Detection of Nucleated Red Blood Cells in Maternal Circulation by Magnetic Sorting and *in situ* Hybridization

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Abstract

Introduction. *Nucleated red blood cells (NRBC) from maternal blood are a frequent researched cell group for noninvasive prenatal diagnosis.* ***Objective.*** *We evaluated the feasibility of analysis of fetal NRBC present in the maternal circulation by fluorescence in situ hybridization (FISH), after enrichment by magnetic sorting, without any other cell localization procedures.* ***Materials and methods.*** *From 31 pregnant women, cells have been isolated from maternal blood with a combination of double-density gradient centrifugation followed by a positive magnetic-activated cell sorting (MACS) enrichment, using anti-CD71 monoclonal antibody. Later, we performed FISH with chromosome X and Y specific probes. After automatic microscopic recording of the whole cell area from the slide, the fluorescent signals have been evaluated by manual analysis of images. The results were compared with fetal sex as results from amniocentesis or at birth.* ***Results.*** *FISH analysis detected at least one nucleus with XY signal in 59% of women bearing male fetuses, with a mean gestational age of 20 weeks (range: 15-30). Small number of fetal cells was found (range: 1-11) with a mean of one erythroblast in one milliliter of maternal blood.* ***Conclusions.*** *Isolation and identification of fetal NRBC from maternal circulation represent a considerable challenge due to their extremely low number. FISH analysis associated with MACS identified these cells in maternal blood from first and second trimester. The efficiency of detection was low, not enough for a clinical diagnosis Thus, although they are considered the best target for non-invasive prenatal diagnosis at the moment, their detection remains problematic, with a great variability of results, due to still incomplete known factors.*

Keywords: *prenatal diagnosis, erythroblast, CD71, magnetic-activated cell sorting (MACS), fluorescent in situ hybridization (FISH)*

Definitive prenatal diagnosis of chromosomal anomalies is currently available only after obtaining fetal genetic material through amniocentesis or chorionic villus sampling. These are invasive procedures, associated with small but quantifiable risks for fetus and/or mother. Thus, there is an increasing interest in developing non-invasive prenatal diagnostic tests.

Fetal cells in maternal blood are now an accepted fact, proved through many studies[(1)]. It is estimated that there are one fetal cell to 105-109 cells in maternal blood[(2)] or approximately one fetal cell per 1 mL of maternal blood[(3)].

The main difficulty lies in choosing the type of fetal cell for efficient use in prenatal diagnosis and developing an appropriate method for detection. This method should be simple, reliable and reproducible due to the few fetal cells present and how difficult it is to detect them.

Nucleated red blood cells (NRBC) are one of the first cell lines produced during fetal development, are abundant in the fetal circulation during early pregnancy and are detectable in maternal blood from the first trimester[(4)]. Transfer of NRBC into maternal circulation predominates over other cell types, including leukocytes and trophoblasts. They also have a short half-life and a limited proliferative capacity, which means they are unlikely to come from a previous pregnancy[(1)]. These characteristics make NRBC particularly suitable for non-invasive prenatal diagnostic testing.

Due to the extremely reduced number of fetal cells in the maternal circulation, enriching and purification procedures are necessary, in order to obtain an adequate number of cells for the study. All these methods are characterized by intermediary stages of preliminary separation of the maternal cellular duns, in general through the centrifugation in the density gradient or by cellular lysis, followed by the antigen-antibody recognition, using monoclonal antibodies specific to the fetal nucleate erythroblasts.

Currently, the precise determination of the fetal origin of erythroblasts requires the emphasis of certain differences from the maternal cells, and the most accessible marker is the chromosome Y. Thus, the cells from the male sex fetuses are identified in the majority of cases in two stages: initially a histological marking is used or they are identified by immunohistochemistry,

and afterwards the cells thus selected are confirmed through the detection of the chromosome Y through in situ hybridization or PCR. These methods require an increased work volume and special cellular localization and/or manipulation technologies.

The purpose of our study was to evaluate the efficacy of identifying fetal nucleated red blood cells in maternal blood through a simplified method. This is based on a single stage of detection and confirmation for fetal cells, using FISH analysis for all cells from enrichment procedure. Thus, different treatments for staining and de-staining of cells are excluded, reducing the alteration risk of DNA chains.

This approach required the automation of the plates in the fluorescence microscope.

Material and methods

Patients

Pregnant women, who attended for routine antenatal care or referred for prenatal diagnosis, were invited into the study. The sex of the fetus was determined by ultrasound examination and confirmed by amniocentesis or at birth. In all the women, gestation age was calculated from the date of the last menstrual period and confirmed by first-trimester ultrasound examination. In all cases, the fetuses/infants were normally grown and did not have any structural or chromosomal abnormalities. Maternal blood samples were taken prior to any fetal invasive procedure. All women gave written informed consent to participate in the study. Ten ml of maternal peripheral venous were collected into EDTA (ethylene diamine tetraacetic acid) vacutainers and processed within 4 hours of collection.

Separation and enrichment of NRBC

Maternal blood samples were diluted 1: 1 with PBS (phosphate buffered saline) Dulbecco without Ca and Mg (Biochrom AG) and layered on a double-density gradient prepared with 10 ml of 1.119 g/ml and 10 ml of 1.077 g/ml Percoll (Fluka). Samples were centrifuged at 500 g for 30 min (2 acceleration, no brake) at room temperature[5].

After centrifugation, the mononuclear cells at the interface of the two density gradients were collected with a Pasteur pipette and washed twice with PBS Dulbecco (1:1 dilution followed by centrifugation at 500 g for 10 minutes)[5]. For a precise harvesting, the mononuclear layer was localized with density marker beads (Sigma). Washed cells were re-suspended in 3 ml buffer containing PBS pH 7.2, 1% BSA (bovine serum albumin) and 2 mM EDTA kept cold at 4–8 °C (1:10 dilution of MACS BSA Stock Solution with autoMACS™ Rinsing Solution, Milteny Biotech). Viable cells were counted using a Bürkner-Türk counting chamber.

Cells from half of the suspension (1.5 ml) were enriched by CD71 magnetic positive sorting technique, following a slightly modified manufacturer protocol. Shortly, washed cells were incubated for 15 min at 4°C with magnetically labeled CD71 antibody to the transferrin receptor antigen (20 µL/107 cells). After incubation, CD71-positive cells were isolated with Mini-MACS MS+ column (Miltenyi Biotech) and eluted with 2.5 ml Hanks solution. After centrifugation at 300g for 10 minutes, the mononuclear cell pellet was re-suspended and incubated in 1 ml pre-warmed KCL 0.075 M (Sigma) at 37°C for 5 minutes, to allow hypotonisation. Viable cells from CD71-positive fraction were counted with a Bürkner-Türk counting chamber.

Fresh Carnoy's fixative (methanol: glacial acetic acid [3:1]) was subsequently added, drop by drop (1-2 ml at 4°C). This was followed by centrifugation at 300 g, for 10 minutes and after the removal of the supernatant, the sediment was re-suspended in 200 µl from the remaining supernatant for at least 2 h. The suspension was stored at 4°C until next day.

Slide preparation

Enriched NRBC were placed on slides with increased adherence (Superfrost®Plus Gold, Menzel-Glaser, Germany) by cytocentrifugation at 200g for 5 min (Rotofix 32, Hettich, Germany) at medium acceleration and brake. For each case, a single cell spot was obtained, with 0.3-3 x 105 total cells/slide. Slides were then centrifuged at 1100g for 1 min, air dried and, if necessary. Although the FISH can be successfully carried out either on fresh slides or slides stored at -20°C for long periods, the highest hybridization efficiency in this study was obtained when the slides were kept at 37°C overnight in an incubator before starting the FISH procedure6.

Florescence *in situ* hybridization analysis

This was done in order to confirm male origin of cells. Slides were pre-treated in 2 x SSC (sodium saline citrate)/0.5% igepal, pH 7.0 at 37°C for 5 minutes, then dehydrated in 70%, 85% and 100% ethanol series for 1 minute and air dried at room temperature. FISH was carried out using a kit with chromosome X and Y satellite enumeration probes (Poseidon™, Kreatech Diagnostics, Amsterdam, Netherlands). DNA probes are supplied as already prepared, in a hybridization solution containing formamide, dextran sulfate sodium in citrate saline. For each cytocentrifugation spot on the slide there were applied 5 µl of probe mix. The area was covered with a glass cover-slip and sealed with Fixogum and slides were placed in a pre-warmed humidified incubator (Hybrite, Vysis). They were incubated for 5 min at 75°C for denaturation and then hybridization was carried out overnight at 37°C. The next day, rubber cement was removed, and post-hybridization washes were done according to manufacturer's protocol. Hence, excess probes were removed in a washing buffer (0.4 x SSC/0.3% igepal) for 2 minutes at a temperature of 72°C (±1°C) without agitation . Subsequently, the slides were washed in the washing buffer number 2 (2 x SSC/0.1% igepal) for 2 minutes at room temperature without agitation. Slides were air dried at room temperature, in the dark. Cells were counterstained with DAPI/antifade solution and fluorescent signals were analyzed using a 1.0 Axioscop microscope (Zeiss, Jena, Germany) with x 100 objective and DAPI/FITC/Texas

Red triple band-pass filters. The images were captured and processed using digital camera and TissueFAXS software (TissueGnostics, Wien, Austria). Enrichment and analysis of fetal cells were carried out without knowledge of the clinical details of the patients.

Only intact cells that were not overlapping were chosen for the analysis. The system performed an automatic recording of cells from the whole cytocentrifugation spot, followed by a manual analysis of images. In cases were a Y chromosome was suspected a manual acquisition of the field was performed. In general, all identified spots were counterchecked for non-specificity in all other filters at a magnification of x 1000.

Results

We processed 31 peripheral blood samples from normal singleton pregnant women with a median maternal age of 26 years (range 16-38). The mean gestational age was 20 weeks (range 12-30), 80% of the women were at their first pregnancy and the rest has no history of a male fetus. Fetal sex distribution at birth or amniocentesis showed that the women carried 27 male and 4 female fetuses. The mean number of mononuclear cells isolated from 10 ml of maternal blood was 9.57 ± 1.22 x 106 cells (mean ± SE) - figure 1.

Anti-CD71 magnetic activated sorting failed to isolate a counting number of cells in 4 of 31 maternal blood samples. For the rest, the mean number was 10.12 ± 1.94 x 104 cells (mean ± SE) - figure 2. There was no association with gestational age and parity for total number of mononuclear cells harvested from double-density gradient centrifugation or for total number of cells from MACS.

FISH showed XY cells in 16 of the 27 (59%) women bearing male fetuses and none in those with female fetuses. The mean number of fetal male cells visualized was 4.1±0.6 (mean ± SE) with a range from one to eleven. This accounts for about one fetal cell per milliliter of maternal blood. There was a slight decrease in identified number of fetal cells with increasing of gestational age (r2=0.09) - figure 3.

We obtained easily identifiable spots for all fluorochromes on male cord blood control slides and the FISH protocol described above gave a hybridization efficiency for X and Y chromosome over 90%. Contamination of the samples with Y-chromosome material during handling of the probes can be excluded because only female examiners processed the samples.

Discussion

Results of this study confirm the presence of fetal nucleated red blood cells in maternal circulation as identified by detection of Y chromosome in euploid nuclei of candidate fetal cells enriched by double-density gradient and magnetic cell sorting with microbeads conjugated to monoclonal anti-human CD71 antibodies.

Double density gradient centrifugation, which removes the majority of neutrophils, platelets and non-nucleated red blood cells, isolated cellular concentrations

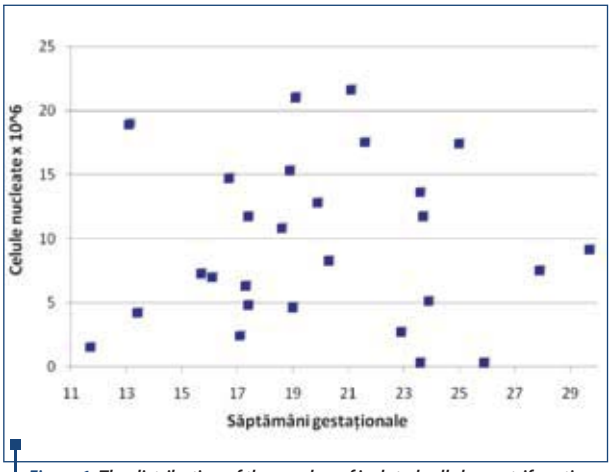

Figure 1. The distribution of the number of isolated cells by centrifugation in the density gradient, according to the gestational age

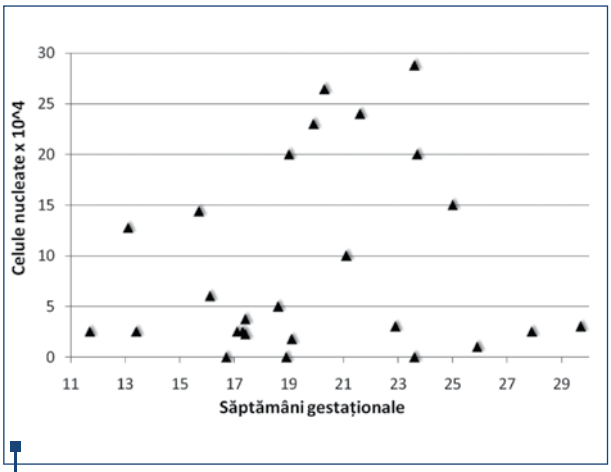

Figure 2. The variation of the number of positive CD71 cells selected through MACS according to the gestational age

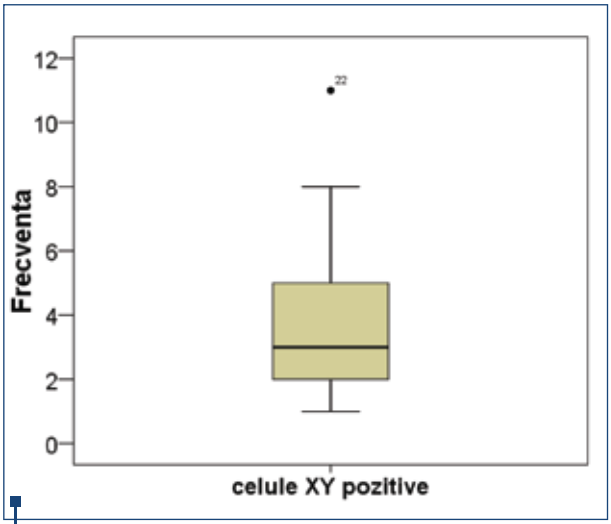

Figure 3. The number of XY nuclei identified by FISH. The superior and inferior limits of the rectangle represent the percentages 75% and 25% respectively. The horizontal line is the median and the inferior and superior bars represent the limits of 10%, respectively 90%. The extreme values are represented separately

with a large range of variation (0,3-21,6 x 106 cells/10 ml blood). Similar results acquired Reading and col.7, using Ficol 1.077 g/ml density gradient centrifugation (mean of 17.47 x 106 cells/10 ml whole blood) and Lim and col. 8, with Histopaque 1.077 g/ml (mean of 11,8 x 106 cells/10 ml blood). Also, Kwon and col. 9, using a double-density gradient identical with ours, but with different osmolarities, obtained similar results, for gestational ages of 17 and 28 weeks (mean of 7, respective 4.9 x 106 cells per 10 ml blood). The number of cells recovered from the double-density gradient varies between the different samples; this may directly influence the number of positive cells obtained after the MACS and probably the number of fetal cells recovered.

The second step was MACS using the transferrin receptor CD71 present on erythroid lineage cells such as nucleated erythrocytes. Positive selection led to a mean depletion of 98.7%, similarly with those obtained by others[7]. However, Prieto[10] and Kwon [9], using a similar method for isolation and magnetic sorting, acquired a significantly greater number of CD71 positive cells: mean of 34 to 149 x 104 cells per 10 ml blood, with a lower depletion rate of only 94-97%.

In this study, FISH analysis showed at least one cell with XY signal only in 59% of women bearing a male fetus. All of those 4 patients with female fetus did not showed XY cells. This result fits in range reported also by other studies.

Thus, the percentage in which the positive CD71 followed by FISH analysis predicts a fetus of male sex varies in the literature in great limits, from 24% to 100%[9]. The majority of studies report values comprised between 50-60%[11,12]. This phenomenon can be explained through: **(1)** only a small part of the linear erythrocyte cells are nucleate[13], **(2)** the fetal erythroblasts are separated in reduced proportion together with the rest of the nucleate, maternal and fetal cells, **(3)** the large variation of the number of erythroblasts, fetal cells available in the maternal circulation, **(5)** the fetal erythroblasts do not survive in an immunological detectable form or through FISH not even to an enrichment process without numerous cellular treatments. In this regard, we ascertained that in the maternal blood, the erythroblasts are subject to a higher oxygen concentration, which favors apoptosis 14 and reduces the nuclei dimensions[15]. Thus, probably a large part of the fetal erythroblasts is not adequate for the FISH analysis.

Moreover, the visualization in fluorescence signaled numerous candidate cells, falsely positive. After the FISH treatment, the automatic scanning of the centrifugation area emphasized some cells with red and green signals. Usually, their number was bigger than 100 and requested a manual analysis.

Generally, all the Y suspect signals were reevaluated for lack of specificity in all the filters, to a magnification of x 1000. The nature of these falsely positive red signals is unknown. They can be given by a non-specific connection of the sample or can be small particles present in the washing solution[16].

Conclusions

The isolation and identification of fetal nucleate erythroblasts in the maternal circulation represents a considerable challenge because of the much reduced number of these cells. However, because of the enormous commercial potential of a non-invasive prenatal screening test, the research is extremely active in this field. The FISH analysis associated with the magnetic classification identified these cells in maternal blood samples from the first and second trimester. Nevertheless, the method efficiency was modest, insufficient for a clinical diagnosis. Although the fetal erythroblasts are considered the best option for the prenatal diagnosis, their detection remains problematic, with a high variability of results, given by the factors incompletely understood in the present. The new technologies of selection and identification of the more specific fetal cells, simple and cheap, will make the non-invasive prenatal diagnosis of the fetal aneuploidies much more feasible. ■

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