Assessment of fetomaternal hemorrhage by Kleihauer-Betke test, flow cytometry and α-fetoprotein after invasive obstetric procedures

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Summary

Purpose: The aim of this study was to evaluate the passage of fetal red blood cells to the maternal circulation, after invasive obstetric procedures, through the Kleihauer-Betke test, flow cytometry and by measurement of maternal serum α-fetoprotein level. *Methods:* This prospective descriptive study with patients submitted to amniocentesis, cordocentesis, chorionic villus sampling (CVS), amnioreduction and ventriculoamniotic shunt was performed for karyotype analysis, treatment of hydrocephalus and polyhydramnios and to assess fetal lung maturity. Maternal blood samples were collected before and 60 minutes after the invasive obstetric procedure to search for fetal erythrocytes using the Kleihauer-Betke test, flow cytometry and serum α-fetoprotein measurement. *Results:* Ten invasive obstetric procedures were performed. The mean age of the patients was 29.2 years and the mean gestational age was 29.6 weeks. The procedures were: five amniocenteses, two cordocenteses, one CVS, one ventriculo-amniotic shunt and one amnioreduction with cephalocentesis. The indications for the procedures were: karyotype analysis in five patients, fetal lung maturity assessment in two patients, amnioreduction in one patient, fetal hydrocephalus shunt in one patient and polyhydramnios related to hydranencephaly in one patient. Regarding the path of puncture, three procedures were accomplished through the placenta and seven apart from it. All punctures were successful at the first attempt. There was no significant increase of fetal erythrocyte quantity in maternal blood samples using the Kleihauer-Betke test. After cordocentesis, a significant increase of fetal erythrocytes was detected by flow cytometry and serum α-fetoprotein measurement. *Conclusion:* Invasive obstetric procedures during prenatal care are safe when performed by experienced professionals using adequate techniques, with minimal chance of passage of fetal erythrocytes from the fetal compartment.

Key words: Fetomaternal hemorrhage; Invasive obstetric procedure, Kleihauer-Betke test; Flow cytometry; α-fetoprotein.

Introduction

When the immune system of a Rh negative person comes in touch with Rh positive blood, antibodies that react against the Rh positive cells are produced. If another exposure occurs, preformed antibodies and new antibodies lead to destruction of Rh positive red cells by antigen-antibody reaction [1].

Pregnancy is a risk situation for allogeneic Rh antigen immunization. Structurally, the fetal red blood cells run through maternal-fetal interface in the capillaries present in the chorionic villus, without direct contact with the maternal circulation. Zipurski and Israels [2] related that when a fetomaternal hemorrhage occurs (FMH) with less than 0.1 ml, the risk of isoimmunization demonstrated at six months after delivery is 3%, and if this blood volume is greater than 0.1 ml the risk rises to 14% over the same period. Procedures and complications such as cesarean delivery, manual extraction of placenta, multiple pregnancy, traumatic birth, the external version, invasive procedures and fetal death are FMH facilitating events [3, 4].

In 1957, Kleihauer *et al*. [5], described the acid-elution method for detection and quantification of fetal red blood cells in maternal circulation [6, 7]. In 1984, Medearis *et al*. [8] proposed the flow cytometry technique to identify and quantify a small number of cells in large cell populations. The dosage of α-fetoprotein (AFP) was first reported by Abelev *et al*. [9] in 1963 in rats with liver disease [10]. It was later identified in patients with hepatocellular carcinoma and during pregnancy.

The aim of this short study was to evaluate the passage of fetal red blood cells to the maternal circulation during pregnancy after invasive obstetric procedures.

Materials and Methods

We conducted a prospective descriptive study from January to August 2010 with ten patients undergoing invasive obstetric procedures. This study was approved by the Ethics Committee of the Federal University of Sao Paulo (UNIFESP) (No. 2009/54508-0). All patients who consented to participate voluntarily signed an informed consent form. Invasive procedures were performed at Sao Paulo Hospital, UNIFESP, and consisted of: amniocentesis, cordocentesis, chorionic villus biopsy, ventriculoamniotic shunt (VAS) and cephalocentesis. Exclusion criteria were fetal death, fetal defects of the neural tube, maternal hemoglobinopathy, multiple pregnancies, vaginal bleeding during pregnancy, pregnant women previously sensitized and prior invasive procedures in the current pregnancy. The passage of fetal red blood cells to the maternal circulation was evaluated two ways: directly by the Kleihauer-Betke test and flow cytometry and indirectly by maternal blood α-fetoprotein dosage.

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Table 1. — *Descriptive analysis of ten invasive obstetric procedures*.

Patient number	Gestational age (week/days)	Procedures	Indication	Trans-placental	K-B before (fetal erythrocytes/ 2,000 erythrocytes)	K-B after (fetal erythrocytes/ 2,000 erythrocytes)	FMH (ml)	FC before (%)	FC after (%)	Increase (%)	AFP before (IU/ml)	AFP after (IU/ml)	Increase (%)
1	13/3	CVB	Karyotype	Yes	0	2	0.05	0	0	0	54.3	68.4	25.96
2	36/3	AMNIOCENTESIS CEPHALOCENTESIS	Hydranencephaly Polyhydramnios	No	1	3	0.05	0	0	0	76.7	91.2	18.9
3	25/4	AMNIOCENTESIS	Karyotype	No	0	0	0	0	0	0	168.8	179.4	6.27
4	29/3	VAS	Hydrocephalus	No	0	3	0.07	0	0	0	157.1	160.5	2.16
5	37	AMNIOCENTESIS	Lung maturity	No	1	2	0.02	0	0	0	53.4	61.3	14.79
6	38/4	AMNIOCENTESIS	Polyhydramnios	No	0	0	0	0	0	0	62.2	73.4	18.0
7	37/3	AMNIOCENTESIS	Lung maturity	No	0	2	0.05	0	0	0	69.0	76.6	11.01
8	28/4	CORDOCENTESIS	Karyotype	Yes	2	5	0.07	0	0	0	125.0	148.9	19.12
9	27/1	CORDOCENTESIS	Karyotype	Yes	0	4	0.10	0	0,1	0,1	125.3	1542.0	≥ 40
10	23/6	AMNIOCENTESIS	Karyotype	No	0	0	0	0	0	0	265.7	284.4	7.03

CVB: choryonic villus biopsy; VAS: ventriculoamniotic shunt; K-B: Kleihauer-Betke test; FMH: fetomaternal hemorrhage; FC: flow cytometry; AFP: α-fetoprotein.

We collected 5 ml of peripheral maternal blood before and 60 minutes after the invasive procedure. Immediately after collection, samples were separated into two EDTA tubes and each tube was dried, lightly homogenized and stored at 4°C for a maximum period of 72 hours.

Kleihauer-Betke test

This technique is based on the fact that fetal hemoglobin is resistant to acid elution, whereas adult hemoglobin is sensitive. The positive control was the mixture of ten parts of adult blood with one part of cord blood for ABO compatible blood types and negative adults only. The technique was based on preparing thin smears of maternal blood samples and controls, leaving them to dry spontaneously, using ethyl alcohol 80% McIlvaine buffer, 0.5% B Erytrosin and Harris hematoxylin.

Microscopic analyses (using 40 x magnification) showed fetal red blood cells (RBCs) were intact with pink color while the adult cells were extremely pale. Ten fields were counted and the percentage of fetal cells were calculated in relation to adult cells. The hemorrhage volume in ml was equal to the percentage of fetal cells multiplied by 50 [11]. FMH was considered significant as established by Lachman *et al*. [12] and taking into account the coefficient of variation of the method, an increase of five or more fetal erythrocytes in 2000 erythrocytes counted in the slides after the procedure in relation to the blades before the procedure, so, 0.25 ml or more of FMH [12].

Flow cytometry

A specific kit was used (The Fetal Cell Count kit - IQ Products - IQP 379). The methodology of the kit was based on a combination of two antibodies. The first reacts directly against fetal hemoglobin and the second against carbonic anhydrase, an enzyme that starts to express in RBCs only after birth, and is always present in adult erythrocytes. Fetal erythrocytes in maternal circulation positive for fetal hemoglobin but negative for carbonic anhydrase were considered. To perform the technique 1 ml of maternal venous blood was collected in EDTA tubes and stored at 4°C. Any increase in the percentage of fetal erythrocytes in relation to maternal cells was considered significant FMH.

α-fetoprotein

The method was based on the reaction between various α-fetoprotein antibodies. The standard serum and serum samples analyzed were added to the respective locations of the microplate, which were previously coated with streptoavidin. Polyclonal anti-α-fetoprotein and peroxidase conjugated monoclonal anti-α-fetoprotein conjugated to biotin is added and the reagents are mixed. The existing antibodies recognize the antigen, forming complexes that bind to molecules of streptoavidin immobilized on the plate. After the incubation step, excess reagents were immobilized and removed during the wash, and the enzyme substrate was added. Hydrolysis of substrate by the peroxidase reaction generates a blue color, transformed into yellow with sulfuric acid addition. The color intensity, whose absorbance is read at 450 nm, is directly proportional to the amount of existing antibodies and α fetoprotein is measured by spectrophotometry. The concentration of protein in the samples was determined by the construction of the standard curve of optical density versus α-fetoprotein. According to Lachman *et al*. [12] and considering the coefficient of variation of the method, an increase of 40% or more of α-fetoprotein concentrations between samples before and after the procedure was considered FMH.

Results

We analyzed ten patients before and after the procedure, and ten blood samples collected before and after procedures for each test by Kleihauer-Betke, flow cytometry and dosage of α-fetoprotein.

Patient ages ranged from 18 to 38 years with a mean age of 29.2 years. The gestational age of the procedures ranged from 13 weeks/3 days to 38 weeks/4 days, with a mean age of 29.6 weeks.

Ten procedures were performed five amniocenteses, two cordocenteses, one chorionic villus biopsy, one ventriculoamniotic shunt and one amniocentesis with cephalocentesis. Of these ten procedures, five were for karyotype analysis, two to assess lung maturity, one for amnioreduction, one for hydrocephalus and one for hydranencephaly with polyhydramnios. According to the placenta localization, three were transplacental and seven did not cross the placenta. All procedures were performed at the first attempt to puncture.

We did not observe a significant increase in fetal red cells in any of the procedures performed by the Kheihauer-Betke test. There was an average increase of 1.8 fetal erythrocytes per procedure, with the largest increase occurring in procedures such as cordocentesis and ventriculoamniotic shunt. Taking into account only the five

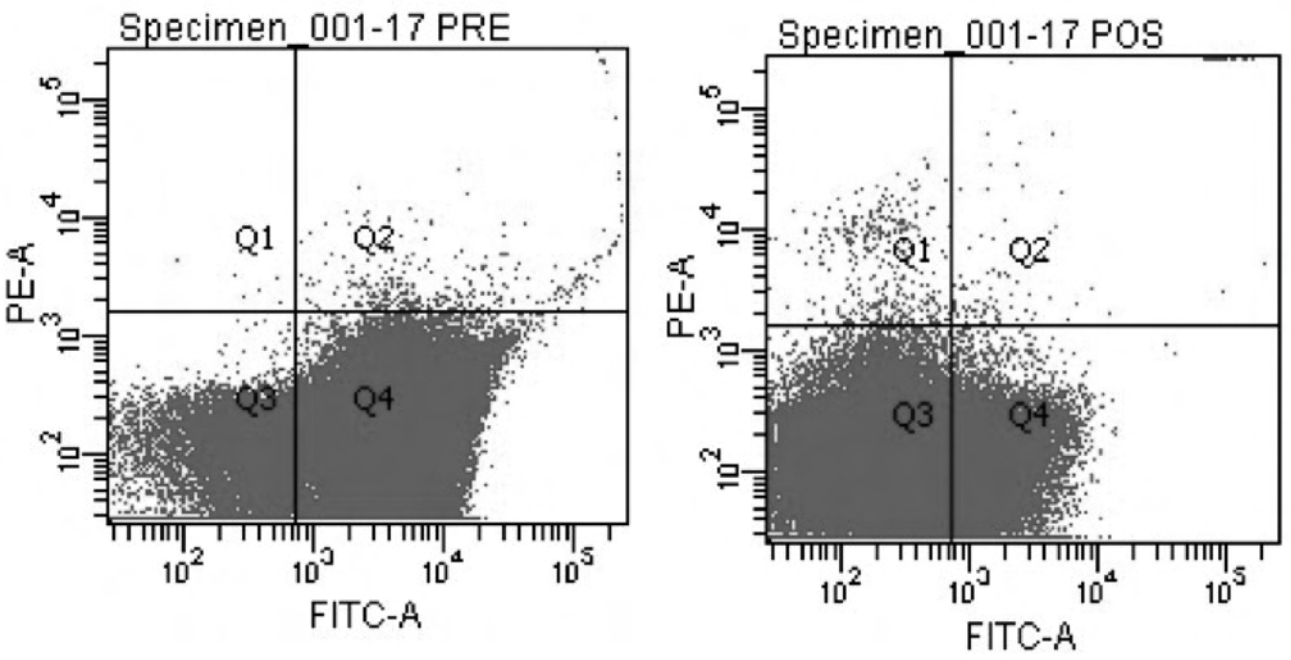

Figure 1. — Cytology sample before and after cordocentesis. Quadrant Q1: fetal erythrocytes.

amniocenteses performed, the increase in fetal red blood cells observed was on average 0.6 erythrocytes per procedure.

In flow cytometry, there was only one cordocentesis in which the presence of fetal erythrocytes had a significant value detectable by the method. The sample before the procedure had no fetal erythrocytes and the sample after the procedure had 0.1% fetal red blood cells. Figure 1 shows the cordocentesis flow cytometry. This same sample was the only one with a significant increase (over 40%) in fetal erythrocytes detected by α-fetoprotein. Table 1 summarizes the results of all ten cases of invasive obstetric procedures.

Discussion

Fetal cells in maternal circulation have been identified in small volumes since the beginning of pregnancy and even increase over the course of pregnancy [13, 14]. A FMH above 30 ml is a rare and catastrophic event in pregnancy and increases the risk of Rh alloimmunization and complications in future pregnancies. Invasive obstetric procedures such as amniocentesis, cordocentesis and chorionic villus biopsy may increase the risk of FMH due to needle transfixation of the maternal abdomen skin to the amniotic membrane and sometimes the placenta and umbilical cord. Factors that increase the likelihood and amount of FMH are the physician's experience, technique, number of punctures and placental trauma [15].

In an attempt to predict and quantify this risk, several studies are found in the literature attempting to quantify the passage of fetal erythrocytes in maternal blood after invasive procedures. Chitrit *et al*. [16] in 2007 proved that the greater the passage of fetal red blood cells to the maternal circulation, the greater the risk of fetal death, induction of preterm labor, need for intensive care unit and neonatal transfusion. In the literature there are some articles that evaluated FMH with different methods and none of them was superior. Only the study by Fernandes *et al*. [17] used flow cytometry. They evaluated 170 chorionic villus biopsies by flow cytometry and Kleihauer-Betke test, and found ten women with fetal cells in maternal peripheral blood in the Kleihauer-Betke test and 26 women in flow cytometry prior to the procedure. Sixty minutes after the procedure, the same patients showed the same cells, respectively, demonstrating good correlation between the two methods. However, flow cytometry was more sensitive and more accurate in determining FMH.

Quantifying the FMH of these procedures is intended to minimize the risks of complications during pregnancy. Among the methods studied, flow cytometry and determination of α-fetoprotein in maternal blood were more accurate in detecting the presence of fetal cells than the Kleihauer-Betke test. The dosage of α-fetoprotein after the procedures seems to be a more sensitive indicator, affordable and accessible to medical practice. Moreover, the result of α-fetoprotein does not have interference from clumping or aggregation of fetal cells.

Conclusion

In summary, we conclude that invasive obstetric procedures are safe to be carried out in fetal medicine when they are followed by standard methods and conducted by trained professionals. Among the invasive obstetric procedures performed, amniocentesis proved to be safe and without risk of passage of fetal erythrocytes to the maternal compartment by any of the three methods. Among

these methods for detection of fetal red blood cells, flow cytometry and determination of α-fetoprotein were more sensitive in detecting FMH.

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