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The Number of Endovascular Trophoblasts in Maternal Blood Increases Overnight and after Physical Activity: An Experimental Study

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Key Words

Fetal cells in maternal blood · Trophoblast · Noninvasive prenatal diagnosis · Physical activity · Diurnal variations · Repeated sampling · Physical manipulation of the uterus

Abstract

Introduction: Fetal cells in maternal blood may be used for noninvasive prenatal diagnostics, although their low number is a challenge. This study's objectives were to evaluate whether physical activity, transabdominal and transvaginal ultrasound scans of the uterus, as well as overnight or day-to-day variation affect the number of isolated fetal cells, more specifically the presumed endovascular trophoblast (pEVT). ***Material and Methods:*** In each of 3 different experiments, 10 normal singleton pregnant women (gestational age 10^{+4}–14^{+4} weeks) participated. The number of pEVTs was assessed in 30–36 ml blood using specific markers for enrichment and identification. ***Results:*** The number of pEVTs increased overnight ($p = 0.001$) from a median of 1.5 to 3.5 and even further to a median of 6.0 after 30 min of physical activity ($p = 0.04$) but was not affected by transabdominal and transvaginal ultrasound scans. Repeated sampling showed that the interindividual variation of pEVTs was higher than the intraindividual variation ($p < 0.001$). However, even in pregnant women with a consistently low number of pEVTs, isolation of the pEVTs for prenatal diagnoses was possible in all cases by doing 2 separate blood samplings a few days apart. ***Discussion:*** The number of pEVTs identified in maternal blood can be increased by presampling conditions or repeated sampling.

Introduction

Noninvasive prenatal diagnosis (NIPD) can potentially be done on either cell-free fetal DNA or fetal cells. Whereas noninvasive prenatal testing (NIPT) based on cell-free fetal DNA is currently being implemented clinically worldwide, numerous attempts to establish NIPD based on fetal cells in maternal blood have failed [1–5]. This is primarily due to the very low number of fetal cells in maternal blood [2]. However, the most promising aspect of using fetal cells for NIPD, compared to NIPT, is that the DNA achieved from fetal cells is uncontaminated and most likely of better quality, probably resulting in a better resolution when looking for microdeletions and duplications.

We have established a method for isolating and identifying a population of fetal cells that is presumed to be an endovascular trophoblast (pEVT), a subgroup of the ex-

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travillous trophoblast from maternal blood [6, 7]. If a sufficient number of pEVTs can be consistently isolated from maternal blood, then analysis of these cells may be developed into a NIPD of fetal chromosomal abnormalities, fetal infections, and placental dysfunction [8–11].

Our method isolated on average only 3 pEVTs per 30 ml blood [12], although it has been estimated that 2–6 fetal cells are circulating in 1 ml of maternal blood [2, 13, 14]. Furthermore, in 12% of cases, we were not able to isolate any pEVTs in maternal blood samples of 30 ml [12]. Thus, it is essential to increase the number of pEVTs in maternal blood. The purpose of this study was therefore to evaluate the number of pEVTs in relation to physical activity, manipulation of the uterus defined as transabdominal and transvaginal ultrasound scans, and overnight and day-to-day variations.

Material and Methods

Participants

A total of 30 participants were recruited, 10 participants in each substudy, when they attended their first-trimester nuchal translucency scanning session. Exclusion criteria were twin pregnancies and pregnancy complications, such as hypertension, at the time of recruitment. The study was approved by the local Danish Scientific Ethical Committee (S-20070045) and the Danish Data Protection Agency (2008-58-0035). All participants signed an informed consent. All included participants gave birth to a chromosomally normal newborn. No pregnant women participated in more than one substudy.

Determination of the Number of pEVTs in Maternal Blood

Depending on the substudy, either 30 ml (overnight difference and physical activity) or 36 ml (transabdominal and transvaginal ultrasound scans of the uterus and day-to-day variation) of maternal blood was drawn into EDTA tubes and processed 2–6 h after they were collected.

The method used to isolate pEVTs was originally developed based on initial isolation by laser capture microdissection of X and Y chromosome-positive fetal cells from the blood of pregnant women carrying a male fetus. From these isolated fetal cells, we purified mRNA and generated a cDNA library, which we used to identify genes highly expressed in the fetal cells. Among the genes significantly overexpressed, more than half have important functions in the placenta and about 25% are expressed in extravillous and/or endovascular trophoblasts [6, 7]. We therefore formulated the hypothesis that a major fraction of the fetal cells in maternal blood was of this type; thus, based on known expression patterns in these cells, we developed a method to enrich the endovascular trophoblasts in maternal blood based on CD105 and CD141 antibodies and final identification based on cytokeratin immunostaining [7].

The method used for fetal cell identification in the present study was a modified version based on that published by Hatt et al. [15], where whole blood samples went through whole blood-positive selection using magnetic cell sorting (MACS; Miltenyi, Germany) with 2 kinds of CD105 antibodies: (1) 75 μl of CD105 antibody microbeads (clone HEC19; Miltenyi) per ml whole blood and (2) 50 μl microbead-coupled (130-048-402; Miltenyi) CD105 antibody (clone SN6; eBioscience, USA) per ml whole blood. Blood samples were incubated with the 2 microbead-coupled CD105 antibodies for 30 min. The mixture was subsequently washed twice in 3.33 ml degassed MACS buffer per ml whole blood mix (Miltenyi). Samples were centrifuged after each wash at 445 *g* for 12 min at 4°C, and supernatants were discarded. Cell pellets were resuspended in degassed MACS buffer to a final volume of 2× the initial blood volume. Samples were then added to prewashed whole blood columns (Miltenyi) at 24 ml sample suspension per column. Each column was washed with 11 ml degassed MACS buffer and eluted in 4 ml whole blood elution buffer (Miltenyi). The eluted fractions from the same sample were collected in one tube and then centrifuged at 445 *g* for 12 min at 4°C.

The supernatant was discarded, and cell pellets were resuspended in 500 μl PBS without $MgCl_2$ and $CaCl_2$ (Gibco, Thermo Fisher Scientific, USA). The 500-μl cell samples were fixed using inside fix (Miltenyi) according to the manufacturer's description. The 500-μl cell suspensions were permabilized in 500 μl of ice-cold methanol for 10 min at 4°C, washed in 14 ml MACS buffer and centrifuged at 500 *g* for 10 min at 4°C. The cell pellets were resuspended in 1,000 μl PBS without $MgCl_2$ and $CaCl_2$ (Gibco, Thermo Fisher Scientific). The cell suspensions were put on prewashed MS columns (Miltenyi) to be stained with a cocktail of cytokeratin antibodies. Initially, the columns were preincubated with 500 μl Imaging Enhancer (Molecular Probes, Thermo Fisher Scientific) for 10 min at room temperature, then washed once in 500 μl of MACS buffer. The cell suspensions were subsequently incubated for 30 min with each of the primary, secondary and tertiary antibodies and washed 3 times with MACS buffer after each incubation. The antibodies used were diluted 1:50 in blocking buffer (4× SSC containing 10% normal goat serum, 1% BSA and 0.5% blocking reagent; Roche, Switzerland). We used 200 μl of a primary antibody solution (Pan Cytokeratin; Sigma-Aldrich, USA, Cytokeratin 7; DAKO, Denmark, and Cytokeratin 8/18; Invitrogen, Thermo Fisher Scientific), 200 μl secondary antibody (AlexaFluor-488-conjugated F(ab)2 fragments of goat anti-mouse IgG; Invitrogen, Thermo Fisher Scientific) and 200 μl tertiary antibody (AlexaFluor-488-conjugated F(ab)2 fragments rabbit anti-goat IgG; Invitrogen, Thermo Fisher Scientific). Columns were washed twice with 500 μl PBS without $MgCl_2$ and $CaCl_2$ (Gibco, Thermo Fisher Scientific). Cells were eluted from the column by using 2 × 500 μl PBS, the first 500 μl by gravity flow and the last 500 μl by plunger. The cells were centrifuged at 500 *g* for 10 min at 4°C, and the supernatant was discarded. Cells were finally smeared onto slides and air-dried overnight, shielded from light. The slides were fixed for 10 min in 2% formaldehyde in PBS, washed 5 min in PBS and then mounted in 150 μl Vectashield with DAPI (Vector Laboratories, USA). Cytokeratin-stained cells were identified by automatic scanning of slides and validated manually as described in Hatt et al. [15]. A pEVT was defined as a cytokeratin-positive stained cell.

The method by Hatt et al. [15] achieved 100% correct gender determination by XY-FISH when 3 or more fetal cells were found. Furthermore, Hatt et al. [15] showed that the fetal cell specificity of the cytokeratin staining in the enriched cell fractions was 96%.

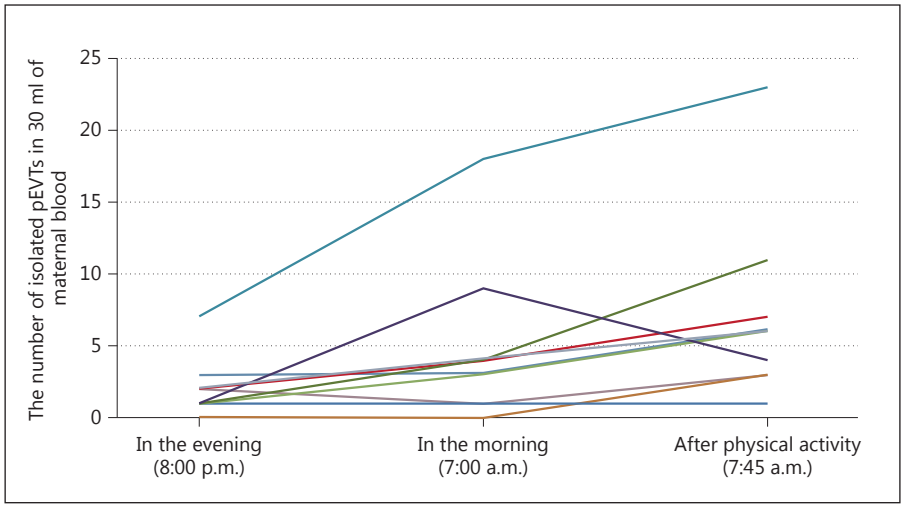

Fig. 1. The number of isolated pEVTs in 30 ml of maternal blood in 10 pregnant women, measured at 8:00 p.m., at 7:00 a.m. the following morning, and after 30 min of physical activity.

Overnight Differences and Physical Activity

Ten participants (gestational age 10^{+4}–14^{+4} weeks), all carrying one male fetus determined through analyses of cell-free fetal DNA, were included. Participants were informed to avoid physical activity 48 h prior to arrival at the hospital where they slept overnight. Blood samples for determination of the number of pEVTs in 30 ml maternal blood were drawn at 8:00 p.m. in the evening and again the following morning at 7:00 a.m. when the participant was still in bed.

Approximately 15 min after the morning blood sampling, the participants performed a defined physical activity (cycling on a stationary bicycle) for 30 min with a pulse rate of 150 beats per minute. Another sample to determine the number of pEVTs in 30 ml maternal blood was drawn immediately after physical activity at 7:45 a.m.

Transabdominal and Transvaginal Ultrasound Scans of the Uterus

Ten participants were included (gestational age 11^{+2}–13^{+6} weeks). Transabdominal and transvaginal ultrasound scans were performed in succession. Total scanning times varied from 17 to 30 min. The same healthcare professional performed all scans. The number of pEVTs in 36 ml maternal blood was determined in blood samples taken immediately before and after the scans.

Day-to-Day Variation

Over a period of 14 days, the number of pEVTs in 36 ml maternal blood was determined in 10 participants (gestational age at first sample 12^{+1}–14^{+0} weeks) 4 times. There was a minimum of 3 days between each sample. Each blood sample was drawn early in the morning when the participant was still in bed.

Statistics

Counts of pEVTs were analyzed by a Poisson model with random person effects to allow over-excess interindividual variation. Data from each experiment were analyzed separately. Statistical analyses were performed with Stata/IC version 13, StataCorp. A 5-percent level of significance was used in all analyses.

Results

Overnight and Physical Activity

The number of pEVTs increased overnight ($p = 0.001$) from a median of 1.5 (IQR 1–2) to 3.5 (IQR 1–4) and even further ($p = 0.04$) to a median of 6.0 (IQR: 3–7) after 30 min of moderate physical activity (fig. 1).

Transabdominal and Transvaginal Ultrasound Scans of the Uterus

The physical manipulation induced by a combination of transabdominal and transvaginal ultrasound examinations did not significantly affect the number of pEVTs in maternal blood ($p = 0.49$; fig. 2).

Day-to-Day Variation

The median number of pEVTs in 36 ml maternal blood was 3 (IQR 2–5), which did not change significantly during the study period of 14 days ($p = 0.44$; fig. 3). At least 1 isolated pEVT was achieved in 8 of the 10 participants after the first blood sampling and in all 10 participants after the second blood sampling when these were taken a minimum of 3 days apart.

It is clear in figure 3 that the number of pEVTs is somewhat consistent within each participant. Statistically, this is also confirmed in that the variance of the random component describing the excess interindividual variation was significantly greater than 0, indicating that the interindividual variation was markedly higher than the intraindividual variation when the number of pEVTs was assessed with days between samplings ($p < 0.001$). This means some participants had consistently low numbers

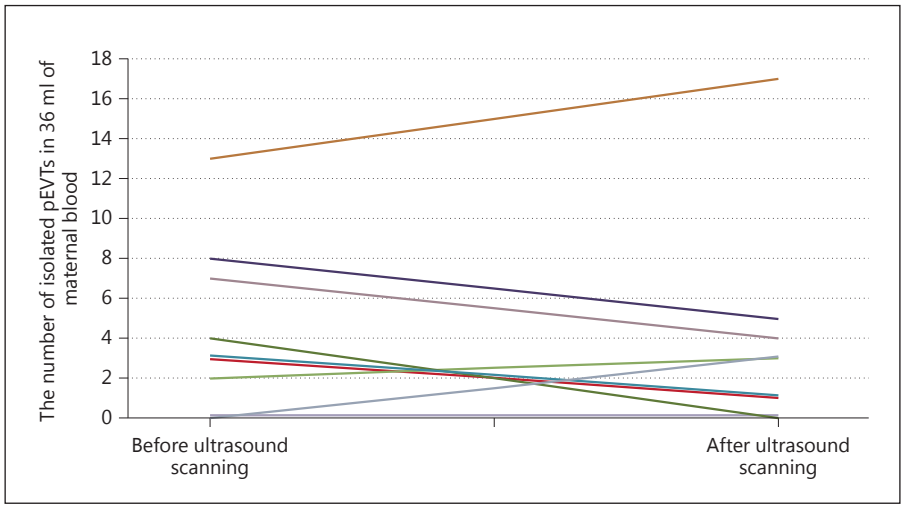

Fig. 2. The number of isolated pEVTs in 36 ml of maternal blood before and after vaginal and abdominal ultrasound scanning.

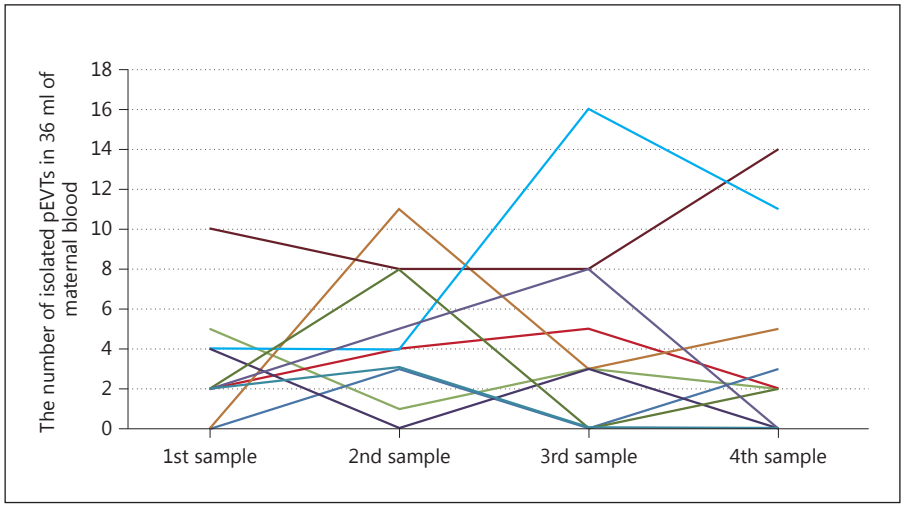

Fig. 3. The number of isolated pEVTs in 36 ml of maternal blood for 10 pregnant women in 4 samples taken in intervals of a minimum of 3 days over a time span of 14 days.

of pEVTs, whereas others had high numbers. The interindividual variation was also significantly higher than the intraindividual variation in the overnight and physical activity study as well as in the study of the transabdominal and transvaginal ultrasound scans of the uterus.

Discussion

The main findings of this study are that the number of pEVTs increased in most participants overnight and in most participants even further after moderate physical activity. Furthermore, we isolated at least 1 pEVT in all participants by the inclusion of a second blood sample of 36 ml taken within an interval of a few days (fig. 3). This was, however, not the case when the second blood sample was taken the following morning or within minutes after the first (fig. 1, 2).

We have not found an explanation for these day-to-day variations or why participants have pEVT counts at different although overlapping levels (fig. 1–3). However, cytokine levels, HLA sharing, and fetal gender may be of importance [12, 16]. Differences in placenta sizes have been previously studied but do not seem to be a factor at early gestational ages [12]. Furthermore, a decreased clearance of fetal cells in the maternal lungs during sleep might contribute to the overnight increase in the number of pEVTs, but such a mechanism does not likely explain the increased number of pEVTs after physical activity [17].

A prior study has demonstrated increased (albeit statistically insignificant) concentrations of cell-free fetal

DNA 30 min after but not immediately after moderate physical activity [18]. This increase might be caused by an intravascular degradation of the increased number of pEVTs demonstrated during physical activity in this study. The increase in the number of fetal cells with increasing gestational age found by prior studies could not be confirmed during the 2-week period of observation in this study [19, 20]. Nonetheless, due to the short period studied, the number of pEVTs may be more pronounced at later gestational ages, and this needs to be further elaborated.

A prior study has suggested that when using the method by Hatt et al. [15], approximately 12% of pregnant women have no pEVTs when only one sample is taken [12, 15]. This is a problem when using single-cell diagnostics as at least one fetal cell is needed in all pregnant women for NIPD. However, this was achieved in this study in all participants by a second sample when taken within a few days. Still, the fact that 12% of patients need a second blood sampling seems unfeasible from a cost-benefit perspective compared to alternative methods such as chorionic villus sampling or NIPT based on cell-free DNA, in a clinical setting, suggesting that improvement of the method is needed. An introduction of moderate physical activity shortly before sampling may improve these numbers.

The main conclusion of the present study is that the number of pEVTs in maternal blood can be increased by specific presampling conditions or repeated sampling. However, it is still important to clarify if these observations are relevant and realistic to implement in a possible NIPD protocol.

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