Improved protection at term with an alternative strategy of prophylactic anti-D administration in pregnancy - a prospective interventional study

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Abstract

Objective: To analyse the proportion of RhD negative women where routine antenatal anti-D prophylaxis (RAADP) in gestational week (gw) 28 is not detectable at delivery and to investigate if a strategy with RAADP administered in gw 28 and 38 result in efficient protection at term, postterm and postdelivery. Design: A retrospective database analysis and a prospective interventional study. Setting: Antenatal centers in the Stockholm region. Sample: RhD negative women carrying an RHD positive fetus, 4280 cases evaluated retrospectively, 39 cases included prospectively. Methods: In a retrospective analysis, RhD negative women with a negative antibody screen at delivery was determined. In 39 pregnancies, quantification of anti-D was analysed before a second dose anti-D was administered in gw 38, and then weekly up to 43 weeks post gestation. Main outcome measures: The proportion of women with non-detectable anti-D at term. The concentration of anti-D measured weekly at term, postterm and postdelivery. Results: In 20,5% (856/4280, retrospective analysis) and 44% (17/39, prospective study) RAADP in gw 28 was below screening detection level, 10 IU/L at term and in 18% (7/39, prospective study) below 1 IU/L in the quantification assay. Anti-D prophylaxis administered in gw 38 showed stable protective levels of anti-D up to 30 days postdelivery, with concentration at delivery 60+34 IU/L (mean+SD). Conclusion: At least one third of the RhD negative women have non-detectable levels of RAADP given at gw 28, at term and postterm. A second dose of RAADP at gw 38 improves the protection.

Background

Despite that anti-D prophylaxis regimens to prevent alloimmunisation during pregnancy are used for fifty years, and are shown effective, anti-D is the most common red cell antibody causing severe hemolytic disease of the fetus and newborn (HDFN).[1] Intrauterine transfusions are in the majority of cases due to anti-D immunisation.[2]

Postnatal anti-D immunoglobulin prophylaxis to RhD negative women with a newborn blood typed RhD positive was introduced in the late 1960s and reduced the risk of immunisation of RhD negative women from approximately 15% to 1-1.5%. [3,4]In the 1990s many countries added routine antenatal anti-D prophylaxis (RAADP) in the third trimester to all RhD negative women, further minimising the risk of immunisation to an incidence of 0.2-0.4%.[5] During the last decade fetal *RHD* -typing combined with targeted RAADP only to those carrying an *RHD* positive fetus, has been proven effective and is implemented in many countries.[6] The strategies of doses and timing of administration of RAADP vary between countries, one single dose of anti-D; 1000-1500 IU in gestational week 28-30 or two doses of 500-625 IU at 28 and 34 weeks are the most common routines.[7,8,9] A second or third dose anti-D is given postdelivery, usually a dose of 1000-1500 IU.

In some programs, testing of fetomaternal hemorrhage (FMH) is recommended at delivery, to determine if additional doses of anti-D are required.[10]

It is known, that one single dose anti-D in gestational week 28 is not detectable at delivery in a proportion of women, varying from 39-56% and up to 78% for those who deliver after 40 weeks of gestation.[7,8] thus not protecting them from immunisation at the end of the third trimester, when the risk of immunisation is increased.[9,11,12] The two-dose strategy gives a higher rate of detectable anti-D at term, 85%, but is associated to higher costs and lower compliance of receiving the two doses.[13,14]

In Sweden the recommendations are to perform fetal *RHD* screening in the first trimester, and to administer targeted RAADP, 1500 IU in gestational week 28 and a second dose of anti-D 1500 IU postdelivery,[15,16] and at situations carrying an increased risk of fetomaternal hemorrhage during pregnancy.

Today, when fetal *RHD* typing is widely implemented and the*RHD* type of the fetus is known with high accuracy[6] an alternative strategy could be to administer two RAADP doses, at gestational week 28 and 38, the latter to cover term and post term of the pregnancy. If the postdelivery anti-D dose could be excluded, this strategy could be cost effective and have the potential of high compliance.

The aim of this study was to retrospectively analyse the proportion of women with undetectable levels of prophylactic anti-D at the time of delivery after one dose of RAADP (1500 IU) at gestational week 28. Secondly, in a prospective study, to investigate if a strategy with administration of the second dose of anti-D in gestational week 38, instead of postdelivery, would improve the protection at term and if the protection would be enough after delivery.

Material and Methods

*Retrospective data analysis*The study population consisted of all consecutive cases of RhD negative pregnant women with request from the delivery ward for type-and-screen test, between October 2010 and October 2012 in the Stockholm region, Sweden. After this period, an RhD-negative test panel for type-and-screen requests in RhD negative women was introduced, in order not to cause delay when blood transfusions were needed. In the laboratory information system, the proportion of RhD negative women, with an *RHD* positive fetus, and a negative type-and-screen test at delivery was identified retrospectively, i.e. those where the anti-D prophylaxis administered in gestational week 28, at that time Rhesonativ®, 1250 IU, (Octapharma, AG, Austria) was not detectable at delivery.

Prospective interventional study

Between 2016 to 2018, RhD negative women with a fetus typed *RHD*positive, were asked to participate in the study, at their appointment to receive RAADP, 1500 IU (Rhophylac®, CSL Behring GmbH, Marburg, Germany) in gestational week 28. Eligible participants were healthy women without previous history of pregnancy complications, with a singleton pregnancy and estimated due date confirmed by ultrasound examination in the second trimester. Exclusion criteria were administration of anti-D prophylaxis earlier in the current pregnancy, a multiple pregnancy, presence of maternal erythrocyte alloantibodies at time of inclusion or medication other than vitamins, folic acid and iron supplement. Before inclusion, a routine obstetric history was checked, as well as blood pressure. Information on body height and weight was received from the first antenatal visit in gestational week 10-14.

The women were given oral and written information from a research midwife and if they agreed they received a second dose of anti-D prophylaxis 1500 IU in gestational week 38. Anti-D concentration was analysed before the anti-D administration in gestational week 38 and then monitored weekly until the correspondent time of 43 weeks gestational age, including postdelivery. The quantitative anti-D analysis was done only daytime weekdays, so administration of anti-D prophylaxis after delivery was based on the type-and-screen test. If the erythrocyte antibody screen was positive at delivery, the women received no more prophylaxis, if the anti-D level was low at delivery, defined as negative or a weak reaction in the routine antibody screen, it was decided to recommend anti-D prophylaxis for safety purposes. 39 pregnant women gave informed consent

and were included in the study. The demographic characteristics of the included women are shown in Table 1.

Type-and-screen analysis

Type-and-screen at delivery was performed in an automated system AutoVue Innova in BioVue Cassettes with anti-IgG (Ortho Clinical Diagnostics, Raritan, NJ, US) in the retrospective analysis, 2010-2012. During the interventional study 2016-2018 the instrument was upgraded to an Ortho Vision, with the same reagents used.

Titration of the 2nd BRITISH STANDARD 1992 Anti-D (Rho) Antibodies (code: 73/515, NIBSC, Potters Bar, UK) in the BioVue Cassettes showed that lower limit of detection (LOD) for anti-D was 10 IU/L (2 μg/L) quantified by flow cytometry. A weak reaction in the screening was defined as $< 1+$ reaction and corresponded to anti-D 10-20 IU/L (2-4 μg/L).

Analysis of anti-D

Anti-D quantification was performed using flow cytometry. To increase sensitivity erythrocytes were pre-treated with the enzyme papain. All dilutions were made in ID-CellStab (Bio-Rad Laboratories Inc) with 0.5% BSA (A7906, Sigma-Aldrich) as additive. The calibration standard was the 2nd BRITISH STANDARD 1992 Anti-D (Rho) Antibodies (code: 73/515, NIBSC, Potters Bar, UK). In short, 50 μL 0.8% erythrocytes (R0r) and 100 μL standard or sample was incubated for 30 min at 37°C. After washing with 3 x 200 μL PBS, 50 μL mouse anti-human IgG-PE (Southern Biotech) diluted 100-fold was added and incubated for 30 min at RT. After an additional wash as above, the erythrocytes were dispersed in 250 μl PBS and analysed on Cytomics FC500 MPL. The mean fluorescense intensity (MFI) on 10,000 gated cells were measured and imported to SoftMaxPro (Molecular Devices) which was used for constructing the calibration curve and calculation of anti-D concentration. The LOD was 1 IU/L (0.2 μg/L).

Analysis of fetomaternal hemorrhage

Determination of fetomaternal hemorrhage (FMH) was done by flow cytometry at time of delivery, using FITC-conjugated Anti-D reagent (9433FI, NHS Blood and Transplant) according to the Guidelines on the estimation of Fetomaternal Hemorrhage.[17] The LOD was 0.05% of fetal erythrocytes, corresponding to 1 mL FMH.

Statistics

Statistics presented as median and IQ range or mean and SD as well as linear regression analysis, were made with GraphPad Prism 5.

Ethical approval

The study was approved by Swedish Ethical Review Authority, D-nr 2016/2:4.

Results

Protective levels of anti-D prophylaxis at delivery

During the period Oct 2010-Oct 2012 type-and-screen were requested from the delivery ward in 4280 RhD negative women carrying an *RHD*positive fetus. According to clinical routine, RAADP 1250 IU (Rhesonativ®, Octapharma, AG, Austria) had been administered in gestational week 28-29. In 876 cases (20.5%) the type-and-screen result was negative, i.e. anti-D was not detectable at delivery, which corresponds to an anti-D concentration less than 10 IU/L (2 μg/L). Information of gestation age at delivery was not available.

Anti-D concentration before and after administration of anti-D prophylaxis at 38 weeks of pregnancy The mean anti-D concentration at inclusion in the prospective interventional study was 14±11 IU/L [mean ± SD, n=39] in gestational week 38±1, Fig 1. In 17 of the 39 (44%) women the anti-D concentration was below LOD in the routine antibody screening, 10 IU/L (2 μg/L), Fig. 1. In 7/39 (18%) the level was below LOD in the anti-D quantification assay, 1 IU/L (0,2 μg/L). The anti-D concentration measured one week after

administration of prophylaxis was 75 ± 47 IU/L [mean ± SD]. The increase of anti-D was 66 ± 45 IU/L [mean ± SD], and showed a significant correlation (p = 0.0118) with body mass index, Fig 2.

Anti-D concentration at delivery

At delivery, the variation of anti-D concentration was large, between 14.4 and 184 IU/L, with mean and SD of 60±34 IU/L, Fig 3. Median gestational age of delivery was 40 weeks (38-42 weeks). Five women were recommended additional prophylaxis due to a weak result in the type and screen test at delivery. They had anti-D concentrations between 14.4 and 30 IU/L (3-6 μg/L). Two of the five women had levels below 15 IU/L (14,4 and 14,5). One of the women had high BMI, 34, and both had low concentrations at all measurements. The other three women had concentrations of 27-30 IU/L. In 25/39 FMH was analysed after delivery and the results were negative in all, i.e. below the LOD, 1 mL fetal blood in maternal circulation.

The women were monitored up to 30 days postpartum, showing stable protective levels of anti-D after delivery, Fig. 3. The concentration of anti-D analysed at delivery and in postdelivery samples (n=59) were 61+ 28 IU/L (mean+ SD) after administration of anti-D 1500 IU in gestational week 38+ 1, excluding the five women who got a new dose anti-D at delivery.

Discussion

Main findings

The retrospective data analysis of more than 4000 type-and-screen tests at delivery in RhD negative women with a fetus typed *RHD*positive, revealed that 20.5 per cent in the cohort had non-detectable anti-D levels after RAADP at gestational week 28-29. In the prospective interventional study including 39 women the proportion was higher. Forty-four per cent had anti-D below limit of detection (10 IU/L) at screening, and eighteen per cent had non-detectable levels (<1 IU/L) with the sensitive quantification assay, at 38 weeks of gestation, after RAADP 1500 IU at 28-29 weeks of gestation. In previous reports, 44-78 per cent of cases had non-detectable levels of anti-D at delivery after one dose RAADP in gestational week 28 and 15-39 per cent after two doses in gestational weeks 28 and 34.[7,8] In pregnancies post 40 weeks, 78 per cent is lacking protection.[8]Small amounts of fetal blood pass into the maternal circulation, with increasing risk during the length of pregnancy and greatest risk at delivery.[18] The volumes are usually low. In an analysis of published studies of more than 20 000 pregnancies, it was found that 74% of the women had less than 0.5 mL, 96% less than 1 mL and 98% had less than 2 mL fetal blood in the circulation.[19] In another study of more than 3000 pregnancies, 99% had FMH less than 2.5 mL and 99,8% had FMH less than 5 mL blood, after vaginal delivery. After caesarian section 99,7% had FMH less than 2.5 mL and 99.5% had FMH less than 5 mL.[20]

In an early recommendation from the World Health Organization[21] supported by the British Medical research Council,[22] 25 μg anti D in the maternal circulation is estimated to protect 2 mL fetal blood. The current product information for anti-D Rhophylac® (CSL Behring), says that 10 μg (50 IU) covers one mL fetal blood.[23] Using an estimated maternal plasma volume of 3750 mL and an extracellular volume of 4500 mL at term,[11] it corresponds to a concentration anti-D of 2,4 μg/L (12 IU/L) to protect from FMH of 2 mL whole blood. A dose of 300 μg (1500 IU) giving a theoretical concentration of 36 μg/L (180 IU/L) protects from FMH of 30 mL. A concentration of 15 IU/L (3 μg/L) protects a FMH of less than 2,5 mL occurring in 99% of pregnancies.[22] In our prospective study 2/39 women had levels below 15 IU/L, 14,4 and 14,5 IU/L respectively. No cases of fetomaternal hemorrhage were found, but the sample size was small. Those who were followed postpartum showed stable protective levels of anti-D concentration up to 30 days postdelivery, 61+ 28 IU/L.

With current anti-D prophylaxis regimens including RAADP in gestational week 28-30 the incidence of anti-D immunisation is low, 0.2-0.4%. Despite this low incidence, anti-D immunisation is still the most common cause of severe fetal anemia requiring intrauterine blood transfusions to the fetus with its inherent risk of complications and perinatal mortality and morbidity. [2,5] Fetal *RHD*screening is now a routine in many countries, resulting in that the fetal *RHD* status is known with high accuracy prenatally, with a sensitivity of the analysis above 99,9%.[6] To add anti-D prophylaxis in gestational week 38, to ensure a protective

concentration of anti-D at term and post term in RhD negative women at risk of immunisation, seems relevant and logistically possible in most programs. Anti-D prophylaxis in gestational week 38 may replace postpartum prophylaxis, and thus make the strategy cost effective.

Strength and limitations

The prospective intervention design, with the possibility to follow each individual, as well as the inter variability of anti-D concentrations strengthen the validity of the present study. On the other hand, the study population of the intervention study was small. There was an obvious difficulty to include subjects with repeated measurements close to term and postdelivery. There is a limitation by the relatively high incidence of protocol violations regarding the analysis of anti-D concentrations postpartum. Only 25 % was monitored weekly postdelivery, but in all of these, stable concentrations were maintained up to 30 days postdelivery. In addition, analyses of FMH at delivery was missing in 14/39 (36 %) of the cases. FMH above the detection level of 1 mL fetal blood is reported to occur in 4% of pregnancies.[20]

A considerable number of women will not have measurable levels of anti-D at term. The reported number varies between studies, 20-80%.[7,8,13] In the present study, with a retrospective data analysis cohort, with over 4000 RhD negative women, with an *RHD* positive fetus, where a type-and-screen test was performed at delivery, 20% had a negative antibody screening at delivery. In the prospective part with 39 pregnancies included, the proportion with a negative antibody screen was higher, 44%. The different result between the two cohorts is unclear, the same method for antibody screening was used during the whole time period, BioVue Cassetttes with anti-IgG (Ortho Clinical Diagnostics) and LOD was estimated to 10 IU/L. Though, the retrospective cohort was from 2010-2012 and the prospective group from 2016-2018 and different anti-D preparations were used, Rhesonativ®, (Octapharma AG),1250 IU, in the first period and Rhophylac®, (CSL Behring) 1500 IU, in the latter period. Minor changes in the screening reagents used cannot be excluded.

Interpretation

Several studies have shown that one single dose anti-D in gestational week 28 is non- detectable at delivery in a considerable proportion of women, thus not protecting them from immunisation at the end of the third trimester. This is confirmed in the present study, with a range of 20-44% of women with undetectable levels at term. The two-dose strategy with RAADP at gestational week 28 and 34, gives a higher rate of detectable anti-D, 86%[8] at term but is associated with higher costs and lower compliance of receiving the two doses.[14] The risk of immunisation after the introduction of RAADP in the third trimester is decreased to an incidence rate of 0.2-0.4% and seems to be the same regardless of one-dose or two-dose strategy.[5] Only a small proportion, approximately 1 %, of women with undetectable anti-D levels at delivery are calculated to be immunised,[18] but they still represent the most common severe immunisations.

The study showed a large inter variation of anti-D concentration at delivery. The variation of anti-D levels may depend on individual IgG clearance from plasma and consumption of anti-D, giving a variability in residual anti-D levels and in half-life. Uptake from muscular compartments and fat tissue may vary as well. As expected, anti-D levels correlated to BMI.[23] The following weeks after delivery the concentration anti-D remained stable.

The results indicate that an alternative strategy with administration of two RAADP doses (1500 IU), at gestational week 28 and 38, may be efficient to protect against immunisations at term, post term as well as postpartum. It has previously been concluded that adding a dose of anti-D in the third trimester cannot be justified due to high costs,[19] but if the postdelivery anti-D dose could be excluded, this strategy may be cost effective. The potential of high compliance at 38 gestational weeks, which is crucial, must be assessed in different antenatal programs, but most women have a visit planned in gestational week 37-38. On the other hand, anti-D prophylaxis may be administered within 72 hours postdelivery for women non-compliant to RAADP at gestational week 38 or delivered preterm. If this strategy will lower the incidence of D-immunisation further is unanswered and has to be addressed in a large prospective trial. Ten out of the 39 women in our small study have been tested for D-immunisation in a subsequent pregnancy, all with negative screening test.

Conclusion A large proportion of RhD negative women have non-detectable levels of protective anti-D at term and post term. In addition to RAADP in gestational week 28 a second dose of RAADP in gestational week 38 to women at risk of anti-D immunisation was evaluated in this study. The gestational week 38 anti-D dose gives protective anti-D concentrations up to 30 days postpartum. If the second dose replaces the postpartum dose, the strategy may be cost-effective and possibly further reduce the risk of immunisation.

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Table 1 Characteristics of included women n=39

	Median [min-max]
Age	32 [19-49]
BMI at gw 10-12	23,9 [18,8-34,8]
GA at delivery (days)	281 [266-295]
No of blood samples*	4 [2-6]
Mode of delivery (%) Vaginal Cesarean Section	87 % 13 %

GA: gestational age; gw: gestational week *: number of samples analysed for anti-D concentration per woman

Legends

Figure 1. Anti-D concentration (IU/L) at inclusion in the study at week 38±1, n=39, after administration of RAADP 1500 IU in gestational week 28. The dotted line represents LOD for detection of Anti-D with type-and-screen, 10 IU/L (2 μg/L), with 17/39 below LOD.

Figure 2. Increase of anti-D IgG after administration of RAADP 1500 IU in pregnancy week 38 was quantified by flow cytometry one week after administration. Linear regression analysis showed a significant correlation to body mass index, p=0.0118, n=39.

Figure 3.

Concentrations of anti-D measured by flow cytometry at different time points after delivery. Data are shown as box plots with median values and quartiles in the box and the range as whiskers. n = no of anti-D analyses performed at delivery and each week after delivery.

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