Non-invasive Prenatal Diagnosis

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INTRODUCTION

Currently used prenatal diagnostic procedures such as amniocentesis, chorionic villus sampling and cordocentesis have the disadvantage of being invasive. Amniocentesis may be the least invasive of the three but it is usually performed in the second trimester and can cause some difficulties to the parents and the obstetrician when termination of pregnancy is needed. This has prompted several investigators to look for new, less invasive techniques which can be performed in the early stages of pregnancy. Two possible interesting sources of embryonic or fetal cells accessible in a non-invasive fashion are maternal peripheral blood and cervical mucus.

Fetal cells in maternal blood

Presence of fetal cells in maternal blood

In the process of implantation, trophoblastic cells actively proliferate and invade the endometrium, causing disruption of maternal blood vessels and glands. With rapid proliferation, several cavities, called lacunae, form inside the syncytiotrophoblast layer and contain maternal blood and secretions from the disrupted blood vessels and gland. The trophoblastic-derived chorionic or placental villi are floating or anchored in lacunae where they are in direct contact with a mixture of maternal blood and glandular secretion. This contact can be a possible pathway for embryonic or fetal cells to get across to the maternal circulation. Many attempts have been made to detect, isolate and test these fetal nucleated cells in maternal peripheral blood. These cells may be lymphocytes, trophoblasts, nucleated erythroblasts and granulocytes.[1,2] Generally, fetal cells are present in a very low concentration in relation to maternal cells. Nakagome et al, using polymerase chain reaction (PCR), could not detect fetal cells at a level of 1 in 25,000.[3] Details of sources of fetal genetic material in maternal blood are described below:

1. Fetal lymphocytes. They are present at concentrations of less than 1:100,000 maternal lymphocytes.[4] It is possible that they are rapidly lysed by the maternal immune response because they express HLA antigen.[1] However, in some cases, fetal lymphocytes are found in maternal blood long after delivery.[5]

2. Trophoblastic cells. These cells are shed into the uterine vein and become trapped in the maternal lungs. Only a few of them reach the peripheral circulation and perhaps only 2 out of 10 mothers have any trophoblastic cells in their peripheral blood.[1] Lim et al, based on samples enriched with MAb340 (monoclonal antibody to trophoblast cells), estimated that the ratio of trophoblast cells to maternal cells was 1:162,162 cells in the first trimester.[6] These cells may be cleared from the circulation by 10-12 weeks of gestation.[7] However, Lim et al still found trophoblast cells in the second and third trimesters but with a less frequency than in the first trimester.[6]

3. Fetal nucleated erythroblasts. They are the most abundant nucleated cells in fetal blood, comprising ~10% of red cells in the 11-week fetus and 0.5% of the 19-week fetus.[8] This makes erythroblasts the most promising cell type to be studied in maternal blood. In a study designed to detect fetal nucleated erythroblasts, it has been calculated that there is only 10 µl of fetal blood in the total maternal circulation.[9] The frequency of fetal cells in the maternal circulation is estimated to be 1 to 91 cells with a mean of 19 cells in 16 ml maternal blood.[5] Lim et al have estimated the mean ratio of fetal erythroblasts to maternal cells to be 1:145, 161 cells in the first trimester.[6]

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4. Recently it has been found that, apart from intact fetal cells, free fetal deoxyribonucleic acid (DNA) can also be detected in maternal plasma or serum.[10]

Isolation, enrichment and identification of fetal cells in maternal blood

In order to be used for prenatal diagnosis, fetal cells first need to be isolated from maternal blood. Based on the differences between fetal cells and maternal white blood cells in size, volume and DNA content, density gradient centrifugation is used for their isolation. However, this technique cannot separate fetal cells from maternal nucleated red blood cells which are present in a higher number in the circulation than in the nonpregnant stage. Other techniques are required to help purify the cells further. Fluorescent activated cell sorting (FACS), which is a technique used to sort a specific subtype of cells from a suspension containing several types of cells, has been used to sort fetal cells from maternal blood.[11] The desired cells are labelled by a fluorescent-tagged antibody. The sorting or flow cytometry is carried out through a narrow tube. The cell suspension is passed through this tube and the fluorescently labelled cells give signals which are analysed using a computer according to the set criteria. The desired cells are then separated for the diagnosis. Magnetic activated cell sorting (MACS) is another technique which has been used to sort fetal cells from maternal blood. It uses tiny metal spheres coated with a monoclonal antibody (immunomagnetic beads), specific to fetal cells to separate the cells from the cell suspension. The fetal cells bind to the beads and are sorted from the suspension by a magnet.[12] The antibody used in FACS or MACS can also be specific for maternal cells and so the fetal cells would be in the negatively sorted portion. Using these strategies, several authors have reported success in the detection of fetal cells in maternal blood by PCR and/or FISH.[4, 13-15] Sometimes, after these "enrichment" techniques, identification of fetal cells may be needed where the analysis has to be performed on pure fetal cells such as in prenatal diagnosis for an autosomal recessive disorder for which the couple is at risk. Identifying cells with fetal haemoglobin has been used for this purpose.[16] Recently, a semi-automated microscopy procedure has been introduced to help scanning for the candidate cells.[17]

Another approach to enrich fetal cells in maternal blood is cell culture. One of the earliest reports of fetal cells in maternal peripheral blood was the discovery of 46, XY cells in lymphocyte cultures from pregnant women.[18] In order to improve the results of culture, a special condition that preferentially promotes proliferation of rare fetal cells in the vast majority of maternal cells is needed. Lo et al and Valerio et al have demonstrated some success using an erythropoietin-containing medium or separating and enriching cells with an erythropoietin receptor before culture.[19,20] Bohmer et al have used charcoal-treated serum to suppress the growth of adult red cells and promote the growth of fetal red cells.[21] Although adult cell proportions were minimised, they could not be completely suppressed. More recently, Han et al have employed a two-phase culture system to enrich erythroid progenitor cells from 10 maternal blood samples at 8-14 weeks gestation and could increase the number of cells positive for hemoglobin F (fetal hemoglobin) to 0.49 x 10^5 cells/ml.[22] In some samples, male DNA could be detected. However, they accepted that a proportion of cells with hemoglobin F could be maternal.

Reported prenatal diagnoses using fetal cells in maternal blood

From maternal blood, several fetal chromosomal abnormalities have been detected such as trisomy 21, trisomy 18, Klinefelter syndrome, and 47, XYY as well as some Mendelian disorders or traits such as sickle cell anaemia and thalassaemia, HLA polymorphism and fetal Rhesus blood type.[8,23] Increased frequency of fetal cells in maternal blood is found in many cases where the fetus is abnormal.[5,8] The preliminary evaluation of a multi-institutional study has shown that up to 41% of cases of aneuploidy can be detected from maternal blood.[24]

In addition to genetic analysis using fetal cells in maternal blood, recently Simchen et al have demonstrated that cells with fetal hemoglobin are found with a higher frequency in pregnancies with intrauterine growth retardation than in controls.[25] The identification of this high frequency may be an early predictor for this complication.

Problems of prenatal diagnosis using fetal cells in maternal blood

Many problems remain to be solved before fetal cells in the maternal circulation can be used for routine prenatal diagnosis.

1. Techniques for the identification and enrichment of fetal cells are not yet perfect. The monoclonal antibodies used are not absolutely fetal specific and there is high maternal cell contamination. Glycophorin A, which is one of the most commonly used antibodies, can cause agglutination of non-nucleated red blood cells leading to a change in the scattering of light and fluorescence signals of cells. Magnetic beads interfere with fluorescent in situ hybridization (FISH), a technique used for cytogenetic analysis, because of residual bead debris after sorting.[26]

2. It is not certain when the cells are shed into, and when they disappear from the maternal circulation. Thomas et al found that the earliest detection of Y-chromosome specific sequences in male pregnancies was at 4 weeks and 5 days and they found no such sequences in any of the 18 male pregnancies in their study 8 weeks after delivery.[27] Moreover, no Y-chromosome sequences were detected in any of the female pregnancies (12 cases). From a recent review by Lamvu and Kuller, looking at several cell types, they stated that trophoblasts can be detected in the maternal circulation very early in pregnancy, but they are cleared from the circulation by 10-12 weeks of gestation.[28] Nucleated red blood cells reach the maternal circulation at 6 weeks and are cleared from the circulation at 16 weeks of gestation. Leukocytes enter the maternal blood at 14 weeks of gestation and can persist for up to 5 years postpartum. This long persistence of fetal cells has been found by other groups as well, as will be described below. More recently, Lim et al have demonstrated that the mean number and mean percentage purity for both fetal trophoblasts and nucleated erythrocytes in maternal blood showed decreasing values with increasing gestational age, even though there was no statistically significant difference.[6]

3. Long persistence of fetal cells may interfere with the analysis of the next pregnancy. Bianchi et al found male DNA in 4 of 13 pregnancies with female fetuses, 2 of the 4 women had prior pregnancies with a male fetus and the other 2 had prior abortions.[29] In addition, they found male DNA in the blood of 6 nonpregnant women, one of them had given birth to her last son 27 years ago. This persistence of fetal DNA long after delivery can be a source of error if fetal cells in maternal blood are to be used in prenatal diagnosis. However, from the enrichment technique used in the study, it is thought that the persistent cells are lymphoid progenitor cells. It has been suggested that error from the persistent cells can be avoided by targeting other cell types such as erythroblasts.[8] Nevertheless, a recent study has shown that only half of the erythroblasts in maternal blood are of fetal origin.[30] It has also been found that fetal DNA may be detected in maternal plasma and serum.[10] Fetal DNA in plasma is rapidly cleared from plasma in the postpartum period, giving less chance that the results are from a previous pregnancy.[31] Nevertheless, this will only help in DNA analysis.

4. Finally, the important problem in using fetal cells from maternal blood is that the methods to purify the fetal cells are laborious, time consuming, sophisticated, expensive, and may not be suitable for practical use in clinical situations. This leads to some groups seeking other possible sources of fetal cells to be achieved non-invasively.

Fetal cells in transcervical cell (TCC) sample

Presence of fetal cells in TCC samples

Early in pregnancy, placental villi undergo different development according to their sites. The ones confronting the decidua basalis form a frond-like structure called the chorion frondosum and this will develop into the placenta. The villi covering the outer part of the gestational sac, facing the uterine cavity and covered by the decidua capsularis, is called the chorion laeve. The fate of villi in this part is not clearly known. It has been suggested that they undergo degeneration, however, it is more likely that they invade through the decidua capsularis as they have invasive properties and fall into the uterine cavity.[32] Either way, this results in the accumulation of trophoblast cells in the lower uterine cavity and the cervical canal (Figure 1). These cells can be collected for prenatal diagnostic genetic studies.

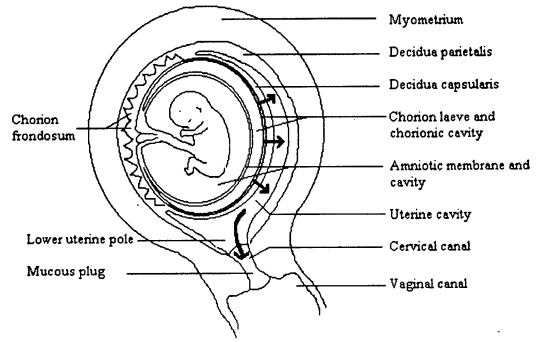


Figure 1. Trophoblasts from the chorion laeve collecting in mucous plug. Trophoblastic cells at the chorion laeve invade through the covering decidua capsularis into the uterine cavity and accumulate in the cervical mucus.

Historical aspect of detection of fetal cells in TCC samples

In 1971, Shettles used cotton swabs inserted into the endocervical canal of pregnant women to sample the mid-cervical mucus, and used a Y-chromosome fluorescent dye (quinacrine.mustard) to detect male cells.[33] He correctly diagnosed the fetal sex of 10 fetuses, 6 males and 4 females. A year later, Warren et al could also predict fetal sex in 50 women at all stages of pregnancy.[34] However, Bobrow and Lewis tried to repeat Shettle's experiment and found no significant difference between the proportion of Y-positive cells in male and female pregnancies.[35] A few further studies have also failed to confirm Shettle's results.[36-38]

Rhine et al repeated Shettle's study.[39] They correctly identified 18 male and 13 female fetuses in 23 male and 13 female pregnancies respectively. In 1978, being less successful in fetal sexing using the same technique, Amankwah and Bond suggested that the conflicting results could be due to the different sites of sampling.[40] By personal communication they found that Rhine's group had obtained samples from the area of the internal cervical os. They expressed their concern about the risk of infection due to the disruption of the mucous plug.[40] They also suggested that the analysis of the Y body in interphase cells might not be adequately reliable.

In 1977, Rhine et al developed a device called the Antenatal Cell Extractor fitted with a syringe containing 5 ml of sterile normal saline to perform lower uterine flushing.[41] TCC samples thus obtained were cultured. They found cells with trophoblast morphology in the culture and, from subsequent karyotyping, some cells were shown to be different from the maternal karyotype by the comparison of Q-band polymorphisms.[41] A 46,XY chromosome spread had been found in a later study.[42] However, these results were not confirmed by Goldberg et al who detected only maternal cells in their cultures of TCC samples obtained by the same technique.[43]

These conflicting results, along with the development of CVS and amniocentesis, made TCC studies less popular for some time. Later, advances in molecular technologies which enable genetic studies to be carried out on a very small amount of sample have prompted several groups to investigate the possibility of using TCC samples as a source of fetal genetic materials. Using PCR to amplify Y-chromosome sequences on samples obtained by cotton swabs, Griffith-Jones et al correctly predicted fetal sex in 25 out of 26 pregnancies.[44] Moreover, using immunohistochemical analysis, syncytial trophoblast fragments were identified in all TCC samples obtained by lower uterine flushing from another 7 pregnancies. On the contrary, in the same issue of the journal, Morris and Williamson reported high rates of false-positive and false-negative results using PCR on TCC samples.[45] However, they admitted that the false-positive results might have been due to possible sperm contamination.

To reduce the chance of false-positive results in the PCR assay which might have arisen from contamination with spermatozoa, in situ hybridisation was employed by Adinolfi et al.[46] This technique has advantages over PCR since 1) it can

give the number of cells present in the sample, thus the frequency of fetal cells can be determined, 2) it can identify the haploid chromosome number of sperm, and 3) it can assess the copy number of the tested chromosome. In their study, lower uterine flushing or lavage with a flexible plastic tube connected to a 10-ml syringe filled with physiologic saline was used to retrieve the TCC samples. Y-derived sequence signals were found in all 6 TCC samples collected from pregnancies with male fetuses, 4 of which had 18-30% of cells containing a Y signal. Also, in a case of trisomy 18 pregnancy diagnosed by CVS performed 8 days earlier, 26% of cells in the TCC sample showed three signals for chromosome 18. Later, using both FISH and PCR for Y chromosome or DNA sequences that are present in fetuses but not in the mothers, more studies have confirmed the presence of fetal cells in TCC samples obtained either by lower uterine lavage or mucous aspirate before any invasive procedures. Examples of DNA sequences used in the studies are RhD sequence in RhD negative pregnant women or polymorphism of short tandem repeats.[47,48]

Recent status of investigation of fetal cells in TCC samples

Subsequently, several studies have been able to detect fetal genetic material in TCC samples either from the lower uterine cavity or endocervical canal. Most of them have used molecular techniques such as PCR or FISH or immunohistochemical techniques.[49-54] Several groups have tried to increase or purify fetal cells for analysis by cell culture or by micromanipulation, i.e., selecting cell clumps that are likely to be trophoblasts.[55-58] Various chromosome anomalies in agreement with the result from the fetus have been detected in TCC samples as well as some haemoglobin mutations.[57-63] With increasing evidence of the presence of fetal cells in TCC samples, some private practitioners in Taiwan even perform uterine lavage for fetal sexing which raises some moral concerns.[64]

However, these promising results are not without a contradiction. In 1996, Overton et al reported a less successful study, using three different PCR techniques to detect Y-derived DNA in 56 pregnancies and FISH to detect the Y chromosome in 36 pregnancies.[65] They were able to detect Y-derived DNA in 79% of male pregnancies but also in 45% of female pregnancies. They correctly predicted fetal sex in 72% of cases using FISH, but fetal cells could be identified in less than 40% of male pregnancies. However, generally speaking, the majority of investigators could demonstrate the presence of fetal genetic material in TCC samples.

Methods used for cervical mucus collection and safety

From previous studies, the methods to collect TCC samples can be divided into 4 groups as follows:[32,33]

1. Cotton swab. This method was used in earlier studies. A cotton swab was inserted into the endocervical canal, rotated, and withdrawn. This technique was associated with some difficulties in cell retrieval from the swab itself and yielded poor results.[49]

2. Cytobrush. A cytology brush was used in the same manner as the aforementioned cotton swab. The end of the brush was cut off into a container which contained physiologic saline solution. The cells, mucus and debris were dislodged into the saline by shaking the container. With this device maternal cellular debris were also collected with the increased maternal cell content and this procedure was considered ineffective.[49]

3. Lavage/flushing/irrigation. This method has been carried out both in the lower uterine portion and the cervical canal. It usually involves a flexible catheter connected to a syringe filled with physiologic saline; after the catheter is inserted into the cervical canal, the fluid is gently injected and aspirated back. The catheter is then withdrawn and the fluid collected. Lower uterine lavage gives a greater chance of obtaining fetal material than collection at the lower level but there are concerns about the safety of injecting saline into the uterine cavity.

4. Aspiration. This technique is similar to lavage but without injection of fluid; instead, the mucus is directly aspirated into the catheter. It is performed in the endocervical canal or at the level of the internal os only. More recently, a catheter which has been used for endometrial sampling had been

employed to collect cervical mucus for detection of fetal genetic material.[66] This catheter has the advantage that it has its own piston and can create negative pressure and does not need to be connected to a syringe, thus is easier to handle.

Currently, the presence of fetal genetic material in cervical mucus is increasingly accepted.[67] Most studies use either lavage or aspiration techniques to collect TCC samples. Many studies have found lavage to be more efficient in retrieving samples with more fetal cells.[32,48] With regard to safety, one follow-up study after aspiration showed no differences between the study group (n=113) and the control cases which were selected from a comparable group of pregnant women undergoing CVS without TCC sampling (n=121) in terms of miscarriages or stillbirths. The miscarriages in both groups occurred at 16 weeks or more, much later than when the procedures (CVS with or without TCC sampling) were performed.[32] At present, other collection techniques should be performed before termination of pregnancy only, due to concerns of the possibility of introducing infection. In addition, there was a report from Taiwan regarding a baby with severe limb reduction defects born to a mother who had undergone uterine lavage at 7-8 weeks' gestation for fetal sexing.[68] This could have been due to the catheter being inserted too high into the uterine cavity.[69]

Fetal cells in TCC samples and prenatal diagnosis

The results so far have not ensured that TCC samples can be used for routine prenatal diagnosis or replace the more invasive procedures being currently employed. Variable frequency of fetal cells in the samples has been found. It can be as low as 2% and as high as 50% from lavage and 0.7-3% from aspirate before CVS.[59] The proportions of cases with fetal cells in the samples also vary. More work is needed to find a more efficient method for the collection and analysis of the samples, to enable TCC to be employed for practical non-invasive prenatal diagnosis in the future.

CONCLUSION

Researchers are trying to look for less invasive techniques to be used for prenatal diagnosis so that the procedures can be applied to more or all pregnant women. Two interesting sources, fetal cells in maternal blood and fetal cells in cervical mucus, are being investigated with some success. There are some problems to be overcome and, hopefully, these two sources for non-invasive prenatal diagnosis will be applicable clinically in the near future.

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