Assessment of Fetomaternal Hemorrhage in Rhesus D-negative Postpartum Women by Kleihauer–Betke Test

OBJECTIVES

Objectives: The aim of this article is to assess fetomaternal hemorrhage (FMH) and determine its volume and also to study the relation of the amount of FMH to various factors.

MATERIALS AND METHODS

Materials and methods: This was a prospective study carried out at Ahmedabad Civil Hospital from October 2012 to March 2013. A total of 75 blood samples were collected from RhD-negative mothers during the postnatal period. Sixteen samples were excluded because the fetus blood group was either Rh negative or unknown. The acid elution or Kleihauer–Betke quantitative test was used to measure the amount of FMH. The data were analyzed using Epi Info version 7.

RESULTS

Results: With Kleihauer–Betke/acid elution test (KBT), 45.76% of women had fetal whole blood in their blood circulation during the postnatal period varying from 1.2 to 9.6 mL. The test was negative (i.e., no fetal cells were identified) in 54.24% of women. The majority of women had hemorrhage less than 4 mL. None of them had a large FMH.

Conclusion: Most of the FMH calculated was <10 mL, which could have been neutralized by lower doses of anti-D immunoglobulin, which have incurred lower costs than the 300-µg dosage. Thus, developing optimized testing and accessing dosing protocols is needed in health care facilities. In the present study, we found no significant relation between the amount of FMH and parity or type of delivery.

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Source of support: Nil

Conflict of interest: None

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INTRODUCTION

Bidirectional passage of minute numbers of cells across the placenta is a physiological event, even though the placenta is considered a barrier separating the maternal and fetal circulations.1,2 It is estimated that less than 1 mL of fetal blood is lost to the maternal circulation during normal labor in around 96% of normal deliveries.3,4 However, the loss of this small amount of blood may be a sensitizing event and stimulate antibody production to the fetal red blood cells, for example, Rh disease of the newborn. Causes of increased fetomaternal hemorrhage (FMH) are seen as a result of trauma, due to placental abruption, or may be spontaneous with no cause found. Loss in excess of this may result in significant morbidity and mortality to the fetus. Fetomaternal hemorrhage is one of the causes of intrauterine death (IUD).

Alloimmune hemolytic disease of the fetus and newborns (HDFN) results from the destruction of red cells by maternal immunoglobulin G antibodies that gain access to the fetal circulation during gestation. The most serious form of HDFN is caused by maternal alloantibody directed against the D-antigen of the Rh blood group system due to the high immunogenicity of D-antigen. Rhesus-D HDFN in Rh-negative women can be prevented if the appropriate dosage of prophylactic anti-D (RhIG) is given at the appropriate time.5-10

All D-negative women who deliver a D-positive fetus should receive at least a single 300-µg dosage of RhIG within 72 hours of delivery. In addition, a maternal sample should be obtained approximately 1 hour after delivery and tested for evidence of FMH in excess of 30 mL of fetal blood. Approximately 17% of Rh D-negative women who deliver Rh D-positive fetus become alloimmunized if RhIG is not administered appropriately. Prophylactic anti-D has reduced the overall risk of Rh immunization from 13.2 to 0.2%, and testing for large FMH has further decreased the risk to 0.14%. Hence, RhD immunization may be further reduced by strict compliance to guidelines concerning determination of FMH and accordingly adjusted RhIG or by routine administration of extra RhIG after a nonspontaneous delivery and/or a complicated or prolonged 3rd stage of labor.11-15 The true incidence of clinically significant FMH is probably underreported, as
an unselected population has not been screened before delivery. The possibility of accurately detecting FMH and precisely determining its volume would enable more effective and less costly prevention of RhD alloimmunization. Anti-D immunoglobulin could be administered only in indicated cases and only in doses essentially necessary for the prevention of RhD alloimmunization. Rh alloimmunization remains a major factor responsible for perinatal morbidity and may result in the compromise of the woman’s obstetric care due to the unaffordability of RhIG. Hence, there is an urgent need for the implementation of universal access to appropriate doses of RhIG for the Rh-negative pregnant women. There is also a need for the availability of FMH measurements following potentially sensitizing events. The common clinical practice is to administer 300-μg dosage to every affording unsensitized woman. This study was, therefore, carried out to overcome the needs described above and due to absence of published study on FMH in the study area and in the country at large.

**MATERIALS AND METHODS**

**Sample Collection and Transport**

This is a prospective study carried out at a tertiary care teaching hospital in India after Institutional Ethics Committee approval. A total of 75 blood samples were collected in ethylenediaminetetraacetic acid (EDTA) bulb from Rh D-negative mothers between 2 and 12 hours after delivery of the placenta. Our study participants were mothers who came for delivery to the Ahmedabad Civil Hospital from October 2012 to March 2013 and willing to give consent to enroll in the study. Sixteen samples were excluded because the fetus blood group was either Rh negative or unknown.

The Kleihauer–Betke/acid elution test (KBT) is used to measure the amount of FMA. Ethylenediaminetetraacetic acid or acid-citrate-dextrose anticoagulated blood samples are required as a cell source for the investigation of FMH. Clotted samples are not reliable. It is desirable that samples for investigation of FMH be maintained between 2 C and 8 C during transit. Temperature extremes may compromise the quality of the sample and should be avoided.

**Slide Preparation for KBT**

Initially, 200 μL of each sample is mixed with 200 μL of phosphate-buffered saline. Conventional blood films are prepared using this prepared sample and air-dried at room temperature. Then, blood film is fixed with 80% alcohol for 5 minutes and air-dried after fixation. Slides are flooded with elusion solution at room temperature for 20 seconds and rinsed in distilled water and air-dried. After that, slides are stained with 1% eosin for 2 minutes and rinsed in tap water and air-dried. Finally, examination of the slide followed.

**Examination of Slides**

Examination of the film and counting of fetal and adult red cells require sufficient cells to be counted to ensure statistical validity at the low end of desired sensitivity. This may be achieved by using an eyepiece graticule to systematically count fetal and adult red cells or to count the fetal cells in a minimum of 50 low-power fields and estimate the number of maternal cells by counting the number of maternal cells present in 2 and 3 low-power fields.

**Calculation of FMH**

When dry blood films are fixed and then immersed in an acid buffer solution, hemoglobin (Hb) A is denatured and eluted, leaving red-cell ghosts. Red cells containing HbF are resistant to the acid and the Hb can be stained; these cells stand out in a sea of ghost maternal cells. Fetal erythrocytes were counted in 2,000 background red cells using a 404 objective. Adult red blood cells that contained small amounts of HbF were distinguished from fetal blood cells by the intensity and intracellular distribution of the pink staining. The following formula and assumptions were used to calculate FMH; the maternal red cell volume of 1,800 mL fetal cells is 22% larger than maternal cells, only 92% of fetal cells stain darkly. The fetal blood should be calculated as follows:

\[
\text{Number of fetal cells per high-power field} = \frac{\text{Number of maternal cells per high-power field} \times 1,800 \times (1 - 0.92)}{100}
\]

Or can be simplified as: Number of fetal cells per high power field \( \frac{2,400}{\text{number of maternal cells per high-power field}} \).

**Data Management and Statistical Analysis**

The data were entered and analyzed by Epi Info version 7. Association of different demographic, clinical, and laboratory parameters to dependent variables was computed (Table 1).

**RESULTS**

A total of 75 blood samples were collected from RhD-negative mothers during the postnatal period. Sixteen samples were excluded because the fetus blood group was either RhD-negative or unknown. The majority of the mothers, 77.96% (46/59), were in the age group of 19 to
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Table 1: Demographic characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>77.96</td>
<td></td>
</tr>
<tr>
<td>19–25</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>26–30</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>55.9</td>
<td></td>
</tr>
<tr>
<td>Primigravida</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Multigravida</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Type of delivery</td>
<td>22.03</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>LSCS</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Instrumental</td>
<td>5.08</td>
<td></td>
</tr>
<tr>
<td>37 weeks or more</td>
<td>96.6</td>
<td></td>
</tr>
<tr>
<td>&lt;37 weeks</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

25 years. Totally 55.9% (33/59) women were primigravida and 44.1% (26/59) had previous history of pregnancy. Out of 26 women, 6 (10.17%) had a history of more than two deliveries. In the present study, 83.1% (49/59) women included were from urban areas. Our data also indicated that 96.6% (57/59) of the participants had a gestational age of >37 weeks and only 3.4% had a premature delivery. Total 72.8% (43/59) mothers delivered normally, 5.08% (3/59) required instrumental delivery, while 22.03% (13/59) underwent lower segment cesarean section (LSCS).

Graph 1 shows that 56% of participants were primipara and rest 44% were multipara.

As shown in Graph 2, 45.76% (27/59) of our participants had fetal whole blood in their blood circulation during the postnatal period. The amount of fetal whole blood calculated was 1.2 to 9.6 mL. The mean of FMH calculated by this method was 2.44 ± 1.95 (mean ± standard deviation [SD]). As depicted in Graph 2, the test was negative (i.e., no fetal cells were identified) in 54.24% (32/59) patients and 45.76% (27/59) showed the amount of hemorrhage varying from 1.2 to 9.6 mL. Out of 27 patients, the majority of women (24/27) had hemorrhage less than 4 mL. None of them had large FMA.

Table 2 shows that there was no significant difference found in the amount of hemorrhage with normal or cesarean delivery as p > 0.05.

Table 3 shows that amount of hemorrhage varies irrespective of parity of patient (p > 0.05).

DISCUSSION

Studies on the prevalence of FMH have shown that some degree of fetal–maternal transplacental hemorrhage occurs in 75% of all pregnancies. This phenomenon is not dangerous to the fetus unless there is an incompatibility between the mother and her fetus with respect to the D-antigen of the red blood cells. Fetomaternal hemorrhage occurs in 3% of pregnancies in the first trimester, 12% in the second trimester, 45% in the third trimester, and 64 to 100% after delivery.6,11 Our result has shown that FMH occurs in 46% (27/59) of our participants after delivery using KBT. The test was negative in 54.24% (32/59) of patients, which was consistent with the study of Urgessa et al16 that concluded FMH was negative in 48% (36/75) of their patients.
The total volume of fetal cells in the maternal circulation is usually small and varies between 0.1 and 0.25 mL in most cases, but large volume of FMH occurs less often, with more than 15 mL of fetal red cells (approximately 30 mL whole blood) detected at a rate of 1.6% after cesarean section or complicated vaginal delivery and 0.7% after spontaneous vaginal delivery. Administration of 100 IU (20 μg) RhD immunoglobulin has been demonstrated to protect against 1 mL of fetal red cells, 500 IU (100 μg) should protect against FMH of up to 5 mL, and 1,500 IU (300 μg) RhD immunoglobulin against FMH of approximately 15 mL of fetal red cells.

In the present study, FMH occurs in 46% (27/59) of our participants after delivery using KBT. The mean amount of FMH was 2.44 ± 1.95 mL (mean SD). None of the patients had FMH greater than 10 mL. Administration of 100 μg of RhD immunoglobulin that is equivalent to 5 mL of FMH would be sufficient for a large number of population. This result was consistent with the result revealed by Augustson et al., in which they concluded that 90.4% (4651/5148) of the women had FMH volume of 1.0 mL or less of Rh D-positive red cells, and 98.5% (5,072/5,148) had a volume of less than 2.5 mL. Only 0.4% of the cases had an FMH volume of 6.0 mL or greater (6.0–92.4 mL).

CONCLUSION

Kleihauer–Betke test is a very simple and easy way to measure the amount of FMH without the need of advanced infrastructure. In our study, 45.76% of women had fetal whole blood in their blood circulation, that too between 1.2 and 9.6 mL. The majority of patients had hemorrhage less than 4 mL. No one had large FMH. In the present study, we found no significant relation between the amount of FMH and parity or type of delivery.

For patients with FMH < 10 mL, the dosage of anti-D immunoglobulin needs to be further modified. In our opinion, anti-D immunoglobulin should be given in appropriate dosage after calculating the amount of FMH. The administration of a relatively large dose of 300 μg to all Rh D-negative patients, who are at risk of alloimmunization, may eventually lead to a future shortage of anti-D immunoglobulin, obtained from volunteers with high circulating antibody levels. In addition, considering cost-effectiveness and the fact that anti-D immunoglobulin is a blood product with a small potential risk of viral transmission, one could discuss another strategy. With the application of a method more reliable in quantifying smaller volumes of FMH, the anti-D immunoglobulin dosage could be adjusted to the detected FMH percentage including a margin of safety. Thus, developing of optimized testing and accessing dosing protocols would be helpful.

REFERENCES