16].

Prenatal diagnostic techniques have been developed to predict the fetal RhD genotype [17]-[19] to improve prevention strategies. Only RhD-negative pregnant women carrying RhD-positive fetuses receive anti-D immunoprophylaxis, optimizing serum availability and minimizing unnecessary risks [20]. Although significant progress has been made in preventing HDFN in developed countries, postnatal prophylaxis remains the primary approach in low- and middle-income countries like Benin. This gap underlines the need for globally coordinated but locally adapted prevention strategies, as emphasized by Zipursky et al., who advocate for equitable access to Rh disease prevention, particularly in resource-limited settings [21]. HDFN remains a major health concern in Africa, whereas its prevalence has significantly decreased in developed countries [21]. We previously showed that approximately 9.7% of women are at risk of delivering infants with HDFN, while postnatal immunoprophylaxis remains the predominant preventive strategy [22]. Similarly, populations in low-income and middle-income countries have a low level of knowledge [23].

Management of HDFN primarily involves red blood cell transfusions, which carry risks of infection and immunohematological complications [24]. Nevertheless, postnatal immunoprophylaxis does not guarantee complete protection for all women. This study evaluates the effectiveness of postnatal anti-D immunoprophylaxis in RhD-negative women in Benin. Addressing this issue is essential to enhancing awareness among healthcare professionals, particularly obstetricians, for better management and monitoring of RhD-negative pregnancies. Ultimately, improved prevention strategies could significantly reduce HDFN cases.

2. Materials and Methods

2.1. Study Population

This longitudinal follow-up study was conducted in Benin from June 2023 to September 2024 and included 174 pregnant women. Among them, 171 were confirmed as RhD-negative, and 131 had RhD-positive partners, forming the final analytical cohort, as illustrated in the flow diagram (**Figure 1**). The required sample size was determined using Schwartz's formula:

$$n = \frac{z^2 \times p \times (1-p)}{m^2}$$

where $z = 1.96$ for a 95% confidence level, $p = 6.2\%$ (estimated prevalence of the target characteristic, based on data from Tesfaye K. Kanko and Woldemariam [25], and $m = 5\%$ margin of error. This yielded a minimum sample size of 72.06. To enhance the reliability and representativeness of the results, the sample size was increased, resulting in the inclusion of 131 RhD-negative pregnant women with RhD-positive partners in the main analysis.

2.2. Ethical Considerations

The study received ethical approval from the Local Ethics Committee for Biomed-

ical Research at the University of Parakou (Ref: 0578/CLERB-UP/P/SP/R/SA, May 9, 2023). Following this, research authorization requests were submitted to the heads of all participating healthcare institutions. All twelve facilities granted approval, ensuring comprehensive geographic representation across Benin's five regions (South, Center, North, East, and West). Samples were collected from a diverse range of healthcare centers, including the Abomey-Calavi University Hospital (CHUZ) in the Atlantic department; the Mother and Child University Hospital (CHUMEL), Hubert Koutoukou Maga National University Hospital (CNHU-HKM), the Humanitarian Health Centers of Saint Jean and Saint Luc, Mênontin District Hospital, and Bethesda Hospital in the Littoral; the Departmental University Hospital (CHUD-OP) in Porto-Novo for the Ouémé and Plateau departments; Lokossa District Hospital in the Mono department; the Zou and Collines Departmental Hospital (CHD ZC) in Abomey and the Ouèssè Health Center in the Collines; and the Natitingou District Hospital in the Atacora department.

Participation in the study was entirely voluntary. All study objectives, procedures, and potential risks were clearly explained to each participant and her spouse in a language they understood. Written informed consent was obtained prior to inclusion. For participants with limited literacy, the consent form was read aloud, and consent was validated by thumbprint, in accordance with ethical guidelines. Confidentiality was strictly maintained by anonymizing all personal data and securing digital access with password protection.

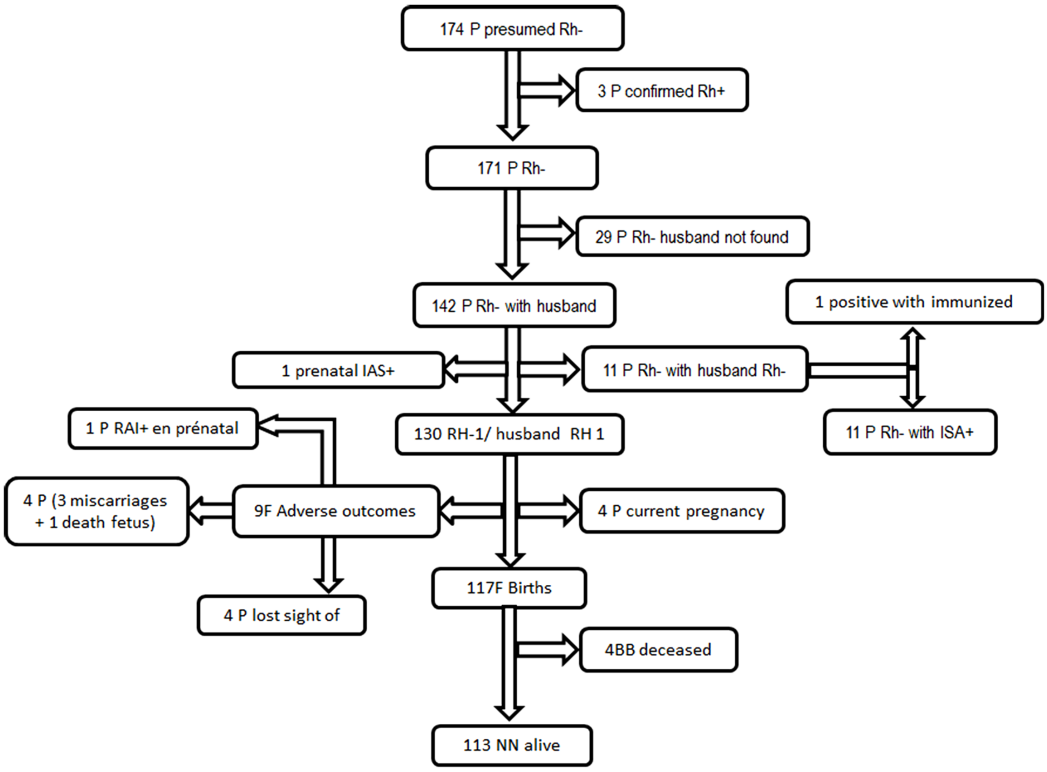

Figure 1. Flow diagram of study participant selection and follow-up.

The diagram illustrates the selection and follow-up process of pregnant women in the study, categorizing them based on their Rh status and the result of Irregular Antibody Screening (IAS). Pregnant women (P) were classified as Rh-negative (Rh−) or Rh-positive (Rh+), with further subgrouping based on partner Rh status and subsequent testing phases.

2.3. Participant Recruitment and Data Collection

Pregnant women were recruited using a structured questionnaire administered via the Kobotoolbox application. The questionnaire collected data on sociodemographic characteristics, history of blood transfusion, history of miscarriage, number of previous pregnancies, gestational age, and contact details for follow-up at different study stages. After explaining the study objectives and potential benefits, the questionnaire was administered upon obtaining informed consent from participants and their spouses. Some participants completed the questionnaire independently. Responses were securely recorded in the application by a single researcher using a unique password.

2.4. Blood Sample Collection and Laboratory Analysis

Five milliliters of blood were collected from each woman and her partner in a dry tube for ABO and RhD blood typing. Only RhD-negative women with RhD-positive partners were included in the study. In addition to RhD blood typing, an Irregular Antibody Screening (IAS) test was performed on the women. A second blood sample (5 mL) was collected from the women one to two months after the initial sampling to reassess IAS. After childbirth, neonatal ABO and RhD blood typing were performed to determine the newborns' Rh phenotypes. Women with RhD-positive newborns who received anti-D immunoprophylaxis within 72 hours postpartum underwent a third blood sample collection at least 30 days after immunoprophylaxis to perform a final IAS. The 30-day interval allows sufficient time for the potential formation of anti-D antibodies if fetal RhD-positive red blood cells enter maternal circulation [26]. This third IAS was conducted only in women with a previously negative IAS to assess seroconversion despite postnatal anti-D immunoprophylaxis.

2.5. Quality Control and Sample Verification

To optimize sample collection, participants were contacted multiple times, and home visits were conducted when necessary. All laboratory tests included quality controls, with positive and negative internal controls for each analysis. ABO blood typing was performed in duplicate using both tube and slide methods. The detection of antigens on the surface of red blood cells was conducted using anti-A, anti-B, and anti-AB sera for the Beth-Vincent globular test. Antibody screening was performed using locally prepared A, B, and O red blood cells. RhD antigen detection was conducted using anti-D sera, and weak D antigen detection was consistently performed using the indirect Coombs test [27].

2.6. Reagents and Analytical Procedures

Two reagent sets were used for quality control: the first technician employed Séraclone (a monoclonal reagent from Bio-Rad®) for the tube method, while the second used DAGAST (a polyclonal reagent from MEDIFF). Irregular antibody screening was also conducted using the indirect Coombs test, which involved two steps. The first step involved incubating the antigen with the immune antibody (if present) in a low-ionic-strength solution (LISS) to accelerate antibody-antigen binding. The second step involved visualizing antigen-antibody complex formation through agglutination using an anti-globulin serum [10] [28].

For antibody screening, panels of three locally prepared group O red blood cells were used, sourced from phenotyped donors with common antigenic profiles: Cell 1 (D+, C+, E−, c−, e+), Cell 2 (D+, C−, E+, c+, e−), and Cell 3 (D−, C−, E−, c+, e+). Antibody identification for positive IAS cases is being conducted as part of a separate study.

3. Data Processing

Results were presented as proportions for variables such as sex, age, gestational age, previous pregnancies, occupation, history of transfusion, and the number of women recruited per healthcare facility. ABO and RhD blood group frequencies were determined for women, their partners, and their newborns using Microsoft Excel 2019. Association measures were calculated using odds ratios with a 95% confidence interval. Associations between immunization status and factors such as parity, transfusion history, and miscarriage history were analyzed using Fisher's exact test with contingency tables, processed using software version 4.4.2 (2016). A $p < 0.05$ was considered statistically significant.

4. Results

4.1. Sociodemographic and Clinical Characteristics

The study population was primarily composed of women aged 25 - 35 years, representing 56.90% of participants, followed by those aged 18 - 24 years, who accounted for 31.61%. The smallest proportion, 11.49%, consisted of women aged 36 years and older (Table 1). Gestational distribution showed that most participants (56.32%) were in the third trimester at the time of enrollment, while 32.18% were in the second trimester. The first trimester had the lowest representation, with 11.49% of participants (Table 1). Awareness of the partner's RhD status varied among participants, with 46.55% knowing their partner's RhD status, whereas 59.45% were unaware (Table 1).

4.2. Immunization Status and Associated Factors

Among the 131 women confirmed as RhD-negative with RhD-positive partners, 5.71% developed anti-D antibodies despite receiving postnatal immunoprophylaxis. Table 2 consolidates data on immunization rates in relation to study period,

parity, history of transfusion, and history of miscarriage. Results showed that one woman was already immunized before delivery, while 6 (5.71%) became immunized postpartum despite receiving anti-D immunoprophylaxis. The data also show that many women were multiparous and non-immunized, with no significant correlation between parity and immunization status ($p > 0.05$) (Table 2). Similarly, no significant associations were observed between immunization and history of blood transfusion or miscarriage ($p > 0.05$) (Table 2).

Table 1. Demographic and clinical profile of participants.

Characteristic	N (%)
Age (years)	
18 - 24	55 (31.61)
25 - 35	99 (56.90)
36+	20(11.49)
Trimester of Pregnancy	
First	20 (11.49)
Second	56 (32.18)
Third	98 (56.32)
Knowledge of Partner's RhD Status	
Aware	81 (46.55)
Unaware	93 (59.45)

Table 2. Immunization rates and contributing factors.

Factor	Category	Non-Immunized N (%)	Immunized N (%)	p-value
Study Period	Prenatal	100 (99.01)	1 (0.99%)	<0.001
	Postnatal	94 (94)	6 (6)	
Parity	Primiparous	26 (81.25)	6 (18.75)	0.44
	Multiparous	68 (98.55)	1 (1.45)	
History of Transfusion	Yes	4 (80)	1 (20)	0.24
	No	90 (93.75)	6 (6.25)	
History of Miscarriage	Yes	8 (100)	0 (0)	0.42
	No	86 (92.47)	7 (7.52)	

4.3. Newborn Blood Typing and RhD Distribution

Among the 114 newborns analyzed, blood group O was the most prevalent, accounting for 48.25% of cases, followed by blood groups B (30.70%) and A (18.42%). Blood group AB was the least frequent, representing only 2.63% of the sample. The distribution of neonatal blood groups and RhD status showed a predominance of group O, with 55 neonates, of whom 49 (89.09%) were RhD-positive and 6 (10.91%) RhD-negative. Blood group B followed with 35 cases, of which 34 (97.14%) were RhD-positive and 1 (2.86%) RhD-negative. Blood group A was

observed in 21 neonates, with 17 (80.95%) being RhD-positive and 4 (19.05%) RhD-negative. Blood group AB had the lowest representation, with only 3 neonates, among whom 2 (66.67%) were RhD-positive and 1 (33.33%) RhD-negative. Overall, RhD-positive status was predominant across all blood groups, particularly in groups B and O (Table 3).

Table 3. Distribution of neonatal blood groups and RhD status.

Neonatal Blood Group	RhD Status	N (%)
A	+	17 (80.95)
	–	4 (19.05)
B	+	34 (97.14)
	–	1 (2.86)
O	+	49 (89.09)
	–	6 (10.91)
AB	+	2 (66.67)
	–	1 (33.33)

A, B, O, AB: Blood groups in the ABO system; +: RhD positive; –: RhD negative.

4. Discussion

This study was conducted across all regions of Benin to provide a comprehensive assessment of the effectiveness of postnatal anti-D immunoprophylaxis, minimizing regional biases. The findings confirm that postnatal immunoprophylaxis does not fully protect all RhD-negative women from alloimmunization, as a non-negligible proportion of participants developed anti-D antibodies despite receiving prophylaxis. This is in line with findings from Cochrane reviews, which indicate that while postnatal administration of anti-D immunoglobulin significantly reduces alloimmunization, it does not guarantee complete prevention—particularly when administration is delayed or when prior sensitization has already occurred [14]. This observation aligns with Hollán (1969), who emphasized that immunoprophylaxis is most effective when administered earlier during pregnancy [16]. A notable finding was the misclassification of some women initially presumed to be RhD-negative but later confirmed as RhD-positive. This discrepancy highlights potential issues with RhD blood grouping accuracy, possibly due to errors in weak D antigen testing or misinterpretation of results. Ensuring high-quality serological testing is critical to prevent misdiagnosis and inappropriate prophylaxis administration.

One woman in the study was already immunized before delivery, despite receiving anti-D immunoprophylaxis after previous pregnancies. This raises concerns about potential failures related to the timing or dosage of prophylaxis [29]. Administering insufficient doses or delaying prophylaxis may contribute to maternal alloimmunization, reinforcing the need for standardized dosing and administration protocols. Furthermore, routine administration of anti-D immuno-

globulin without prior irregular antibody screening could lead to unnecessary use of the prophylactic agent, increasing healthcare costs and depleting resources [29]. This practice may also contribute to shortages of anti-D immunoglobulin, which should be prioritized for RhD-negative women who genuinely require it. These findings are consistent with previous reports from low-resource settings, where postnatal prophylaxis alone has shown limited effectiveness in preventing alloimmunization. For instance, studies in Nigeria [30] and Ethiopia [25] have reported seroconversion rates ranging from 4% to over 6% despite postpartum administration of anti-D immunoglobulin, reflecting gaps in timing, dosage, or immunization before delivery. This suggests that reliance solely on postnatal prophylaxis may not be sufficient in similar contexts.

The distribution of ABO and RhD blood groups observed in this study aligns with trends reported in other African countries, such as Guinea [31], Tunisia [32], and Cameroon [33]. However, the relatively high prevalence of RhD-negative individuals compared to studies in Algeria [34] but lower than values reported in European populations, such as those in France [35], is expected given the known genetic differences in RhD antigen distribution between African and Caucasian populations. Additionally, some newborns were RhD-negative despite having RhD-positive fathers, likely due to heterozygosity in the paternal genotype [11].

Given the limitations of postnatal prophylaxis, the administration of anti-D immunoprophylaxis during pregnancy is warranted for RhD-negative women. Implementing routine fetal RHD genotyping through non-invasive prenatal testing would allow for targeted prophylaxis, ensuring that only women carrying RhD-positive fetuses receive immunoglobulin [28]. This would enhance both the efficiency and cost-effectiveness of immunoprophylaxis while preventing unnecessary exposure to blood-derived products. Additionally, healthcare providers, particularly obstetricians and midwives, should receive adequate training in RhD-negative pregnancy management to improve adherence to best practices.

Despite its contributions, this study has several limitations. Although the sample size was increased beyond the calculated minimum to enhance reliability, it may still not fully capture the heterogeneity of the target population across Benin. Differences in laboratory infrastructure, personnel expertise, and sample handling protocols across collection sites may have introduced inter-laboratory variability, despite efforts to standardize procedures. The exclusive use of serological methods for RhD typing -while routine in many clinical settings- can lead to variability in detecting weak D variants, potentially resulting in misclassification and inappropriate administration of prophylaxis. Similar limitations have been reported in other sub-Saharan studies, highlighting the challenges of relying solely on conventional blood group testing in genetically diverse populations. Population-based studies in high-income countries, such as those conducted in Sweden, have also shown persistent alloimmunization despite established prophylaxis protocols, suggesting the need for individualized risk assessment and earlier intervention strategies [20].

Moreover, this study did not account for potentially confounding factors such as the timing and dosage of prophylaxis, access to prenatal care, maternal education, or socioeconomic status -all of which may influence the risk of alloimmunization, as suggested by previous research. Future studies should aim to integrate these clinical and social determinants while also employing molecular RhD typing to improve diagnostic accuracy and better capture the dynamics of alloimmunization. Indeed, the clinical utility of non-invasive fetal RHD genotyping has been well-documented. Studies have shown that it can improve the precision of antenatal immunoprophylaxis, helping to avoid unnecessary anti-D administration while ensuring protection for truly at-risk pregnancies [15].

Beyond methodological considerations, the findings carry important implications for clinical practice and national policy. In clinical settings, they emphasize the need to move beyond reliance on postnatal prophylaxis alone and adopt a more proactive approach through early identification of RhD incompatibility. Incorporating non-invasive fetal RhD genotyping during pregnancy would enable targeted antenatal prophylaxis, thereby optimizing anti-D immunoglobulin use and minimizing unnecessary exposure. The potential value of such an approach is currently under investigation by our research team in an ongoing complementary study involving the same population. From a policy standpoint, implementing this strategy in Benin would require careful evaluation of its feasibility and long-term benefits. While molecular genotyping methods are currently limited to centralized laboratories, their decentralization through regional reference centers could improve accessibility. Therefore, cost-effectiveness analyses are warranted to assess the viability of integrating routine fetal RhD genotyping into the national maternal health system. Such strategies could significantly reduce the burden of Hemolytic Disease of the Fetus and Newborn in low- and middle-income countries and align with global efforts to eliminate preventable causes of perinatal morbidity.

5. Conclusion

This study demonstrates that postnatal anti-D immunoprophylaxis in RhD-negative pregnant women is not entirely effective, as a proportion of women still developed anti-D antibodies despite receiving prophylaxis. These findings highlight the need for improved preventive strategies, including earlier administration of immunoprophylaxis and routine fetal RHD genotyping. Increased awareness and training among healthcare providers are crucial for optimizing patient care. Policymakers should consider integrating fetal genotyping into standard prenatal care to ensure selective and efficient prophylaxis, ultimately reducing the risk of alloimmunization and improving maternal and neonatal health outcomes.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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