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Comparing Evaluation of Feto Maternal Hemorrhage (FMH) Using Fetal HbF Determination by Bicolor and D Antigen Using Flow Cytometry

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ABSTRACT

Quantitative measuring of red blood cells of infant in mother's bloodstream plays an important role in determining the exact amount of anti-globulins RhIg to prevent active immunization in RhD negative mother with positive RhD baby birth.
flow Cytometry method is convenient, fast, accurate and reliable for evaluation of fetal-maternal hemorrhage. The aim of this study is to evaluate the extent of fetomaternal hemorrhage (FMH) with RhD antigenic index and fetal hemoglobin HbF with flow Cytometry method.
Materials and methods: The study conducted was experimental. Thirty four blood samples of Rh-positive umbilical cord with an RhD negative adult blood in serial dilution form in 6 dilutions (0.125, 0.25, 0.5, 1, 5 and 10 percent), which represented 99.9% of fetal maternal hemorrhage, were simulated in laboratory, and studied by HbF bicolor flow Cytometry and carbonic anhydrase (CA), and mono-color RhD. T test, regression, and ANOVA statistical tests were used for result analysis.
Findings: According to the statistical results, there is an acceptable correlation between the results of RhD and HbF with flow cytometry method (r = 0.897). The amount of bleeding calculated by two HbF and RhD parameters compared with the expected blood values showed that dual-color analysis of HbF and CA has more sensitivity compared to single color analysis of RhD in determining small amount of bleeding. In determining the amount of FMH, the results obtained from RhD method, despite the high correlation (r = 0.984), showed unreal increase compared to the amount of bleeding obtained from HbF/CA method false showed increased bleeding, but its ability in determining higher values of bleeding compared with HbF/CA was remarkable.
Conclusion: Using monoclonal of exclusive antibodies in flow Cytometry method creates the detailed evaluation of bleeding, and hence determining the exact dose of RhIg immunoglobulin to protect women from all immunization against D antigen. Results showed that staining red blood cells by dual -color method and simultaneous use of Anti-CA and Anti- HbF to evaluate FMH caused getting more accurate estimates than FMH compared to RhD.
KEYWORDS: Measuring fetomaternal hemorrhage (FMH) HbF / / antigen D / Flo Cytometry

INTRODUCTION

When fetal red blood cells find a way to the mother's body, through the blood stream of placenta, this incident is called fetomaternal hemorrhage (FMH) (1).

FMH is caused by loss of integrity of the natural physiological barrier between maternal and fetal circulation. FMH positive arteriovenous gradient causes red blood cells of fetus to find way to maternal blood circulation, and create a condition similar to transient chimerism in the mother's bloodstream. Of the known causes of FMH delivery, strikes, especially to abdominal area of pregnant women, vascular abnormalities in the placenta and placental abruption can be noted (2). FMH in more than 75% of pregnancies is usually observed in the third trimester of pregnancy and immediately after birth. (3, 4)

D anti globulin injected for prevention to Rh-negative mothers with a history of Rh-positive delivery, at a minimum dose of 500 to 1,500 of international units (500-1500 IU) is different in different countries is only enough to thwart up to 4-to12 ml of RhD-positive red blood cells.

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Accurate assessment of bleeding volume will be of great help in the calculation and determination of possible additional dose (5).

In the past, the only way to measure FMH was using elution acid method, which was introduced by Kleihauer and his colleagues in 1957. In this method, the basis for measuring bleeding was hemoglobin F resistance to washing in alkaline solution. (6)

In recent years, with the increasing use of techniques such as flow cytometry and conjugated monoclonal of antibodies against antigenic characteristics of red blood cells, the possibility of use of this technique to assess the FMH is provided.

Quantitative assessment of wide FMH using flow Cytometry has shown that this technique has higher accuracy compared to the older method (elution acid method). Johnson and colleagues in 1995 and Lubenko and colleagues 1997published this result (7-8).

Over the past 10 years, several studies have been carried out on the use of flow Cytometry technique in quantitative assessment of fetal red blood cells circulating in maternal blood using polyclonal antibodies against the surface indicator of D antigen (AgD) and monoclonal antibodies against intracellular indicator of fetal hemoglobin (HbF). In this research, flow Cytometry has proved high sensitivity and accuracy in detection and determination of fetal-maternal bleeding. (10-12)

The simultaneous use of a monoclonal antibody against fetal hemoglobin and polyclonal antibody against carbonic anhydrase enzyme (CA) in precise determination of the amount of fetal red blood cells in maternal blood will be very accurate. Carbonic anhydrase isoenzymes, which exist in two main forms carbonic anhydrase type 1 (CA I) and carbonic anhydrase type 2 (CA II), that are present on the surface of red blood cells, are fully expressed only after birth. By simultaneous use of Anti-HbF in monoclonal way and Anti-CA II, the distinction between true fetal cell population of red blood cells containing hemoglobin HbF in maternal red blood cells referred to as F-Cells has been made possible that they made possible, this population can be separated from the embryonic cell populations.

The aim of this study was to evaluate the FMH through simultaneous staining of fetal hemoglobin and carbonic anhydrase enzyme and its comparison with results related to staining from stained with monoclonal antibodies against the D antigen. Research on 34 series of laboratory samples was done by simulated serial dilutions and in the dilutions between 0.125 to 10% for FMH. (13-15).

MATERIALS AND METHODS

Determining ABO and RhD groups of samples:

To determine blood group, all cord blood samples, positive control and negative control were washed for 3 times with normal saline (PH =7.2-7.4), and then 3 percent suspension was provided. Then equal proportions of 3% suspension and antisera of A, B, AB and RhD blood groups (Iranian Blood Transfusion) were added to the samples in separate tubes and were centrifuged and examined for the creation of agglutination.

Preparing the suspension of red blood cells:

To prepare red blood cell suspension, 4 ml of whole cord blood of RhD-positive fetus on anticoagulant EDTA (ethylene-diamide tetra-acetate) was collected and kept at refrigerator temperature (4° C). The whole blood of an adult healthy male with no history of blood transfusion or underlying medical conditions that was O negative blood type was used to produce red blood cell suspension at different concentrations.

Counting RBC of samples of umbilical cord blood and maternal blood was done with automatic counter (Sysmex-Japan). To produce suspension, serial dilution method of umbilical cord in RhD negative adult blood red blood cells was used, and 10, 5 , 1, 0.5, 0.25 and 0.125 percent dilution, which respectively represent 220, 110, 22, 11, 6 and 3 ml of FMH hemorrhage were simulated.

All suspension were counted accurately with automated cell counter (Sysmex-Japan) before carrying out flow Cytometry technique to make sure of the accuracy of providing suspensions according to mean cell volume (MCV). Then they were washed in phosphate buffer (PBS) (Merck-Germany) three times with PH 7.2 – 7.4 to oust all the confounding factors from the environment.

Positive control was gotten from adult males with O positive and negative control sample was gotten from adult males with O negative in every working shift.

Staining red blood cell hemoglobin contents by dual color staining method

Staining hemoglobin contents of red blood cells was performed according to kit protocol Fetal Cell Count (AQ Product, the Netherlands), which included three fixation, making red blood cells penetrable and staining stages for the final cell suspension as follows.

Five micro liters of red blood cells was fixed in formalin for 30 minutes at room temperature. After this period, the red blood cells were washed in PBS solution, were made permeable in a solution of sodium dodecyl sulfate (SDS) for 4 minutes at room temperature, and after two rounds of washing in phosphate-buffered saline (PBS) were given suspension state.

To stain, immuno-fluorescence of 50 ml of red blood cells from each suspension with a mouse monoclonal antibody conjugated with 50 ml of murine monoclonal antibody conjugated with phycoerythrin (PE) (clone NaM16-2F4) against HbF and 50 micro liter rabbit polyclonal antibodies against carbonic anhydrase enzyme conjugated with Fluorescein isothiocyanate (FITC) were incubated for 15 min at room temperature in the dark.

After the incubation time, tubes containing stained red blood cells in 500 micro-liters of PBS were made suspension.

All samples were analyzed with Flow Cytometer set (Partek- Germany) with 50000-100000 cell count. To analyze the results Flomax-version 2.4 software was used.

Negative control was gained from an adult consisting of RhD-negative who has had no history of hemoglobinopathies and blood transfusions. This sample, like all red blood cell suspension, was evaluated in every work shift. In addition, positive control was used from O positive person in each shift.

Evaluation of FMH by dual color staining of red blood cells

To evaluate FMH volume the following formula was used:

$$\text{FMH hemorrhage (ml)} : \frac{\text{The percentage of fetal red blood cells}}{\text{Percentage of maternal red blood cells + F-Cell maternal cell percentage)}} \times 2400^{*}$$

The percentage of fetal red blood cells: percentage of HbF + / CA - cell

The percentage of maternal red blood cells: the percentage of HbF - / CA + cells

Percentage of maternal F -Cell: the percentage of HbF + / CA + cells

* 2400 Factor: As the size of fetal red blood cell is 122% of maternal red blood cells size and in the traditional Kleihauer-Betke method on average only 92% of fetal red blood cells can be detected and the volume of maternal red blood cells during childbirth on average is about 1,800 ml, so the correction factor in FMH is calculated as follows. (16)

Correction factor: $122 \times 1800/92 = 2389 \approx 2400$

Immunofluorescence staining using anti-D:

To stain red blood cell suspension, Anti-D monoclonal antibody conjugated with Phycoerythrin (IQ Product – the Netherlands), which is done by direct staining, was used. Samples provided on EDTA anticoagulant, after three times wash in phosphate buffer solution (PBS) were prepared as 10% suspension of red blood cells in PBS.

Monoclonal Antibody (Anti-D RPE) was diluted with a ratio of 1 to 10 with phosphate buffer solution containing 1.0% bovine serum albumin (Merck -Germany). Then 100 µL of diluted antibody was added to 10 µL 10% suspension of each dilution and incubated for 15 min at 37 ° C. After incubation, the samples were washed twice with PBS and then were made suspension in 500 µL of phosphate buffer.

Negative control was obtained from the samples of RhD-negative adults who have no history of hemoglobinopathies and blood transfusions. This example, like all red blood cell suspension, was evaluated in every work shift. In addition, positive control of O positive person was used in each shift.

All samples were analyzed with Flow Cytometer set (Partek - Germany) with 50000-100000 cell count. To analyze the results Flomax-version 2.4 software was used.

Number and percentage of red blood cells containing Anti-D^{+} or D that was placed in the special Gate for red blood cells were counted.

Calculations of volume of hemorrhage were calculated with the following formula:

1200 X 1800 (percentage of events in negative control - Anti-D+ cell percentage)/100=FMH (ml)

Statistical analysis:

For statistical analysis Pearson correlation coefficient (r) and statistical test of Student's T-test were used. Statistical tests were performed at the 0.05 significance level and the results $P < 0.05$ were considered as significant.

Findings:

Suspension of red blood cells were evaluated in dilutions of 10, 5, 1, 0.5, 0.25 and 0.125 percent with both Anti-D and Anti-HbF staining methods.

Staining umbilical cord red blood cells was done using HbF monoclonal antibody and carbonic anhydrase polyclonal antibody. Dot point obtained from the flow Cytometer, including different populations of embryonic red blood cell (HbF +, CA-), maternal F-Cell globule (HbF +, CA +), colorless cells (HbF-, CA-) adult red blood cells, (HbF-, CA +) was evaluated.

Univariate analysis of variance for each parameter (HbF) and (RhD) showed that there is a significant difference between the results obtained from different suspension and it showed that suspensions had been prepared well. This was calculated by MCV before and after the suspension.

The correlation between the results of readings percent by dual staining methods of red blood cells was examined using monoclonal antibodies against RhD and HbF and showed that this correlation is meaningful and correlation coefficient determined was ($r = 0.897$, $p < 0.05$). The implication was that the changes in the percentage of each suspension of red blood cell in both parameters occurred in parallel with each other, and this relationship was significant.

In another study, the information on the percentage of suspensions prepared in the laboratory and FMH volume determination formula (Mollison formula) made it possible to estimate the volume of hemorrhage (FMH) where the calculation was done and considered as expected value from each suspension. After immunofluorescence staining with Anti-RhD and Anti-HbF monoclonal antibodies, the percentage of red blood cells was obtained after flow Cytometry analysis, this time the observed value was calculated with the formula and analyzed with test T-test - every two parameters were evaluated at each dilution pair.

The calculated results of volume of FMH in various dilutions of red blood cell suspension and the expected values in HbF parameter showed that there is no significant difference in hemorrhage volume of 10,5, 1 , and 0.05 suspensions in calculated percentage and expected values ($P > 0.05$) and the differences were significant only in 0.25 and 0.125 suspensions. In addition, this significance was observed towards untrue increase. And staining of red blood cells with Anti-HbF monoclonal antibodies only showed 0.25 and 0.125 percent of blood volume more than the expected value. (Diagram 1-1)

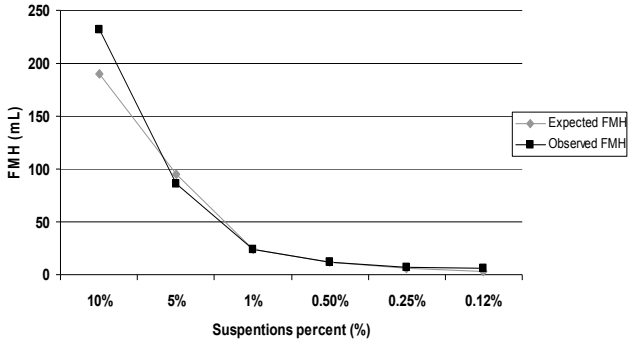

Diagram 1-1: Comparison of FMH volume calculated by Mollison formula in 10, 5, 1,0.50, 0.25, 0.12 percent suspensions and the calculated hemorrhage after flow Cytometry analysis and immunofluorescence staining using Anti-HbF monoclonal antibody

The results calculated of the volume of FMH at various dilutions of red blood cells and the expected values in RhD parameter showed that there is no significant difference in hemorrhage volume of 5 and 10% suspensions calculated from the expected values ($P > 0.05$) and the rest of the suspensions which contained 5, 1, 0.25 and 0.125 percent had a little false value compared to the expected values. ($P < 0.05$) (Diagram 1-2)

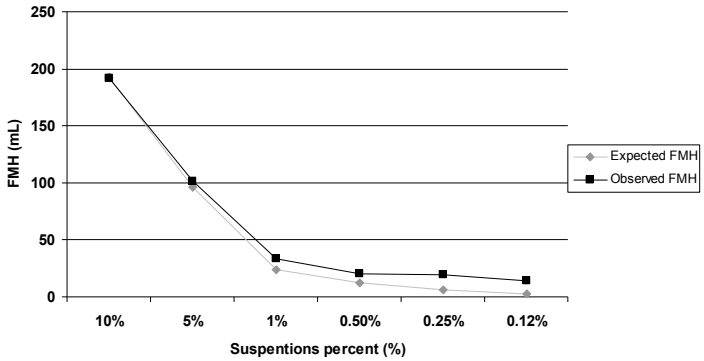

Diagram 1-2: The expected FMH and the FMH obtained from different suspensions by flow Cytometry of RhD

Regression obtained from FMH using both monoclonal Anti-D and Anti-HbF antibodies in hemorrhage volume calculated for different percentage of red blood cell suspensions was investigated and found that this correlation is significant and the correlation coefficient in red blood cell staining by immunofluorescence staining with monoclonal antibody Anti-HbF (r =0.874) and the monoclonal antibody Anti-RhD (r = 0.990), was calculated. The implication of this correlation was that the changes in the results of hemorrhage volume calculated for each percentage of red blood cell suspension in both parameters occurred in parallel with each other and this relationship was statistically significant. (Diagram 3.1 and 4.1)

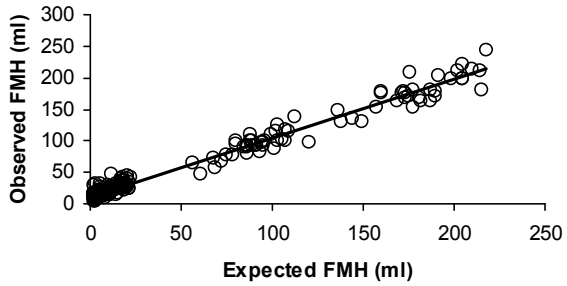

Diagram 3-1: Regression of FMH volume by staining red blood cells by single color with monoclonal antibody Anti-RhD (r = 0.990)

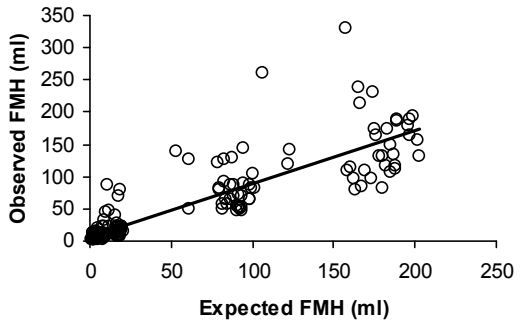

Diagram 4-1: Regression of FMH volume by staining red blood dual -color cells with Anti-HbF anti- Monoclonal (r=0.874)

Regression analysis of variance to assess the results obtained for hemorrhage volume using RhD single-color staining and dual -color HbF was done. It was found that there is a significant difference between the results in both parameters and diagram of the average results of suspensions showed this point. The average results of the two methods in any dilution did not

intersect and the normal range of each parameter RhD and HbF were different. (P> 0.05, r = 0.875) (Diagram 5-1)

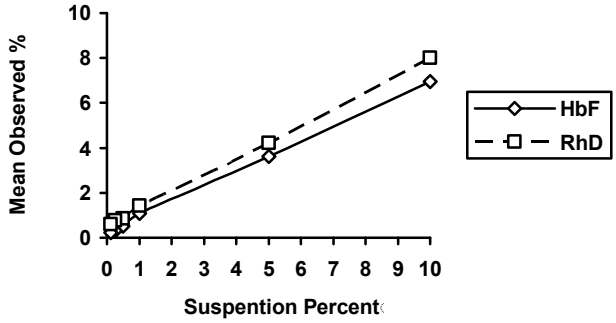

Diagram 5-1 : Comparison of the average results related to single color RhD immunofluorescence staining and HbF dual -color for different percent of 0.125, 0.25, 0.5, 1,5 and 10 of red blood cell suspension by flow Cytometry method

DISCUSSION

The method considered by researchers in recent years in the field of FMH volume measurement is using flow Cytometry technique, where various monoclonal antibodies including Anti-D and Anti-HbF are used. Of the advantages of this technique are sensitivity and speeding up the analysis of large number of cells that did not exist in previous methods. (17-20)

Dual staining method were used in this study allows accurate measurement of fetal cells containing HbF from the maternal F-Cell, and compared with RhD single-color staining have higher ability to detect small amounts of bleeding.

According to the results obtained from the percentage of HbF readings in suspension of red blood cells carried out by intracellular staining, a significant correlation between the dilutions of 0.125,0.250,0.5,1,5 and 10 percent of red blood cell suspension was observed, indicating linearity and accuracy of the analysis. ($r = 0.874$, $p < 0.05$)

Porra and colleagues, who had used red blood cells suspension by serial (0.01 to 5%) method for the measurement of HbF intracellular dual-color staining also, confirmed linearity of the results and the correlation between different dilutions. ($r = 0.95$, $p < 0.05$). These results were similar to the results of above study. (21)

In another investigation carried out in this study, it was found that determining the amount of HbF by dual color staining in 0.25 and 0.125 percent dilutions, the red blood cell had false increase compared to the expected results, which indicates the lack of accuracy of this method in measurement of HbF in the above dilutions. In a study by Pelikan and colleagues, it was reported that Cytometry flow technique in the diagnosis of HbF levels in dilutions less than 0.01 percent of fetal red blood cells is not sufficiently accurate (22).

This is while Porra et al did not observe significant differences in HbF volume in (0.02-0.1) dilutions suspension of red blood cells compared to the expected values (21). It seems that one of the reasons causing false increase in the volume of HbF in (0.25-0.125) low dilutions is placing of small percentage of maternal F-Cell within the red blood cells with a high content of HbF and the gate of the red blood cells of the fetus. The report provided by Nelson and colleagues indicated that about 0.16 to 0.20 per cent of F-Cells can be place in the range of red blood cells with a high content of HbF and in fact in the place of fetal red blood cells (23).

In another analysis that was done in this study, the measurement of RhD surface antigen was conducted in the different dilutions (5, 1, 5, 25.10, 0.0 and 0.125 %) of red blood cell suspension by one color cell surface staining. According to these results, despite a significant correlation between different dilutions in RhD reading ($R = 0.990$, $P < 0.05$), the results obtained were consistent with the expected result only in a dilution of 10% and in other dilution a significant increase was seen compared to the expected results. It seems that this method cannot have sufficient accuracy in lower than 10% dilutions. This is while, in the study of Kumple et al., in examining RhD with dual color method using anti-glycophorin A, could increase the sensitivity in reading RhD in less than 10% dilution (24).

In another study conducted by Ochenbein-Imhof and colleagues, using flow Cytometry method and Anti-RhD antibody, they were able to recognize the presence of red blood cells D^+ in suspensions 0-1 percent and this result is contrary to the result of the current study (25).

According to the results of this study, it was shown that dual color immunofluorescence staining HbF and carbonic anhydrase staining in measurement of laboratory samples in the range 0.5 to 10% could determine hemorrhage volume with good precision, which were consistent with the results of recent years studies (26,27). This is while in checking hemorrhage surface coloring of the cell using anti-RhD antibody compared to HbF dual color is less accurate, and it seems that using dual color HbF can be raised as an efficient method to determine FMH volume.

Conclusion

This study, like previous studies carried out by Davis and his colleagues in 2001 and Pora and colleagues in 2007, suggests using HbF dual-color staining with flow Cytometry method in determining FMH volume, which is due to accuracy of this method. Although the entry of fetal red blood cells is probable in the first trimester, its occurrence particularly increases at delivery time (21, 28).

All samples of suspicious mothers should be sent to reference centers like the central laboratory and medical laboratory of Iranian Blood Transfusion Research Center that is equipped with flow Cytometry equipment for accurate assessment of FMH volume. In addition, for more accurate calculations of bleeding volume, it is better to calculate the mother's weight or red blood cell volume and the necessary information be sent to the laboratory with samples (29).

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