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Original research article

"Provoked" feto-maternal hemorrhage may represent insensible cell exchange in pregnancies from 6 to 22 weeks gestational age☆,☆☆,★

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ABSTRACT

Objective: To quantify spontaneous and provoked fetal to maternal cell exchange in the first half of pregnancy. Transfer of fetal red blood cells (FRBCs) into the maternal circulation during the first half of pregnancy is poorly characterized but of clinical relevance for miscarriage management and invasive procedures.
Study design: Prospective, descriptive cohort study of women presenting for surgical termination of pregnancy with sonographically confirmed gestational age (GA). Pre-procedural and post-procedural blood samples were collected to characterize both spontaneous (pre) and provoked (post) cell exchange with analysis via flow cytometry to quantify FRBC count.
Results: A total of 100 patients at 6–22 weeks GA contributed 200 matched pre- and post-procedural samples. FRBCs were identified in 69 patients including 4 who exhibited FRBCs pre-procedure only and 9 post-procedure only, for a total of 65 patients having post-procedural FRBCs. Of patients with FRBCs following their procedure, the majority (n=56, 86%) also exhibited evidence of cells before the procedure with just 9 patients (14%) exhibiting FRBCs only after. No dose–response relationship was appreciable between GA and FRBC count.
Conclusion: After experiencing disruption of the placenta with instrumentation, roughly two thirds of patients had detectable FRBCs in maternal circulation following their procedure but—among those that did—the majority also exhibited cell presence prior to the procedure. This leads to further questions regarding the relationship between risk events and alloimmunization potential in previable pregnancies as the rate of spontaneous transplacental cell exchange may be underappreciated and the magnitude of provoked transfer may be overestimated.
Implications: The relationship between feto-maternal hemorrhage risk events and alloimmunization potential in previable pregnancies has previously been poorly characterized but these data reveal spontaneous transplacental cell exchange may be underappreciated and the magnitude of provoked transfer may be overestimated.

1. Introduction

The human immune system functions to detect and destroy foreign material as a protective mechanism against infection. Pregnancy is a unique state where the body must support a fetus containing antigens discordant from the mother's, such that there must be modulation of the maternal immune system to dampen the response to these fetal cells. However, Rh alloimmunization occurs when an Rh-negative woman becomes sensitized to Rh-positive fetal cells that enter her circulation (feto-maternal hemorrhage). Without prophylaxis with anti-D immune globulin, alloimmunization occurs in 9–10% of at-risk pregnancies [1].

Disruption of the feto-maternal interface increases the likelihood of passage of fetal red blood cells (FRBCs) from fetus to mother [2,3]. While the majority of these sensitizing events occur in late pregnancy or at delivery, sensitization can occur earlier if sufficient numbers of fetal cells pass and are detected by the maternal immune system [4,5]. The absolute threshold for sensitization is unknown but likely influenced by both fetal and maternal factors.

Characterization of the immune response to fetal cells was first performed in male volunteers. Subsequent studies in later pregnancy were then performed in pregnant women but far less is known surrounding feto-maternal hemorrhage in the first half of pregnancy [6,7]. Only three studies characterizing early cell exchange have been published and these studies were small with gestational ages not universally confirmed with ultrasound [8–10]. Moreover, the characterization of early antigenic expression and thus the immunologic significance of fetal red blood cells has also been limited [11–13]. Regardless, it is common

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★ Findings previously presented in poster form at the Society for Maternal Fetal Medicine Annual Pregnancy meeting in 2017 (partial cohort) and 2018 (complete cohort).
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for some small volume of fetal cells to enter the maternal circulation during normal pregnancy [14–16]. Given the uncertainty surrounding risk for sensitization across gestational ages and risk events, the American College of Obstetricians and Gynecologists (ACOG) recommends consideration of treatment for all Rh-negative women with vaginal bleeding, first-trimester miscarriage, amniocentesis, CVS, and ectopic pregnancy at any point in gestation [17].

Thus the objective of our study was to document the frequency of detectable cell exchange before and after surgical termination of first and second trimester pregnancies. This cohort illustrates the incidence of spontaneous (not provoked by traditional risk events) passage of FRBCs in the first half of pregnancy (represented in the pre-procedural group) and the degree to which surgical disruption of the placenta affects passage of FRBCs into the maternal circulation.

2. Materials and methods

We performed a prospective, descriptive cohort study of women presenting for surgical termination of pregnancy (dilation and suction or evacuation). All had sonographically confirmed gestational age (GA). The University of Rochester Research Subjects Review Board approved the study and each study subject gave informed consent.

2.1. Enrollment and descriptive data collection

Women aged 18–45 years presenting to the University of Rochester's Women's Health Practice Family Planning Clinic for terminations of pregnancy in the first or second trimester were eligible for inclusion in this prospective, descriptive cohort. Exclusion criteria included non-proficiency in reading, writing, or comprehending English and those patients otherwise unable to give informed consent. A goal enrollment of 100 was sought for this exploratory, descriptive cohort.

On enrollment, patients provided extensive descriptive information. This included traditional demographic information and any history of recent risk events (e.g. vaginal bleeding, chorionic villus sampling) as well as previously diagnosed maternal conditions that increase de novo synthesis of fetal hemoglobin in the maternal red cell population such as sickle cell anemia [18].

2.2. Approach to sample collection

Pre-procedural samples were drawn in conjunction with standard pre-operative labs 1–2 days prior to the patient's procedure. Post-procedural samples were drawn 5–10 min after placental extraction. The blood drawn before the procedure characterized the baseline incidence of spontaneous cell transfer and provided a within-subject control for the quantity of cells identified after the procedure. The blood drawn after the procedure characterized fetal cells exchanged following the feto-maternal hemorrhage risk event. Based on prior nuclear medicine studies using radiolabeled red blood cells which demonstrated maximum target tissue activity at 4–5 min, we timed the post-procedure blood draw for 5–10 min from placental extraction [19].

2.3. Flow cytometric analysis of samples and statistical approach

Characterization of fetal cells was completed with flow cytometric analysis of the number of fetal cells in a given sample of maternal blood. Percentage counts were reported rather than extrapolated volume of maternal-fetal hemorrhage to provide the data obtained without introducing the additional confounders induced through standard formulae from maternal hematocrit and plasma volume [20].

Flow cytometry was completed using a commercially available fetal cell detection assay (Trillium Diagnostics FMH QuikQuant Flow Cytometry version). This assay is able to quantify fetal red blood cells in the maternal sample with a sensitivity to 4.3 FRBCs per 10,000 maternal red blood cells. In addition to providing a quantitative estimation of the volume of fetal blood transferred to the maternal circulation, this assay uses evidence of exposure to environmental oxygen and evidence of nucleation to discriminate maternally derived fetal hemoglobin from fetal hemoglobin truly originating in the fetal circulation.

Descriptive characteristics were compiled for the entire population then by subgroups. Comparative statistics were performed between groups by T-tests for continuous data or Fisher's exact tests for categorical data.

Table 1
Overall patient demographics and procedural characteristics

Patient Demographics and Procedure Characteristics (n=100)	
GA at procedure	
Mean and standard deviation	17w4d±3.4
Median	18w1d
Range	6w-22w
Maternal characteristics	
Age (years)	26±5.5
Weight (lb)	162±40.7
Gravidity (range)	1–12
Parity — all deliveries (range)	0–6
Maternal blood type characteristics	
A	41%
B	13%
O	43%
AB	3%
Rh negative	17%
Preceded by invasive diagnostic procedure	
CVS	3%
Amniocentesis	0%
Preoperative vaginal bleeding	26%
Cervical dilator placement	71%
Mechanical dilation	65%
Intact extraction	3%

3. Results

From October 2015 through October 2017, we enrolled 110 patients presenting for terminations of pregnancy between 6w1d and 22w6d ultrasound confirmed gestational age, 100 of which contributed matched pre- and post-procedural samples. Ten additional patients were initially enrolled but were excluded due to inability to complete procedural blood draws.

The median gestational age at the time of the procedure was 18w1d and the median patient age was 25 years. Approximately one quarter (26%) of patients reported pre-procedural vaginal bleeding and 3% of women had previous chorionic villus sampling. Table 1 provides a tabulated representation of patient and procedural characteristics.

FRBCs were identified in 69 patients (Fig. 1). Of the women with any cells detected, 65 demonstrated FRBCs following their procedure but

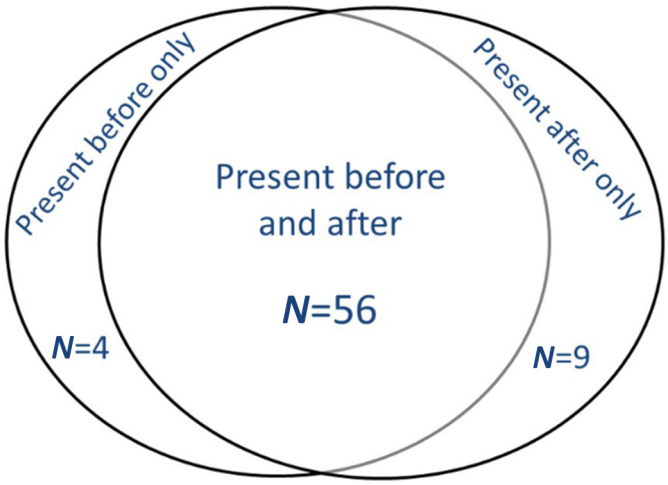

Fig. 1. Distribution of FRBC detection pre- and post-procedure.

Table 2
Patient demographics overall and classified by fetal cell detection groupings. All data represented as mean±S.D.

Patient demographics within cell detection groups					
	Total cohort, n=100	No FBRCs detected, n=31	FRBCs before only, n=4	FRBCs before/after, n=56	FRBCs after only, n=9
GA	17w4d±3.4	17w1d±4w	19w1d±2w	17w4d±3w	18w0d±2.1w
Pt age (y)	26±5.5	27.1±5.0	30.5±4.8	25.0±5.3	30.1±6.2
Pt wt (lb)	162±40.7	168.9±45.3	167.5±21.8	158.7±39.5	162.6±42.8

Table 3
Procedural characteristics overall and classified by fetal cell detection groupings. Incidence of risk associations represented as absolute number in group and percentage of that group

Procedural characteristics within cell detection groups					
	Total Cohort, n=100	No FBRCs detected, n=31	FRBCs before only, n=4	FRBCs before/after, n=56	FRBCs after only, n=9
Preop VB	26 (26%)	6 (19%)	2 (50%)	17 (30%)	1 (11%)
Cervical dilators	71 (71%)	22 (71%)	4 (100%)	37 (66%)	8 (89%)
Mechanical dilation	65 (65%)	23 (74%)	2 (50%)	33 (59%)	7 (78%)

Table 4
Cell detection overall and classified by incidence of history of spontaneous vaginal bleeding. Incidence of cell detection groupings represented as absolute number in group and percentage of that group. No significant difference between groups (with p<.05) on Fisher's Exact testing

Cell detection characteristics within spontaneous vaginal bleeding (VB) incidence groups					
	Total cohort, n=100	No VB, n=74	Prior spotting, n=13	Prior bleeding — soaking pads, n=7	Prior bleeding — volume unspecified, n=6
No FBRCs detected	31 (31%)	25 (34%)	3 (23%)	2 (29%)	1 (17%)
FRBCs before only	4 (4%)	2 (3%)	1 (8%)	1 (14%)	0 (0%)
FRBCs before/after	56 (56%)	39 (53%)	8 (62%)	4 (57%)	5 (83%)
FRBCs after only	9 (9%)	8 (11%)	1 (8%)	0 (0%)	0 (0%)

only 9 of these women demonstrated cell appearance only after their procedure with the remainder of women with cells present post-procedure demonstrating a continuation of cell presence from the pre-procedure state. Interestingly, 4 women demonstrated FRBCs before their procedure which could not be detected post-procedure.

When grouped by cell detection before and/or after their procedure, there was no significant association with gestational age, maternal age, patient weight, use of cervical dilators, or pre-procedural vaginal bleeding (Tables 2 and 3).

On specific subgroup analysis of those patients with pre-procedural bleeding (Table 4), there was no significant association between presence of FRBCs and incidence of bleeding, volume of bleeding specified by the patient, or time since the bleeding event occurred. Of note, patients were considered to have pre-procedural bleeding if they reported any vaginal bleeding in the pregnancy prior to enrollment thus the range of time since last bleeding event was large with an average time elapse since last bleeding event of 30 days.

Of the 56 concordantly positive patients, 36 (64%) demonstrated an increase in the cell count, 18 (32%) demonstrated a decrease in the cell count, and 2 (4%) demonstrated an equivalent value (Fig. 2). No dose–response relationship was appreciable between GA and FRBC count (Fig. 3).

4. Discussion

These data represent quantification of the incidence of spontaneous and provoked feto-maternal red blood cell exchange in ultrasound-dated previable pregnancies.

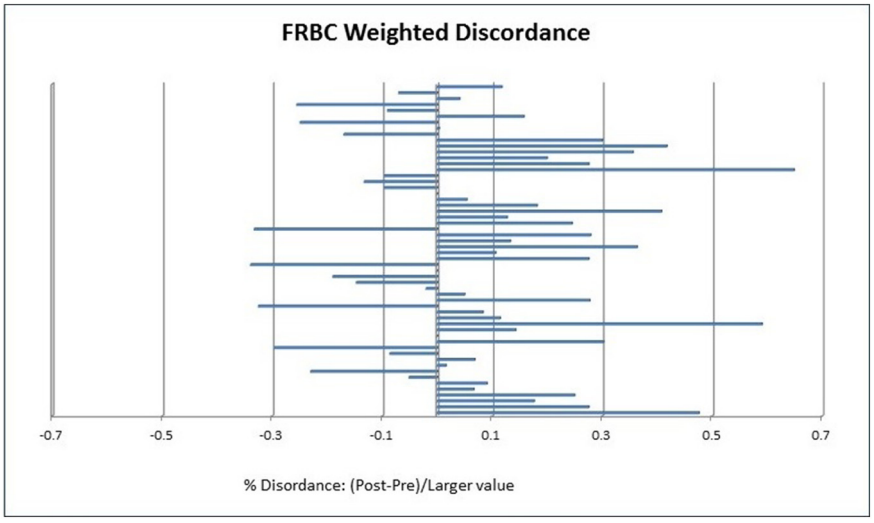

Fig. 2. Weighted discordance representation of change in magnitude of cell count before and after procedure in subjects with cells detected at both time points. The x-axis represents the percentage change between each patient's sample and the y-axis represents each subject organized by chronologic study enrollment.

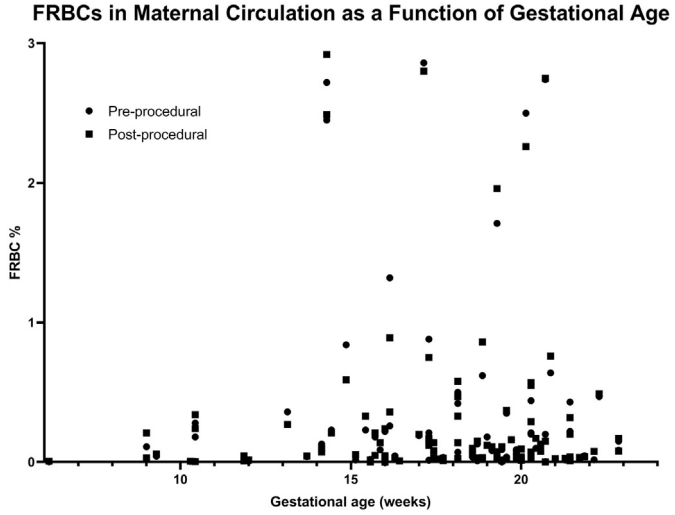

Fig. 3. Fetal red blood cells in maternal circulation as a function of gestational age.

Following all patients undergoing instrumental disruption of the placenta, roughly two thirds of patients had detectable fetal red blood cells in the maternal circulation following their procedure but — among those that did — the majority (86%) also demonstrated cells present prior to the procedure. This is in contrast to just 9% of the entire cohort who demonstrated FRBCs only after their procedure. On analysis of the magnitude of FRBCs detected among women with cells present before and after their procedure, two thirds of patients experienced an increase in the magnitude of cells present and one third of patients experienced a decrease in cells present following the procedure. For visual representation of the change in magnitude of FRBC counts pre- and post-procedure for women with cells present on both samples, the weighted discordance was calculated which demonstrates a relative magnitude increase or decrease in count normalized to the higher count magnitude (Fig. 2).

The cohort of patients experiencing a decrease in cells from their pre-procedural draw to their post-procedural draw is of particular interest. These likely represent patients who experienced little procedural bleeding but experienced spontaneous clearance of the background cells present in the 1–2 days between their pre-procedural draw and their post-procedural draw.

Our study was subject to the procedural limitation of the need to collect the pre-procedural blood sample following the placement of cervical dilators. While there is a theoretical risk of placental disruption — especially for low lying placenta or placenta previa — we saw a similar pattern of pre-procedural cell presence in the patients who did not require cervical dilators and specifically no significant interaction between the pre-procedural presence of fetal cells and the use of cervical dilators for the procedure. Our study was also unable to address the natural history of appearance and clearance of fetal cells following a provocative event as only one post-procedural draw was obtained.

This was an ideal cohort for characterization because these women have excellent ultrasound dating and are undergoing a procedure classically associated with the provoked transfer of fetal cells into the maternal circulation. Moreover, the temporal relationship to this risk event was clearly demarcated unlike women who experience spontaneous pregnancy loss.

A strength of this study is using matched pairs to compare pre- and post-procedure FRBC counts. The pre-procedure count is the baseline for early pregnancy, and serves as a control for the post-procedure value, allowing assessment of the direction of FRBC change.

This matched pair design was of particular importance in addressing the issue of interpreting the counts of maternal and fetal red blood cells. This flow cytometry assay allows for the count of both maternal and fetal cells, similar to the traditional acid elution method but with improved reliability of counts. For either manual or automated cell counting methods though, the counts generated are translated into estimated fetal blood volumes for clinical purposes with equations that estimate maternal blood volume to extrapolate the fetal blood volume from a given count. These estimations however reflect neither obstetric nor maternal physiology introducing a strong theoretical effect of maternal body mass and advancing gestation on maternal plasma volume. For the purposes of this study, the decision was made to report the percentage of counted fetal and maternal red blood cells rather than estimated volumes of fetal bleed to avoid introduction of additional, unaccounted for assumptions. These data are therefore similar to the clinically utilized values in the inability to address the role of plasma volume changes related to maternal habitus, pregnancy-related plasma expansion, or fluid hydration in fluctuations of counts. However, we were able to control for some of this variation within individual subjects with the comparison of each patient's FRBC percentages before and after their procedure.

These data lead to further questions regarding the relationship between risk events and alloimmunization potential in the first half of pregnancy as the rate of spontaneous transplacental cell exchange may be underappreciated and the magnitude of provoked transfer may be overestimated. Prior inattention to the background presence of fetal cells likely lead to overstating the magnitude of cell exchange from risk events.

These findings elicit clinically relevant questions regarding the appropriate allocation of resources as there has been little exploration of the role of RhIG administration in the first half of pregnancy to prevent alloimmunization. Given that the magnitude of cellular exposure is similar following invasive procedures compared to prior to these procedures — or sometimes more outside of the procedure — this calls into question the physiologic need for immune prophylaxis early in pregnancy. This is consistent with the observation that, although the residual seroconversion rate was decreased when routine 28 week prophylaxis was added to standard delivery administration, it was not further decreased when RhIG use was extended beyond these indications [1,17].

These data are hypothesis generating for further research. Additional studies could explore the immunogenicity of early fetal cells including the onset of expression of Rh antigen by fetal cells and the maternal immune components which may be reacting to the background presence of fetal cells. The dynamics of cell distribution and clearance would also be of interest and could be studied by serial assessments of fetal cell counts at periodic intervals following provocative events. Moreover, given the improved awareness of background cell presence in women without traditional risk factors, serial assessments of asymptomatic women would also contribute to the physiologic understanding of insensible cell exchange throughout pregnancy.

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