Special Article

Fetal Blood Sampling in Prenatal Diagnosis of Thalassemia at Late Pregnancy

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Fetal blood sampling is a procedure that involves the drawing of a blood sample from the umbilical vein of the umbilical cord, which can be performed after 18 weeks gestation. Fetal blood sampling is a preferable method for prenatal diagnosis of thalassemia in second trimester or late pregnancy. Additionally, it is suggested to be performed in cases in which mosaicisms are identified by amniocentesis or chorionic villus sampling (CVS), areas where DNA analysis is not available, and when mutations of the parents are not known. Laboratory steps regarding prenatal diagnosis by fetal blood sampling were summarized, including the ensuring of fetal origin, determination of red blood cell parameters, fetal hemoglobin analysis, and finally fetal DNA analysis. The objective of this review is to present an overview of procedures in terms of benefits, laboratory interpretations, and some limitations.

Keywords: *Fetal blood, Prenatal diagnosis, Thalassemia*

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Fetal blood sampling (FBS), also called percutaneous umbilical blood sampling (PUBS), cordocentesis or funiculocentesis, can be performed after 18 weeks gestation[1,2]. FBS is obtained by the insertion of a needle through the mother's abdomen under ultrasound guidance, like an amniocentesis, for drawing a blood sample from the umbilical vein of the umbilical cord. Immediately after the first drop of blood is obtained, the syringe is replaced by a new one containing no additive. The blood sample is immediately transferred into special tubes containing adequate anti-coagulant for biological study. Clinical guidelines for this technique were summarized in Society for Maternal-Fetal Medicine (SMFM)[3]. The most common reason for FBS is to obtain fetal blood for prenatal diagnosis of karyo-type (chromosome analysis), hemoglobinopathies, fetal infections, and fetal hematologic studies. Another reason is in the management of Rh isoimmunization in the case of suspicion of anemia, aiding confirmation and allowing transfusion while the needle is in place[4].

Prenatal diagnosis of thalassemia

The possibility of prenatal diagnosis of inherited hemoglobinopathies was first suggested by the discovery that β-globin chain synthesis in cord blood of β-thalassemia heterozygotes was significantly lower than normal[5] and that adult hemoglobin synthesis could be detected in fetuses at mid-trimester[6]. Later, the β^S-chain was detected in the blood of fetuses[7]. FBS by fetoscopy or placentocentesis was then set up[8,9] and prenatal diagnosis was performed by globin chain synthesis on fetal blood[10]. These techniques were used for five years until DNA analysis was applied in prenatal diagnosis in the late 1970s and early 1980s, first using DNA from amniotic fluid cell and later, chorionicvillus. However, FBS remained popular throughout the 1980s because of the complex and sophisticated nature of DNA analysis techniques, particularly Southern blotting combined with the linkage analysis of restriction-fragment-length polymorphism (RFLP) or hybridization of oligonucleotide probes. Thus, prenatal diagnosis programs were restricted to a few specialized centers until the advent of the polymerase chain reaction (PCR) and the subsequent development of simple and rapid techniques for prenatal diagnosis[11].

Nowadays, prenatal diagnosis of thalassemia is usually done using standard DNA analysis by PCR of fetal tissues obtained by chorionic villus sampling (CVS) between 8 and 12 weeks of gestation, amniocentesis between 14 and 20 weeks of gestation, or cordocentesis after 18 weeks of gestation[1,2]. FBS is now rarely used. Historically, it was recommended in many cases in which mosaicisms were identified by

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amniocentesis or CVS, but the limited prognostic utility of this approach led to a decrease in procedures done for this indication[12]. However, numbers of cordocentesis procedures in some areas, such as India, Malaysia, and especially Thailand, were reported. The study of Deepika D et al[13] revealed that FBS is still being performed in very few centers in India. A total of 1,342 procedures with 53 procedures for prenatal diagnosis of thalassemia were done from 1990 to 2009. Evidence of 39 procedures performed in Malaysia from 2003 to 2010 were reported by Valayatham V et al[14]. In Thailand Yamsri S et al[15] showed that approximately 22.2% of prenatal diagnoses were done by cordocentesis. Surprisingly, in one recent study reported from Thailand, more than 2000 cordocentesis were performed from 1989 to 2010 and more than 75% of these were done due to a risk of fetal thalassemia[16]. Although not a common practice, fetal blood specimens obtained with cordocentesis can be used for both hemoglobin (Hb) and DNA analyses. This is especially useful in areas where DNA analysis is not available and when mutations of the parents are not known.

Laboratory steps in prenatal diagnosis of thalassemia

To ensure fetal origin of specimen

Once the needle has been successfully inserted, blood is aspirated into a previously heparinized syringe. Initially, many centers determine if the blood obtained is fetal in origin[17,18]. Methods to determine the origin of fetal blood include measuring mean corpuscular volume (MCV) and the acid elution test[19]. Fetal erythrocytes usually have an MCV in the range of 120-140 fl while those of maternal cells typically have an MCV of less than 80 fl[20], so MCV can be used to confirm if the initial sample is fetal blood. For application of the acid elution test for Hb F, Hemoglobin F is precipitated and fixed in red blood cells on a peripheral smear. Each cell containing fetal Hb gives a positive staining reaction. Specimens that appear as adult ghost cells likely indicate maternal contamination. The acid elution test is a simple method, requires less time and equipment, and is suitable for routine screening tests. Additionally, some centers take and evaluate a maternal sample drawn prior to the procedure for comparison of MCV and hemoglobin/hematocrit, since the fetal MCV is usually larger, and fetal hemoglobin/hematocrit values are typically different from corresponding maternal values. The purposes of these tests establish the absence of maternal contamination and to ensure the fetal origin of the specimen, a necessary requirement for prenatal diagnosis.

Determination of red blood cell parameters in fetuses

Although there is extensive experience in prenatal diagnosis of severe thalassemia, little is known about red blood cell parameters of fetuses with various thalassemia syndromes, which may be due to the lack of fetal blood specimens in routine practice. However, a few recent reports[21-25] revealed the hematologic parameters of normal and various thalassemic fetuses. A summary of red blood cell (RBC) parameters from these reports is demonstrated in Table 1. Normal fetuses have MCV of about 120 fl. No significant differences in RBC parameters from a non-thalassemic group were observed in fetuses with β-thalassemia trait, homozygous β-thalassemia, Hb E trait, and β-thalassemia/Hb E disease. This finding is consistent with the theory that γ-globin genes are the main functioning non α-globin genes during the fetal and neo-natal periods, and mutations of β-globin genes are expected to cause less hematological changes. This is useful in clinical practice to confirm that fetuses with affected β-thalassemia are not anemic *in utero*[23-25]. The significant differences from normal mean Hb levels, MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were found in fetuses with α-thalassemia 1 trait and especially Hb Bart's hydrops fetalis[22,23]. This, therefore, suggests that simple analysis of fetal RBC parameters is useful for making presumptive prenatal diagnosis of Hb Bart's hydrops fetalis, which can then be confirmed by Hb and DNA analyses. However, determination of RBC parameters is suitable for automated blood counters with the use of small amounts of specimens or having pre-diluted modes.

Analysis of fetal hemoglobin

Prenatal diagnosis of thalassemia by analysis of fetal hemoglobin is used as another alternative when DNA analysis is not routinely performed and mutations of the parents are unknown[2,26,27]. Several researchers were interested in prenatal diagnosis of β-thalassemia in fetus by the determination of the amount of Hb A[28,29]. Homozygote or compound heterozygote for β^0-thalassemia, β^0-thalassemia/Hb E disease and homozygote for Hb E are associated with the absence of Hb A. Compound heterozygote for β^0-thalassemia/β^+-thalassemia show very small amounts of HbA ($\leq$0.5%). The ranges of Hb A in β-thalassemia heterozygote's and normal cases determined by different machines are summarized in Table 2. There is an overlap between normal and heterozygous states but no overlap is observed between Hb A of affected

Table 1. Summary of studies regarding fetal red blood cell parameters (the values were presented as mean ± SD)

Thalassemia genotype	Study	No.	Rbc ($x10^{12}$/L)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)
Normal	Forestier et al[21], 1986	163	3.04±0.17	12.4±0.24	37.9±0.7	125.4±1.2	41±2.27	32.8±1.65	19.4±1.86
	Srisupundit et al[22], 2008	22	n/a	11.34±0.83	n/a	123.4±7.76	41.39±3.35	33.44±1.3	n/a
	Karnpean et al[23], 2013	10	2.9±0.3	10.8±1.8	33.5±4.0	119.6±6.7	37.4±4.6	31.3±3.4	14.0±0.5
	Srisupundit et al[24], 2013	43	n/a	11.1 (6.8-12.6)*	33.4 (20-38)*	124.86±1.3	41.44±3.4	33.17±0.9	7.4±1.3
	Srisupundit et al[25], 2013	64	n/a	10.87±1.04	n/a	122.34±8.77	39.95±3.17	32.66±0.89	7.72±1.3
β-thalassemia trait	Srisupundit et al[24], 2013	18	n/a	10.85 (9.2-13.2)*	32.9 (27.5-38.7)*	126.67±5.7	41.77±1.9	33.0±0.6	7.36±0.9
	Karnpean et al[23], 2013	6	2.8±0.3	11.2±1.7	34.0±3.7	123.7±11.4	40.7±4.6	33.0±2.5	14.2±0.3
Homozygous β-thalassemia	Srisupundit et al[24], 2013	25	n/a	11.4 (9.6-13.2)*	34.2 (29-39.9)*	123.48±8.5	40.37±3.2	32.67±0.8	7.64±1.2
Hb E trait	Srisupundit et al[25], 2013	50	n/a	10.86±0.79	n/a	120.7±9.24	39.59±3.54	32.75±0.88	8.03±1.42
	Karnpean et al[23], 2013	15	2.9±0.4	11.0±1.4	34.7±3.7	122.6±8.2	38.9±4.5	31.7±3.2	13.7±0.5
β-thalassemia/Hb E	Srisupundit et al[25], 2013	57	n/a	10.77±1.24	n/a	121.19±9.37	39.63±3.56	32.66±0.96	7.80±1.24
	Karnpean et al[23], 2013	6	3.0±0.4	11.2±2.0	33.9±4.2	121.0±7.0	37.7±5.7	31.1±3.5	14.0±1.6
α-thalassemia 1 trait	Srisupundit et al[22], 2008	40	n/a	10.42±0.91	n/a	105.95±7.35	33.71±2.74	31.81±1.09	n/a
	Karnpean et al[23], 2013	6	3.1±0.4	10.1±1.6	32.6±3.6	104.7±5.7	32.3±4.6	31.0±4.2	12.8±0.6
Hb Bart's hydrops fetalis	Srisupundit et al[22], 2008	26	n/a	6.4±1.64	n/a	96.26±8.19	26.23±2.6	27.29±1.78	n/a
	Karnpean et al[23], 2013	20	2.5±0.6	6.0±1.8	27.2±6.5	111.0±10.3	23.8±4.9	21.8±4.4	19.1±3.9

n/a = not available

* Median (range) for nonparametric data

Table 2. Summary of hemoglobin analysis in fetus by different methods

Thalassemia type	Method	Fetal Hb				
		Hb Type	Hb E (%)	Hb F (%)	Hb A (%)	Hb Bart's (%)
Normal	AX-HPLC[29], 1997	FA	n/a	n/a	8.2-9.5	n/a
	BTS-HPLC[26], 1998	FA	0	94	5.8	n/a
	CZE[31], 2009	FA	0	92.8-95.7	4.3-7.2	n/a
β-thalassemia trait	AX-HPLC[29], 1997	FA	n/a	n/a	3.09-5.2	n/a
	BTS-HPLC[26], 1998	FA	0	94.8-96.3	4.0-5.2	n/a
	CZE[31], 2009	FA	0	90.0-97.9	2.1-8.9	n/a
Homozygous β-thalassemia	AX-HPLC[29], 1997	n/a	n/a	n/a	0	n/a
Hb E trait	BTS-HPLC[26], 1998	EFA	0.8-1.4	94.0-97.2	2.0-2.8	n/a
	CZE[31], 2009	EFA	0.8-1.6	95.4-98.2	1.0-3.0	n/a
Homozygous Hb E	BTS-HPLC[26], 1998	EF	3.0, 2.1	96.8, 97.3	0	n/a
	CZE[31], 2009	EF	2.5	97.5	0	n/a
β-thalassemia/Hb E	BTS-HPLC[26], 1998	EF	1.0-1.7	89.4-98.6	0	n/a
	CZE[31], 2009	EF	1.1-1.8	94.9-98.9	0	n/a
Hb Bart's hydrops fetalis	BTS-HPLC[26], 1998	Bart's	0	0	0	100
	CZE[31], 2009	Bart's	0	0	0	78.4-81.3

n/a = not available; AX-HPLC = Anion exchange-high pressure liquid chromatography; BTS-HPLC = Beta-thalassemia short program-high pressure liquid chromatography; CZE = Capillary zone electrophoresis

and unaffected fetuses. Afterwards, the analysis of fetal Hb is usually applied to detect α-thalassemia disease with the presence of high amount of Hb Bart's[2,30,31]. A normal fetus has predominant Hb F with a small amount (<10%) of Hb A. In Hb Bart's hydrops fetalis fetus with the absence of α-chain synthesis, no Hb F ($\alpha_2\gamma_2$) is synthesized and excess γ chains form soluble tetramers (γ_4, Hb Bart's), which constitute 80-90% of the total Hb, the remainder being embryonic Hb such as Hb Portland. With the decrease of α-chain synthesis, the amount of Hb Bart's is raised in corresponding with the number of α-globin gene defects[32]. The fetal Hb analysis using automated Hb analyzer is a simple method for accurate prenatal diagnosis of severe thalassemia. However, in an area with high prevalence of Hb E, such as Thailand, definite distinction between β^0-thalassemia/Hb E disease and homozygous Hb E (in which no Hb A is observed from hemoglobin analysis) should be a concern.

Fetal DNA analysis

After presumptive identification of thalassemia and, particularly for the purpose of genetic counseling, determination of the mutation or deletion may be required. Fetal DNA is analyzed by one of the methods described for the detection of known mutations in the process of carrier identification. Fetal DNA can be conducted from trophoblast, amniocytes, chorionic villi, and fetal leukocytes in fetal blood. DNA obtained from fetal blood can be simply prepared using a standard method like adult peripheral blood[33]. Prenatal diagnosis by DNA analysis is currently available in many at-risk populations. The PCR-based technique of prenatal diagnosis is highly reliable and widely used. Several molecular techniques for DNA analysis are available, such as Allele Specific Polymerase Chain Reaction (ASPCR), Gap-PCR, Real-time PCR, PCR-RFLP, DNA sequencing, Denaturing High-Performance Liquid Chromatography (dHPLC), and Multiplex Ligation-dependent Probe Amplification (MLPA)[34-41]. Current molecular methods, advantages, and limitations of them were summarized by Harteveld C et al[42]. However, misdiagnosis may occur for several reasons, such as failure to amplify the DNA fragment, mis-paternity, maternal contamination, and sample exchange. To limit the possibility of misdiagnosis, several researchers suggest the analysis of fetal DNA by two different techniques[11,36,43] and the use of variable number of tandem repeat (VNTR) analysis to detect maternal contamination and miss paternity[44].

Conclusion

Although chorionic villus sampling is a method of choice in prenatal diagnosis of thalassemia in the first trimester, FBS is still used in some centers. This indication is preferable for prenatal diagnosis in

the second trimester or late pregnancy. Additionally, it is suggested to be performed in cases in which mosaicisms are identified by amniocentesis or CVS, in areas where DNA analysis is not available, and when mutations of the parents are not known. The ability to complete all the steps of prenatal diagnosis is ideal providing conclusive diagnosis. However, small amounts of fetal blood generally obtained should be a concern in the performance of laboratory procedures. Prenatal diagnosis by fetal blood sampling could help in prevention and control programs for severe thalassemia diseases in regions with high prevalence of thalassemia.

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Potential conflicts of interest

None.

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การวินิจฉัยธาลัสซีเมียของทารกก่อนคลอดในหญิงที่มีอายุครรภ์มากโดยวิธีการเจาะเลือดจากสายสะดือ

รสริน การเพียร

การเจาะเก็บตัวอย่างเลือดทารกก่อนคลอดจากสายสะดือ สามารถทำได้เมื่ออายุครรภ์ 18 สัปดาห์ขึ้นไป การวินิจฉัยธาลัสซีเมียของทารกในครรภ์ก่อนคลอดจากตัวอย่างชนิดนี้นอกจากจะใช้ในกรณีที่หญิงตั้งครรภ์ มีอายุครรภ์มาก ยังใช้ในกรณีที่ผลการตรวจวินิจฉัยโดยการเจาะน้ำคร่ำหรือชิ้นเนื้อทารกมีปัญหา ใช้ในห้องปฏิบัติการที่ไม่สามารถตรวจวิเคราะห์ ดี เอ็น เอ ได้หรือใช้ในกรณีที่ไม่ทราบมิวเทชั่นของพ่อแม่ การทบทวนครั้งนี้ได้รวบรวมสรุปขั้นตอนในการตรวจวินิจฉัยทารกในครรภ์ก่อนคลอดในห้องปฏิบัติการ ตั้งแต่การตรวจยืนยันว่าเป็นตัวอย่างเลือดทารกในครรภ์จริง การตรวจค่าพารามิเตอร์ของเม็ดเลือดแดง การตรวจวิเคราะห์ฮีโมโกลบิน และการตรวจวิเคราะห์ ดี เอ็น เอ ของทารกในครรภ์ โดยนำเสนอขั้นตอนต่าง ๆ ในภาพรวม แสดงให้เห็นประโยชน์และการพิจารณาแปลผลทางห้องปฏิบัติการ เพื่อนำมาใช้ในการวินิจฉัยธาลัสซีเมียตลอดจนข้อจำกัดในแต่ละขั้นตอน