



Recombinant anti-D for prevention of maternal-foetal Rh(D) alloimmunization: a randomized multi-centre clinical trial

Rahul Vishwanath Mayekar, MD¹, Gopalkrishna Vinayak Paradkar, MD², Archana Anilkumar Bhosale, MD¹, Rekha Sachan, MS³, Sumalatha Beeram, MS⁴, Ashok Ramachandra Anand, MD⁵, Shuchita Ramesh Mundle, MD⁶, Yamini Trivedi, MD⁷, Rashmi MD, MS⁸, Kiran Pandharinath Patole, MD⁹, Pradip Wamanrao Sambarey, MD¹⁰, Gautam Vinod Daftary, MBBS¹¹, James John, MD¹¹, Ganesh Harishchandra Divekar, MBBS¹¹

Department of Obstetrics and Gynaecology, ¹Lokmanya Tilak Municipal Medical College & Lokmanya Tilak Municipal General Hospital, Mumbai, ²Rajiv Gandhi Medical College & Chhatrapati Shivaji Maharaj Hospital, Thane, ³King George's Medical University, Lucknow, ⁴Gandhi Medical College and Hospital, Secunderabad, ⁵Grant Medical College and Sir J.J. Group of Hospitals, Mumbai, ⁶Government Medical College and Hospital, Nagpur, ⁷AMC MET Medical College and Sheth LG General Hospital, Ahmedabad, ⁸Apollo BGS Hospital, Mysore, ⁹Dr. Vasant Rao Pawar Medical College and Research Centre, Nashik, ¹⁰Byramjee Jeejeebhoy Government Medical College and Sassoon General Hospitals, Pune; ¹¹Clinical Research and Pharmacovigilance, Bharat Serums and Vaccines Limited, Navi Mumbai, India

Objective

To compare the efficacy and safety of recombinant anti-D (R-anti-D) with conventional polyclonal anti-D (Poly anti-D) in preventing maternal-fetal rhesus D (RhD) alloimmunization and to investigate the immunogenicity of R-anti-D.

Methods

This was a randomized, open-label, multi-center clinical trial conducted in RhD-negative pregnant women who did not receive antenatal anti-D who delivered RhD-positive babies and showed negative indirect Coombs tests (ICTs) at baseline. The women were randomized in a 2:1 ratio to R-anti-D or Poly anti-D groups and were administered 300 mcg (IM) of the corresponding drug within 72 hours of delivery. ICT was performed 72 hours, 90 days, and 180 days after anti-D injection. Serum samples were collected to check for the development of antibodies against R-anti-D at days 90 and 180, using bridging enzyme-linked immunosorbent assay. The proportion of subjects who had positive ICT results at days 90 and 180 were compared between the groups using Fisher's exact test.

Results

A total of 144 women were randomized to the R-anti-D group and 71 to the Poly anti-D group. Three women in the R-anti-D and none in the Poly anti-D group had a positive ICT result at day 90. No woman in either group had positive ICT result at day 180. Both drugs were well tolerated with only 4 reports of adverse events in each group—all were mild, non-serious, and resolved without sequelae. No subject developed antibodies against R-anti-D.

Conclusion

The studied R-anti-D is comparable in efficacy to conventional Poly anti-D and is safe and non-immunogenic.

Trial Registration

Clinical Trials Registry of India Identifier: CTRI/2017/03/008101

Keywords: Rho(D) immune globulin; Recombinant proteins; Newborn hemolytic disease; Rh isoimmunization

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Corresponding author: James John, MD

Clinical Research and Pharmacovigilance, Bharat Serums and Vaccines Limited, Liberty Tower, Plot K-10, Airoli, Navi Mumbai 400708, India

E-mail: dr.jamesjohn85@gmail.com

https://orcid.org/0000-0002-7168-269X

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Introduction

Hemolytic disease of the fetus and newborn (HDFN) results in the destruction of the fetus or newborn's red blood cells by preformed maternal immunoglobulin G (IgG) antibodies against red cell antigens. The anti-D alloantibody against the rhesus D (RhD) antigen is most frequently responsible for HDFN [1] and causes the most widespread form of severe HDFN, as there is a relatively high frequency of the RhD-negative phenotype and the RhD antigen is highly immunogenic [2]. RhD alloimmunization in the pregnant mother may cause anemia in the fetus or newborn, and severe cases may ultimately lead to baby's demise. The most effective strategy used to reduce the incidence of RhD alloimmunization is the introduction of anti-D IgG prophylaxis [3].

Routine use of postpartum anti-D IgG in RhD-negative women has decreased the rate of alloimmunization from 16% to 2% and this was further reduced to <0.1% by additional antepartum administration of anti-D [4,5].

Conventionally, anti-D is produced via fractionation of IgG from the pooled plasma of donors who are primarily RhD-negative men deliberately immunized with RhD-positive red blood cells [6]. The resultant IgG is polyclonal in nature and referred to as polyclonal anti-D (Poly anti-D). An inherent limitation of this method is the requirement for human donors, limited capacity of production [7], theoretical risk of transmission of viral/prion diseases, and periodic shortages [8]. Most of these limitations were addressed by the introduction of monoclonal anti-D (Mono anti-D), manufactured using the hybridoma technique. A commercial preparation (Rhoclone[®]) is available in some countries, including India. However, technologically, maintaining hybridoma in a stable culture is difficult and the generation of monoclonal antibodies is a time-consuming and laborious process [9]. Furthermore, with the advent of recombinant DNA technology, the availability of growth supplements for hybridoma such as fetal bovine serum (FBS) is declining, thus limiting the possibility of increasing monoclonal antibody outputs [10].

Considering the advancements made in technologies used for antibody manufacture, the natural successors to hybridoma-derived antibodies are recombinant DNA-derived antibodies. The manufacturer of Rhoclone[®] (Bharat Serums and Vaccines Limited, Navi Mumbai, India) developed anti-D IgG antibodies using recombinant DNA technology and genes for anti-D derived from the hybridoma used to manufacture

Rhoclone[®]. The antibody genes from this hybridoma were isolated and introduced in Chinese hamster ovarian cells (CHO), thus enabling the cells to express recombinant anti-D (R-anti-D). The steps in the manufacture of R-anti-D using antibody genes from Rhoclone[®] hybridoma are illustrated in Fig. 1.

In this clinical trial, we aimed to compare the efficacy and safety of this R-anti-D preparation with those of conventional Poly anti-D when used in post-partum immunoprophylaxis. Antidrug antibodies (ADAs) may be generated *in vivo* as part of an immune response to therapeutic antibody drugs and may significantly affect the efficacy and safety of these drugs. Thus, for such drugs, in addition to efficacy and safety evaluation, assessment of the immunogenic potential is essential before approval for use in humans and is required by regulatory agencies. This trial, therefore, had the additional objective of assessing the immunogenicity of R-anti-D.

Materials and methods

1. Study design

This was a randomized, controlled, open-label, multi-center trial comparing an R-anti-D preparation with a conventional Poly anti-D preparation. The comparator, Poly anti-D, was selected because of its efficacy and safety profile, established over the last six decades, as well as its universal availability and acceptance. The overall study was designed according to the European Medicines Agency's "Guideline on the clinical investigation of human anti-D immunoglobulin for intravenous and/or intramuscular use - CPMP/BPWG/575/99 Rev. 1" [11]. The trial was conducted at obstetric in-patient departments in 10 tertiary care hospitals in India.

2. Study participants

RhD-negative pregnant women who did not receive antenatal anti-D, who delivered RhD-positive babies, and whose indirect Coombs test (ICT) test results were negative at baseline were eligible for the study. The main exclusion criteria were positive ICT test results at baseline, the husband/partner having an RhD-negative blood group, a history of incompatible blood transfusion, allergic reaction to immunoglobulins, or IgA deficiency, anticipated requirement for blood transfusion after delivery and diagnosis of abruptio placentae, placenta previa, or intrauterine death. Study subjects were random-

ized in a 2:1 ratio to one of 2 groups, with a total sample size of 210 subjects (140 subjects in the R-anti-D group and 70 subjects in the Poly anti-D group). A 2:1 ratio was chosen to generate data regarding the new R-anti-D preparation, as the comparator Poly anti-D's efficacy and safety has already been established in numerous studies and could be referenced from literature [12,13].

3. Subject randomization

Subjects were randomly assigned in a 2:1 ratio to either the R-anti-D or Poly anti-D group using a computer-generated randomization code. A 2:1 ratio was acceptable as the reference product Poly anti-D is well established with ample scientific data confirming its efficacy and safety. Additionally, more data (especially safety data) could be obtained with the new recombinant preparation. Codes were provided to the study sites in sealed envelopes.

4. Intervention

Subjects received 300 mcg of R-anti-D (manufactured by Bharat Serums and Vaccines Limited) or Poly anti-D (RhoGAM®; Kedrion Biopharma Inc., Melville, NY, USA) within

72 hours of delivery.

5. Study outcomes

The primary efficacy variable was the proportion of subjects with a positive ICT result on day 180 following administration of anti-D. ICT is used to detect circulating antibodies to red cell antigens. A positive ICT result at day 180 in a subject who showed a negative ICT result before anti-D administration would indicate that the subject had become immunized to the RhD antigen. ICT results obtained after 72 hours and at day 90 were also assessed, although because administered anti-D IgG is present in detectable quantities for up to 12 weeks after an anti-D injection [14] and as it is not possible to distinguish between administered and immune anti-D IgG, these results were considered as supportive evidence and were not carried forward for day 180. Only serial increases in titers were considered positive results.

The safety variables assessed included the incidence of adverse events (AEs), such as injection site reactions in both groups, and the incidence of immunogenicity (development of ADAs) in the R-anti-D group.

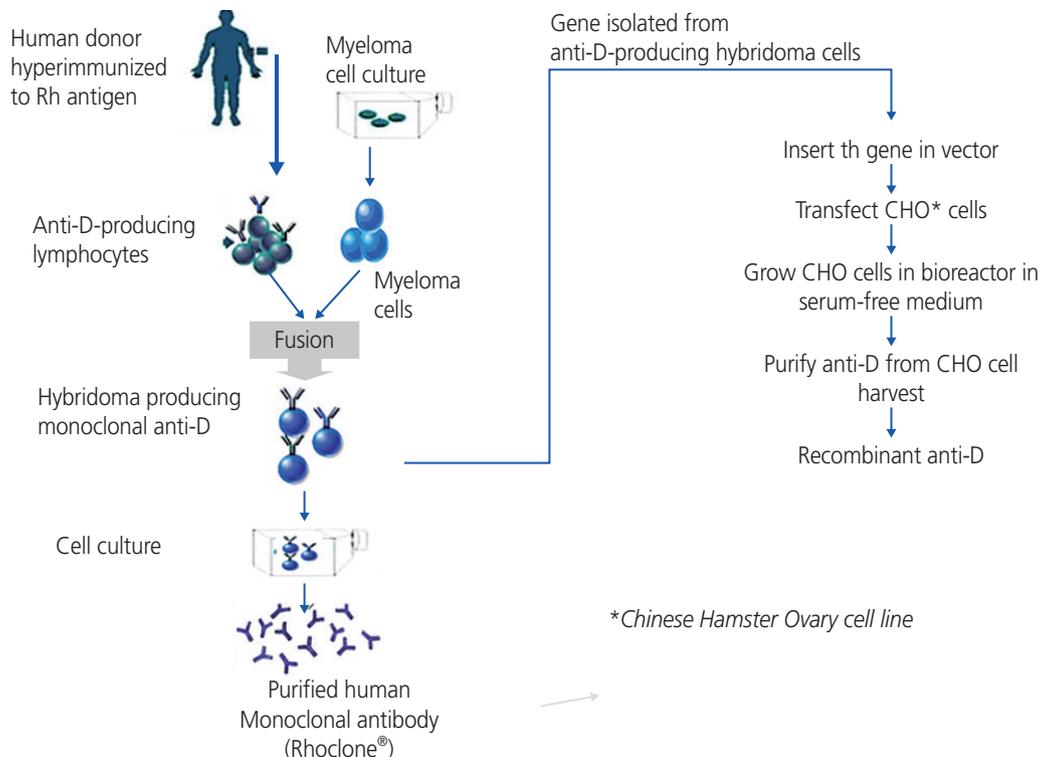


Fig. 1. Manufacturing process and link between monoclonal anti-D (Rhoclone®) and recombinant anti-D.

6. Study procedures

Each eligible subject received a single intramuscular injection of anti-D IgG within 72 hours of delivery. Blood samples were collected before administration of the study drug (baseline) as well as 72 hours, 90 days, and 180 days after anti-D administration, as recommended by the European Medicines Agency guidelines. ICT was performed on all samples. AEs were recorded throughout the study. Subjects lost to follow-up were considered as failure of therapy (ICT positive) for the purpose of this analysis.

7. Immunogenicity assessment

The immunogenic responses of subjects allocated to the R-anti-D group were evaluated. This was accomplished by detection of ADAs in the subjects' sera at baseline as well as at 30, 90, and 180 days after R-anti-D administration.

The assay used for immunogenicity assessment was validated according to the 2016 "Assay development and validation for immunogenicity testing of therapeutic proteins" guidelines laid down by the United States Food and Drug Administration [15]. ADAs were detected using an acid dissociation bridging enzyme-linked immunosorbent assay (ELISA). To quantitate antibodies against R-anti-D, a biotin-digoxigenin complex-based format of the bridging ELISA was used (Fig. 2). Biotin-tagged anti-D was coated on streptavidin-coated 96-well plates. Following incubation, washing, and blocking, a mixture of the sera (containing antibodies against anti-D) and digoxigenin-tagged anti-D was added to designated wells. Samples prepared via acid dissociation were then added to the wells. This complex was further reacted with anti-digoxigenin streptavidin-horseradish peroxidase conjugate. After incubation, tetramethylbenzidine substrate was added. The

reaction was stopped via the addition of 2N H₂SO₄, and absorbance was recorded on an ELISA plate-reader at 450 and 570 nm. The samples were assigned potentially positive or negative status based on the "cut point" generated by the negative controls used in the specific run. Potentially positive samples were subjected to a confirmatory assay against positive controls, and the amounts of antibodies present in the confirmed positive samples were evaluated via titer-based analysis using serial dilutions of positive control samples.

8. Statistical analysis

The sample size was calculated considering the 2:1 allocation ratio of subjects to the R-anti-D and Poly anti-D groups. It is reported that approximately 2% of women achieve seroconversion after the administration of postpartum anti-D [5]. With a statistical power of 80%, a non-inferiority margin of 2% treatment effect, and with expected incidences of RhD sensitization of 4% and 1% in the R-anti-D and Poly anti-D groups, respectively, a sample size of 210 subjects (140 in the R-anti-D group and 70 in the Poly anti-D group) was deemed appropriate for this study. Results were analyzed to assess the non-inferiority of R-anti-D to Poly anti-D with regard to the primary efficacy variable using Fisher's exact test. SPSS[®] version 18.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis and *P*<0.05 was considered to indicate statistically significant differences.

Results

1. Baseline characteristics

The trial was conducted between June 30, 2017 and July 4,

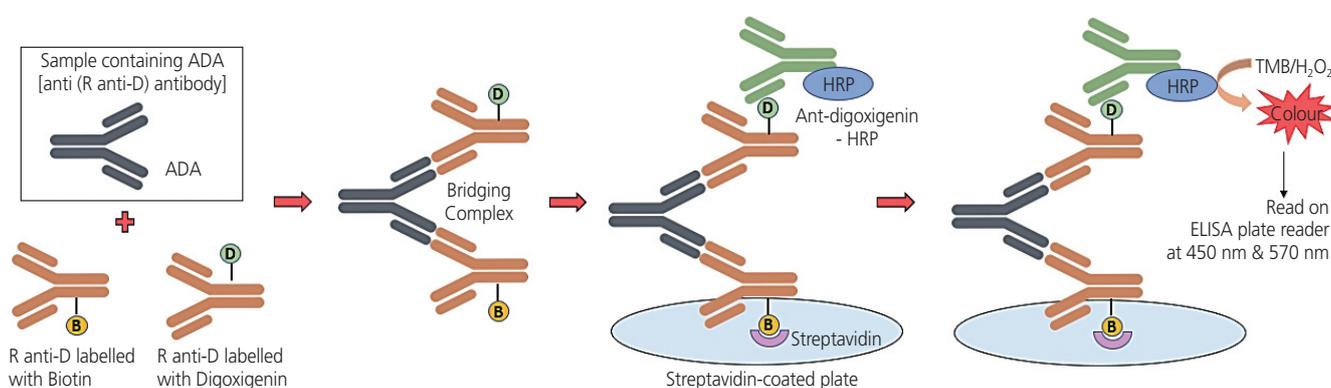


Fig. 2. Principle of biotin-digoxigenin complex-based bridging ELISA to test the immunogenicity of R-anti-D. ADA, antidrug antibody; R-anti-D, recombinant anti-D; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; ELISA, enzyme-linked immunosorbent assay.

2018. A total of 228 women were screened across 10 study centers, and 215 were randomized to a group and received one of the study drugs: 144 were administered R-anti-D and 71 received Poly anti-D. The study participant flow is shown in Fig. 3.

Demographics and baseline characteristics were comparable between the 2 treatment groups (Table 1). The population analyzed for safety assessment (safety population) included all 215 subjects who received either of the study drugs and the population analyzed for efficacy assessments (efficacy population) comprised 210 subjects. Five subjects enrolled in the study were subject to protocol deviations,

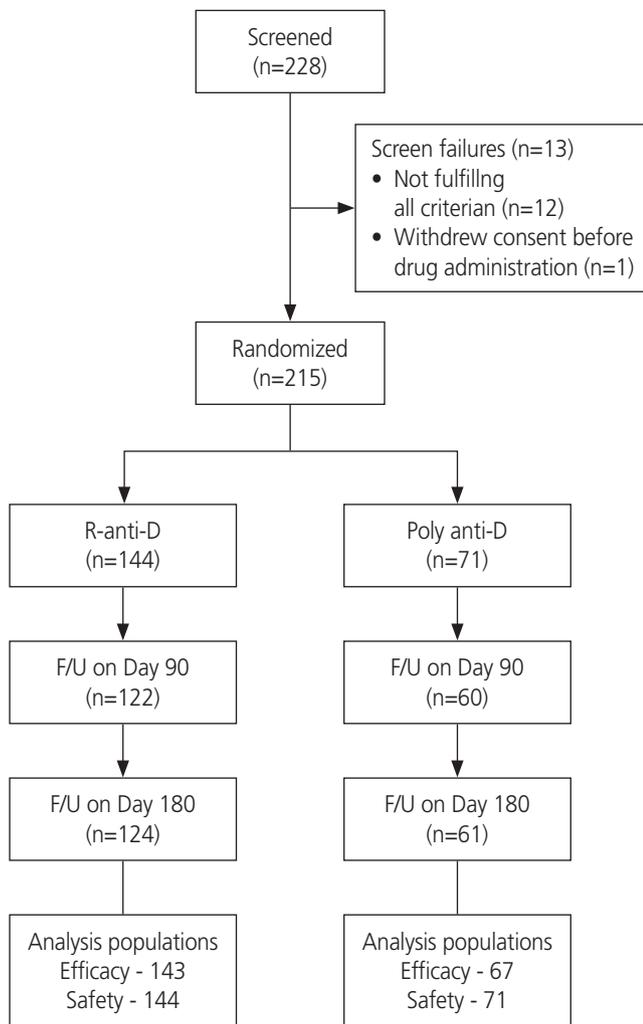


Fig. 3. Trial and participant flow. One subject from the R-anti-D group and 4 subjects from the Poly anti-D group were excluded from efficacy analysis owing to major protocol deviations. R-anti-D, recombinant anti-D; Poly anti-D, polyclonal anti-D; F/U, followed-up.

Table 1. Demographic and baseline characteristics

Characteristics	R-anti-D	Poly anti-D
Subjects randomized	144	71
Age (yr)	25.95±4.0	25.71±4.2
Gravidity		
1	50 (34.7)	22 (31.0)
2	55 (38.2)	24 (33.8)
3	28 (19.4)	16 (22.5)
4	9 (6.3)	6 (8.5)
5	2 (1.4)	2 (2.8)
6	0 (0)	1 (1.4)
Parity		
0	34 (23.6)	15 (21.1)
1	71 (49.3)	32 (45.1)
2	26 (18.1)	21 (29.6)
3	11 (7.6)	3 (4.2)
4	2 (1.4)	0 (0)
Gestational age (wk)		
<28	2 (1.4)	1 (1.4)
28–32	7 (4.9)	3 (4.2)
33–37	61 (42.4)	36 (50.7)
38–42	74 (51.4)	29 (40.8)
>42	0 (0)	0 (0)
Not known	0 (0)	2 (2.8)
Type of delivery		
Vaginal	82	39
Caesarean section	60	32
Forceps	2	0
Delivery outcome		
Single live birth	141	69
Twin live birth	3	2
Neonatal DCT		
Negative	144 (100)	71 (100)
Neonatal blood group		
A+ve	41	14
B+ve	44	19
AB+ve	9	5
O+ve	53	34
B–ve	0	1 ^{a)}

Data are shown as mean±standard deviation or number (%). R-anti-D, recombinant anti-D; Poly anti-D, polyclonal anti-D; DCT, direct Coombs test.

^{a)}Excluded from efficacy analysis.

including delivery of an RhD-negative baby, positive ICT result at baseline ICT, and receiving the study drug beyond the stipulated study time period, which rendered these subjects ineligible for efficacy analysis. The exclusion of these subjects from efficacy assessment did not impact the findings of the study as the study drug does not impact the subject's underlying condition. Of the 210 randomized subjects, 183 (87.13%) completed the day 90 visit, and 185 (88.10%) completed the day 180 visit. No subject was discontinued because of safety reasons. Among the randomized subjects, 13.29% of the R-anti-D group subjects and 8.96% of the Poly anti-D group subjects were lost to follow-up before completion of the study.

2. Efficacy endpoints

On day 90, 3 subjects (2.09%) from the R-anti-D group showed positive ICT results and none of the subjects from the Poly anti-D group showed positive ICT results. On day 180, none of the subjects from the R-anti-D or Poly anti-D groups reported a positive ICT result.

In the efficacy population, a negative ICT result at day 180 was reported in 86.71% of subjects in the R-anti-D group and 91.04% in the Poly anti-D group.

The *P*-values for ICT results calculated using Fisher's exact test for day 90 (*P*=0.30) and 180 (*P*=0.49) indicated that the differences were not statistically significant and thus confirmed similar efficacy between the recombinant and Poly anti-D groups (Table 2).

3. Safety outcomes

Eight AEs were reported by 8 subjects, 4 in each of the 2 groups. The details are shown in Table 3. All AEs reported were mild and deemed unrelated to study medications by the investigators.

4. Immunogenicity

A total of 493 samples from subjects who were administered R-anti-D, obtained at baseline as well as on day 30, 90, and 180 visits, were available for analysis. These were subjected to screening ELISA, in which 14 samples tested positive. These samples were further subjected to confirmatory analyses against positive controls and all samples returned negative results, thus confirming that none of the samples were positive for anti-R-anti-D antibodies.

Discussion

The worldwide prevalence of RhD disease is estimated to be 276 per 100,000 live births, and it is estimated that 50% of babies with untreated HDFN will either die or develop brain damage as a result of the disease [16]. Rhesus disease may be prevented by avoiding pregnancy-related RhD alloimmunization of mothers by anti-D administration. However, considering the problems faced in Poly anti-D production and the potential risk of transmission of infectious diseases [17], alternative methods of obtaining rhesus immunoglobulin were investigated. Mono anti-D is manufactured using anti-D-producing hybridomas. These are made from anti-D-producing lymphocytes obtained from a hyperimmunized human donor fused with myeloma cells. However, considering the inherent limitations of hybridoma technology [9], newer methods are

Table 2. Efficacy data: indirect Coombs test results

Time-point & result	R-anti-D (n=143)	Poly anti-D (n=67)	P-value (Fisher's exact test)
Day 90			
Positive	3	0	0.30 (NS) ^a
Negative	119	61	(0.55) ^b
LTF/not performed	21	6	
Day 180			
Positive	0	0	0.49 (NS) ^a
Negative	124	61	(1.00) ^b
LTF/not performed	19	6	

R-anti-D, recombinant anti-D; Poly anti-D, polyclonal anti-D; LTF, lost to follow-up; NS, not statistically significant.

^aCalculated for efficacy population with "LTF/not performed" subjects considered as failure of therapy; ^bThe *P*-value in parenthesis is obtained after excluding "LTF/not performed" subjects from analysis.

Table 3. Safety data: AEs

AE	R-anti-D group	Poly anti-D group
Pyrexia	1	2
Abdominal pain	2	0
Itching	0	1
Hypertension	0	1
Hypotension	0	2
Deranged leucocyte count	1	0

AE, adverse event; R-anti-D, recombinant anti-D; Poly anti-D, polyclonal anti-D.

currently being explored. There is also the concern of global shortage of transmissible spongiform encephalopathies/bovine spongiform encephalopathy-certified FBS, which is used as growth supplement for hybridoma cells [10]. Additionally, this FBS may contain protein contaminants. Recombinant DNA technology offers major advantages over hybridoma technology by obviating the need for FBS and the problem of cell line changes and mutations associated with classical hybridoma production and storage, as well as providing better yield.

Among common red cell alloantibodies, anti-D is the most long-lived [18]. Immune anti-D IgG is developed by the body in response to Rh antigen and appears 6 weeks to 6 months after antigen exposure [19]. Conversely, passive anti-D (administered anti-D) may be detected in enzyme tests and ICT for 12 weeks or longer after administration, and cannot be differentiated from immune anti-D [20]. This is why a 6-month (day 180) time-point was considered suitable for evaluating the effectiveness of anti-D prophylaxis. Antibody detection at other time-points provides supportive data only and should be followed-up to check for rising titers and confirm that these are due to immune anti-D.

This study demonstrated that the efficacy of R-anti-D matches that of Poly anti-D in preventing the development of RhD alloimmunization. None of the subjects in either group developed immune anti-D antibodies at the end of 6 months. This demonstrates that R-anti-D may serve as a suitable substitute for Poly anti-D in preventing RhD alloimmunization.

The safety profile of Poly anti-D is excellent, with approximately 0.7% of women report minor, predictable, and transient AEs [21,22]. Therefore, a new anti-D replacing the existing Poly anti-D must match its safety and efficacy. In our study, less than 1% of participants in each group reported AEs, and none of the reported AEs were deemed to be related to the study drug. This indicates that R-anti-D was well tolerated by the subjects and that its safety profile is in line with that of Poly anti-D.

The findings of the study indicate that the new R-anti-D not only matches Poly anti-D in efficacy but that it is also safe and non-immunogenic. It provides a potentially limitless supply of safe and effective anti-D IgG. R-anti-D, as it is obtained from serum-free medium, also quells the concerns of transmission of infectious diseases. R-anti-D may be a suitable alternative to existing anti-D preparations in the market

for the prevention of maternal alloimmunization, especially as the supply of human-sourced Poly anti-D is expected to diminish.

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Conflict of Interest

The author Gautam Vinod Daftary is the managing director at Bharat Serums and Vaccines Limited. The authors James John and Ganesh Harishchandra Divekar are full-time paid employees of Bharat Serums and Vaccines Limited.

Ethical approval

The trial protocol (code: BSV/r-ANTI D/10) was approved by the Indian drug regulatory authority (Central Drugs Standard Control Organization) and the institutional ethics committees of all the participating centers. The trial was prospectively registered on the Clinical Trials Registry of India (CTRI No. CTRI/2017/03/008101). The trial was performed in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization Guidelines for Good Clinical Practice, and local regulatory requirements.

Patient consent

All study subjects provided voluntary written informed con-

sent to participate in the study.

No other consent was obtained as no material pertaining to the identity of subjects was included in this article.

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