Fetal Diagnosis and Therapy

Research Article

Fetal Diagn Ther 2021;48:227–234
DOI: 10.1159/000513747

Received: July 2, 2020
Accepted: December 10, 2020
Published online: March 11, 2021

Fetal-Maternal Hemorrhage in First-Trimester Intrauterine Hematoma

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Keywords
Fetal-maternal hemorrhage · Intrauterine hematoma · Flow cytometry · First-trimester bleeding · Threatened miscarriage

Abstract
Objective: The objective of this study was to compare the frequency and percentage of fetal hemoglobin (HbF%) by flow cytometry of (1) first-trimester asymptomatic patients with intrauterine hematoma (IUH), (2) first-trimester pregnant patients with vaginal bleeding (VB), and (3) first-trimester asymptomatic pregnant women without hematoma. ***Methods:*** Prospective study involving pregnant women in the first trimester of pregnancy. Patients with ultrasound findings of asymptomatic hematoma and with VB were paired with asymptomatic pregnant women of same gestational age without hematoma (control group [CG]). Maternal blood HbF% was evaluated by flow cytometry. The groups were compared in terms of circulating fetal hemoglobin and HbF%. ***Results:*** Sixty-six patients were selected, 22 with hematoma, 17 with bleeding, and 27 in the CG. Fetal hemoglobin was detected in 15 patients with hematoma (68.2%) and 13 with bleeding (76.5%) and in 20 of the control (74.1%) ($p = 0.830$). The mean HbF% of each group was 0.054, 0.012, and 0.042 for hematoma, bleeding, and control, respectively, and differences were not significant ($p = 0.141$). There was a moderate negative correlation between the volume of hematoma and HbF% ($r_{Spearman} = -0.527$; $p = 0.012$). ***Conclusions:*** The fetal-maternal hemorrhage expressed by Hbf% in first-trimester pregnancies did not seem to differ between patients with and without ultrasound findings of IUH.

Introduction

Fetal-maternal hemorrhage (FMH) is the transfer of fetal erythrocytes to maternal circulation during pregnancy and/or delivery [1]. This process emerges in the first trimester and the volume of FMH increases as the pregnancy progresses [1, 2]. Although FMH is not of clinical importance in most cases [1–4], it is well known that even very small amounts of fetal red cells in maternal cir-

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culation can lead to pregnancy complications, especially in a RhD-negative woman carrying a RhD-positive fetus [1, 2].

Any situation that breaks the placental barrier can increase blood transfer and, as a consequence, FMH. A rupture in the coriodecidual interface that occurs in threatened miscarriages, miscarriages, pregnancy terminations, and ectopic pregnancies is associated with increased rates of fetal erythrocytes in maternal circulation [5–7]. Presence of an intrauterine hematoma (IUH) is a consequence of this rupture, and it has been reported in 4–39% of pregnancies [8–10].

About 3–11% of RhD-negative women with first-trimester bleeding can develop significant FMH [11] and are thus at increased risk of alloimmunization. In first-trimester threatened miscarriages, the amount of fetal erythrocytes in maternal circulation is low (<1 mL) in the vast majority of cases [2]. While some studies have shown alloimmunization in RhD-negative patients caused by inoculation of a very small sample of blood [11–14], others advocate that this small amount of blood would not be able to sensitize a RhD-negative woman [11, 12, 15, 16]. Hence, there is no universal recommendation of anti-D immunoglobulin for the prevention of RhD sensitization in an early first-trimester threatened miscarriage [11, 12, 15, 17].

The frequency and volume of FMH occurring in asymptomatic patients with a hematoma in the first trimester of pregnancy are unknown. Therefore, the objective of this study was to compare the presence and percentage of fetal hemoglobin (HbF%) in maternal circulation, using flow cytometry for first-trimester asymptomatic pregnancies with IUH, pregnancies with threatened miscarriage, and asymptomatic pregnancies without hematoma on sonographic examination.

Methods

Study Design and Participants

This is a prospective study involving first-trimester pregnant women who underwent ultrasound in 2 referral hospitals (Hospital das Clínicas and Hospital Universitário, both affiliated to Sao Paulo University Medical School, Sao Paulo, Brazil). The data were collected between February 2019 and February 2020. The study protocol was approved by the Ethical Committee of both hospitals (CAAE: 63241616.9.0000.0068 and CAAE: 63241616.9.3001.0076), and all patients provided an informed consent statement.

The patients were consecutively selected from the routine prenatal care unit and the antenatal ultrasound unit of the hospitals during a routine early dating scan or first-trimester anomaly scan, when they presented with vaginal bleeding (VB) or IUH, regardless of their RhD status. Only patients with single topic pregnancies with an alive embryo/fetus up to 13 weeks and 6 days were included. The patients were categorized into 2 groups: (1) hematoma (asymptomatic patients with IUH) and (2) bleeding (patients presenting VB regardless of the status of hematoma). Consecutively to the addition of a patient in the hematoma or bleeding group, at least one asymptomatic pregnant woman of same gestational age (GA) without hematoma was selected as a control. If the last patient included in the bleeding and hematoma group were at the same GA, only one patient was added to the control group (CG).

All patients underwent first-trimester ultrasound scanning. In all cases with VB and GA <10 weeks or in cases where the hematoma was first seen by the transabdominal route, a transvaginal scan was performed. The GA was calculated based on the last menstrual period, or it was corrected if the embryo/fetus crow-rump length measurement had a 5-day difference or more from amenorrhea. The ultrasound measurements and data also included the mean gestational sac diameter, estimated gestational sac volume, hematoma volume, and its position in relation to the uterus (if anterior, posterior, cervical, or fundal) and to the placenta (if it lay underneath the placental bed or opposite the placental bed). The hematoma and gestational sac volumes were estimated by measuring the maximum anteroposterior, transverse, and longitudinal diameters and multiplying these values by 0.52. The total volume of the hematoma was compared to the gestational sac volume to characterize it as small (<20% of the gestational sac), medium (20–50% of the gestational sac), or large hematoma (>50% of the gestational sac).

The IUH was defined as an anechoic or hypoechogenic crescent-shaped collection located between the chorion and the uterine wall, involving only the chorion laeve or both the chorion laeve and frondosum [18, 19]. When the collection was situated between the chorion laeve or edge of the chorion frondosum and the myometrium, it was classified as a subchorionic hematoma, and when it was fully situated between the chorion frondosum and the myometrium, it was classified as a retrochorionic hematoma.

The patient's obstetric history, demographic details, and BMI were also obtained. For HbF% identification and quantification, a 5-mL peripheral venous blood sample was collected from each patient and placed in EDTA tubes. The blood was kept refrigerated at 4°C for a maximum interval of 72 h until flow cytometry analysis. To validate the test, a sample from umbilical cord and an adult male was as technical controls in each experiment.

Flow Cytometry Technique

The detection and quantification of fetal erythrocytes in maternal circulation were performed using the Fetal Cell Count Kit II (IQ Products, the Netherlands) [20] following the manufacturer's instructions. First, for the cell fixation and permeabilization processes, blood samples (10 μL) were mixed with 100 μL of fixative solutions (A and B reagents). After 30 min of incubation at room temperature, the RBCs were washed with 2 mL of D reagent. Then the cell pellet was resuspended in 100 μL of reagent D, mixed with 100 μL of permeabilization solution (reagent C), and incubated for 3 min. Subsequently, the RBCs were washed with 2 mL of reagent D and resuspended in 1 mL of the same reagent.

For fluorescence staining, 50 μL of cell suspension was incubated for 15 min with 50 μL of polyclonal anti-human CA antibodies (reagent E) and 50 μL of monoclonal anti-human HbF-PE antibodies (reagent F). After the staining procedure, RBCs were washed with reagent D and the cell pellet was ready to be analyzed using flow cytometry.

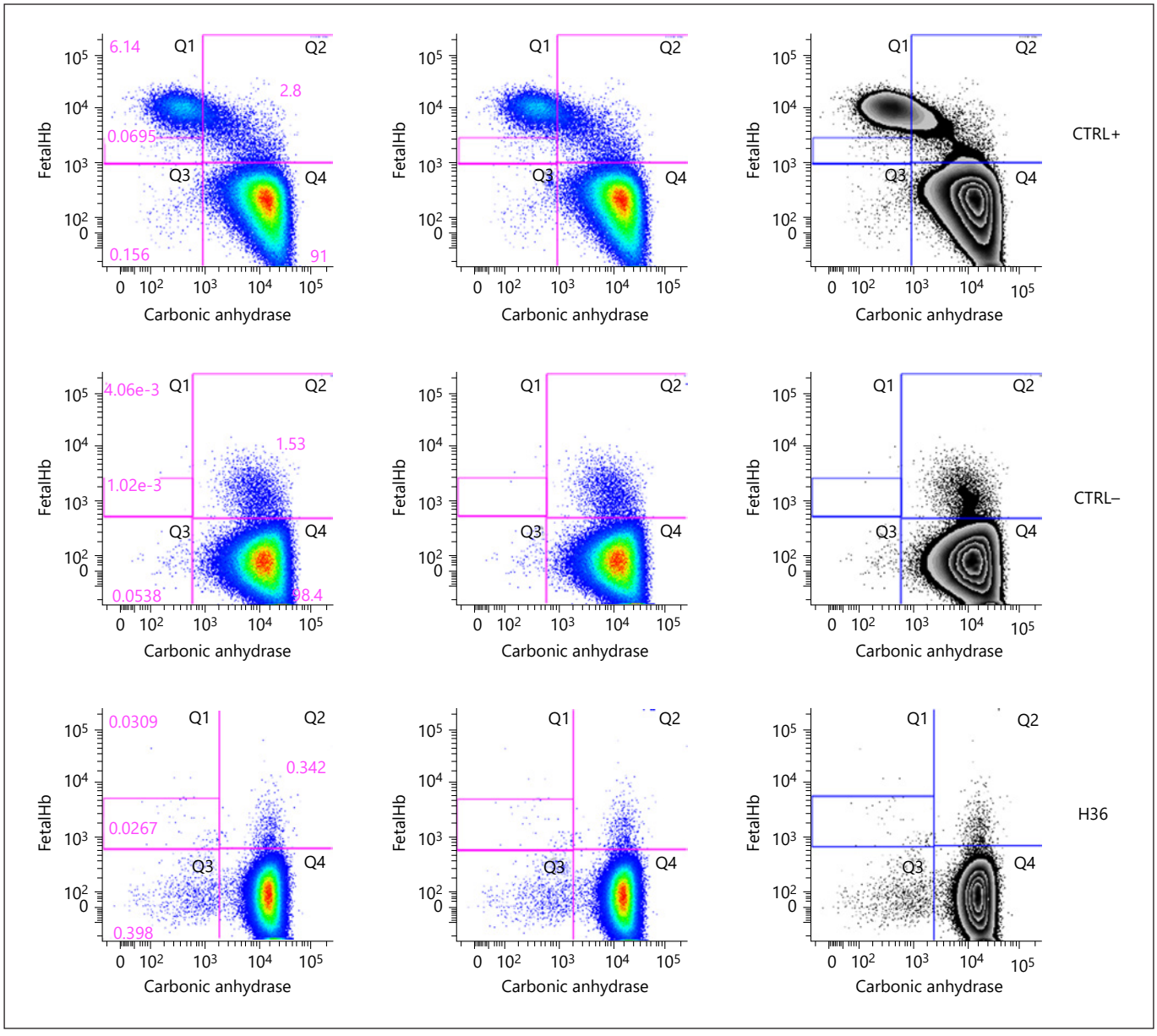

Fig. 1. Cytograms from flow cytometry analysis of a case (H36) with FMH and controls (CTRL+ and CTRL−). FMH, fetal-maternal hemorrhage.

The cytometer was calibrated according to the manufacturer's instruction. The samples were analyzed using the FACS CANTO II™ cytometer and FlowJo™ Software (Becton Dickinson Biosciences, San Jose, CA, EUA). The analysis led to the identification of 3 groups of RBCs in the cytometer charts: (i) negative or weakly positive for HbF and positive for anhydrase (adult erythrocytes), (ii) strongly positive for HbF and negative for anhydrase (fetal erythrocytes), and (iii) positive for HbF and positive for anhydrase (adult F cells) (shown in Fig. 1). The occurrence of FMH was shown by the presence of circulating fetal hemoglobin in maternal circulation.

Sample Size

Sample size was calculated in accordance with Von Stein et al. [7]. The authors compared FMH of patients with threatened miscarriage with those of asymptomatic patients admitted for elective termination. A cutoff point of 0.07% of HbF was adopted for a positive case using the Kleihauer-Betke test for analysis. A FMH was observed in 11.24% of the patients with VB and in 4.26% of the elective termination patients. Assuming that the FMH frequency in the group of asymptomatic IUH women would be similar to that in the group with VB and considering a test power of 80, the sample size was 18 patients with IUH.

Table 1. Demographic characteristics, obstetric history, and ultrasound parameters of the study population

	Hematoma, $n = 22$	VB, $n = 17$	Control, $n = 27$	p value
Maternal age, years, medium ± SD	31.18±4.84	30.94±6.36	30.59±5.79	0.935*
Race, *n* (%)				
White	14 (63.6)	10 (58.8)	18 (66.7)	0.139§
Black	4 (18.2)	3 (17.6)	9 (33.3)	
Mixed	4 (18.2)	4 (23.5)	0 (0.0)	
Parity, *n* (%)				
Nulliparous	11 (50.0)	8 (47.1)	12 (44.4)	0.928§
Multiparous	11 (50.0)	9 (52.9)	15 (55.6)	
Previous abortions, *n* (%)	4 (18.2)	3 (17.6)	7 (25.9)	0.800§
BMI ± SD[a]	27.42±4.94	25.24±5.40	27.80±4.78	0.234*
Tabagism, *n* (%)	0 (0.0)	2 (11.8)	3 (11.1)	0.251§
Comorbidities, *n* (%)[b]	8 (36.4)	8 (47.1)	18 (66.7)	0.102€
Chronic hypertension	3 (13.6)	1 (5.9)	4 (14.8)	0.797§
DM	0 (0.0)	0 (0.0)	1 (3.7)	1.000§
Thrombophilia	0 (0.0)	3 (17.6)	1 (3.7)	0.084§
Blood analysis <24 h, *n* (%)	16 (72.7)	13 (76.5)	22 (81.5)	0.053§
GA weeks; mean ± SD	8.94±1.24	9.18±1.28	8.89±1.35	0.630&
CRL, mean ± SD, mm	22.59±12.03	25.24±9.48	23.78±11.53	0.767*
GSV, mean ± SD, cm^3	20.06±14.82	20.47±14.12	27.61±26.79	0.376*

GA, gestational age; CRL, crown-rump length; GSV, gestational sac volume; VB, vaginal bleeding. [a] BMI (kg/m^2). [b] Chronic hypertension, diabetes, and clinical conditions prior to pregnancy: chronic hypertension, diabetes, thyroid disorders, heart disease, thrombophilia, and previous thromboembolic event. * ANOVA. & Kruskal-Wallis. § Fisher's exact test. € Likelihood ratio.

Statistical Analysis

Quantitative variables were displayed as means and standard deviations or medians and minimum and maximum values. Qualitative variables were represented by absolute frequencies (*n*) and percentages (%).

Quantitative variables were analyzed with the Mann-Whitney nonparametric test for 2-group comparisons and with the Kolmogorov-Smirnov test for assessment of data distribution. For normal distribution, the ANOVA test was used, while for non-normal distribution, the Kruskal-Wallis nonparametric test was applied.

Association analysis of qualitative variables was performed using the Fisher exact test and the correlation between the variables was assessed by the Spearman correlation coefficient. The difference was considered significant when the p value <0.05. The data were analyzed using the Statistical Package for the Social Sciences (SPSS version 20 IBM, Armonk, NY, USA).

Results

A total of 66 first-trimester pregnant women were recruited, 22 of whom were allocated to the hematoma group, 17 to the bleeding group, and 27 to the CG. Ten patients presented with both bleeding and hematoma, being allocated to the bleeding group. There was no significant difference between the groups regarding demographic characteristics, obstetric history, and ultrasound parameters at study recruitment. No difference was observed in terms of interval of blood sample analysis: all samples were analyzed in <72 h and over 70% in <24 h (Table 1).

The distribution of FMH and the percentage of circulating HbF% among the groups are presented in Table 2. In all 3 groups, more than half of the patients had FMH. No difference was found between the groups regarding the percentage of cases with FMH ($p = 0.83$, Fisher exact test) and HbF% ($p = 0.141$, Kruskal-Wallis).

Overall, no significant correlation was observed between HbF% and GA (Spearman, $r = 0.128$; $p = 0.304$) (shown in Fig. 2) or maternal BMI (Spearman, $r = 0.010$; $p = 0.938$) (shown in Fig. 3). In the IUH group, a moderate negative correlation between hematoma volume and HbF% was observed (Spearman, $r = -0.527$; $p = 0.012$) (shown in Fig. 4).

Table 2. Distribution of cases with positive fetal hemoglobin and percentage of circulating fetal hemoglobin (HbF%) among the 3 groups: patients with IUH, VB, and CG

Group	Cases with positive HbF (%)	*p* value	HbF%		*p* value
			mean (SD)	median (min–max)	
IUH (*n* = 22)	15 (68.2)		0.054 (0.067)	0.043 (0.000–0.238)	
VB (*n* = 17)	13 (76.5)	0.830*	0.012 (0.019)	0.003 (0.000–0.072)	0.141[&]
CG (*n* = 27)	20 (74.1)		0.042 (0.045)	0.031 (0.000–0.164)	

IUH, intrauterine hematoma; VB, vaginal bleeding; CG, control group. * Fisher exact test. [&] Kruskal-Wallis.

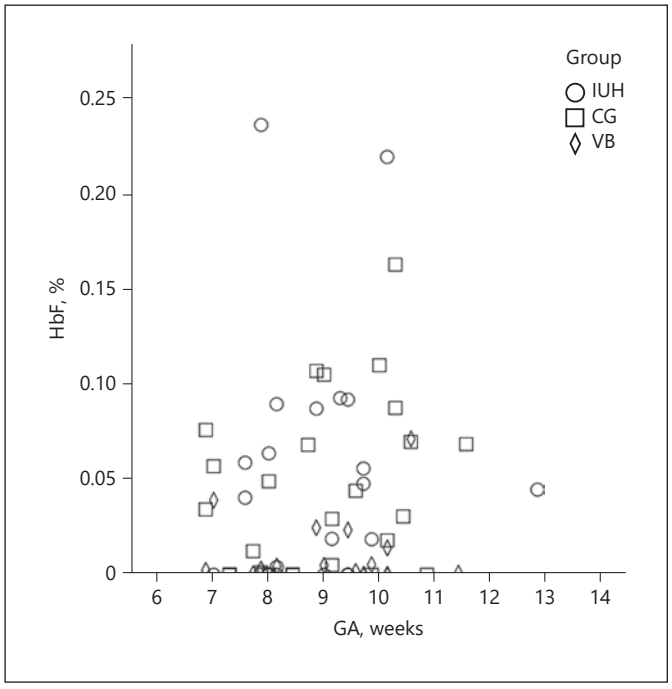

Fig. 2. Percentage of fetal hemoglobin (HbF%) relative to GA among patients with IUH (*n* = 22) and patients with VB (*n* = 17) and CG (*n* = 27). GA, gestational age; CG, control group; IUH, intrauterine hematoma; VB, vaginal bleeding.

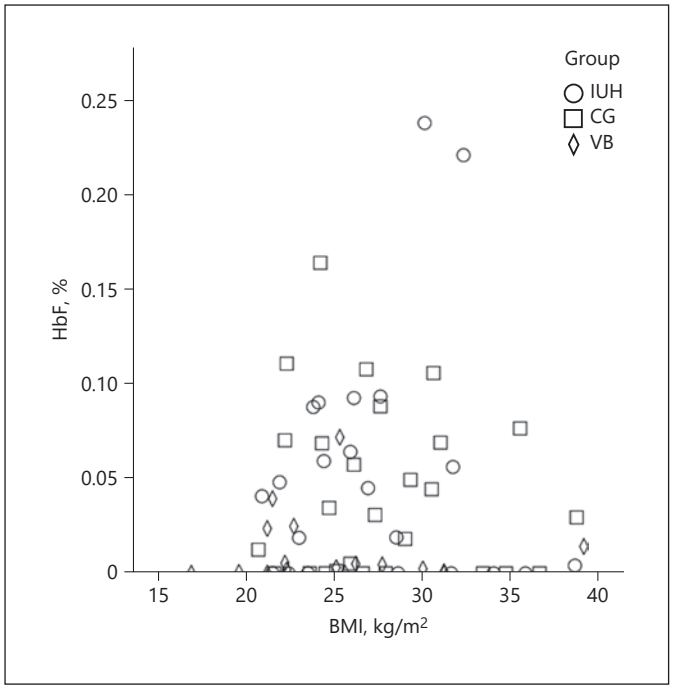

Fig. 3. Percentage of fetal hemoglobin (HbF%) relative to BMI in patients with IUH (*n* = 22) and patients with VB (*n* = 17) and CG (*n* = 27).

Discussion

Major Findings

Our results show that, in the study population, the HbF% and thus the FMH volume did not differ among patients with IUH, VB, and asymptomatic patients. Besides, contrary to some initial expectations, no correlation was found between GA and FMH rates, and a negative correlation was observed between the hematoma volume and FMH rates in that the patients with smaller hematomas exhibited higher rates of circulating fetal hemoglobin.

Interpretation of Findings and Comparison with Existing Literature

It has been reported that all pregnancies are subject to the transfer of fetal cells to the maternal circulation [1]; however, there is no consensus on the volume of fetal erythrocytes expected to be or considered normal in the first trimester of pregnancy. Hence, we opted to compare

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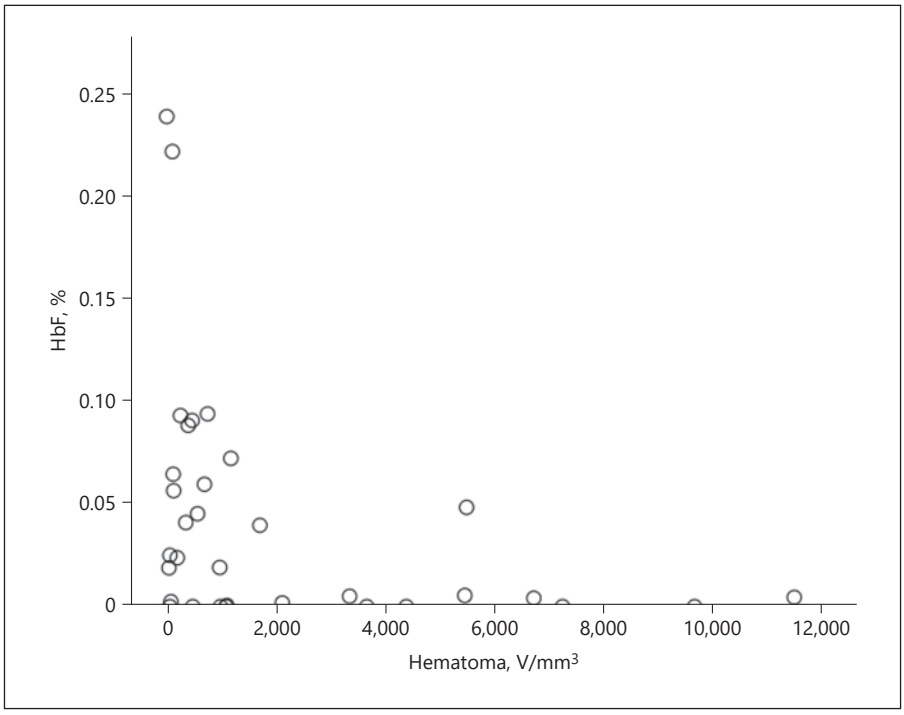

Fig. 4. Percentage of fetal hemoglobin (HbF%) relative to hematoma volume in patients with IUH ($n = 22$).

patients presenting with IUH with those with VB and asymptomatic pregnant women at the same GA. Our results showed the groups did not differ in relation to FMH evaluated by flow cytometry.

While investigating the intervillous circulation development, Jauniaux et al. [21] pondered that, in a normal pregnancy, significant placental blood flow is not expected until the end of the first trimester. The authors argued that at such GA there would be migration of the extravillous trophoblast and formation of cellular plugs that occludes the spiral arteries. The trophoblastic barrier created by the cell plugs would make it difficult for blood to enter the intervillous space, thus hindering maternal and fetal blood exchanges. A break in the placental barrier in the presence of IUH and VB would entail higher oxidative stress and maternal and fetal blood inflow into the chorionic villi and a likely higher HbF% and FMH rates in such groups than in asymptomatic women. However, this is not what we observed in the present study. The groups did not differ in relation to the percentage of patients who showed circulating fetal erythrocytes and the group with the lowest HbF% was that of patients with VB. The lack of difference might be explained by the possibility that some of the sonographic hematomas might represent intrauterine fluid rather than blood or that the hematomas could be composed primarily by maternal blood. However, even if the difference between the groups was not significant, the previously conducted studies with threatened miscarriage patients [7, 12] led us to expect the group with VB to have the highest HbF% rates.

Von Stein et al. [7] compared threatened miscarriage patients with those who desired elective termination and had no history of bleeding. In addition, they evaluated a group of paired-age nonpregnant women using Kleihauer-Betke test and established the cutoff value of at least 0.07 HbF% as positive. There was a higher rate of patients with FMH in the VB group (11.24%) compared to the asymptomatic patients (4.26%); however, the difference was not significant ($p = 0.13$). In a methodologically similar study, Kuller et al. [22] compared patients with threatened miscarriage and asymptomatic control patients using Kleihauer-Betke test and deemed positive the HbF% greater than or equal to 0.04. They found a positive rate for FMH of 4.1 and 4.4% in the bleeding and CG, respectively, and as Von Stein et al. [7], they did not find significant difference between the 2 groups.

The option to use flow cytometry was based on the fact that this technique is automated, more precise, and less operator-dependent [23, 24], which would be a better fit for early GA of the cases (<14 weeks), when a very small number of circulating fetal cells are expected. Furthermore, there are no first-trimester studies using the flow cytometry to estimate HbF%. De Wit et al. [23], when analyzing asymptomatic pregnant women, reported a

0.047% mean and a 0.025% HbF% for GAs between 21 and 41 weeks and failed to show a correlation between GA and HbF%. Similar to De Wit et al. [23], we did not find in the present study a correlation between HbF% and GA.

We observed a moderate negative correlation between the estimated volume of the hematoma and the circulating fetal hemoglobin, which was the opposite of our initial expectation. The patients with the smallest hematomas had the highest rate of HbF%. Our hypothesis for this result is that the patients with the smallest hematomas, especially those with a more advanced GA, are those with a subacute, chronic, or slow-paced evolving lesions, which, given the nonexternalized blood collected, are exposed to fetal blood for a longer time until complete reabsorption of the hematoma. Kuller et al. [22] also raised the hypothesis of chronic/repetitive exposure in response to threatened miscarriage with HbF% values inconsistent with the total fetal blood volume for GA.

Applicability of the Study

There is still much controversy in the literature surrounding the risk of alloimmunization and the prophylaxis with RhD immunoglobulin in cases of first-trimester threatened miscarriage [12, 15, 16, 25]. In the diverse publications of the topic, those advocating administration of immunoglobulin claims the low risk of the medication compared to the high risk of gestational complications with alloimmunization. However, considering that there was no significant difference in FMH between the 3 study groups, there is no evidence in this study to support the administration of anti-D immunoglobulin to RhD-negative patients with asymptomatic IUH.

Strong Points and Limitations of This Study

The strong point of this study is that it provides information not yet available in the literature regarding the data on FMH in patients with asymptomatic IUHs diagnosed by ultrasound in first-trimester pregnancies. Even though the number of patients in this study was small, it satisfied the estimated sample size. Furthermore, previous studies showed no significant differences in HbF% in maternal circulation in the first trimester in patients with threatened miscarriage [12, 14, 15, 22].

Conclusion

FMH expressed by Hbf% in the first trimester of pregnancy appears not to differ between patients with and without ultrasound findings of IUH.

Statement of Ethics

This research complies with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The study protocol was approved by the Ethical Committee of Hospital das Clínicas and the Hospital Universitario (CAAE: 63241616.9.0000.0068 and CAAE: 63241616.9.3001.0076), both affiliated to Sao Paulo University Medical School, Sao Paulo, Brazil. All patients signed an informed consent statement.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

There was no specific funding for this study.

Author Contributions

Thaisa A.R.M. Narciso contributed with study design, data acquisition and interpretation, and manuscript writing and revision. Mara S. Hoshida contributed with data analysis and interpretation and manuscript revision. Priscilla R. Costa contributed with data analysis and interpretation and manuscript revision. Andrea Niquirilo contributed with data analysis and interpretation and manuscript revision. Sckarlet E. Biancolin contributed with study conception and design and manuscript writing and revision. Lawrence H. Lin contributed with study conception and design and manuscript writing and revision. Rossana P.V. Francisco with study conception and design and manuscript writing and revision. Maria L. Brizot contributed with study conception and design, data acquisition, manuscript revision, and supervision.

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