Fetal cells in maternal circulation:

Fetal Cell Separation and FISH Analysis

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Summary

This work focuses on the isolation of fetal cells from the blood of pregnant women, with the aim of developing safe, efficacious, non-invasive alternatives for prenatal diagnosis. Although the fetal cells were first detected in maternal blood in the 1893, an effective protocol for non-invasive analysis is still not firmly established. This is due, on the one hand, to the scarcity of fetal cells in maternal blood, which is of the order of 1 fetal cell to 10^6 - 10^7 maternal nucleated cells and on the other hand, to the fact that fetal cells have no specific cell markers.

Efforts were made to improve development and evaluation of new fetal cell enrichment procedures. One of the tasks of this study was to evaluate galactose specific enrichment via soybean agglutinin, a galactose-specific lectin for isolation of erythroblast from maternal blood, and to compare this new technique with the conventional CD71 enrichment technique.

Another technical obstacle which had to be overcome was how to analyze the chromosomal content of few fetal cells enriched from the maternal circulation. Since these fetal erythroblasts were not actively dividing it was impossible to use standard cytogenetic methods. To address this issue multicolor fluorescence in situ hybridisation (FISH), or single cell polymerase chain reaction (PCR) procedures for analysis of fetal cells were developed and optimized. In the largest series of articles published to date, the efficacy of detecting fetal cells by the use of FISH for X and Y chromosomes was below what was needed. We tried to optimize the FISH procedure by applying different treatments to the nucleus and using different kinds of fluorescent probes firstly on cord blood erythroblasts as a model system. Then, after optimization, the best FISH protocols were applied to electronically marked erythroblasts from maternal blood. The analysis of FISH signals in maternal blood revealed that about half of erythroblasts did not hybridize. Additionally, we checked whether the ability to successfully perform FISH depended on chromosome choice. FISH analysis for chromosome 18 gave the same result. Thereafter we searched for possible reasons of FISH signal absence. The morphometric analysis of erythroblasts indicated that erythroblasts which had hybridized efficiently were of larger nuclear size than those which had been impervious to the FISH procedure; that is, the

efficiency of FISH procedure is connected with nuclear size. We then compared the erythroblasts from maternal blood with those from cord blood. The morphometric analysis indicated a significant difference in size between erythroblasts circulating in maternal blood and cord blood.

Additionally, we wanted to determine whether the presence of fragmented DNA hindered the FISH analysis. The results of Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) analysis suggested that effective FISH analysis had been hindered by the presence of dense nuclei rather than nuclei containing fragmented DNA.

A point of interest was for us to look for possible reasons for nucleus size reduction of erythroblasts in the maternal circulation. We hypothesized that the changes in the nuclear size of erythroblasts could be attributed to the different oxygen tensions in the fetal and maternal circulatory systems. We checked and confirmed this hypothesis on model systems such as culture at low and normal oxygen conditions.

Another interesting issue for us was to look in detail at the chromatin and cytoplasm organization of erythroblasts on a spectral level, using spectral imaging analysis, and then to compare the results for erythroblasts from maternal and cord blood. This analysis also confirmed differences between erythroblasts from maternal and cord blood.

Which fetal target cell is best suited for analysis remains an open question. Our task was to evaluate the ability to perform FISH analysis of fetal cells in whole blood without any enrichment. One further task was to improve fetal cell recovery by applying XYY FISH as alternative to conventional XY FISH

As reported by many researchers, single cell PCR analysis of fetal erythroblasts is more effective than FISH analysis. We checked the fetal status of small dense erythroblasts which appeared to be refractory to FISH analysis by Taqman PCR after laser microdissection capture (pool). Furthermore, we explored the opportunity of single cell Taqman analysis of erythroblasts enriched by soybean lectin-based (SBA) method and microdissected by laser microdissection and pressure catapulting (LMPC) technology from membrane covered slides.

All the above–listed questions comprised the subject matter for our investigation. In what follows, we give a detailed description of the studies performed.

Aim

Main purpose of this study is to development of non-invasive analysis of fetal cells from maternal blood for prenatal diagnosis.

In particular, the purposes of our study are:

1) To evaluate galactose-specific enrichment via soybean agglutinin for isolation of erythroblasts from maternal blood and to compare it with conventional CD71 enrichment;
2) To optimize FISH on cord blood by comparing different types of probes and different protocols;
3) To apply the FISH protocols after optimization on maternal blood;
4) To check whether the efficacy of FISH depend on chromosome choice (18 chromosome FISH);
5) To compare the morphometrical measurements of erythroblasts, accordingly the FISH signals, from maternal blood and cord blood;
6) To check apoptosis status of erythroblasts in maternal blood and cord blood, applying TUNEL;
7) To check the effect of oxygen concentration on the number of erythroblasts and their morphometric properties at culture at low and normal oxygen conditions;
8) To evaluate and to compare the ability to perform XY-FISH, YY-FISH and XYY-FISH analysis of fetal cells in whole blood;
9) To analyze and compare the chromatin and cytoplasm organization of erythroblasts from maternal and cord blood on a spectral level;
10) To check the fetal status of erythroblasts without any FISH signals by Taqman-PCR after laser microdissection capture (pool);
11) To optimize the single cell RT-PCR after soybean lectin-based enrichment

1. Introduction

1.1. Disadvantages of diagnosis by invasive testing and blood screening

Currently, prenatal diagnosis of fetal genetic traits relies on invasive procedures such as amniocentesis, chorionic villus sampling (CVS) or fetal blood sampling. However, all these approaches are connected with increased risk of fetal loss or maternal injury and face the problems of reliability and accuracy of the results.

Amniocentesis is time consuming and faces problems of culture failure and culture artefact. Culture failure can occur in up to 1% of amniotic fluid samples and chromosomal mosaicism in 0.5% (Hsu and Perlis, 1984). Serious maternal complications such as lower abdominal cramps, vaginal blood loss and amniotic fluid leakage occur in up to 3% of women. Occasionally, significant loss of amniotic fluid may be associated with neonatal complications (Finegan et al., 1990). In the large randomised controlled trial, done in 4,606 low-risk healthy women between 25-34 years, it was shown that the excess risk of miscarriage due to amniocentesis was as high as 1% (Tabor et al., 1986).

The problem with ***Chorion Villus Sampling (CVS)*** is that the discrepancies exist between the cytogenetic results of culture and the actual fetal karyotype. One reason for this is that placental mosaicism due to the structure of chorionic villi (Slunga-Tallberg and Knuutila, 1995). The incidence of confined placental mosaicism in CVS specimens is 1.5% (Hahnemann and Vejerslev, 1997). Another reason for the discrepancies is phenomenon called trisomy rescue (Ledbetter and Engel, 1995). Natural selection against aneuploidy often leads to loss of the supernumerary chromosome in an originally trisomic embryo. In addition, it is accepted that CVS is a slightly more risky procedure than amniocentesis, with a procedure-related miscarriage rate around 2% (Rhoads et al., MRC Working Party, 1991).

The ***Fetal Blood Sampling*** procedure is associated with a risk of miscarriage of about 2% (Buscaglia et al., 1996). The main causes of fetal loss are rupture of membranes, chorioamniotis, and puncture of the umbilical artery, bleeding from the puncture site and prolonged bradycardia. The frequency of procedure-related losses can be reduced by methods such as serum screening, which identify women with an increased risk of bearing abnormal fetuses. However, serum screening is a statistical method that identifies only 60-70% of fetuses with Down syndrome (with a 5% false positive rate) (Phillips et al., 1992).

Main disadvantage of all those tests is that they cannot be used to provide cells for chromosomal analysis. Because of the uncertainties of screening and the procedure–related risks of invasive diagnosis methods, there is considerable interest in developing alternatives risk free non-invasive test for prenatal diagnostic.

1.2. Alternative non-invasive diagnosis

In quest for the development of non-invasive methods for prenatal diagnosis two strategies have emerged; the enrichment of rare circulatory fetal cells from maternal blood and the analysis of cell free fetal DNA in maternal plasma.

1.2.1. History of recovery of fetal cells and cell-free DNA in maternal circulation

In 1893, Schmorl first have described the appearance of fetal trophoblasts in the maternal pulmonary vasculature (Schmorl, 1893). Then, Douglas et al. (1959) have identified circulating trophoblasts in maternal blood. The definitive proof that fetal cells circulate in maternal blood only came when lymphocytes bearing the Y-chromosome were detected in the peripheral blood of mother carrying male foetuses (Walknowska et al., 1969). Herzenberg et al. (1979) first have isolated the fetal cells using fluorescence-activated cell sorting (FACS). Bianchi et al. (1990) have identified the fetal erythroblast cells by Y chromosome sequences in male pregnancies after FACS-enrichment. Many researchers have demonstrated the opportunity of identification fetal aneuploidies by applying fluorescence in situ hybridisation (FISH) after FACS-enrichment (Price et al., 1991; Bianchi et al., 1992; Elias et al., 1992b; Simpson and Elias, 1993). The similar results were obtained after magnetic-activated cell sorting (MACS) of the fetal erythroblasts (Ganshirt-Ahlert et al., 1992).

Convincing evidence of the existence of fetal DNA in maternal blood came in 1990 with the application of sensitive molecular technique such as polymerase chain reaction (PCR). Lo et al. (1989) have demonstrated by PCR the existence of fetal cell-free DNA in maternal plasma, which was detected in relatively much more quantity than intact fetal cells. Although the PCR method has rapidly established itself as the method of choice for the analysis of more facile fetal genetic loci, such as the presence of the Y chromosome in pregnancies at risk for an X-linked disorder or the fetal RhD gene in pregnancies with a Rhesus constellation, it is currently not possible to examine for more complex fetal

genetic disorders such as chromosomal anomalies by this approach. On the contrary, the analysis of intact fetal cells would allow a wider range of diagnoses to be made, including the detection the fetal aneuploidy.

Unfortunately, the fetal cells are present relatively rarely in maternal blood, they have not specific fetal cell markers, that makes difficult (but not impossible) to isolate and physically identify them. Currently, the research in fetal cell area focuses on aims how to isolate and use these ones for non-invasive prenatal diagnosis. Also it is of interest to know their biological role and effect in the mother.

1.2.2. Variety of fetal cells in the maternal circulation

Trophoblasts are epithelial cells, which shed in the maternal blood as early as sixth week of gestation (Ganshirt et al., 1995); (van Wijk et al., 1996), but unlike lymphoid and myeloid fetal cells, they do not persist for years after delivery (Bianchi, 1999). Trophoblast cells are unique and of critical importance for development and functioning of the placenta. The trophoblasts form cytoblasts layer and multinucleate trophoblastic syncytium of placental villi. In addition, a certain population of cytotrophoblas invades the walls of uterus and its spiral arteries. It is a long known fact that syncytiotrophoblasts find their way into maternal circulation and are at least partly trapped in the capillaries of the lungs, but the extent of cytotrophoblasts invasion is still unclear.

The trophoblasts express the cytokeratins (Zvaifler et al., 2000) and they have relatively large size that permits definitive microscopic identification. The use of trophoblast cells for non-invasive prenatal diagnosis however has met with several difficulties. Trophoblast deportation into the maternal circulation does not appear to be a phenomenon common to all pregnancies (Sargent et al., 1994). These cells are invariably trapped in the lungs and rapidly cleared by the pulmonary circulation (ATTWOOD and PARK, 1961). Furthermore, they are originating from the placenta, which is known from chorionic villus sampling studies to have 1% incidence of chromosomal mosaicism (Henderson et al., 1996), which severely restricts the use of trophoblasts for accurate genetic diagnosis. The enrichment for these cells has often been hindered by the lack of specific antibodies (Covone et al., 1984; Covone et al., 1988; Bertero et al., 1988). However, some groups have successfully isolated the trophoblasts from maternal blood

by using the specific monoclonal antibodies against trophoblast cell surface antigens (Mueller et al., 1990) or by “isolation by size of epithelial tumor cells” (ISET) (Vona et al., 2002; Beroud et al., 2003).

One of the earlier attractions of fetal ***leukocytes*** was their ability to proliferate in vivo. Walknowska et al. (1969) demonstrated the presence of the Y chromosome in mitogen-stimulated leukocytes obtained from pregnant women who were carrying male fetus. Now this propensity is regarded as a disadvantage, since leukocytes can proliferate in vivo in maternal organs and persist in the maternal blood (Schroder, 1974; Ciaranfi et al., 1977). Thus, the case in point is that enriched leukocytes may be the vestiges of previous pregnancies and they do not represent fetal genetic status in the current pregnancy.

Further limitation of using fetal leucocytes for non-invasive diagnosis is the lack of monoclonal antibodies specific to fetal leukocyte antigens. However, Herzenberg et al. (1979) have recovered fetal leukocytes from maternal blood by FACS with antibodies against paternally-derived HLA-A2 antigens. The fetal gender and HLA type were successfully predicted also by Iverson et al. (1981).

However, none of the resulting metaphases contained a fetal karyotype when the separated leukocytes were grown in tissue culture in another study (Tharapel et al., 1993). Zilliacus et al. (1975) and Wessman et al. (1992) have raised the possibility of using fetal ***granulocytes*** as targets for non-invasive prenatal diagnosis, and suggested that fetal granulocytes are transferred into the maternal compartment from as early as seven weeks and on a regular basis. The fetal granulocytes comprised on average 0.13% and occasionally as much as 0.26%, of all mononuclear cells in maternal blood. Available data do not support the use of granulocytes as suitable targets.

Relatively recently, Campagnoli et al. (2001) identified a novel population of ***mesenchymal stem cells (MSC)***. These cells are present in fetal blood from 7 to 14 weeks of gestation where they account for 0.4% of fetal nucleated cells. The frequency of these cells declines with gestational age. Their immunophenotype is non-haemopoietic (CD45 negative, CD14 negative), non-endothelial (CD31 negative, CD34 negative, no expression of von Willebrand factor) and myofibroblastic. Circulating fetal MSC express a number of adhesion molecules (e.g. Vimentin, Fibronectin, VCAM-1), are HLA-DR

negative, and are positive for the mesenchymal markers, SH2, SH3 and SH4 (Macek et al., 2002).

The multilineage potential of MSCs can be demonstrated under appropriate culture conditions by their differentiation into osteocytes, adipocytes, neurons, muscle cells and chondrocytes, but they assume fibroblastic appearance and growth pattern when cultured in 10% fetal calf serum. Furthermore, these cells synthesise collagen when placed in culture. MSC are not known to be normally present in adult or maternal blood, they are currently only one cell type to be present in fetal blood and not in maternal blood. Although the presence and quantity of MSCs in maternal circulation remain to be elucidated (Zvaifler et al., 2000) and still no well-defined protocol for isolation of fetal MSC from maternal blood exist, the ease by which these cells can be propagated in culture suggests they are another possible target cell for non-invasive prenatal testing (Uitto et al., 2003).

Haematopoietic progenitor cells are present in the first trimester fetal blood and account for 5% of the total CD45 positive cell population. They can be enriched based on the expression of the CD34 antigen and expanded in vitro (Campagnoli et al., 2000). Despite culture conditions favouring fetal cells, only limited expansion of these cells over maternal cells in vitro has been achieved, and with few exceptions, fetal haematopoietic progenitors have not been successfully identified in the maternal circulation before 16 weeks of gestation (Jansen et al., 2000).

Thus, the cell type chosen for non-invasive prenatal diagnosis should be short lived within the mother, have no or only limited capacity to proliferate, and have unique cell surface markers to facilitate enrichment. These requirements led to the choice of the erythroblasts as target cell.

The ***erythroblasts (Nucleated red blood cells (NRBCs)*** are very abundant in the early fetal circulation, virtually absent in normal adult blood and by having a short finite life-span, there is no risk of obtaining fetal cells which may persist from a previous pregnancy. In contrast to the leukocyte, they have many cell surface markers (the transferrin receptor and the glycophorin A cell surface molecule) and cytoplasmic markers (embryonic (ζ- and ε-) or fetal (γ-) hemoglobin) that can help them to

differentiate. Furthermore, unlike trophoblast cells, fetal erythroblasts are not prone to mosaic characteristics.

Initially, all researchers believed that all erythroblasts isolated from a maternal sample were fetal in origin. Later on, the newer and more sensitive techniques of enrichment used to detect fetal cells have allowed discovering previously under-appreciated population of maternal erythroblasts that circulate during pregnancy (Slunga-Tallberg et al., 1995; Slunga-Tallberg et al., 1996). Thus, if fetal erythroblasts are to be used as target cells for accurate non-invasive prenatal diagnosis, a more specific identification system needs to be developed.

1.3. Non-invasive prenatal diagnosis using fetal erythroblasts in maternal blood: current state of the art

1.3.1. Number of fetal cells in maternal blood at normal and aneuploid pregnancies

The presence of fetal cells in maternal blood is a rare event - only one fetal cell per 10^5-10^9 of maternal cells (Ganshirt-Ahlert et al., 1990; Price et al., 1991). Takabayashi et al. (1995) reported on average of 2 fetal cells in 1 ml of maternal blood. Bianchi et al. (1997) evidenced by applying quantitative PCR, that the average number of fetal cells that can be isolated from maternal blood is 1.6 cells per ml when the fetal karyotype is normal. Fetal cell number estimation based on PCR on whole blood includes all fetal cell types, whilst most studies are restricted to fetal erythroblasts but nevertheless they confirm the observation of Bianchi et al. (1997).

Simpson and Elias (1993) and Ganshirt-Ahlert et al. (1993) have described that the number of fetal cells in maternal blood increases in aneuploid pregnancies. Increased numbers of fetal cells in maternal blood in aneuploid pregnancies could be due to several mechanisms. Firstly, the placental feto-maternal barrier could be impaired due to disturbed development or function of placental villi, leading to an increased feto-maternal transfusion (Genest et al., 1995; van Lijnschoten et al., 1993). Secondly, in the trisomic fetus itself, more fetal cells could be present. Genest et al. (1995) have described a lack of nucleated red blood cells in trisomic spontaneous abortions compared with eusomic ones. This could indicate altered haemotopoiesis in early trisomic embryo (Thilaganathan et al., 1995), leading to an increased erythroblasts content in feto-maternal transfusion. Thirdly, the number of trisomic erythroblasts in maternal blood could be higher because these ones have a longer survival.

The fact that the number of fetal cells increases at aneuploid pregnancies probably may contribute to diagnosing the numerical chromosomal anomalies.

1.3.2. Enrichment of fetal erythroblasts from maternal blood

Since the erythroblasts are very rare in maternal venous blood (Oosterwijk et al., 1998a; Pertl and Bianchi, 1999; Ganshirt-Ahlert et al., 1990), various enrichment methods exist to

overcome this limitation: discontinuous density gradients, magnetic activated cell sorting (MACS), fluorescence activated cell sorting (FACS) and lectin-based enrichment.

1.3.2.1. Discontinuous density gradient centrifugation

The trend to use density gradient centrifugation as the first enrichment step to eliminate/reduce the abundance of maternal red blood cells followed a publication by Bhat et al. (1993). They demonstrated, using cord blood samples, that if Histopaque-1077 was used alone, 82.6% of erythroblasts would pellet. Nowadays, most groups use continuous Ficoll gradients of different specific densities varying from 1077 to 1119. Troeger et al. (1999a) have found in comparative study on artificial mixture of cord and adult blood that the most erythroblasts was recovered when Ficoll 1119 was used as compared with lower density gradients 1077, 1098, 1110. Bhat et al. (1993) also have shown that if a discontinuous double density gradient of Histopaque-1077 and 1119 was used, less than 0.5% of erythroblasts would collect at the top interface but 78.6% would settle at 1119.

In 1993 Bhat et al. were again the first to report the use of triple density-gradient centrifugation; they have demonstrated a 25-fold enrichment of fetal nucleated red blood cells (Bhat et al., 1993).

In comparative study, Al Mufti et al. (2004) have demonstrated that triple density-gradient separation is more effective with regard to fetal cell yield than single density gradient separation on maternal blood samples.

Since then, density gradient centrifugation has been used either alone or as the first step in most enrichment protocols (Cheung et al., 1996; Oosterwijk et al., 1998b).

1.3.2.2. Surface antigens

Erythroblasts express on their surface several antigens: the transferin receptor (CD71), thrombospondin receptor (CD36), blood type antigens (ABO, rhesus, MN, li, etc.), erythropoietin receptor (Valerio et al., 1996), the fetal liver surface antigens HAE9, FB3-2 or H3-3 (Savion et al., 1997; Zheng et al., 1997) and possibly HLA-G (Steele et al., 1996). Potentially-useful surface antigens for positive selection of erythroblasts are glycophorin A (GPA) expressed on cells of erythroid differentiation; transferin receptor (CD71) expressed on all proliferating cells of both red and white cell lines and the thrombospondin receptor (CD36), being expressed on monocytes, platelets and erythroid cells (Table 1; Figure 1).

The surface antigens present on the surface of other blood cells can be used for negative selection, for example: CD35 is present on white blood cells; CD47 is present on erythrocytes, and many others.

Fetal erythroblasts were first isolated from the peripheral blood of pregnant women using anti-CD71 (Bianchi et al., 1990). The transferin receptor, present on all cells that incorporate iron including activated lymphocytes and trophoblasts, is known to be expressed on erythroid cells from BFU-e to the reticulocyte stage (Loken et al., 1987). Zheng et al. (1997) have found that 89.6% of erythroblasts from multiple or structurally abnormal pregnancy in the first trimester fetal blood were CD71-positive.

Most groups have used CD71 to enrich the fetal cells based upon these findings (Cheung et al., 1996; Durrant et al., 1996; Zhong et al., 2000; Al Mufti et al., 2001). However, the disadvantages of CD71-enrichment are poor yield, which has been attributed to low number of target cells within maternal blood, and the lack of purity due to the fact that other cell types also express CD71.

Bianchi et al. (1993) have demonstrated that GPA alone or in combination with CD71 or CD36 improves the retrieval of fetal NRBC from maternal blood. Troeger et al. (1999a) have found the using of CD36 in MACS-based protocol to be less effective than GPA or CD71. Two other antigens that could achieve the same objective using negative selection are CD35 and CD47. CD35 (complement receptor type 1 (CR1)) is present on white blood cells and to a lesser degree on mature erythrocytes. CD47 (integrin-associated protein) has recently been identified as a marker of `self` on erythrocytes (Oldenborg et al., 2000).

A major drawback of the above antibodies is that they do not distinguish between fetal and maternal erythroid cells, whereas most of the erythroblasts in maternal blood are of maternal origin (Slunga-Tallberg and Knuutila, 1995; Holzgreve et al., 1998).

The disadvantages of all antibody enrichments are poor yield, which has been attributed to low number of target cells within maternal blood, and the lack of purity of target cells in positive population.

Type of cell	Antibody
erythrocytes	CD 35, CD 44, CD 55, CD 59, CD 147
CFU-E	CD 36, CDw 123, CDw 131
BFU-E	CD 33, CD 34, CDw 123, CDw 131
myeloid stem cell	CD 33, CD 34, CD 117, CDw 123, CDw 131
pluripotent stem cell	CD 90, CD 123, CD 117, CD 135
lymphoid stem cell	CD 34, CD 10, CD 38, CD 90, CD 117, CD 124, CD 127
granulocytes	CD 11b, CD 11c, CDw 12, CD 13, CD 31, CD 43, CD 45RO, CD 52, CD 66b, CD 66c, CD 87, CD 88, CD 114, CDw 101, CD 123

Table 1. Surface antigens.

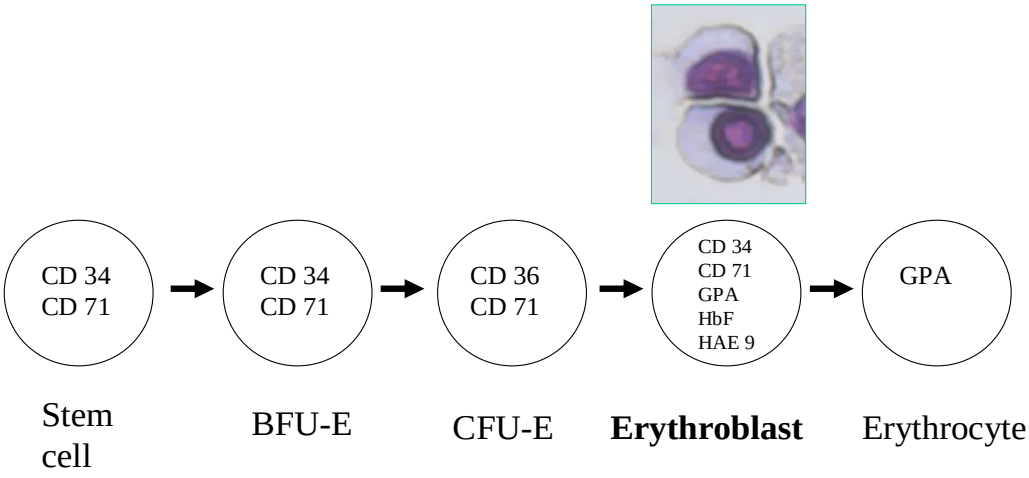


Figure 1. Surface antigens

1.3.2.3. Devices for antibody labeled cell sorting

Fluorescence-activated cell sorting (FACS) and magnetic activated cell sorting (MACS) are the two most commonly used systems for antibody labeled cell sorting.

FACS: Herzenberg et al. (1979) were the first who have used the FACS. The advantages of this technique are its ability to enrich cells with high purity, to perform multiparameter sorting i.e. simultaneously analyzing several criteria on a single cell; and to be adapted for use with intracytoplasmic antigens. Its disadvantages are its cost, expensive maintenance, requirement for specially–trained laboratory personnel, limitation in the number of cells that can be put through the system in a reasonable amount of time, fading of the fluorochrome if exposed to FISH after sorting and cell loss (Wang et al., 2000).

MACS: The use of MACS for non-invasive prenatal diagnosis was first suggested by Ganshirt-Ahler et al. (1992). It is a faster, less expensive, bench-top technique better suited to process large cell numbers and can be performed in most laboratories without the need to trained staff and high maintenance costs. Its major disadvantage is that the cell selection can be based upon only a single criterion. Negative and positive selection can be performed on the same population of cells within the same experiment but the enrichments need to be carried out one at a time. Its disadvantages are: the yield and purity poorer as compared with FACS, slides had more maternal cell contamination (Wang et al., 2000). However, the absolute numbers of fetal cells recovered are comparable.

1.3.2.4. RosetteSep enrichment

Bischoff et al. (2003) have reported on improved isolation using a simple whole blood progenitor cell enrichment approach (RosetteSep). They enriched the NRBCs by removing unwanted mature T-cells, B-cells, granulocytes, natural killer cells, neutrophils and myelomonocytic cells using CD2, CD3, CD14, CD16, CD19, CD24, CD56 and CD66 RosetteSep progenitor antibody cocktail (StemCell Technologies, Canada). The detection (FISH) rate in this pilot study was 53%, which suggests great promise.

1.3.2.5. Soybean Lectin-based enrichment

Kitagawa et al. (2002) have offered the new enrichment of erythroblasts from maternal blood using the adsorption of erythroblasts to slides containing galactose sites by galactose-bearing conjugation via soybean agglutinin (SBA), a galactose-specific lectin.

Blood cells express saccharides on their surface, which play an important role in the control of cellular behavior and fate (Raedler et al., 1981). Cell-surface galactose associated with the development and maturation of the erythrocytes is highly expressed on the erythroid precursor cells (Skutelsky and Bayer, 1983).

The selective attachment of the cells with the cell-surface galactose to substrate coated with a galactose-containing polymer (PV-MeA, Ne Tech) via galactose-bearing conjugation with soybean agglutinin (SBA – a galactose-specific lectin), allows to enrich the erythroblasts with good yield. As well as erythroblasts, leukocytes and erythrocytes are also adsorbed to the slides via SBA (Figure 2). The optimal conditions for isolating erythroblasts are possible to be obtained by adjusting the concentration of SBA.

Using this lectin enrichment based on the FISH analysis, Kitagawa et al. (2002) have estimated that 65% of erythroblasts were of fetal origin and average number of erythroblasts was 3.4 per 1 ml of maternal blood during normal pregnancy.

In comparative study Babochkina et al. (2005c) have found 7-fold increase in the yield of erythroblasts after lectin enrichment as compared with CD71 enrichment. This indicates that the lectin-based method is more efficacious than a MACS approach for the enrichment of erythroblasts from maternal blood and that this method may be a promising alternative for future investigations concerned with non-invasive prenatal diagnosis.

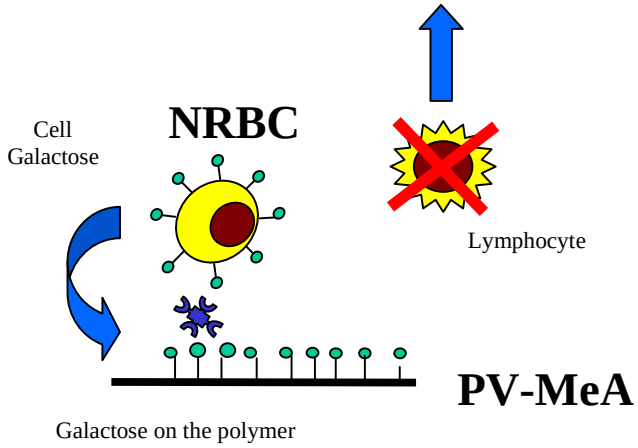


Figure 2. Scheme of galactose-bearing conjugation of blood cells via soybean agglutinin (SBA), a galactose-specific lectin.

Blood cells are attached to the solid support with PV-MeA, carrying α-1.6 galactose terminal, via SBA. Erythroblasts preferentially bind to the solid support with a low concentration of SBA.

1.3.3. Identification and analysis of the enriched cells

1.3.3.1. The identification of fetal origin of erythroblasts enriched from maternal blood

To identify the fetal origin of enriched candidate cells it is possible to use morphological criteria and labeling for embryonic and fetal haemoglobin (Zheng et al., 1993; Mesker et al., 1998; Parano et al., 2001).

1.3.3.1.1. Morphological properties

The erythroblasts have distinct morphometric characteristics such as small, condensed, rounded nuclei, big nucleus-cytoplasm (N/C) ratio, which allow differentiate them from all other cells.

Many investigators have suggested that erythroblasts originating from the fetus have properties of nuclear morphology that are distinguishable from erythroblasts of maternal

origin (Wang et al., 2000; Samura et al., 2000; Samura et al., 2001; Ikawa et al., 2001). Cha et al. (2003) have presented a scoring system based on the distinct phenotypic characteristics of the fetal erythroblasts. Two of them are morphological characteristics, and another two – characteristics of gamma haemoglobin staining. The study demonstrates the ability to identify fetal cells without relying on the presence of the Y chromosome.

1.3.3.1.1. Fetal haemoglobin labeling

Yolk sac erythroblasts synthesize ε-, ζ-, γ- and α-globins, these combine to form the embryonic haemoglobins. Between six and eight weeks, the primary site of erythropoiesis shifts from the yolk sac to the liver, the three embryonic haemoglobins are replaced by fetal haemoglobin (HbF) as the predominant oxygen transport system, and ε- and ζ-globin production gives way to γ-,α- and β-globin production within definitive erythrocytes (Peschle et al., 1985). HbF remains the principal haemoglobin until birth, when the second globin switch occurs and β-globin production accelerates.

Zheng et al. (1993) suggest that fetal origin of erythroblasts can be confirmed by labeling intracytoplasmic, developmentally-specific fetal haemoglobin. Since then, there have been several attempts to develop fetal erythroblast specific antibodies for both enrichment and/or identification (Zheng et al., 1997; Zheng et al., 1999; Huie et al., 2001). Presently, most investigators use γ-globin for fetal cell identification. However, not only fetal erythroblasts but also maternal erythroblasts express γ-globin (Pembrey et al., 1973). The "leaky" expression of γ-globin in adults prompted Cheung et al. (1996) to suggest the use of the embryonic ζ-globin instead. They have demonstrated this principle in the case of sickle cell anaemia and also in the case of β-thalassaemia on micromanipulated, ζ-globin positive NRBCs enriched from maternal blood between 10-12 weeks. Luo et al. (1999) have shown that ζ-globin was present in 53% of definitive erythrocytes between 15-22 weeks and 34% at term, because its expression is not completely switched off after embryonic period. Also, ζ-globin chains exist in adults with the α-thalassaemia trait (Chung et al., 1984).

Mesker et al. (1998) have demonstrated the presence of other embryonic ε-globin positive NRBCs in male fetal erythroblasts from two post-CVS maternal blood samples. Mavrou et

al. (1999) in comparative study have found that ε-globin was more reliable and specific for the detection of the fetal NRBCs than ζ-globin.

1.3.3.2. The analysis of fetal erythroblasts enriched from maternal blood

The enriched fetal cells can be analyzed predominantly by polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH).

1.3.3.2.1. Polymerase Chain Reaction (PCR)

The main advantage of single-cell PCR based analysis is the good efficiency and specificity. The disadvantage is that the collection of single cells needs a lot of experience with regard to manipulation and transferring the cells to reaction tubes and is associated with a significant loss of the rare fetal cells.

The ability of PCR to amplify minute quantities of DNA has been exploited by several investigators to demonstrate the possibility of prenatal diagnosis of monogenic disorders using fetal cells enriched from maternal blood.

Sekizawa et al. (1996) have demonstrated that it was possible to select single fetal erythroblasts by micromanipulation and to potentially diagnose genetic conditions such as Duchenne muscular dystrophy by PCR. One limitation of that study was that erythroblasts identified by morphology alone were presumed to be fetal. In contrast, Cheung et al. (1996) used micromanipulation to pick ζ-globin positive fetal erythroblasts and diagnosed fetal haemoglobinopathy in two pregnancies between 10-12 weeks. They avoided the problem of allele dropout by pooling several erythroblasts in each PCR reaction. The usability of micromanipulation prior to PCR has since been confirmed by Watanabe et al. (1998) who demonstrated the prenatal diagnosis of the X-linked ornithine transcarbamylase deficiency syndrome and by Samura et al. (2001) who demonstrated the adjunctive use of PCR after FISH.

1.3.3.2.2. Fluorescence in situ hybridisation (FISH)

The main advantage of the FISH is that the counting of fetal cells could be realized automatically in the future. With such automatic analysis it could be possible to examine

large number of cells in short time. The disadvantage of FISH for fetal cells is low efficiency.

The ability of FISH to detect fetal aneuploidies within fetal cells isolated from maternal blood was demonstrated by many researches. Price et al. (1991) have demonstrated the use of FISH technique for diagnosis of the trisomies 18 and 21. Elias et al. (1992a) have extended these observations by diagnosing fetal trisomy 21 in the maternal blood taken before CVS. The first series was reported by Ganshirt-Ahlert et al. (1993) who confirmed five cases of trisomy 18 and ten cases of trisomy 21 in erythroblasts enriched from maternal blood. Cacheux et al. (1992) have shown that sex chromosome aneuploidy could also be detected reliably and Bischoff et al. (1995) have detected sex chromosome mosaicism by FISH in fetal cells enriched from maternal blood. Finally, Pezzolo et al. (1997) have described one case in which fetal triploidy was diagnosed by FISH on enriched fetal cells and confirmed by CVS. Some investigators have attempted to increase the number of chromosomes that could be analyzed by interphase FISH by performing simultaneous multicolour FISH (Bischoff et al., 1998) or by sequential hybridisation of chromosome pairs (Zhen et al., 1998).

1.3.4. Clinical trials of non-invasive prenatal diagnosis

The NICHD (National Institute for Child and Development) funded NIFTY (National Institute for Child and Development Fetal Cell Isolation Study), which began in 1994. The trial aimed to recruit 3000 women considered to be at "high risk" (women that are over 35 years, have serum screening results indicating an increased risk of Down Syndrome, have fetal anomalies detected on ultrasound and have familial chromosomal rearrangement) for fetal aneuploidy. However, studies performed have indicated that fetal erythroblasts cannot be reliably isolated and analyzed despite the use of current state-of-the-art technologies. In these studies sensitivities as low as 16% were recorded by groups using flow cytometrical (FACS) enrichment strategies, whereas those using a magnetic cell sorting (MACS) approach were able to detect fetal cells with a sensitivity of 45% (Bianchi et al., 2002).

1.3.5. Limitation of clinical application of non-invasive diagnosis

The disappointing results of detection and analysis of erythroblasts in maternal blood have initially been attributed to very low occurrence of erythroblasts within maternal blood (Hamada et al., 1993) and to the lack of suitable refined enrichment procedure using fetal cell markers specific enough to enrich and identify them.

The recent studies have, however, indicated that fetal erythroblasts may express certain apoptotic traits, such as fragmentation of their nuclear DNA as detected by the TUNEL assay (Sekizawa et al., 2000; Kolialexi et al., 2004). This has raised the question of whether fetal erythroblasts are suitable for molecular genetic analysis, and have again voiced the concern whether these cells can indeed be detectable at all in the maternal circulation.

1.3.6. Place of fetal cells in non-invasive prenatal diagnosis

Currently it is envisaged that fetal cells derived from maternal blood could be used as a screening tool, alone or (more likely) in combination with other modalities such as cell-free DNA biochemical tests and nuchal translucency scans.

However, two changes in the current state of the art would allow enriched fetal cells to be used for screening and, more important, diagnosis. These include reliable enrichment of fetal erythroblasts in the first trimester and specific identification of the fetal origin of these cells.

Fetal cells can be used for diagnosis only if a pure population of these cells can be obtained, or if there is a method of confirming the fetal origin of the erythroblasts being tested and distinguishing them from background maternal (Goldberg, 1997).

The complete system for using fetal cells from maternal circulation for prenatal diagnosis of specific genes as well as chromosomal disorders is likely to be derived from investigating the following functional components, some of which may be procedurally combined: first, segregation of a population of nucleated blood cells enriched in fetal cells of one or more types; second, identification of cells that are probably of fetal origin within this population; third, isolation of the cells likely to be of fetal origin; fourth, confirmation of the cell(s) as fetal; and, fifth, analysis of the fetal cells (Bayrak-Toydemir et al., 2003).

Over the past two decades, investigators have developed and pursued strategies that draw upon several or all of these components in various combinations and sequences of step, but still today performing the cytogenetic and molecular analysis of fetal cell obtained non-invasively from maternal circulation is very desirable though elusive goal, and so far we have not yet an optimal protocol.

2. Results

2.1. FISH optimization on cord blood samples

Introduction

The enriched fetal cells can be analyzed by fluorescence in situ hybridisation (FISH) (Bianchi et al., 1992; Bischoff et al., 1998; Oosterwijk et al., 1998b; Al Mufti et al., 1999; Parano et al., 2001).

The main advantage of the FISH is that the counting of fetal cells could be realized in the future automatically. The disadvantage of FISH for fetal cells is the low efficiency.

As Troeger et al. (1999b) and many other authors have previously shown the erythroblasts isolated by micromanipulation could be reliably analyzed by PCR for a number of fetal genetic loci with a disparity appeared to exist between the analysis of fetal erythroblasts by PCR and FISH.

This restriction for successful FISH analysis of erythroblasts circulating in maternal blood may be due to the fact that the nuclei in fetal cells are very small, the DNA in these cells is very dense, and it means, that access for penetration and hybridization of probes is difficult. In such case the major aspects for successful FISH are the kind of fixation, pretreatment of cells, choice of probes and optimization of hybridisation conditions. In our study we tried to improve the FISH efficiency on MGG stained slides by optimization of the FISH protocol.

The choice of MGG staining was made, because such approach allows the cell morphology to be unchanged. The cell morphology helps to recognize the erythroblasts.

To make easier the access to DNA of erythroblasts we have compared different kinds of pretreatment of cells: pepsin, HCl, pro K, and microwave activation.

A further aspect, which plays a critical role in success of FISH is the choice of hybridization probes. We have compared Vysis two-color cocktail: X centromeric α-satellite (spectrum green)/Y centromeric α-satellite (spectrum orange) and Qbiogene two-color cocktail: X centromeric a-satellite (fluorescein)/Y centromeric a-satellite (rhodamine). Also we checked the probes for Y chromosome for centromeric region Yp11.1-q11.1 (α-satellite) and for region q12 (satellite III), alone and in combination. Then we used the combination of Qbiogene probes: two-color cocktail: X α-satellite (fluorescein) and Y α-satellite (rhodamine) in combination with Y III-satellite

(rhodamine) in one–step two–color XYY-FISH-hybridization. To optimize the FISH protocols we also checked different conditions of hybridization: melting temperature and time, duration of hybridization and washing conditions also.

2.1.1. Pepsin, Proteinase K and HCL pretreatment

Introduction

The pepsin and proteinase K digest the protein matrix that surrounds the nucleic acid. The precise action of the HCL acid is not known, but it has been suggested, that it is connected with extraction of proteins and partial hydrolysis of the target sequence.

Aim

We have applied these pretreatments to improve access of the probe to target nucleic acid and to reduce non-specific background signals. The pretreatments were supposed to improve the efficiency of the FISH on erythroblasts enriched from maternal blood.

Results

By examination of different concentrations, temperature and duration of time, the following optimal conditions for pepsin pretreatment were determined: 0,005% solution of pepsin in water, for 30 sec at 37^0C.

The efficiency for FISH on erythroblasts from cord blood under these experimental conditions with Vysis probes for MGG slides was 98%. The signals were bright and very good recognizable, but the morphology of cells was destroyed. The cells represented only nuclei with small rim of cytoplasm (Figure 6).

Proteinase K and HCL treatments did not produce good results for MGG slides. They have demonstrated good FISH signals, but have destroyed the morphology of cells completely. After hybridization the slides have high non-specific background signals.

2.1.2. Microwave pretreatment

Introduction

The microwave treatment has been suggested to enhance the exposure of the chromosomes to FISH probes (Durm et al., 1997).

The mechanism and the reason for the accelerating of FISH efficiency by microwave treatment still are not well understood. It is considered that the microwave treatment

produces the local thermal effects which may contribute to DNA denaturation and exerts a considerable influence on proteins.

Aim

To improve the efficiency of the FISH analysis by the microwave treatment.

Results

1) Microwave conditions optimization

The experimental conditions were systematically optimized using a household microwave oven. It was important to place the specimen slide onto a glass filled with PBS buffer in order to prevent "overdenaturation" of the sample and to maintain the nuclei architecture. The best position of the slide was determined in the centre of circled plate since the microwave field is not homogeneous. The best denaturation conditions were determined as 700 W for 40 sec (Table 2; Figure 4A).

	180 W	360 W	700 W
5 sec			
10 sec			
15 sec			
20 sec			
25 sec			
30 sec			
35 sec			
40 sec			
45 sec			
50 sec			
55 sec			
60 sec			

Table 2. Choice of conditions of microwave pretreatment.

Red color – best conditions; orange – good; yellow – satisfactory; blue – bad.

2) The conventional XY Vysis FISH with microwave pretreatment on MGG stained erythroblasts from cord blood

Hybridization was performed with conventional Vysis two-color cocktail XY probes for 6 hours. The average efficiency of X- and Y- signals on erythroblasts enriched from cord blood was 99% (SDev 0.01136, Var 0.00013) (8 cases). Under the same experimental conditions without the microwave pretreatment, the average efficiency for MGG-slides

was 73%. The fluorescence signals for Y- and X-chromosome were equal and bright, without dim, in nucleus position. Morphology of MGG-staining was kept well.

3) The conventional XY Vysis FISH with microwave pretreatment on immunocytochemical (ICC) stained erythroblasts from cord blood

Microwave pretreatment of the slides was used before FISH under the same experimental conditions also for ICC staining slides. The average efficiency of X- and Y-signals on erythroblasts enriched from cord blood was 83% (SDev 0.14, Var 0.02) (5 cases). Under the same experimental conditions without microwave pretreatment the average efficiency for ICC-slides was 65% (Table 3; Figure 3).

2.1.3. Choice of probes for FISH analysis of erythroblasts

Aim

To compare Vysis and Qbiogene probes.

Results

For the comparison of Vysis and Qbiogene probes we have performed hybridisation with Vysis two-color cocktail probes: X centromeric a-satellite (spectrum green)/Y centromeric a-satellite (spectrum orange) and Qbiogene two-color cocktail probes: X centromeric a-satellite (fluorescein)/Y centromeric a-satellite (rhodamine). The average efficiencies were 75% and 79% for the Vysis and Qbiogene probes, respectively. Further we tried to use for our research the Qbiogene probes, because they are more suitable in hybridization and less expensive; in addition, the company offers the wide range of different fluorochromes and their combinations (Figure 5).

2.1.4. The labeling of Y chromosome by two different probes in the one-step two-color XYY-FISH

The mathematical model

The mathematical calculation of probability of labeling the fetal cells by XY-and XYY-probes combinations gives the values 0.810 and 0.891, respectively (in the mathematical model we took equal FISH efficiency of 90% for X, Y (α-satellite) and Y (III-satellite) probes in the mathematical model, as described by the manufacturer). Thus, the labeling of Y chromosome by two different probes (α- and III-satellite) of the same color in the

one-step two-color XYY-FISH has the advantage in detecting fetal Y-chromosome. Theoretically the increase in the number of Y chromosome probes of the same color leads to increase in the probability of detection of Y-chromosome.

Aim

To test a possible improvement in the detection of Y-chromosome signal by applying one–step two–color XYY-FISH-hybridization in which the Y-chromosome was labeled by two different probes (α- and III-satellite) of the same color.

Results

Firstly, we have checked hybridization efficiency for Y α- and Y III-satellite Qbiogene probes. The average efficiencies on cord blood from male were 100% and 90% for Y α- and Y III-satellite Qbiogene probes, respectively.

At the next step, we have compared the efficiency of Qbiogene probes: Y α- (fluorescein) and Y III-satellite (rhodamine) in one-step YY-FISH. The hybridization efficiency for both probes was the same and was equal to 98% (Figure 7).

Then, we used the combination of probes: two-color cocktail: X α-satellite (fluorescein) and Y α-satellite (rhodamine) in combination with Y III-satellite (rhodamine) in one–step two–color XYY-FISH-hybridization. The average efficiency on cord blood from male was 100% (Figure 4 B, C, D, E).

2.1.5. Development of a combined immunocytochemistry (ICC) for glycophorin A (GPA) (fetal cell identification) and FISH (as genetic diagnosis) protocol on cord blood samples

Introduction

The glycophorin-A (GPA) antigen has been shown to be expressed over a broad spectrum of erythropoietic development, and had previously been used for the highly successful enrichment of erythroblasts from maternal blood samples (Troeger et al., 1999a; Al Mufti et al., 2004; Choolani et al., 2003). A reason for choosing the GPA antigen and not other possible fetal erythroblast specific antigens, such as gamma and epsilon globin molecules, is that these latter proteins are frequently not expressed with equal abundance in all fetal erythroblasts.

The immunocytochemistry and FISH are fundamentally incompatible techniques. The harsh fixatives, high temperatures, formamide and stringent washes commonly used in FISH results in the protein damage. Conversely, the gentler fixatives used during immunocytochemistry hinders probe penetration into the nucleus and give poor FISH efficiency (Oosterwijk et al., 1998c). In order to combine the two techniques, every step needed to be analyzed separately.

Aim

To combine the immunocytochemistry and FISH techniques and to check the limitation of sensitivity and the specificity of the combined technique.

Results

The hybridisation efficiency of 65% was achieved when applying conventional XY Vysis FISH protocol on ICC slides. Unfortunately, cell morphology was poor and most of the nuclei and cytoplasm were damaged. In contrast, FISH efficiency when using MGG staining was 98%. The hybridisation efficiency on ICC slides can be improved up to 83% by using microwave pretreatment (Table 3; Figure3) (See also results of microwave pretreatment)

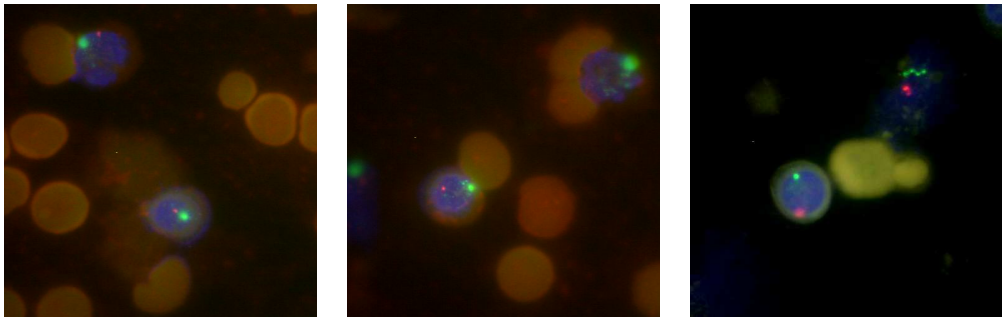


Figure 3. XY Vysis FISH on ICC (GPA)-stained erythroblasts from cord blood.

Case	Time of hybridisation, hours	Probe	Efficiency, %
1	9	Vysis XY	97
2	5	Vysis XY	78
3	5	Vysis XY	76
4	5	Vysis XY	98
5	5	Vysis XY	67
		Average	***83***

Table 3. XY Vysis FISH with microwave pretreatment on ICC (GPA)-stained erythroblasts from cord blood.

2.1.6. Conclusion of FISH optimization on cord blood samples

In order to optimize the FISH protocol for hybridization of erythroblast we have tried different pretreatments of slides: pepsin, HCl, proteinase K and microwave activation. The best results were demonstrated by the microwave activation. This pretreatment allows achieving good FISH efficiency with keeping of good cell morphology. All other kind of applied pretreatments resulted in destroying the cell morphology which is significant disadvantage.

Next critical aspect, which plays a critical role in the success of the FISH, is the choice of hybridisation probes. According to our results, the best FISH efficiency was demonstrated by the combination of two probes of the same color (rhodamine) for Y chromosome (α- and III-satellite) in combination with X (α-satellite) chromosome probe (fluorescein) in one–step two–colour XYY-FISH-hybridisation (Figure 4).

The combination of the immunocytochemistry and FISH resulted in poor cell morphology and reduction of FISH efficiency. The applying of microwave pretreatment to ICC slides increases the FISH efficiency, but destroys the cell morphology to a great extent.

Further, microwave and pepsin pretreatments as the best of investigated ones were used with XYY-FISH for erythroblasts enriched from maternal blood (See also results of “FISH analysis on maternal blood samples”).

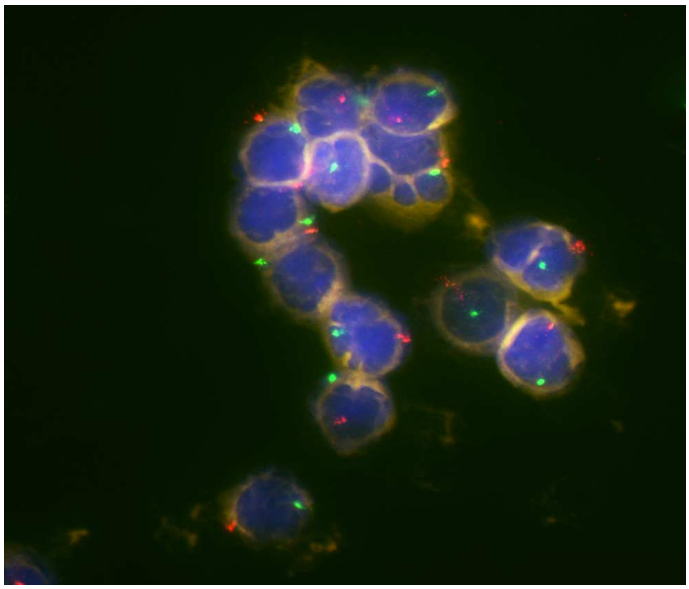


Figure 6. Vysis XY FISH on cells from cord blood after pepsin preatreatment.

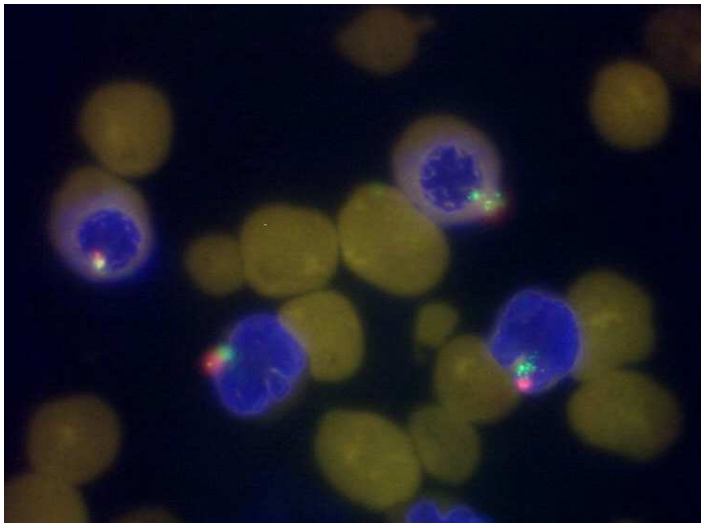


Figure 7. YcenY III Qbiogene FISH on erythroblasts enriched from cord blood

2.2. FISH analysis on maternal blood samples

2.2.1. XY Vysis FISH

Aim

To perform conventional XY Vysis FISH analysis on memorized erythroblasts enriched from maternal blood and to analyze the structure of erythroblast population after FISH.

Design

The FISH signal was individually analyzed for every memorized erythroblast. The detected signals were noted as XX, XO, or OO (no signal), XY and XYY signals. Also the cases of loss of the cells were noted.

Results

1) Pregnancy cases with male fetus

Seven maternal blood samples from pregnant women with male fetus were hybridized with Vysis probes, without microwave pretreatment according to the optimized protocols. Average number of erythroblasts was 16, ranging from 11 to 20 per slide. We found specific XX signal on average in 50.9% of the erythroblasts; XO signal on average in 16.7% of the erythroblasts; OO signal on average in 21.0% of the erythroblasts; XY signal on average in 5.3% of the erythroblasts; XXY signal on average in 2.6% of the erythroblasts and loss of cells was determined on average in 3.5% of all memorized erythroblasts enriched from maternal blood samples (Table 5).

2) Pregnancy cases with female fetus

Seven maternal blood samples from pregnant women with female fetus were hybridized with Vysis probes following the optimized protocol. Average number of erythroblasts was 8, ranging from 2 to 12 per slide. XX signal was found on average in 41.1% of the erythroblasts, XO signal was found on average in 5.4% of the erythroblasts, OO signal was found on average in 26.8% of the erythroblasts after applying Vysis FISH protocol and loss of cells was found on average in 26.6% of all memorized erythroblasts enriched from maternal blood samples (Table 6).

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number of memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells						Another cell (not memorized) with Y signal
					XX	XO	OO	XY	XXY	Loss of cell	
3054-4	Vysis	No	6	15	9	4	1	1			2
3074-1	Vysis	No	6	11	7	1	1	1	1		3
3065	Vysis	No	6	13	9	2	1	1			2
3064	Vysis	No	6	14	12	1		1			2
3096	Vysis	No	6	11	5	5	1				2
3054-1	Vysis	No	6	14	5	2	1	2	2	2	1
3107-2	Vysis	No	6	15	4	2	7			2	6
3087-2	Vysis	No	6	20	6	2	12				2
	Average			15.7	8.4	2.2	4.2	1.6	1.2	2.8	3.0
	Sum			113	58	19	24	6	3	4	20
	In %			100%	50.9%	16.7%	21.0%	5.3%	2.6%	3.5%	

Table 5. XY-Vysis-FISH analysis of morphologically assessed erythroblasts enriched from maternal blood with male fetus

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number of memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells			
					XX	XO	OO	loss of cell
3073	Vysis	No	16	2	1			1
3663-4	Vysis	No	17	7	4		3	
3055	Vysis	No	17	12	1		5	6
3023	Vysis	No	9	6	2	1	2	1
3114-1	Vysis	No	9	8	3		3	2
3105	Vysis	No	9	10	6	1	0	3
3104	Vysis	No	9	11	6	1	2	2
	Average			8.0	3.3	1.0	2.5	2.5
	Sum			56	23	3	15	15
	in %			100.0%	41.1%	5.4%	26.8%	26.6%

Table 6. XY-Vysis-FISH analysis of morphologically assessed erythroblasts enriched from maternal blood with female fetus

2.2.2. XYY Qbiogene FISH

Aim

To improve the FISH efficiency on erythroblasts enriched from maternal blood by applying XYY Qbiogene FISH protocol following the optimization on cord blood and to analyze the structure of memorized erythroblast population after FISH.

Results

1) Pregnancy cases with male fetus

Eight maternal blood samples from male pregnant women were hybridized with the mixture of Qbiogene two-color cocktail: X centromeric α-satellite (fluorescein)/Y centromeric α-satellite (rhodamine) and Qbiogene Y III-satellite (rhodamine) probes, following the protocols optimized on cord blood. Average number of erythroblasts was 14, ranging from 6 to 19. We found specific XX signal on average in 61.5% of the erythroblasts, XO signal on average in 8.3% of the erythroblasts, OO signal on average in 14.7% of the erythroblasts, XY signal on average in 6.4% of the erythroblasts, XXY signal on average in 0.9% of the erythroblasts and loss of cells was determined on average in 8.1% cells from population of memorized erythroblasts (Table 7).

2) Pregnancy cases with female fetus

Eight maternal blood samples from female pregnant women were hybridized with the mixture of Qbiogene two-color cocktail: X centromeric α-satellite (fluorescein)/Y centromeric α-satellite (rhodamine) and Qbiogene Y III-satellite (rhodamine) probes, following the protocols optimized on cord blood. Average number of erythroblasts was 14, ranging from 5 to 35.

XX signal was found on average in 47.8% of the erythroblasts, XO signal was found on average in 8.8% of the erythroblasts, OO signal was found on average in 38.9% of the erythroblasts and the loss of cells was found on average in 4.4% of the erythroblasts after Qbiogene FISH (Table 8).

Case ID	Type of probes	Pretre atment	Time of hybridisati on, hour	Number of memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells						Another cell (not memorize d) with Y signal
					XX	XO	OO	XY	XXY	Loss of cell	
3084	QbioXY+Y	No	15	16	11		2			3	2
3085	QbioXY+Y	No	15	17	13	1	3				3
3011	QbioXY+Y	No	15	11	5	3	3				2
3063	QbioXY+Y	No	15	11	5	3		3			3
3089	QbioXY+Y	No	16	16	8	1	1	1	1	4	2
3091	QbioXY+Y	No	16	6	6						5
3083	QbioXY+Y	No	16	13	8	1	2	1		1	2
3077	QbioXY+Y	No	16	19	11		5	2		1	3
	Average			13.6	8.4	1.8	2.7	1.8	1.0	2.3	2.8
	Sum			109	67	9	16	7	1	9	22
	In %			100.0%	61.5%	8.3%	14.7%	6.4%	0.9%	8.1%	

Table 7. XYY-Qbiogene-FISH analysis of morphologically assessed erythroblasts enriched from maternal blood with male fetus

Case ID	Type of probes	Pretreat ment	Time of hybridisati on, hour	Number of memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells			
					XX	XO	OO	Loss of cell
3117	Qbiogene XY-Y	No	9	8	7	1		
3110	Qbiogene XY-Y	No	9	5	5			
3111	Qbiogene XY-Y	No	9	9	3	1	5	
3109	Qbiogene XY-Y	No	9	12	8	1	2	1
3062	Qbiogene XY-Y	No	9	35	10	2	21	2
3055-2	Qbiogene XY-Y	No	9	20	6	1	11	2
3663-2	Qbiogene XY-Y	No	9	18	11	3	4	
3023-2	Qbiogene XY-Y	No	9	6	4	1	1	
	Average			14.1	6.8	1.4	7.3	1.7
	Sum			113	54	10	44	5
	In %			100.0%	47.8%	8.8%	38.9%	4.4%

Table 8. XYY-Qbiogene-FISH analysis of morphologically assessed erythroblasts enriched from maternal blood with female fetus

2.2.3. XY Vysis FISH on microwave pretreated slides

Aim

To improve FISH efficiency on erythroblasts enriched from maternal blood by applying microwave pretreatment of slides following the results of optimization on cord blood in conventional XY Vysis FISH procedure and to analyze the structure of memorized erythroblast population after FISH.

Results

Six maternal blood samples from male pregnant women were hybridized with XY Vysis probes, with microwave pretreatment following the optimized protocols. Average number of erythroblasts was 16, ranging from 10 to 24 per slide. We found specific XX signal on average in 50.4% of the erythroblasts, XO signal on average in 14.4% of the erythroblasts, OO signal on average in 17.1% of the erythroblasts, XY signal on average in 3.6% of the erythroblasts, XXY signal on average in 5.4% of the erythroblasts and loss of cells was determined on average in 9.0% of memorized erythroblasts (Table 9; Figure 8A).

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells						Another cell (not memorized) with Y signal
					XX	XO	OO	XY	XXY	loss of cell	
3054-3	Vysis	microwave	6	17	10	3		1	1	2	0
3074-2	Vysis	microwave	6	17	8	4	0	3	2		1
3054-2	Vysis	microwave	9	14	6	4	2	0	2		3
3094	Vysis	microwave	6	10	5	2	1			2	1
3092	Vysis	microwave	6	10	10						2
3107-1	Vysis	microwave	6	24	8	3	6		1	6	5
3087-1	Vysis	microwave	6	19	9		10				3
	Average			15.6	8.4	2.4	3.6	1.5	1.3	2.7	2.9
	Sum			111	56	16	19	4	6	10	15
	In %				50.4%	14.4%	17.1%	3.6%	5.4%	9.0%	

Table 9. XY-Vysis-FISH analysis (microwave pretreatment) of morphologically assessed erythroblasts enriched from maternal blood with male fetus

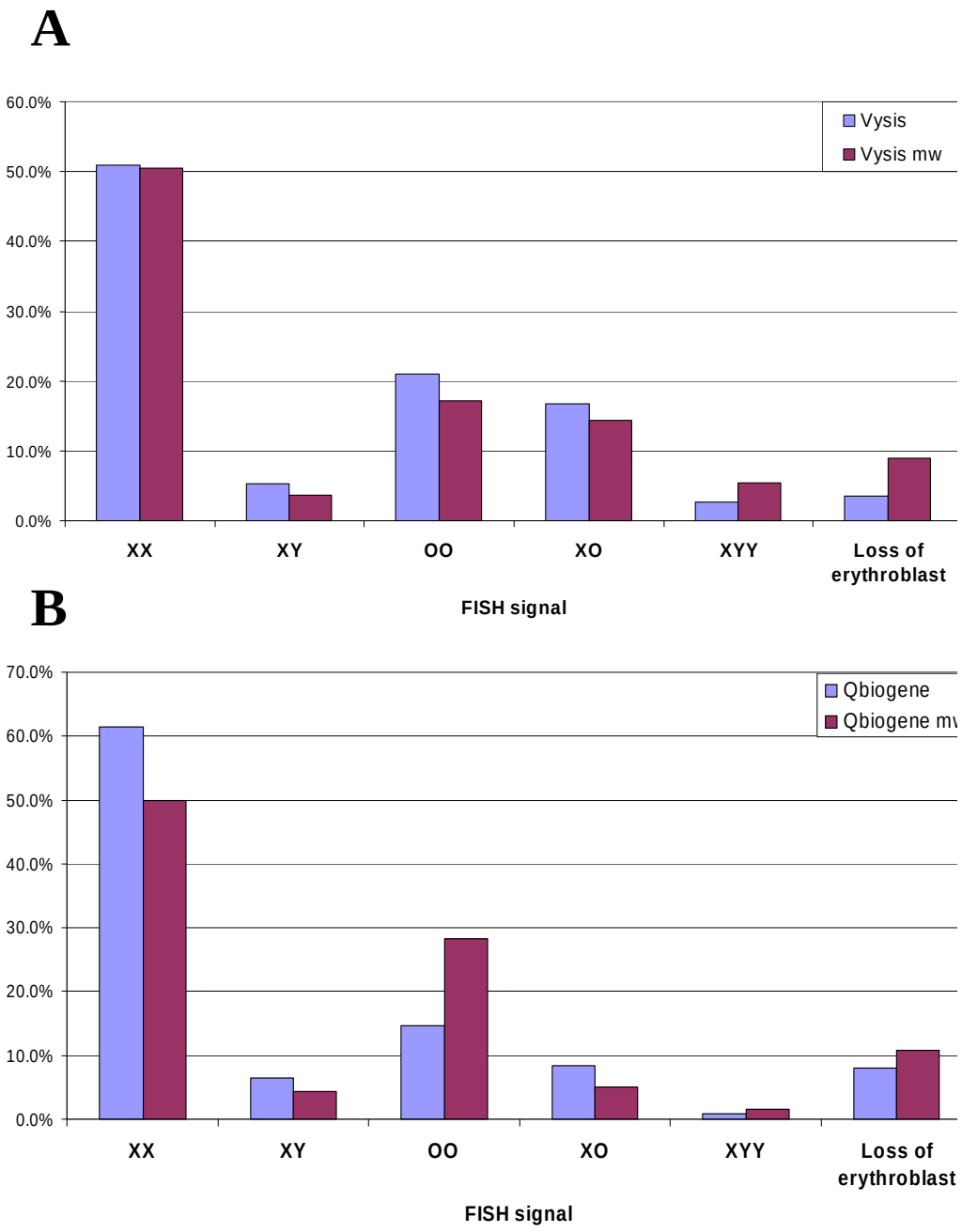


Figure 8. The comparison of microwave pretreatment on morphologically assessed erythroblasts enriched from maternal blood samples from male pregnancy cases

A. Fish analysis with Vysis XY-probes

B. Fish analysis with Qbiogene XYY-probes

2.2.4. XYY Qbiogene FISH on microwave pretreated slides

Aim

To improve the FISH efficiency on erythroblasts enriched from maternal blood with male fetus using simultaneously the two protocols which show best results on cord blood: microwave treatment and applying of Qbiogene probes with double labeling of Y chromosome.

Results

Seven maternal blood samples from male pregnant women were hybridized with mixture of Qbiogene two-color cocktail: X centromeric α-satellite (fluoresceine)/Y centromeric α-satellite (rhodamine) and Qbiogene Y III-satellite (rhodamine) probes, with pretreatment by microwave, following the optimized protocols. Average number of erythroblasts was 20, ranging from 14 to 24. We found specific XX signal on average in 50.0% of the erythroblasts, XO signal on average in 5.0% of the erythroblasts, OO signal on average in 28.3% of the erythroblasts, XY signal on average in 4.4% of the erythroblasts, XXY signal on average in 1.5% of the erythroblasts and loss of cells was determined on average 10.8% of all memorized erythroblasts (Table 10; Figure 8B).

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number of memorized erythroblasts after MGG	Result of XYY Qbiogene FISH on memorized cells						Another cell (not memorized) with Y signal
					XX	XO	OO	XY	XXY	Loss of cell	
3087-3	QbioXY+Y	microwave	19	21	3	1	11	1		5	3
3077-2	QbioXY+Y	microwave	19	24	5		13			6	5
3083-2	QbioXY+Y	microwave	18	14	11	1	1	1			4
3107	QbioXY+Y	microwave	18	22	8	1	6	4	1	2	3
3089-2	QbioXY+Y	microwave	19	19	13	3	3				4
3084-2	QbioXY+Y	microwave	19	18	14		3			1	3
3091-2	QbioXY+Y	microwave	18	20	15	1	2		1	1	5
	Average			19.71	9.86	1.40	5.57	2.00	1.00	3.00	3.86
	Sum			138	69	7	39	6	2	15	
	In %			100.0%	50.0%	5.0%	28.3%	4.4%	1.5%	10.8%	

Table 10. XYY-Qbiogene-FISH analysis (microwave pretreatment) of morphologically assessed erythroblasts enriched from maternal blood with male fetus

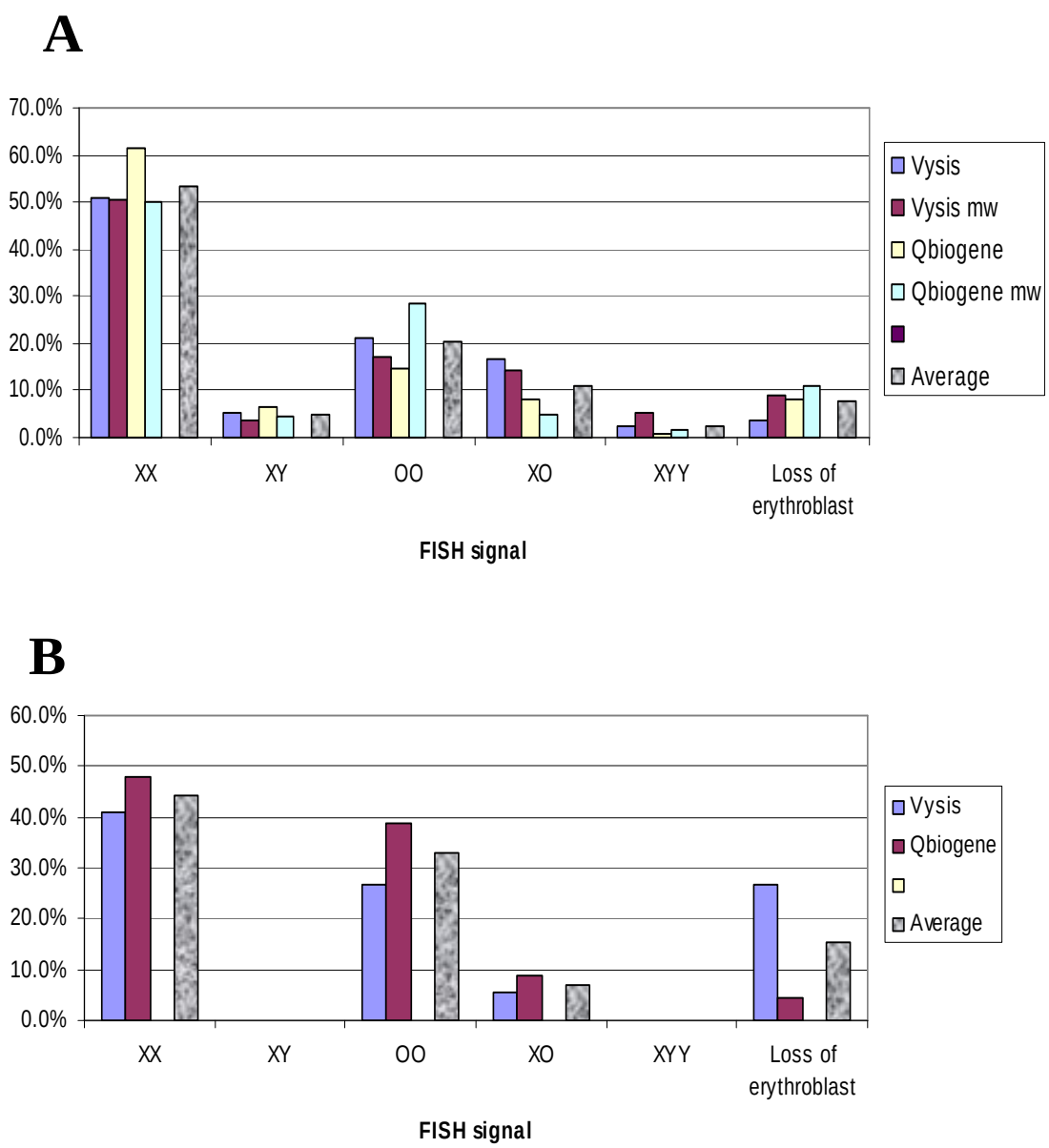


Figure 9. Comparison of the different NRBC-populations, enriched from maternal blood samples analysed with different XY-FISH protocols (%)

A. Different FISH protocols on erythroblasts from pregnancy with male fetus.

B. Different FISH protocols on erythroblasts from pregnancy with female fetus.

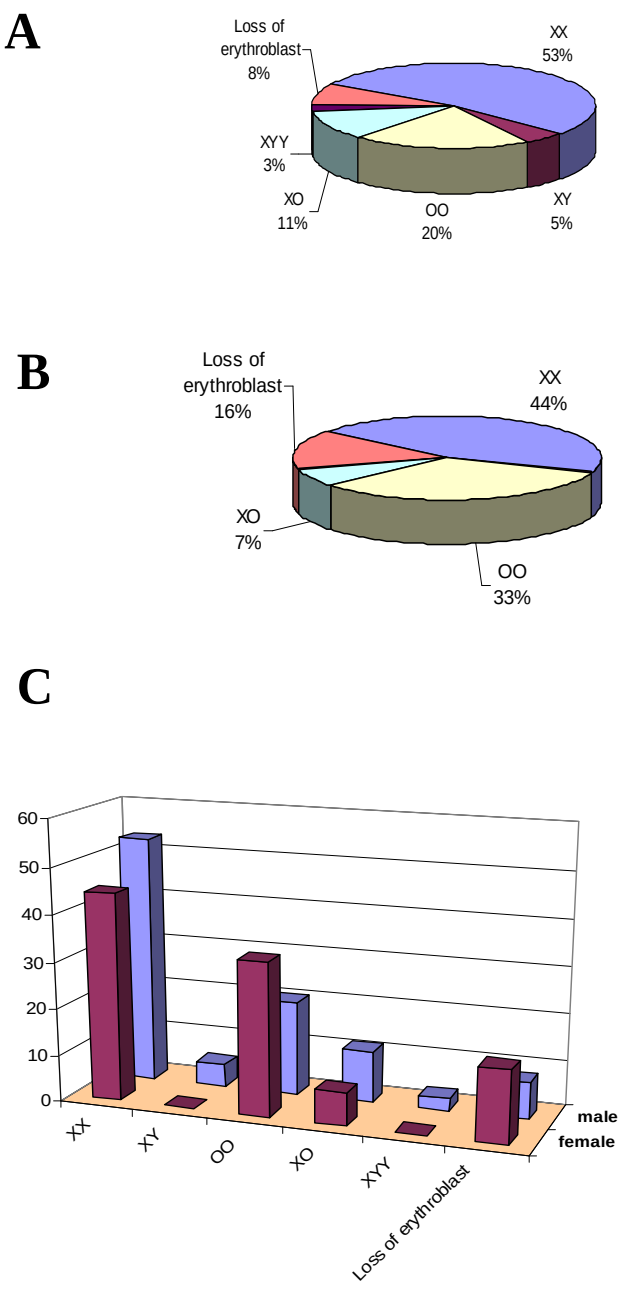


Figure 10.

A. The structure of erythroblast population from maternal blood with male fetus.

B. The structure of erythroblast population from maternal blood with female fetus.

C. Comparison of of FISH result on erythroblasts enriched from pregnancies with male and female.

2.2.5. The separate analysis of erythroblasts population structure for pregnancies with male fetus with and without Y signal detected

Aim

To compare the structure of memorized erythroblasts population in the case of pregnancy with male fetus when the Y signal was detected and those with no Y signal detected.

Results

We have separately analyzed the cases of pregnancy (for which XY Vysis, XY Vysis mw, XYY Qbiogene, XYY Qbiogene mw protocols were applied) with male fetus (Table 11), in which the Y signal was determined, and the results of hybridisation, in which no Y signal was detected. The analysis of population structure of erythroblasts with Y signal shows XX signal on average in 53% and XY signals on average in 10% of erythroblasts. The XO was determined on average in 13% of erythroblasts. XXY signal was determined on average in 5% of erythroblasts. The absence of any signals was observed on average in 13% of erythroblasts and 7% of the cells were lost. The analysis of structure of populations with no Y signal has shown that the XX signal was determined on average in 56% of erythroblasts. The XO signal was determined on average in 12% of erythroblasts. The XXY signal was found on average in 1% of erythroblasts and the absence of signals was determined on average in 24% of erythroblasts. 7% of the cells were lost during the hybridization procedure.

From the comparison of the structure of populations with Y signals and without those we can see considerable increase in percentage of erythroblasts without any signals (OO) in those cases when Y signal was not detected up to 24% compared with 13% in cases when the Y signal was detected. We can see also the decrease of XXY signals down to 1% in cases in which Y signal was not detected compare with 5% in cases in which Y signal was detected. All other data are approximately similar (Table 11; Figure 11).

FISH	Vysis, %		Vysis, mw, %		Qbiogene XcYc-Y III, %		Qbiogene XcYc-Y III, mw, %		Average, %	
Signal detected	No Y signal	Y signal	No Y signal	Y signal	No Y signal	Y signal	No Y signal	Y signal	No Y signal	Y signal
XX	34	64	55	50	73	54	61	43	56	53
XO	23	15	12	23	8	10	5	5	12	13
OO	39	6	20	5	14	12	24	29	24	13
XY	0	9	0	8	0	13	0	10	0	10
XXY	0	5	4	11	0	2	1	2	1	5
Lost cells	4	3	9	4	5	9	9	11	7	7

Table 11. Comparison of the structure of erythroblast population in pregnancies with male fetus with and without detected Y signal (mw refers to microwave treatment)

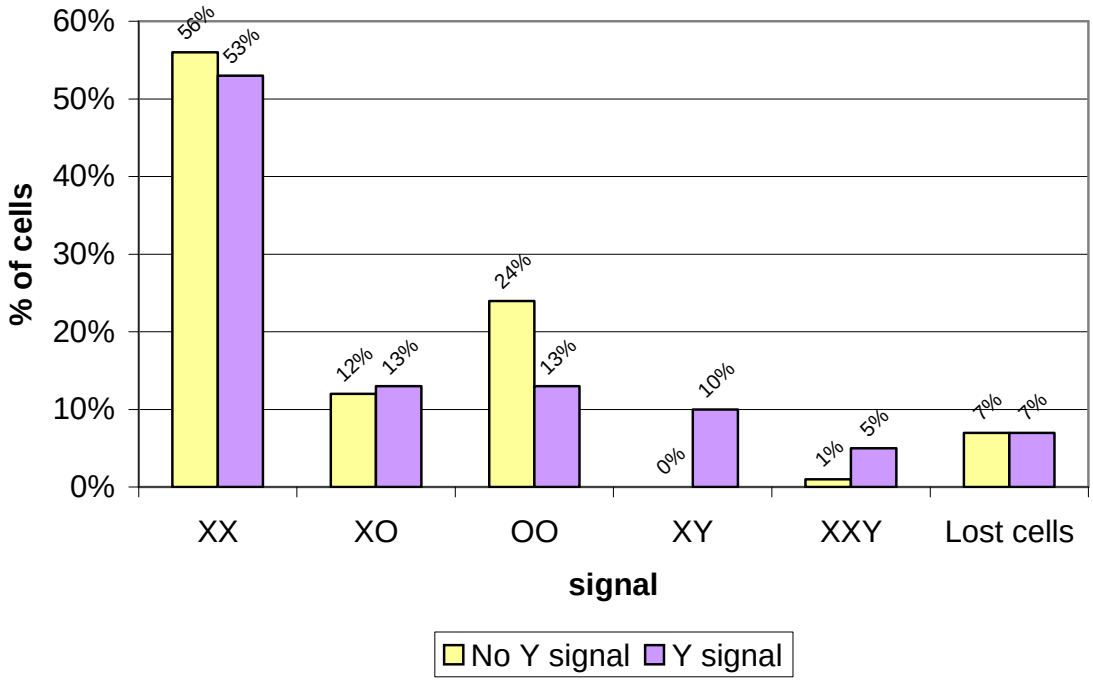


Figure 11. Comparison of the structure of erythroblast population in pregnancies with male fetus in which the Y signal was detected and was not.

2.2.6. XYY Qbiogene FISH on pepsin pretreated slides

Aim

To improve the FISH efficiency on erythroblasts enriched from maternal blood with male fetus using together the two protocols which show best results on cord blood: pepsin treatment and applying of Qbiogene probes with double labeling of Y chromosome.

Results

Eight maternal blood samples from male pregnant women were hybridized with mixture of Qbiogene two-color cocktail: X centromeric α-satellite (fluorescein)/Y centromeric α-satellite (rhodamine) and Qbiogene Y III-satellite (rhodamine) probes, with pepsin pretreatment, following the optimized protocols. Average number of erythroblasts was 23, ranging from 8 to 69. We found specific XX signal on average in 31.0% of the erythroblasts, XO signal on average in 4.4% of the erythroblasts, OO signal on average in 45.1% of the erythroblasts, XY signal on average in 1.0% of the erythroblasts, XXY signal on average in 0% of the erythroblasts with on average 18.5% of all memorized erythroblasts being lost (Table 12).

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number of memorized erythroblasts after MGG identification	Result of XY Vysis FISH on memorized cells					
					XX	XO	OO	XY	XXY	Loss of cell
3089-3	QbioXY+Y	pepsin	15	17	12	1	3	1		
3083-3	QbioXY+Y	pepsin	15	17	13	1				3
3087-4	QbioXY+Y	pepsin	16	27	4	3	19			1
3002-2	QbioXY+Y	pepsin	16	12	7		4			1
2988	QbioXY+Y	pepsin	16	8	1		1			1
3084-3	QbioXY+Y	pepsin	16	19	8	1				10
3085-2	QbioXY+Y	pepsin	16	15	12	1	1			1
2970	QbioXY+Y	pepsin	16	69	0	1	55			17
	Average			23	7.13	1.33	13.83	1		4.86
	Sum			184	57	8	83	1		34
	In %			100.0%	31.0%	4.4%	45.1%	1.0%		18.5%

Table 12. XYY-Qbiogene-FISH analysis (pepsin pretreatment) of morphologically assessed erythroblasts enriched from maternal blood with male fetus

2.2.7. FISH analysis with Qbiogene Xc and 18c chromosome probes

Introduction

Every chromosome in interphase nuclei has special localization, and if, for example, Y chromosome was located in the center of the nuclei, the access to it is difficult. On the other hand, if Y chromosome is at the periphery of nuclei, it can be lost during procedure of hybridization. We performed the hybridization with chromosome 18 centromeric probe Qbiogene. Such choice was made, because chromosome 18 has the same size like Y chromosome and was established earlier to be located at nuclear periphery (Cremer and Cremer, 2001).

Aim

To check the hypothesis that efficiency of hybridization depends on chromosome choice for the probe.

Results

Six maternal blood samples from male pregnant women were hybridized with the mixture of Qbiogene X centromeric (rhodamine) and Qbiogene 18 chromosome (fluorescein) probes following the optimized protocols. Average number of erythroblasts was 16, ranging from 15 to 18. We found specific XX1818 signal on average in 52% of the erythroblasts, XX18O signal - on average in 5% of the erythroblasts, XXOO signal - on average in 1% of the erythroblasts, XO1818 signal - on average in 9% of the erythroblasts, XO18O signal - on average in 8% of the erythroblasts, XOOO signal - on average in 1% of erythroblasts and OO1818 signal on average in 1% of erythroblasts. OOOO signal was determined on average in 13% with on average 10% of erythroblasts from the population of fixed erythroblasts being lost (Table 13; Figure 12).

The separate analysis of FISH efficiency for X and 18 chromosomes has shown, that XX signal was found in 58% erythroblasts, XO signal was found in 18% of erythroblasts and OO signal was found in 14% of erythroblasts. We found 1818 FISH signals in 62% of erythroblasts, O18 signal - in 13% of erythroblasts and OO signal was found in 15% of erythroblasts (Table 14).

Case ID	Type of probes	Pretreatment	Time of hybridisation, hour	Number of memorized erythroblasts after MGG identification	Result of XX1818 Qbiogene FISH on memorized cells								
					XX1818	XX18O	XXOO	XO1818	XO18O	XOOO	OO1818	OOOO	Loss of cell
3016	QbioXY+Y	No	19	18	3	2		1	3	1		8	
3020	QbioXY+Y	No	19	18	11	1	1		3			2	
3014	QbioXY+Y	No	22	15	8			2	2			1	2
3064	QbioXY+Y	No	22	16	9						1		6
3063	QbioXY+Y	No	22	15	6	2		3				2	2
3065	QbioXY+Y	No	20	16	13			3					
	Average			16.3	8.3	1.7	1	2.3	2.7	1	1	3.3	33.3
	Sum			98	50	5	1	9	8	1	1	13	10
	In %			100.0%	52.0%	5.1%	1.0%	9.2%	8.2%	1.0%	1.0%	13.3%	10.2%

Table 13. XX1818-Qbiogene-FISH analysis of morphologically assessed erythroblasts enriched from maternal blood with male fetus

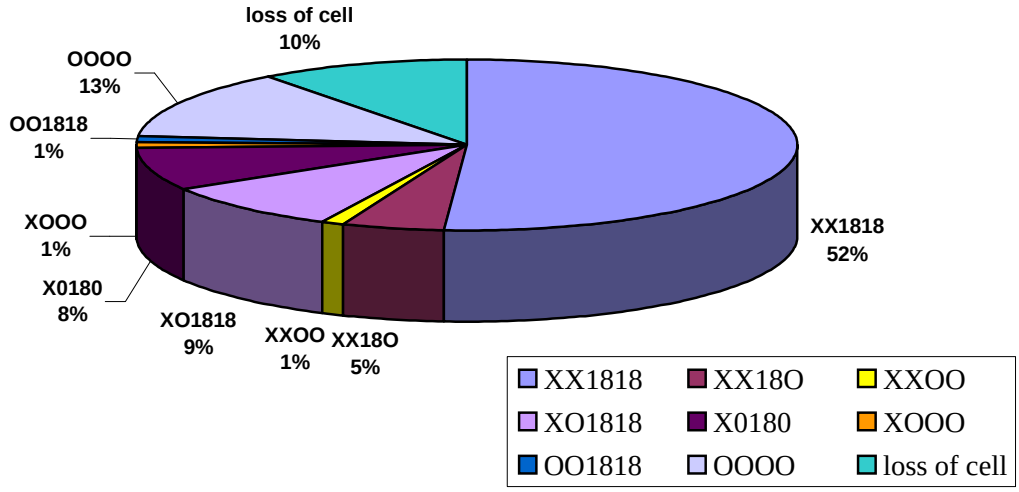


Figure 12. Result of Qbiogene FISH with X centromeric and 18 chromosome probes.

Analyzed FISH signal	FISH signal (in nuclei)	FISH signal, %	Sum for analyzed FISH signal, %
XX	XX1818	*52*	*58*
	XX18O	*5*	
	XXOO	*1*	
XO	XO1818	*9*	*18*
	XO18O	*8*	
	XOOO	*1*	
OO	OO1818	*1*	*14*
	OOOO	*13*	
Loss of cells		*10*	*10*
1818	XX1818	*52*	*62*
	XO1818	*9*	
	OO1818	*1*	
O18	XX18O	*5*	*13*
	XO18O	*8*	
	OO18O	*0*	
OO	XOOO	*1*	*15*
	XXOO	*1*	
	OOOO	*13*	

Table 14. Separate analysis of FISH with X centromeric and 18 chromosome probes (X- and 18 chromosome signals were analyzed separately).

2.2.8. Conclusion: FISH on maternal blood samples

On average 13 (range 2-24) erythroblasts were recovered from each maternal blood sample analyzed following the enrichment with our standard anti-CD71 MACS protocol. Following positive identification, the position of each erythroblast on the glass slide was electronically marked using an automated location finder. Each cell was then individually examined by conventional FISH using conventional Vysis centromeric probes for the X and Y chromosomes. This analysis has indicated that in samples obtained from pregnancies with male fetuses, on average only 5.3% of the erythroblasts were XY positive, while 50.9% were clearly of maternal origin as they contained XX signals.

The remaining erythroblasts (>40%) had aberrant or no FISH signals (Table 15; Figure 13; Figure 14). Although our hybridisation efficiencies on normal lymphoid cells were greater than 95%, we consider that these results may have been due to not completely optimal FISH procedure. Hence, we tested a variety of conditions, including the use of microwave and pepsin pretreatment, as well as a different source of FISH probes (Qbiogene) in which two different Y probes (α- and classical-satellite III) were used in combination with an α-satellite probe for the X chromosome. These examinations did not yield any significant improvements (Table 15). The best result was received by using XYY Qbiogene FISH protocol, when on average only 6.4% of the erythroblasts being identified as male, while more than 30% of the cells had aberrant or no FISH signals. We also have observed that in those cases when no Y positive erythroblasts were detected, the number of erythroblasts with no FISH signals increased. These data suggest that some erythroblasts can not be hybridized and as a result we can not detect Y chromosome signal in pregnancies with the male fetus.

The inability to examine all erythroblasts by FISH did not appear to be restricted to pregnancies with male fetuses, as in pregnancies with female fetuses we also have observed that on average only 44.5% of the erythroblasts were XX positive, while the remaining erythroblasts had aberrant or no FISH signals (Table 15, Figure 13). In addition, this phenomenon was not restricted to the sex chromosomes, as we have observed similar results in an analysis of 6 cases for chromosome 18 in FISH hybridisation with X- and 18-chromosome probes, since only 52% of the erythroblasts were correctly hybridized. It means that the efficiency of hybridization is independent on chromosome choice for the probe (Babochkina et al., 2005a).

FISH probe	Fetal sex	Nr. of cases	Total nr. of erythroblast	XX %	XY %	OO %	XO %	XYY %	Loss of erythroblasts %
XY Vysis	male	7	113	*50.9*	*5.3*	*21.0*	*16.7*	*2.6*	*3.5*
XY Vysis mw	male	6	111	*50.4*	*3.6*	*17.1*	*14.4*	*5.4*	*9.0*
XYY Qbiogene	male	8	109	*61.5*	*6.4*	*14.7*	*8.3*	*0.9*	*8.1*
XYY Qbiogene mw	male	7	138	*50.0*	*4.4*	*28.3*	*5.0*	*1.5*	*10.8*
XYY Qbiogene pepsin	male	8	184	*31.0*	*1.0*	*45.1*	*4.4*	*0.0*	*18.5*
XY Vysis	female	7	56	*41.1*	*0.0*	*26.8*	*5.4*	*0.0*	*26.6*
XYY Qbiogene	female	8	113	*47.8*	*0.0*	*38.9*	*8.8*	*0.0*	*4.4*

Table 15. FISH analysis of morphologically assessed erythroblasts enriched from maternal blood samples (mw refers to microwave treatment).

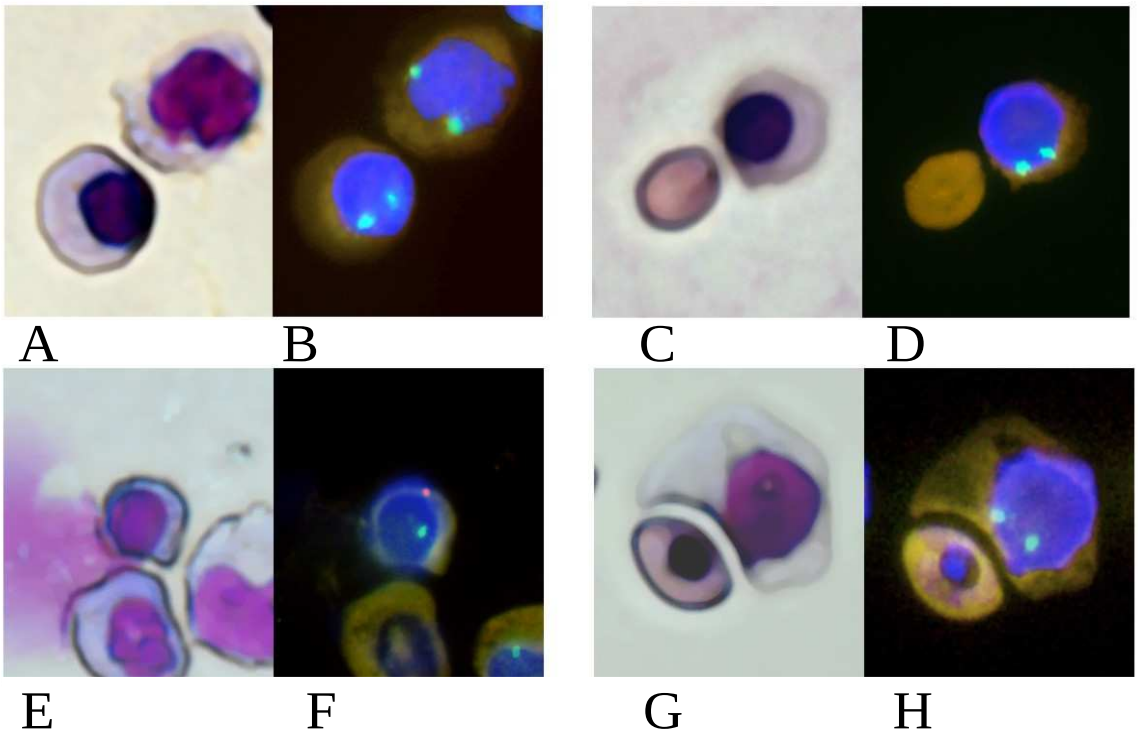


Figure 13. FISH analysis of erythroblasts enriched from maternal blood.

A, B. XX positive erythroblast, characterized by a large nuclear diameter, enriched from a pregnancy with a male fetus.
C, D. XX positive erythroblast, characterized by a large nuclear diameter, enriched from a pregnancy with a female fetus.
E, F. XY positive fetal erythroblast, characterized by an intermediate nuclear diameter, enriched from a pregnancy with a male fetus.
G, H. FISH negative erythroblast, characterized by very small nuclear diameter.

All images were taken at 630X magnification.

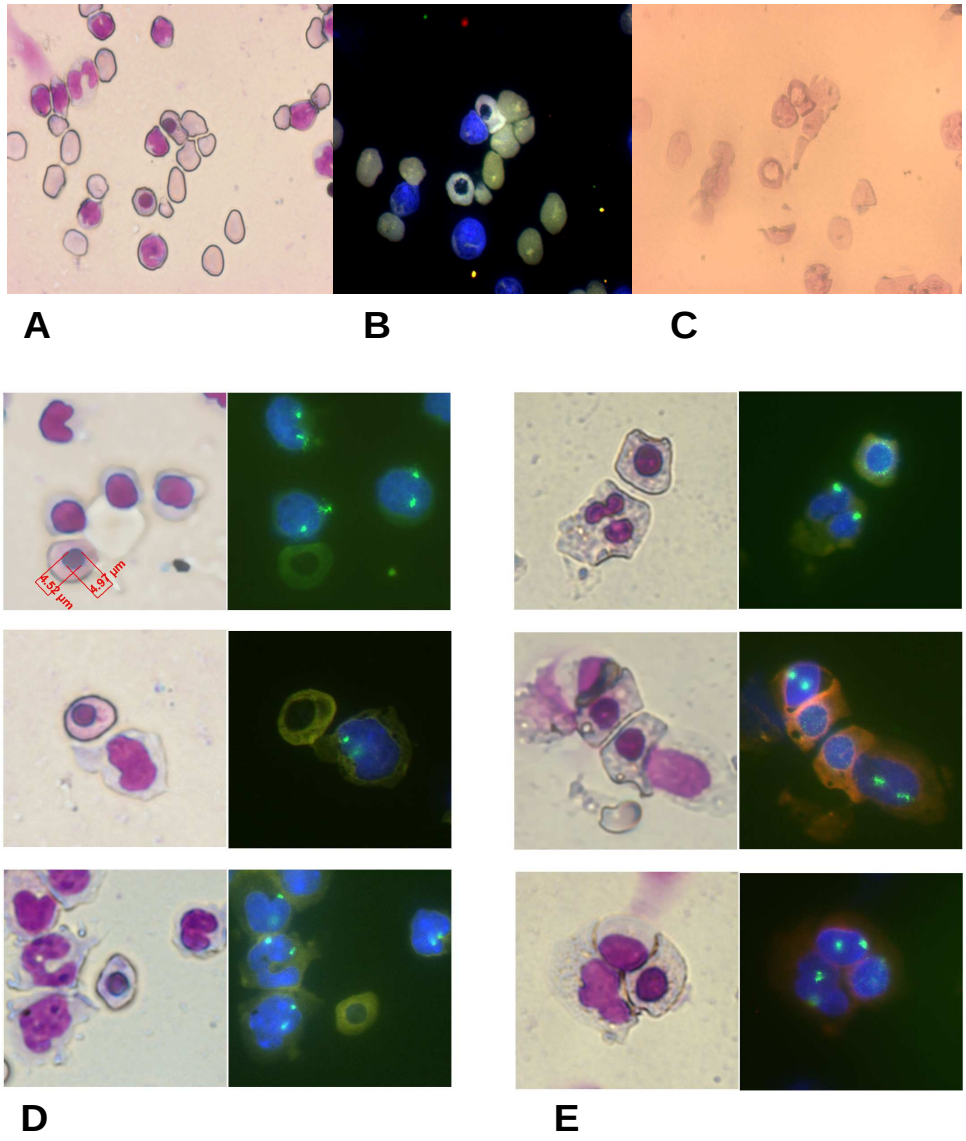


Figure 14. Loss of nucleus of erythroblasts during FISH procedure and OO FISH signal.

A. MGG stained cells
B. FISH (signals are bleached) on same cells
C. Gimsa staining of same cells after FISH
D. Examples of loss of nucleus
E. OO FISH signal

2.3. The comparative analysis of the erythroblasts at different oxygen concentrations

Hypothesis

We suggested that fetal erythroblasts undergo a fundamental change when they enter the maternal circulation, and that this change in phenotype involves an alteration in the morphology of the cells, particularly an alteration in nucleus structure.

Aim

To examine whether this change could be attributed to the different oxygen tensions in the fetal and maternal circulatory systems. To this aim we have cultured and analyzed post-partum cord blood samples under different oxygen concentrations mimicking those in fetal and maternal blood (3 and 20 %, respectively).

Design

Preincubated and incubated blood cells were thin smeared on glass slides. After drying, they were MGG stained. Erythroblasts were then morphologically identified and counted. The relative number of erythroblasts was calculated as the number of erythroblasts per 100 nucleated cells. Then the diameter of nucleus and its circumference for every erythroblast were measured.

Results

1) Effect of oxygen concentration on the number of erythroblasts

With the preincubation erythroblasts number designated as 100%, the average number of erythroblasts after incubation at 20% oxygen was 50% (range 33–57%). The average number of erythroblasts after incubation at 3% oxygen was 85% (range 58–100%). The number of erythroblasts decreased significantly after incubation at 20% oxygen ($P=0.027<0.05$; Wilcoxon sign-rank test), whereas the number of erythroblasts did not decrease significantly after incubation at 3% oxygen ($P=0.066<0.05$; Wilcoxon sign-rank test) (Table 16; Figure 15). The difference between the results at 3% and 20% is significant ($P=0.027<0.05$; Wilcoxon sign-rank test).

Case	Post-partum blood		After 3% O_2 (24h)		After 20% O_2 (24h)	
	Number of erythroblasts per 100 nucleated cells	%	Number of erythroblasts per 100 nucleated cells	%	Number of erythroblasts per 100 nucleated cells	%
1	8	100%	8	100%	4	50%
2	13	100%	13	100%	5	38%
3	9	100%	8	89%	4	44%
4	25	100%	23	92%	20	80%
5	7	100%	5	71%	4	57%
6	12	100%	7	58%	4	33%
average			11	85%	7	50%

Table 16. Effect of oxygen concentration on the number of erythroblasts.

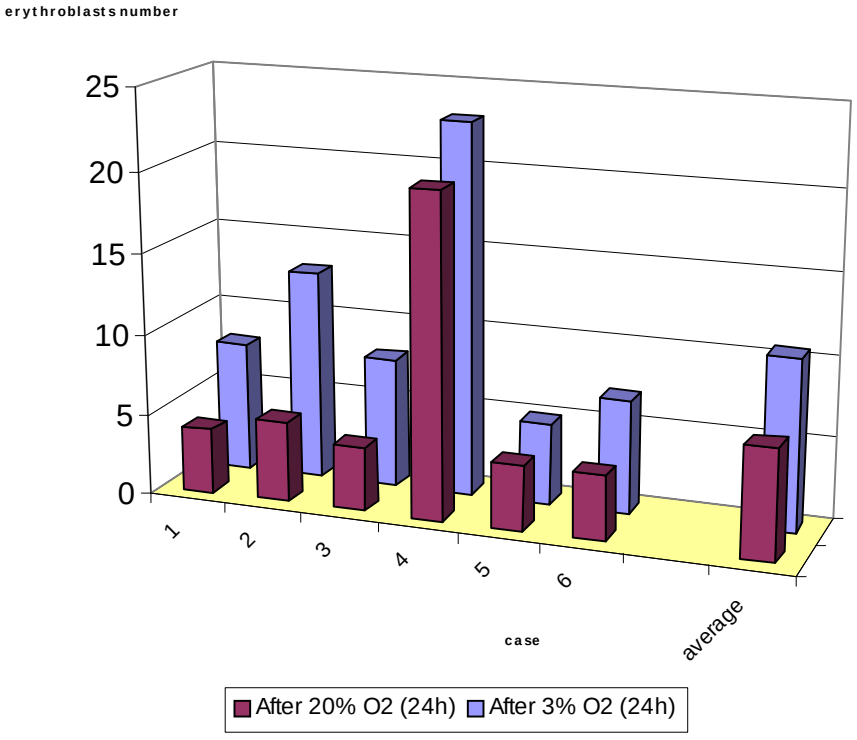


Figure 15. Comparison of erythroblasts (percentage) after incubation at 3% and 20% oxygen.

2) Effect of oxygen concentration on erythroblast nuclear size

With preincubation diameter of erythroblasts designated as 100%, the average diameter of erythroblasts after incubation at 20% oxygen was 88% (range 84-94%). The average diameter of erythroblasts after incubation at 3% oxygen was 99% (range 92-113%).

This experiment clearly showed that the erythroblast nuclei did indeed shrink significantly ($P=0.028<0.05$; Wilcoxon sign-rank test) at the higher oxygen concentration (20%) when compared to the lower oxygen concentration (3%) ($P=0.463>0.05$; Wilcoxon sign-rank test) (Table 17; Figure 16).

The difference between the results at 3% and 20% is also significant ($P=0.028<0.05$; Wilcoxon sign-rank test).

Conclusion

As the maternal circulation is higher in oxygen concentration than fetal circulation, the present results suggest that the high oxygen environment of maternal circulation induces morphometric changes in fetal erythroblasts transferred to the maternal circulation. This morphometric changes can be connected with apoptotic changes resulted in nuclei loss. This suggests that high oxygen concentration induces significant decrease in erythroblasts number. All this leads to clearance of erythroblasts from the maternal circulation.

This phenomenon may be important to maintain the pregnancy because persistence of fetal cells in maternal circulation would stimulate the maternal immune system (Babochkina et al., 2005a).

Case	Post-partum blood		After 3% O2 (24h)		After 20% O2 (24h)	
	Diameter, µm	in %	Diameter, µm	in %	Diameter, µm	in %
1	5.11	100%	5.76	113%	4.45	87%
2	5.46	100%	5.17	95%	4.60	84%
3	5.33	100%	4.89	92%	4.63	87%
4	5.37	100%	5.10	95%	4.63	86%
5	5.24	100%	4.96	95%	4.66	89%
6	5.26	100%	5.45	104%	4.93	94%
average			5.22	99%	4.65	88%

Table 17. Influence of oxygen concentration on erythroblast nuclear size (average diameter, in µm).

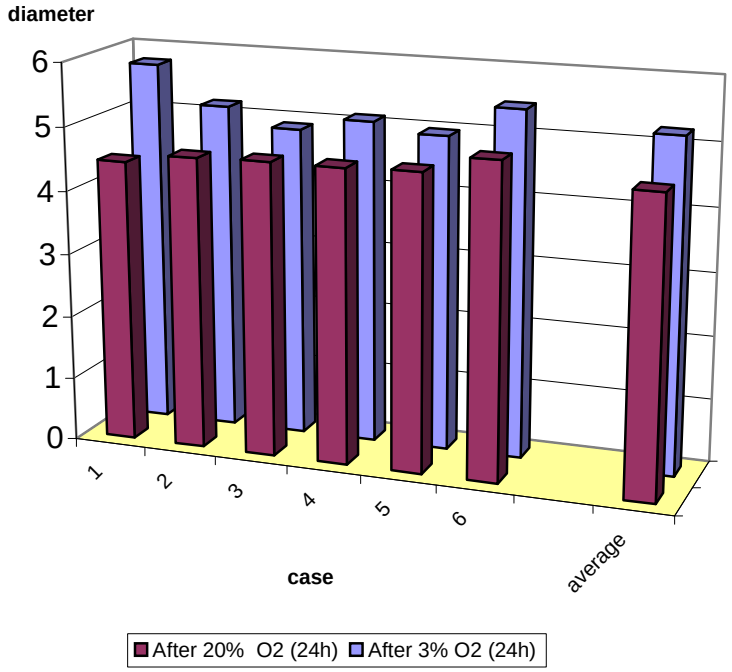


Figure 16. Comparison of erythroblast diameter (µm) after incubation at 3% and 20% oxygen.

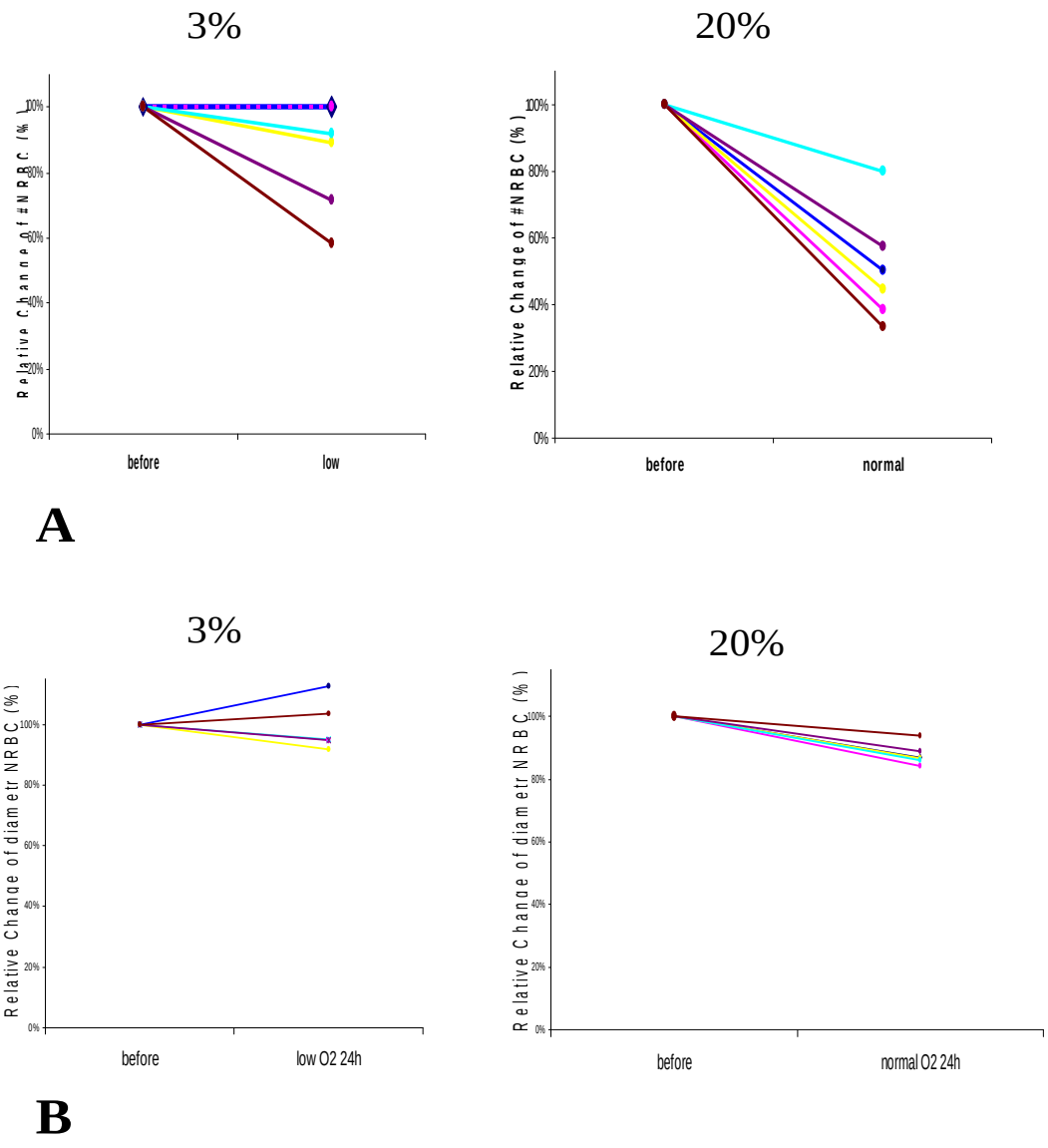


Figura 17. Effect of oxygen concentration on NRBCs number (A) and diameter of NRBCs nuclei

A. The relative number of NRBCs decreased significantly after incubation under 20% oxygen, whereas the number of NRBCs did not decrease significantly after incubation under 3% oxygen (Wilcoxon sign-rank test, $P<0.05$).

B. The diameter of NRBCs decreased significantly after incubation under 20% oxygen, whereas the diameter of NRBCs did not decrease significantly after incubation under 3% oxygen (Wilcoxon sign-rank test, $P<0.05$).

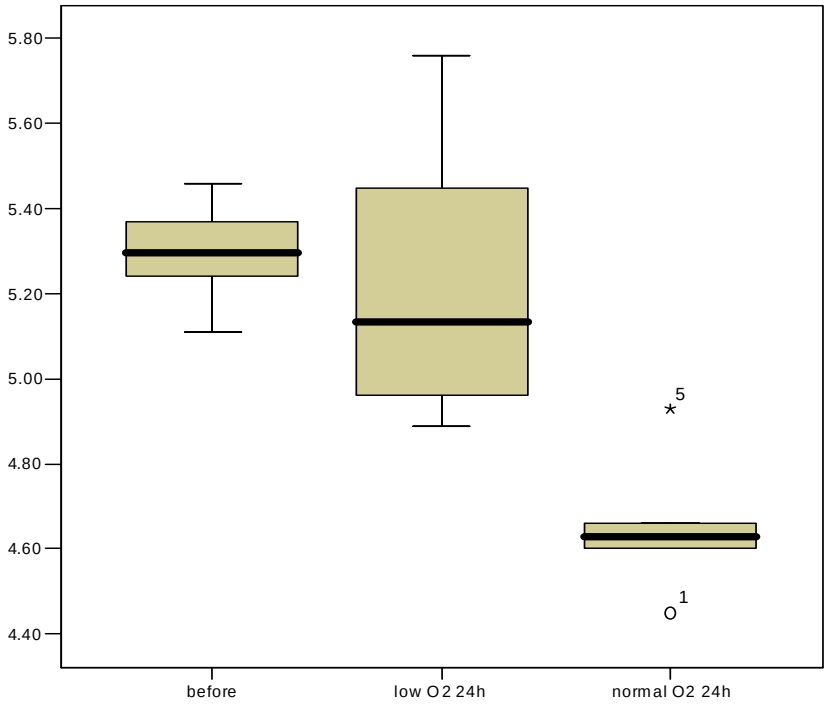


Figure 18. Influence of oxygen concentration on erythroblast nuclear size. Cord blood erythroblasts were briefly cultured at 3% and 20% oxygen concentrations, after which morphometric characteristics of their nuclei were examined. The data are presented by box plots, indicating the median value (line in the box), the 75th and 25th percentiles (limits of the box), and the 10 th and 90 th percentiles (upper and lower horizontal bars). Outliers are indicated by empty circles.

2.4. FISH after incubation at 3% and 20% oxygen concentrations

Aim

To determine whether the 24 hours exposure to high oxygen concentration hampered the analysis of erythroblasts by FISH.

Results

The FISH (vysis XY probe) signals at high oxygen concentration were found in 59% (range 25-77%) of erythroblasts, when compared to those at low oxygen concentration (average 88%; range 69-94%) and untreated control samples (average 91%; range 86-95%) (Table 18).

Conclusion

The 24 hours exposure at high oxygen concentration has hampered reliable analysis of these erythroblasts by FISH (Babochkina et al., 2005a).

Case	Post-partum blood	3% oxygen culture	20% oxygen culture
1	90%	69%	25%
2	95%	93%	77%
3	95%	96%	68%
4	86%	85%	68%
5	88%	94%	65%
6	91%	92%	53%
average	*91%*	*88%*	*59%*
min	*86%*	*69%*	*25%*
max	*95%*	*94%*	*77%*

Table 18. The comparison of FISH efficiency (%) before and after incubation at 3% and 20% oxygen.

2.5. Measurements of erythroblasts

Aim and design

In order to determine why only one fraction of the circulatory erythroblasts was readily analyzable by FISH we have examined their morphological characteristics more closely.

Then we have compared the morphometric characteristics of FISH positive and FISH negative erythroblasts from maternal blood with those of erythroblasts from cord blood.

Results

The measuring of 286 erythroblasts from maternal blood was performed. The average diameter of nuclei of erythroblasts with XX- and XX1818chromosome signals (in case of X 18 chromosome FISH) were 6.5 μm, average circumference of nuclei was 23.17 μm and average nucleus/cytoplasm ratio was 0.47 (Table 19).

The measuring of 10 erythroblasts from maternal blood with XY signal was performed. The average diameter of nuclei of erythroblasts with XY signals was 6.22 μm, average circumference of nuclei was 22.95 μm and average nucleus/cytoplasm ratio was 0.51.

The measuring of 21 erythroblasts from maternal blood with XO signal was performed. The average diameter of nuclei of erythroblasts with XO signals was 6.22 μm, average circumference of nuclei was 22.67 μm and average nucleus/cytoplasm ratio was 0.43.

127 cells without any hybridization signals (OO) show average diameter of nuclei 4.92 μm, average circumference 18.10 μm and average nucleus/cytoplasm ratio was 0.28 (Table 19).

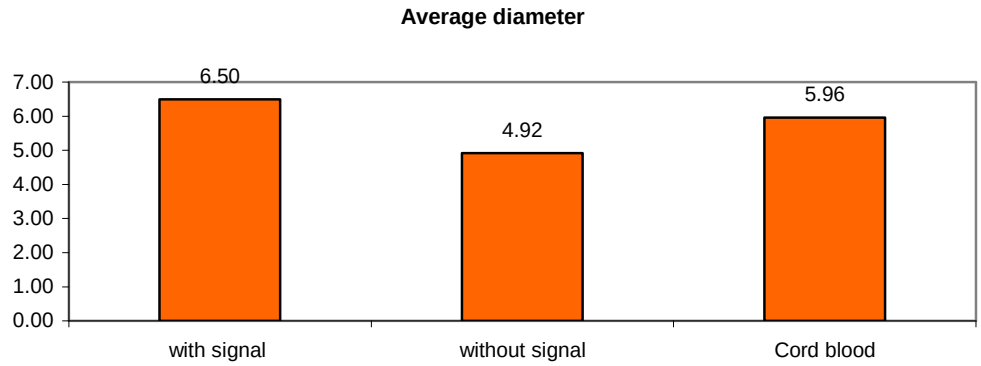
The statistic analysis using the T-test shows that differences in the diameter average size of nuclei with XX FISH signals and those without FISH signals (OO) are statistically significant ($p=1.044exp-14<0.05$). Also, T-test shows that the differences in the average circumference of nuclei with XX FISH signals and those without FISH signals are statistically significant ($p2.31exp-17=<0.05$). The statistic analysis using the ANOVA test shows that differences in the diameter, circumference and nucleus/cytoplasm (N/C) ratio of erythroblasts from maternal blood: with XX signals, those without FISH signal and erythroblasts from cord blood are statistically significant ($P<0.0001$ for all cases).

Conclusion

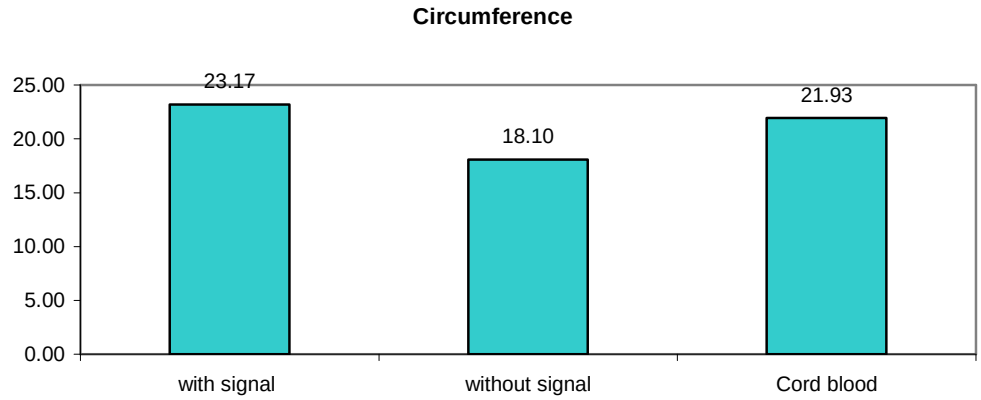
The morphometric analysis has indicated that those erythroblasts in maternal blood which had been hybridized efficiently had a significantly larger nuclear diameter and circumference and N/C ratio than those which had been impervious to the FISH procedure (Babochkina et al., 2005a). The erythroblasts which had been impervious to the FISH procedure are significantly different also from erythroblasts in cord blood

Type of measurements		***Maternal blood***				***Cord blood***	
		with XX signal (number of measured cells)		***without signal (number of measured cells)***			
Average diameter	female pregnancy	average mode (n=84)	6.31 6.60	average mode (n=60)	4.53 4.20		
	male pregnancy 18 X chromosome hybridization	average mode (n=155) average mode (n=47)	6.62 6.70 6.59 6.24	average mode (n=59) average mode (n=8)	4.92 5.20 5.32 5.50	average mode (n=106)	5.96 5.80
	average	***average mode (n=286)***	***6.50 6.51***	***average mode (n=127)***	***4.92 4.97***	***average mode (n=106)***	***5.96 5.80***
Circumference	female pregnancy	average mode	22.39 21.70	average mode	16.45 16.70		
	male pregnancy 18 X chromosome hybridisation	average mode average mode	24.22 22.60 22.90 24.71	average mode average mode	18.61 16.60 19.24 19.00	average mode	21.93 21.60
	average	***average mode (n=286)***	***23.17 23.00***	***average mode (n=127)***	***18.10 17.43***	***average mode (n=106)***	***21.93 21.60***
N/C ratio	female pregnancy	average mode	0.49 0.50	average mode	0.26 0.30		
	male pregnancy 18 X chromosome hybridisation	average mode average mode	0.48 0.50 0.45 0.50	average mode average mode	0.26 0.20 0.31 0.30	average mode	0.32 0.30
	average	***average mode (n= 286)***	***0.47 0.50***	***average mode (n= 127)***	***0.28 0.27***	***average mode (n= 106)***	***0.32 0.30***

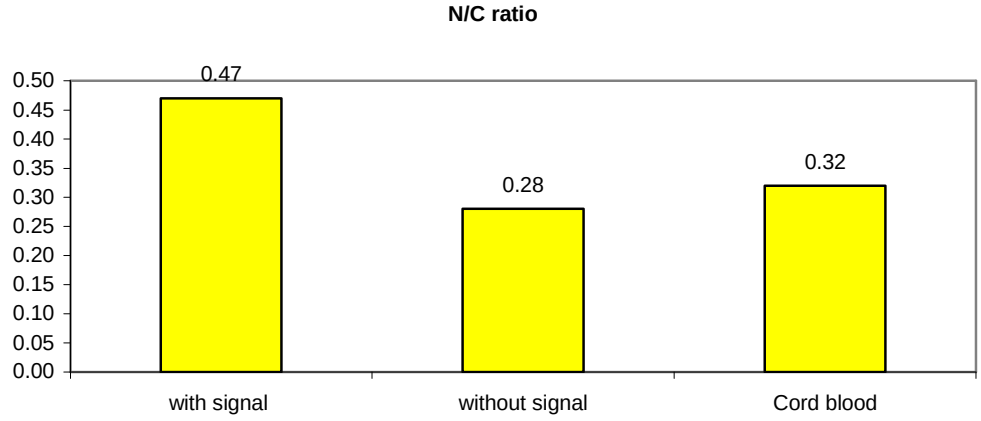
Table 19. Measurements of diameter, circumference and nucleus/cytoplasm (N/C) ratio of erythroblasts in maternal and cord blood.



A



B



C

Figure 20.
A. Comparison of erythroblasts diameter measurement in maternal blood (cells with and without signal) and in cord blood.
B. Comparison of erythroblasts circumference measurement in maternal blood (cells with and without signal) and in cord blood.
C. Comparison of erythroblasts N/C measurement in maternal blood (cells with and without signal) and in cord blood.

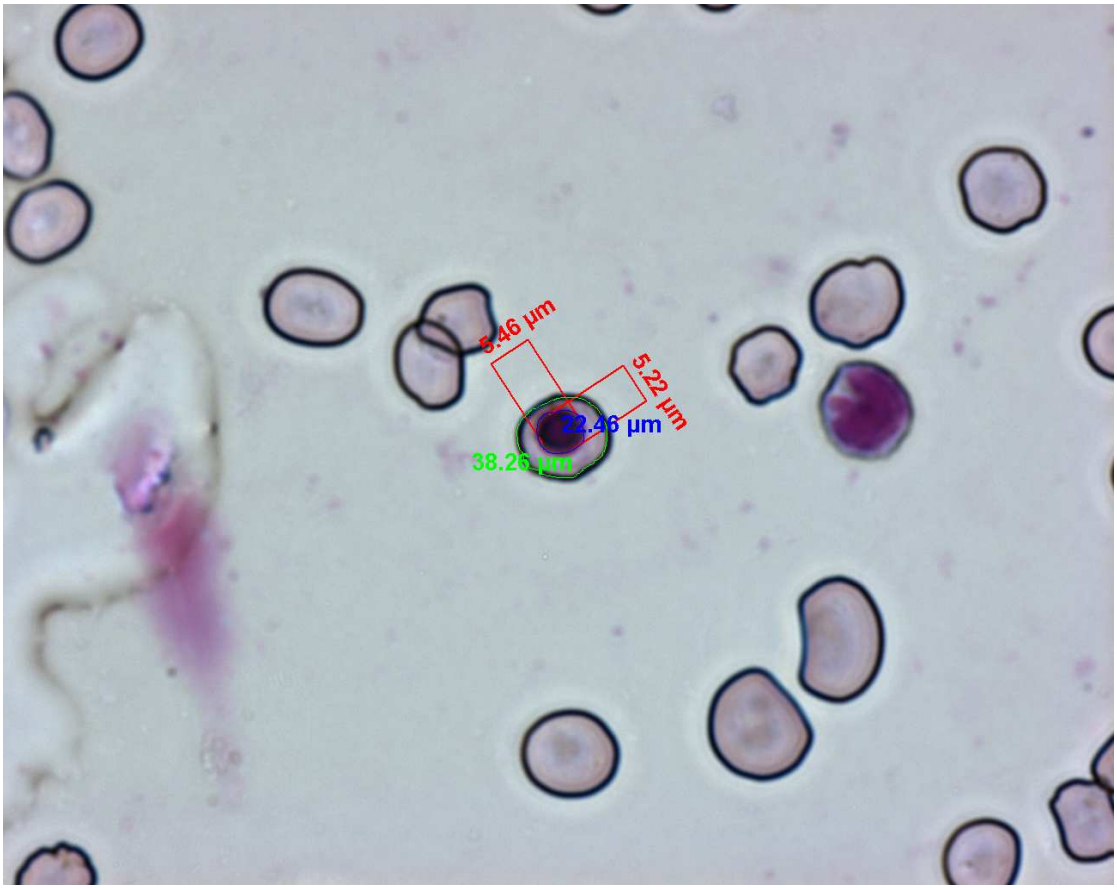


Figure 21. Measurement of erythroblasts
Red pointers. Measurement of diameter.
Blue pointer. Measurement of circumference of nucleus.
Green pointer. Measurement of circumference of cell.

2.6. Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) analysis of erythroblasts

2.6.1. TUNEL and FISH analysis of erythroblast nuclei in cord blood

Aim

Since the differentiation of erythroblasts into enucleated erythrocytes involves fragmentation of the nuclear DNA, which can be detected by the TUNEL assay, we next examined the presence of fragmented DNA (TUNEL positive signal in the nucleus) in erythroblasts from cord blood in correlation with result of FISH analysis of these cells.

Design

Lymphocyte fraction was isolated by Ficoll, MGG–stained and 2 slides from each case were used for FISH (Vysis XY probe) and 2 slides for TUNEL.

Results

Our analysis of erythroblasts in cord blood indicated that although a high proportion of these erythroblasts were TUNEL positive (57.3%), almost all erythroblasts could be reliably analyzed by FISH (83.2%) irrespective of being TUNEL positive or negative. Of interest is that our analysis of these post-partum cord blood erythroblasts has indicated that these cells had relatively large nuclear diameter and circumference (average = 6.0 μm and 21.93 μm), which is larger than the group of erythroblasts in maternal blood which could not be analyzed by FISH (average diameter = 4.7 μm and average circumference = 17.5 μm) (Table 20).

Case	Gender	TUNEL positive cells (%)	FISH positive cells (%)
1	male	64.0	94.0
2	male	65.0	84.0
3	male	61.5	100.0
4	male	60.5	66.0
5	male	71.0	83.0
6	male	56.0	60.0
Average male		*63.0*	*81.2*

Case	Gender	TUNEL positive cells (%)	FISH positive cells (%)
8	female	43.0	86.5
9	female	49.0	86.0
10	female	53.0	87.5
11	female	44.0	83.0
12	female	63.5	85.5
Average female		*50.5*	*85.7*
Total average		**57.3**	**83.2**

Table 20. TUNEL and FISH analysis of erythroblasts in cord blood.

2.6.2. TUNEL analysis of erythroblast nuclei in maternal blood

Aim

To examine the presence of fragmented DNA (TUNEL positive signal in the nucleus) in erythroblasts enriched from maternal blood and to analyze these results in correlation with nuclear size of these cells.

Results

69 erythroblasts enriched from 10 maternal blood samples were recorded and measured (Axiovision 3.1, Carl Zeiss, Zürich, Switzerland), and then the TUNEL assay was applied. This analysis has demonstrated that approximately 69% of erythroblasts with nuclei having the diameter larger than 5.6 μm were negative for the TUNEL assay, whereas almost 92% of those with the diameter smaller than this value contained fragmented DNA (Table 21; Figure 22; Figure 23).

This latter phenomenon supports the notion that erythroblasts with a small nuclei containing fragmented DNA are of fetal origin, as it has previously been shown that these cells become TUNEL positive upon entering the maternal circulation.

Conclusion

These results suggest that the presence of a dense nucleus rather than a nucleus containing fragmented DNA hinders the effective analysis of erythroblasts by FISH (Babochkina et al., 2005a).

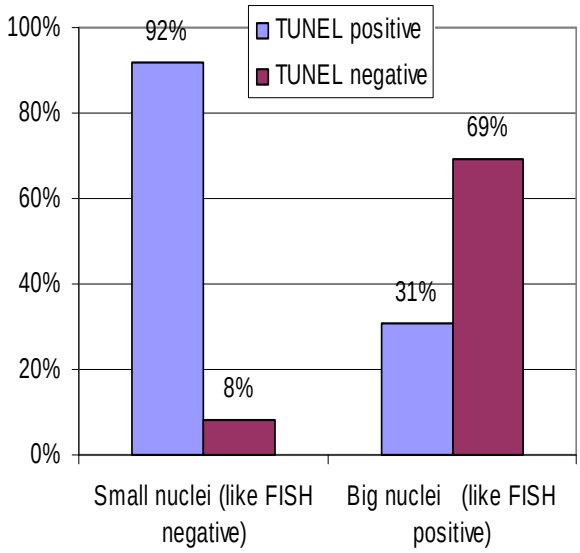


Figure 22. TUNEL analysis and diameter of erythroblast nuclei.

Parameter	Small nuclei (like FISH negative)	Big nuclei (like FISH positive)
Average diameter	under or equal 5.6	above 5.6
Number of cells	24	45
TUNEL positive	***92%***	***31%***
TUNEL negative	***8%***	***69%***

Table 21. TUNEL analysis and diameter of erythrocytes.

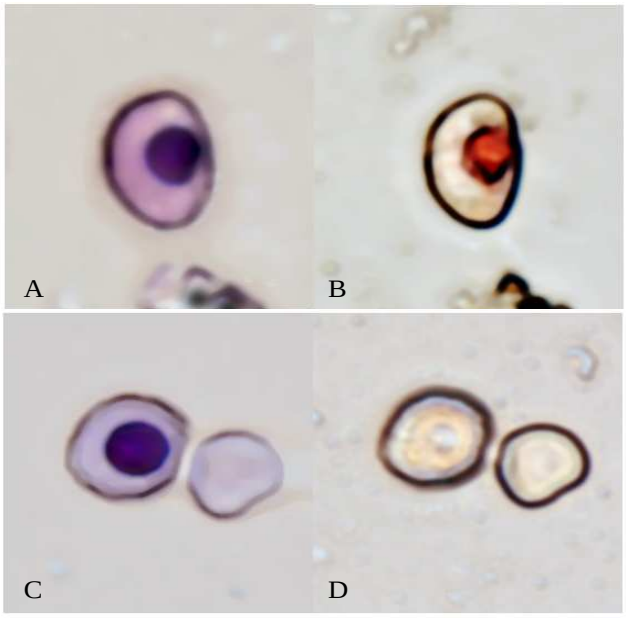


Figure 23. Examination of erythroblast nuclear size and presence of fragmented nuclear DNA in maternal blood.
A, B**.** Erythroblast with a small dense nucleus which is TUNEL positive.
C, D. Erythroblast with a large nucleus which is TUNEL negative. All image were taken at 630X magnification.

2.7. Detection of fetal cells in whole blood

(Detection of fetal cells in maternal blood using a combination of two different Y chromosome-specific and a single X chromosome-specific FISH probes)

The mathematical model

The mathematical calculation of probability of labeling the fetal cells by XY-, YY-and XYY-probes combinations gives the values 0.81, 0.81 and 0.891, respectively (we took equal FISH efficiency of 90% for X, Y (α-satellite) and Y (III-satellite) probes in the mathematical model, as described by the manufacturer). Thus, the labeling of Y chromosome by two different probes (α- and III-satellite) of the same color in the one-step two-color XYY-FISH has the advantage in detecting fetal Y-chromosome over other methods of labeling mentioned above. Theoretically the increase in the number of Y chromosome probes of the same color leads to increase in the probability of Y-chromosome detection.

Aim

To test a possible improvement in the detection of Y-chromosome signal of fetal cells in maternal blood by applying one–step two–color XY-FISH-hybridization in which the Y-chromosome was labeled by two different probes (α- and III-satellite) of the same color and to compare the results of XYY-FISH with conventional two-color XY-FISH protocol.

Results

Our blinded manner analysis of the 19 maternal blood samples by the combination of two Y chromosome-specific FISH probes in combination with a FISH probe for the X chromosome (XYY-FISH) has indicated that male fetal cells could be detected with 75% specificity and 91% sensitivity (Tables 22; Table 23), indicating that male fetal cells could be detected in 10 out of the 11 pregnancies with male fetuses.

Our parallel analysis of 9 of these samples by conventional XY-FISH has indicated that male fetal cells could be detected with 50% specificity and 60% sensitivity (Tables 22; Table 23). On the other hand, when the same samples were analyzed by our new XYY-FISH approach male fetal cells could be detected with 100% specificity and 100% sensitivity (Table 23). Therefore, the use of two different Y chromosome-specific FISH

probes in combination with an X chromosome-specific FISH does appear to yield a better result than that obtained by conventional XY-FISH. Furthermore, it appeared that more male fetal cells were detected by the XYY-FISH approach, where 1 to 4 fetal cells were recorded per slide, (yielding an average concentration of between 8 to 32 fetal cells per ml of maternal blood), compared to 1 to 2 fetal cells recorded per slide using conventional XY-FISH (Table 22; Table 23). We also have observed that the signals for the Y chromosome were more readily identifiable using the XYY-FISH approach when compared to those detected by conventional XY-FISH (Figure 24; Figure 25).

Conclusion

In our present study we demonstrated the results which confirm our mathematical prediction. This XYY detection system has demonstrated the real improvement as compared to conventional one (Babochkina et al., 2005b).

Case	**Gestation**	**Number of male fetal cells detected by XYY-FISH (in 1 ml)**	**Number of male fetal cells detected by XY-FISH (in 1 ml)**	**Gender**
1	37+2	24	12	male
2	26+4	16	12	male
3	12+1	0	8 f.p.	female
4	12+4	0	0	female
5	12+0	0	8 f.p.	female
6	14+3	0	0	female
7	26+4	32	0	male
8	25+1	32	0	male
9	28+2	32	20	male
10	12+3	8 f.p.	n.t.	female
11	16+1	0	n.t.	male
12	35+0	0	n.t.	female
13	20+0	8	n.t.	male
14	20+2	16	n.t.	male
15	37+2	32	n.t.	male
16	14+5	8	n.t.	male
17	26+2	0	n.t.	female
18	25+3	24	n.t.	male
19	13+3	8 f.p.	n.t.	female

Table 22. Detection of male fetal cells by two different FISH strategies. (f.p. – false positive, n.t. – not tested).

QbioXY+Y

19 cases		gender		
		male	female	
FISH	male	10	2	12
	female	1	6	7
		11	8	19

false positive	25%
false negative	9%
sensitivity	**91%**
specificity	**75%**

Vysis XY

9 cases		gender		
		male	female	
FISH	male	3	2	5
	female	2	2	4
		5	4	9

false positive	40%
false negative	50%
sensitivity	**60%**
specificity	**50%**

QbioXY+Y

9 cases		gender		
		male	female	
FISH	male	5	0	5
	female	0	4	4
		5	4	9

false positive	0%
false negative	0%
sensitivity	**100%**
specificity	**100%**

Table 23. Sensitivity and specificity of XYY- and XY- FISH.

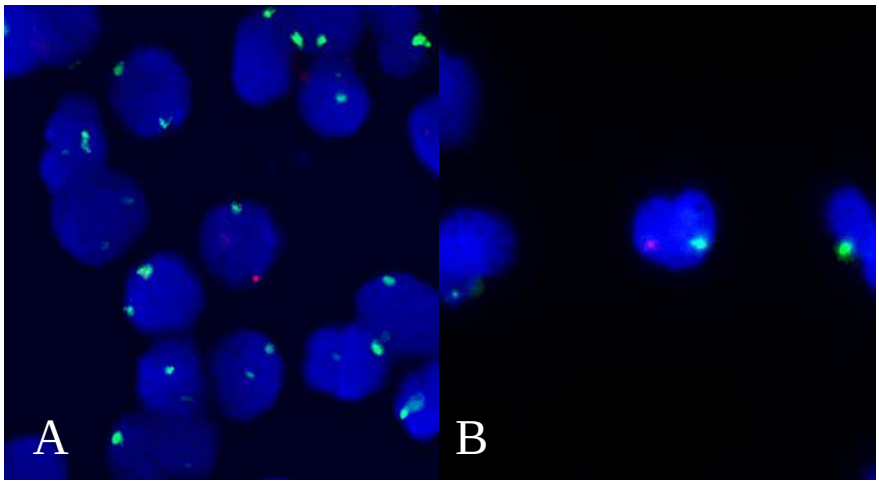


Figure 24 A, B. Detection of a male fetal cell in maternal blood by conventional XY-FISH.

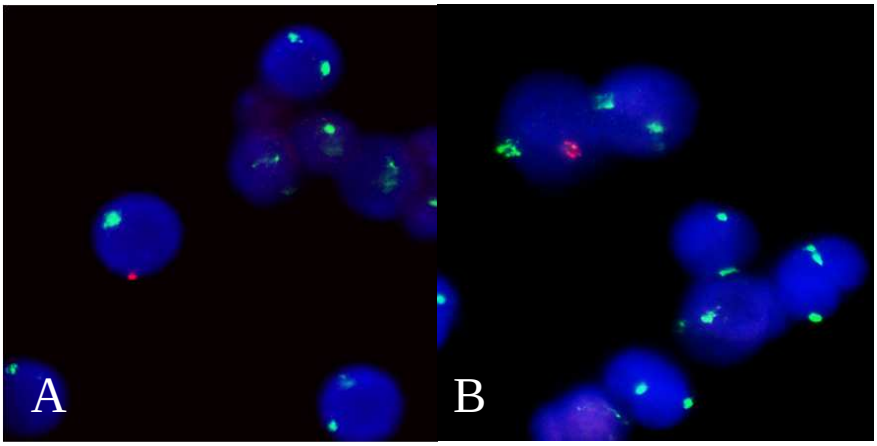


Figure 25 A, B. Detection of a male fetal cell in maternal blood by the use of two Y chromosome-specific (α- and III-satellite) FISH probes in combination with a FISH probe specific for the X chromosome (XYY-FISH).

2.8. Evaluation of Soybean Lectin-based method for the Enrichment of erythroblasts

Introduction

Different approaches have been proposed for the enrichment of erythroblasts from maternal blood. However, none of these methods has been shown to obtain fetal cells from maternal blood with sufficient reliability for routine prenatal diagnosis. Recent studies have indicated that enrichment based on galactose-bearing conjugation may lead to more promising results.

Erythroblasts express galactose on cell surface to a larger extent than other cell types.

The selective attachment to substrate coated with a galactose-containing polymer (PV-MeA, Ne Tech) via soybean agglutinin, a galactose-specific lectin, allows enrich the erythroblasts (Kitagawa et al., 2002).

Aim

To evaluate Soybean Lectin-based method we have performed a comparative study on maternal blood samples, with one half of the blood sample being subjected to enrichment by standard CD-71 MACS protocol and the other half being enriched by the lectin protocol.

Design

The lectin enrichment and the MACS/CD 71 methods were performed as described previously (Kitagawa et al., 2002) and (Troeger et al., 1999a). In both instances the enriched cells were stained with MGG and the number of recovered erythroblasts was scored. The cells with low nucleus-to-cytoplasm ratio, dense and small nucleus, and orthochromatic, non-granular cytoplasm were considered as erythroblasts at light microscopy analysis (Axioplan 2, Carl Zeiss, Zürich, Switzerland).

The number of erythroblasts on slides isolated by CD-71 MACS enrichment was accounted for 2 cytospin slides (2 x (1 x 10^5 cells)) and recalculated for 1 ml of maternal blood. The number of erythroblasts in 1 ml of maternal blood after SBA-lectin enrichment was recalculated after analysis for 1 chamber (2 x 10^6 cells)

Results

The average number of erythroblasts after CD 71 enrichment was 2.4 cells (range 0-6) per 1 ml of maternal blood. The average number of erythroblasts after SBA-lectin

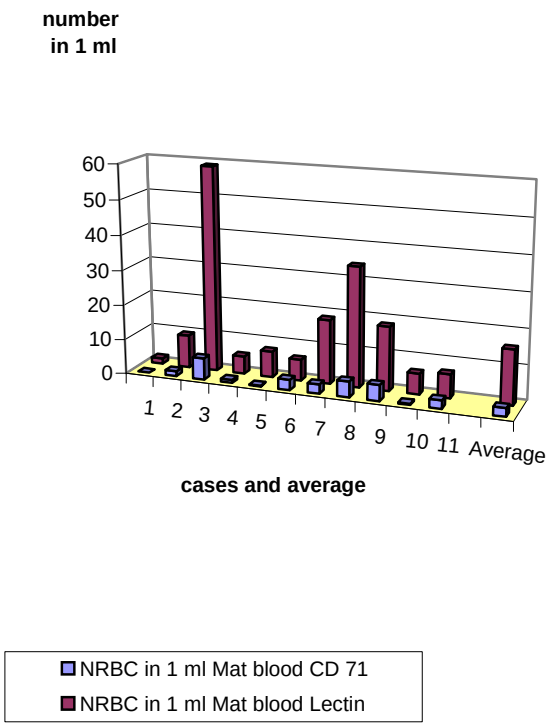
enrichment was 15.7 cells (range 2-59) per 1 ml of maternal blood. Our results (Table 23; Figure 25; Figure 26) indicate that significant differences (p=0.0033<0.05; Wilcoxon signed rank test for paired samples) exist between the two enrichment methods, with almost 7 fold more erythroblasts being recovered per ml of maternal blood by the SBA-lectin method when compared with standard MACS approach.

Another apparent advantage of the SBA-lectin method is that the enriched cells appeared to have a better morphology, facilitating easier recognition and analysis, than cells which had been treated by the MACS protocol. This aspect is to be explored in an extended study.

Conclusion

Although the efficiency of the erythroblast recovery by MACS that we have achieved in this study is comparable with the data from other studies, it is clear that the SBA-lectin method offers a better recovery. The present study also serves to indicate that numerous erythroblasts are therefore being lost during the MACS procedure. In this regard, it has previously been calculated, that cell loss due to centrifugation can be as high as 2-4% and that the most significant loss (almost 10-20%) occurred during the transfer of enriched cells onto microscope slides by cyto-centrifugation (Oosterwijk et al., 1998a). It is also likely that target cells are lost during the MACS procedure, in that they may be retained by the column or lost during the washing steps. Hence, it is possible, that the lack of such steps may contribute to the overall improved recovery by the SBA-lectin method.

Our results suggest that SBA lectin-mediated procedures may provide a better opportunity for the enrichment of rare fetal erythroblasts from maternal blood. Our preliminary FISH results on lectin- enriched erythroblasts demonstrated a better FISH signal quality (Figure 27).



Case	Gestation age	Erythroblasts in 1 ml Maternal blood CD 71	Erythroblasts in 1 ml Maternal blood Lectin
1	36+6	0.0	1.6
2	15+4	1.4	9.4
3	27+6	6.2	58.9
4	12+5	0.9	5.1
5	12+3	0.2	7.4
6	14+6	3.0	6.0
7	40+0	2.7	18.4
8	36+1	4.5	34.3
9	35+6	4.6	18.3
10	14+2	0.4	6.0
11	12+4	2.4	6.8
	Average	***2.4***	***15.7***

Figure 25. Comparison of enrichment efficiency: CD-71 MACs vs Lectin.

Table 23. Comparison of enrichment efficiency: CD-71 MACs vs Lectin

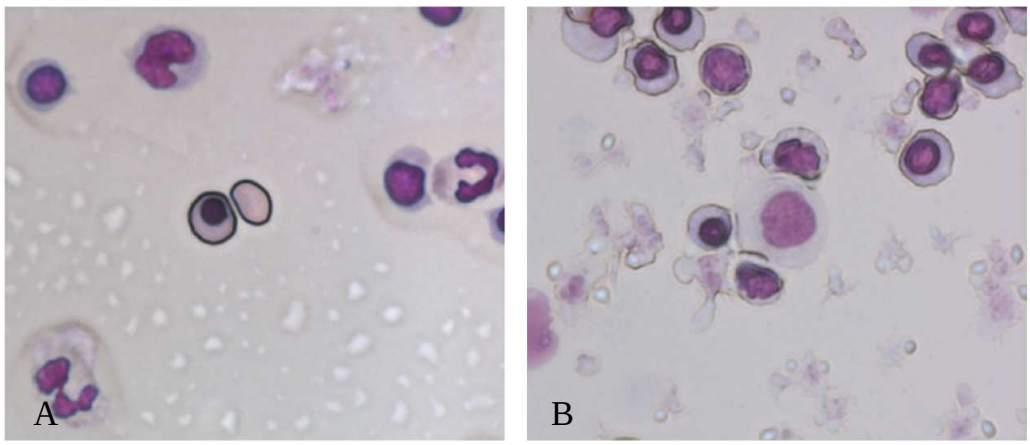


Figure 26. A. MGG stained erythroblasts from maternal blood after CD-71 B. Lectin-based enrichment.

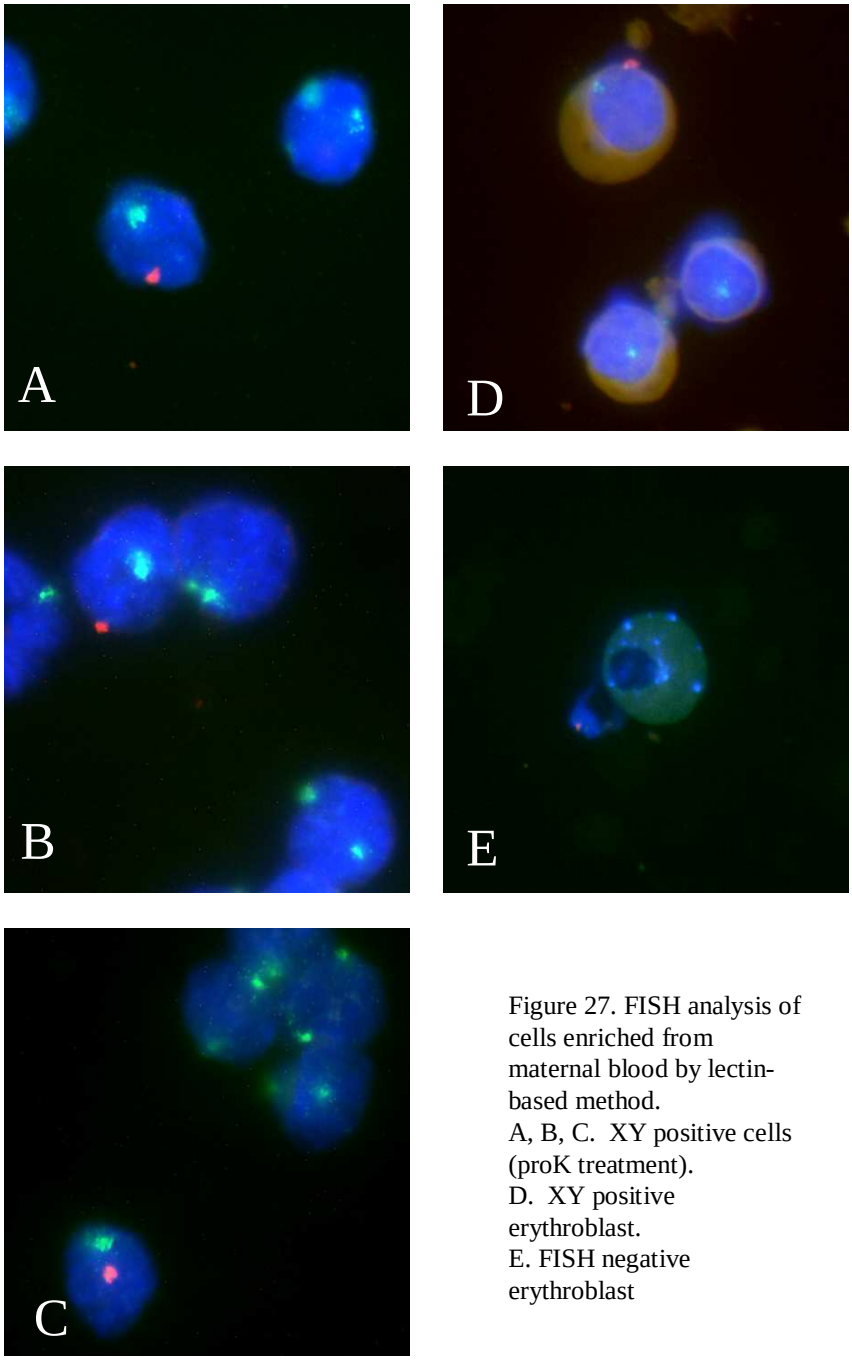


Figure 27. FISH analysis of cells enriched from maternal blood by lectin-based method.
A, B, C. XY positive cells (proK treatment).
D. XY positive erythroblast.
E. FISH negative erythroblast

2.9. Spectral morphometric comparative analysis of erythroblasts from maternal and cord blood

Aim

The present study is the attempt to identify and evaluate erythroblasts from maternal and cord blood using technique of spectrally resolved image analysis.

Design

Firstly we determined the localization of different spectral components in nuclei and cytoplasm of erythroblasts cells from maternal and cord blood stained with MGG employing classification algorithm based on minimal square error matching (MSE). Then we compared the chromatin and cytoplasm organization for erythroblasts from maternal and cord blood using the computational morphometrical analysis of spectral map.

Results

Spectral imaging was performed on MGG-stained blood specimens from 8 cord blood (93 cells) and from 12 maternal blood samples (53 cells).

Figure 29A and 29C show the spectral cube image of some MGG-stained erythroblasts from cord and maternal blood, respectively. Using the spectra library, all cells in the database were scanned and to each nuclear domain there were assigned the color of the library spectrum most similar to its spectral characteristics. The scan produced a classified image for each cell using a classification algorithm, as described in Materials and Methods.

Several examples of pseudo-colored spectral mapped images of cord and maternal blood cells are shown in Figure 29B and 29D, respectively. The classified images represent the spectrally similar regions with the match to the reference spectra of the library.

The spectral classified image reveals the visually distinct spectral regions in each cellular compartment and has demonstrated that erythroblasts from maternal and cord blood displayed distinct spectral characteristics.

To create the histograms of spectral distribution, the computational morphometric measurements were performed on the spectrally classified images for 53 erythroblasts from maternal blood and 14 ones from cord blood. The area of all spectra domains of the nuclei was calculated. The pixels within the distinct nuclear spectral domains were used

also to generate histograms that represent relative area of spectral domain in percentage. An analysis of the nuclear color content is shown in Figure 28. An inspection of the color histograms reveals that the dominant spectrum (67%) in maternal blood erythroblasts was the spectrum designated as number 1 (No. 1) in spectral library, while in cord blood erythroblasts the spectrum No. 1 was not so dominant, (40% only). Spectrum No. 2 was represented by contrast in 16% of maternal blood and in 42% of cord blood erythroblasts. Spectrum No.3 was represented in approximately same percentages for maternal and cord blood, 17% and 18%, respectively. It means that difference between the nuclei of erythroblasts from maternal and cord blood on spectral level is in the distribution of spectra 1 and 2. We consider that spectrum No. 1 can correspond to condensed chromatin in the nuclei.

For detailed analysis of spectral domain No.1, as marker of condensed chromatin, we have performed measurements on 93 erythroblasts from cord blood and 53 erythroblasts from maternal blood. The spectral domain was found in 97% of erythroblasts from cord blood and in 100% of erythroblasts from maternal blood. The spectral domain No.1 did not appear in the nucleus of 3% of the cord blood erythroblasts, for which only spectral domains No. 2, 3 were found.

Statistical analysis (t-Test) has revealed significant differences ($p \leq 0.0001$) in the distribution of spectra No.1 between maternal and cord blood erythroblasts.

Table 24 presents relative area in % of the spectral domain No.1 in the nuclei of erythroblasts from maternal and cord blood in connection with results of morphometric measurements of diameter, circumference and nuclei-cytoplasm ratio and FISH results (results partially published by (Babochkina et al., 2005a).

For analysis of spectrum No. 4, as marker of cytoplasm content, we performed measurements on same cells (53 and 93 erythroblasts from maternal and cord blood, respectively).

The morphometrical analysis of distribution spectrum of No.4 as a marker of cytoplasmic content, for this cells has revealed that this spectrum was observed for 93% of erythroblasts from cord blood (on average 26% of cytoplasm area) and for 99% of erythroblasts from maternal blood (on average 45% of cytoplasm area). The difference in

distribution of spectrum No.4 in cells from the two classes is statistically significant (t-Test, $p \leq 0.0001$).

Analysis		Cord blood	Maternal blood	
FISH analysis	*vysis XY probe, efficiency , %*	98%	xx-51% xy- 5%	oo-21%
Morphometric analysis	*diameter*	5.96	6.47	4.73
	circumference	21.93	23.31	17.53
	N/C	32%	47%	28%
Spectral analysis	*Spectrum No.1 (red in nuclei) on average*	40%		67%
	Spectrum No.4 (green in cytoplasm) on average	26%		45%

Table 24. Comparison of erythroblasts in cord and maternal blood by FISH, morphometric measurements and spectral imaging analysis.

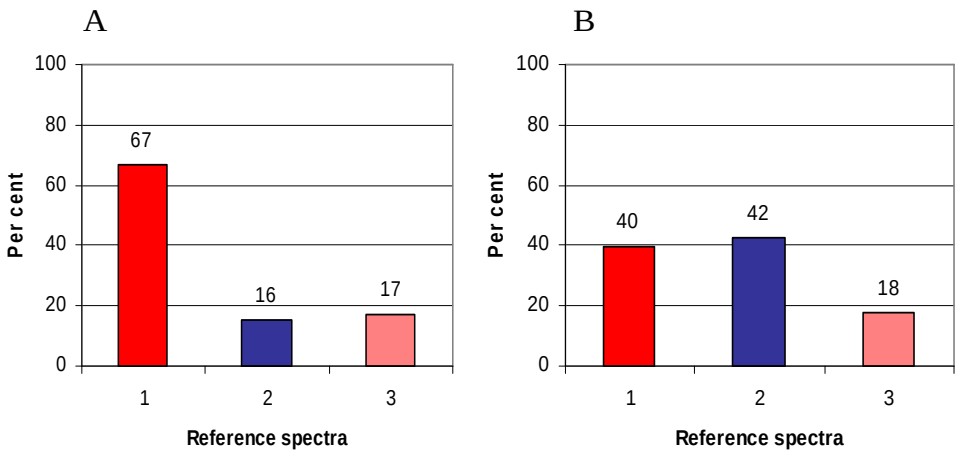


Figure 28. Spectral arrays for erythroblasts from: A. maternal blood; B. cord blood. Area of each spectrally similar region in the nucleus was calculated and represented as the percentage of the nuclear area

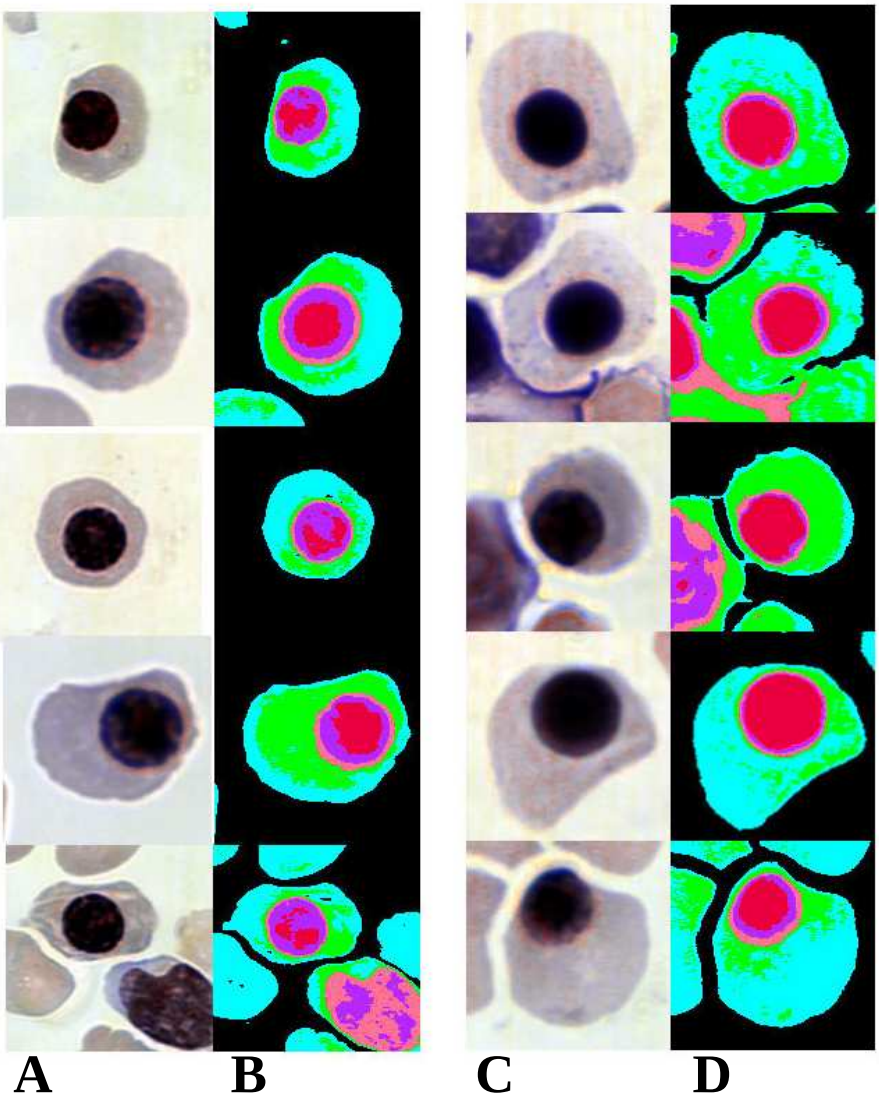


Figure 29. Spectral imaging and spectral classification of erytroblasts
A, C. Spectral cube imaging of erythroblasts from cord and maternal blood, correspondingly.
B, D. Pseudo-colored spectral mapped images same cells from cord and maternal blood, correspondingly, after applying a classification algorithm. Each coloured domain represents a spectrally similar region.

2.10. Single cell Taqman PCR analysis of erythroblasts isolated using Laser Microdissection and Pressure Catapulting (LMPC) technology

Introduction

The enriched fetal cells can be isolated by micromanipulation and analyzed by single cell Taqman PCR (Troeger et al., 1999b). The analysis of single cells will always be hampered by limiting amount of target template. The several strategies can be adopted to overcome this problem, including optimization of microdissection (using slides with and without membrane), application of different cell lysis protocols, choice of Taqman probes, raising the denaturation temperature.

In our study we tried to optimize the single cell Taqman PCR analysis firstly on cord blood. Next we have applied the best protocol for analysis of erythroblasts with small dense nucleus from maternal blood.

2.10.1. Taqman PCR analysis of erythroblasts microdissected from cord blood

Aim

To optimize the Taqman PCR analysis of erythroblasts microdissected by PALM laser-mediated micromanipulation system from cord blood (from slides with and without membrane) by using different choice of probes.

Design

The erythroblasts from cord blood were transferred onto glass slides with and without membrane by cytocentrifugation and MGG stained. Single erythroblasts were isolated by the LPC microdissection method and examined by a multiplex Taqman PCR which had been optimized for the analysis of single cells.

In order to optimize protocol of cell collection we have tried the PALM microdissection of erythroblasts from glass slides with and without membrane. To optimize the Taqman protocol for amplification of single cells we have used SRY labeled with fluorochrome FAM (SRY-FAM) and GAPDH labeled with fluorochrome VIC (GAPDH-VIC) probes for to detect the fetal loci and to control the PCR reaction, respectively. The control loci had previously been shown to be very effective in monitoring the efficacy of the PCR reaction and in ascertaining whether a single cell had indeed been transferred to the

reaction vessel. The efficiency for single cell Taqman PCR analysis was tested for every probe alone and for their combination. Besides, we have tested the 21 chromosome and 18 chromosome specific probes as control of DNA amplification in combination with SRY probe.

Results

Thirty two erythroblasts were collected by PALM microdissection from membrane slides and analyzed by single cell Taqman PCR analysis with GAPDH–VIC probes. The amplification efficiency was 43.3% (Table 25).

Case	Number of microdissected cell	Number of GAPDH-VIC positive cells	Efficiency, %
1	20	9	45.00
2	12	5	41.67
SUM	32	14	43.33

Table 25. Single cell Taqman PCR analysis with GAPDH-VIC probe on erythroblasts microdissected from cord blood

Forty erythroblasts were collected by PALM microdissection from membrane slides and analyzed by single cell Taqman PCR analysis with SRY-FAM probes. The amplification efficiency was 64.6% (Table 26).

Case	Number of microdissected cell	Number of SRY-FAM positive cells	Efficiency, %
1	24	16	66.67
2	16	10	62.50
SUM	40	26	64.58

Table 26. Single cell Taqman PCR analysis with SRY-FAM probe on erythroblasts microdissected from cord blood

The multiplex Taqman PCR analysis with SRY-FAM and GAPDH–VIC probes was performed for 62 erythroblasts, collected in the same way. The amplification efficiency was 28.0% and 53.8% for GAPDH and for SRY loci, respectively. In total, the amount of Taqman positive cells was 53.8% (Table 27).

Case	Number of microdissected cell	Number of GAPDH-VIC positive cells	GAPDH efficiency, %	Number of SRY-FAM positive cells	SRY efficiency, %	Taqman positive cells, %
1	30	3	10.00	9	30.00	30.00
2	16	4	25.00	9	56.25	56.25
3	16	8	50.00	12	75.00	75.00
SUM	62	15	28.33	30	53.75	53.75

Table 27. Single cell multiplex Taqman PCR analysis with GAPDH-VIC and SRY-FAM probes on erythroblasts microdissected from cord blood

The multiplex Taqman PCR analysis with SRY-VIC and 21 chromosome-FAM probes was performed for 32 erythroblasts, collected by PALM microdissection from membrane slides. The amplification efficiency was 34.4% and 87.5% for SRY and for 21 chromosome loci, respectively. In total the amount of Taqman positive cells was 87.50% (Table 28).

Case	Number of microdissected cell	Number of SRY-VIC positive cells	SRY efficiency, %	Number of 21chromosome-FAM positive cells	21 chromosome efficiency, %	Taqman positive cells, %
1	16	2	12.50	14	87.50	87.50
2	16	9	56.25	14	87.50	87.50
SUM	32	11	34.38	28	87.50	87.50

Table 28. Single cell multiplex Taqman PCR analysis with SRY-VIC and 21 chromosome FAM probes on erythroblasts microdissected from cord blood.

The multiplex Taqman PCR analysis with SRY-FAM and 18 chromosome-VIC probes was performed for 68 erythroblasts, collected by PALM microdissection from slides without membrane. The amplification efficiency was 18.1% and 32.5% for SRY and for 18 chromosome loci, respectively. In total the amount of Taqman positive cells was 35.56% (Table 29).

Case	Number of microdissected cell	Number of 18 chromosome-VIC positive cells	18 chromosome efficiency, %	Number of SRY-FAM positive cells	SRY efficiency, %	Taqman positive cells, %
1	24	7	29.17	4	16.67	33.33
2	20	7	35.00	5	25.00	40.00
3	24	8	33.33	3	12.50	33.33
SUM	68	22	32.50		18.06	35.56

Table 29. Single cell multiplex Taqman PCR analysis with 18 chromosome VIC and SRY-FAM probes on erythroblasts microdissected from cord blood.

Conclusion

The best efficiency of single cell Taqman PCR for detection of Y chromosome was achieved when SRY probe labeling by FAM was applied. Therefore, the isolation of single cells by PALM from slides with membrane is more effective than that from slides without membrane. A major factor to consider here is that membranes prevent the cell from damaging during transfer.

2.10.2. Taqman PCR analysis of erythroblasts microdissected from maternal blood

Aim

To analyze the origin of erythroblasts from maternal blood with small dense nuclei and resistant for FISH analysis.

Design

For experiment 1 the CD 71 enriched erythroblasts from maternal blood were transferred onto glass slides without membrane by cytocentrifugation and MGG stained. Single

erythroblasts with small dense nucleus, which were resistant to FISH analysis were isolated by the LPC microdissection method, collected in pool and examined by a multiplex Taqman PCR.

For experiment 2 the erythroblasts enriched from maternal blood by Soybean Lectin-based method were transferred onto membrane slides and MGG stained.

Single erythroblasts with small dense nucleus, which were resistant to FISH analysis, were isolated by the LPC microdissection method and every cell was examined by a multiplex single cell Taqman PCR which had been optimized for the analysis of single cells.

Results

1) Taqman PCR analysis of pooled erythroblasts microdissected from maternal blood

Our in blinded way examination of 6 samples obtained from pregnant women has indicated that in 2 from 4 cases the fetal gender was determined by Taqman PCR analysis on pool of collected erythroblasts. The sensitivity and specificity of our analysis were 50% and 100%, respectively.

Thirty two erythroblasts were collected by PALM microdissection from membrane slides and analyzed by single cell Taqman PCR analysis with GAPDH–VIC probes. The amplification efficiency was 43.3%. On average 28 erythroblasts was microdissected (Table 30).

Cases ID	Gender	Number of cells in the pool	GAPDH	SRY
3087	male	31	positive	positive
3107	male	10	positive	negative
3697	male	20	positive	negative
3699	female	29	positive	negative
3031	male	31	positive	positive
3039	female	45	positive	negative

Table 30. Taqman PCR analysis of pooled erythroblasts microdissected from maternal blood

2) Single cell Taqman PCR analysis of erythroblasts microdissected from maternal blood

Our in blinded way examination of 6 samples obtained from pregnant women has indicated that in 2 from 2 cases the fetal gender was determined by single cell Taqman PCR analysis with GAPDH-VIC and SRY-FAM probes. On average 14 erythroblasts (range from 4 to 24) were microdissected. The average amplification efficiency was 52.0%. The sensitivity of our analysis for detection fetal gender was 100% and specificity 100% (Table 31). But the proportion of fetal cells from all successfully amplified erythroblasts was in the first case (ID 3842) 33%, in the second case (ID 3843) 20%.

Case ID	Gender	Number of microdissected cell	Number of GAPDH-VIC positive	Number of SRY-FAM negative	Tagman efficiency, %
3841	female	20	10	0	50.00
3842	male	16	2	1	18.75
3843	male	24	5	1	25.00
3868	female	4	3	0	75.00
3885	female	6	5	0	83.33
3886	female	20	12	0	60.00
	Average				52.01%

Table 31. Single cell Taqman PCR analysis of erythroblasts enriched by Lectin method from maternal blood and microdissected from membrane covered slides.

Conclusion

We have demonstrated that the use of membrane-coated slides for lectin enrichment for LPC microdissection restricts severely the use of this approach because of the very high level of background of those slides that influences the quality of analysis. The percentage of fetal erythroblasts among all those amplified successfully was on average only 25%. Our preliminary data have indicated that the single cell Taqman PCR analysis on erythroblasts microdissected by PALM technique is reliable for the detection of fetal origin of erythroblasts but this issue will have to be explored in an extended study.

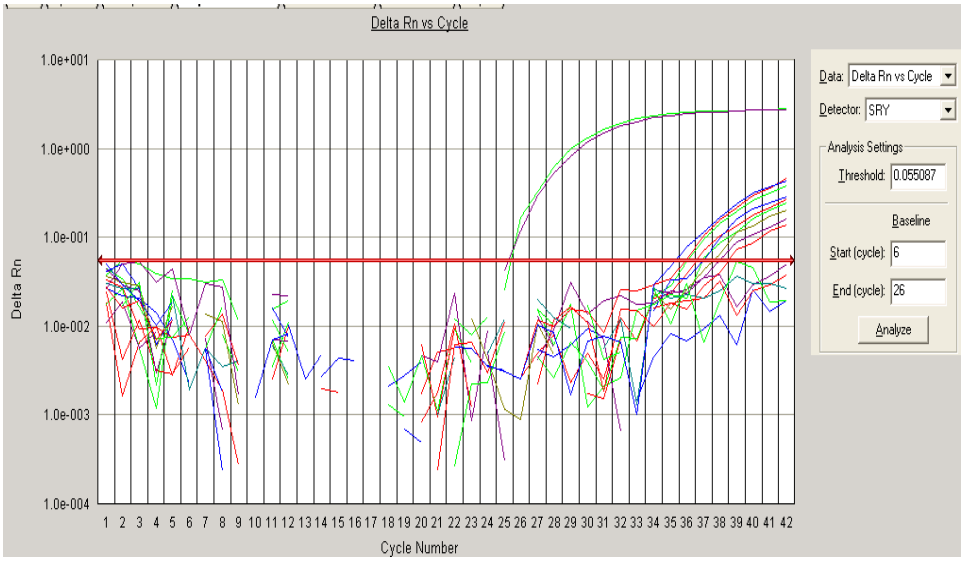


Figure 30. Single cell singleplex Taqman PCR analysis on erythroblasts from cord blood (amplification efficiency for SRY locus is 63%).

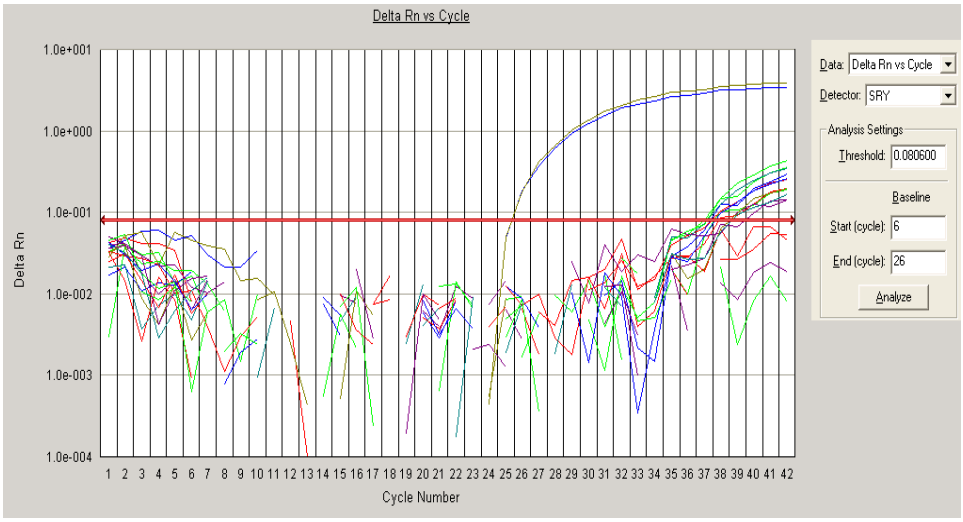


Figure 31. Single cell multipley Taqman PCR analysis on erythroblasts from cord blood (amplification efficiency for SRY locus is 75%).

3. Discussion

3.1. The majority of erythroblasts in maternal blood are impervious to the analysis by FISH

The results of our study of erythroblasts in maternal blood have indicated that only half of the erythroblasts could be efficiently analyzed by FISH, and that these were largely of maternal origin, as they had a XX genotype. Only a small fraction of the erythroblasts (<5%) in maternal blood could be reliably identified as being of fetal origin on the basis of clear FISH signals for the X and Y chromosomes. The remaining portion of this pool of circulating erythroblasts appeared to be refractory to FISH analysis.

This feature was evident not only for the Y chromosome, but also for the analysis of chromosomes X and 18. Furthermore, this feature could not be overcome by employing microwave treatment, which hade previously been shown to be effective for the FISH analysis (Durm et al., 1997).

Our data further indicate that this group of erythroblasts differs from those which are clearly of maternal origin in that they have a small dense nucleus containing fragmented DNA. However, we have determined that the inability to analyze these cells efficiently by FISH was most likely not due to the presence of fragmented DNA but rather due to a small dense compact nucleus.

This result was most evident in our analysis of cord blood erythroblasts, whose nuclei could all be efficiently analyzed by FISH despite being TUNEL positive. In previous studies using single-cell PCR on individual erythroblasts isolated by micromanipulation, it was observed that almost 50% of the erythroblasts in the maternal circulation were of fetal origin (Troeger et al., 1999b). Although these cells were not selected on the basis of having a small dense TUNEL positive nucleus, it would appear that the erythroblasts, which are impervious to FISH analysis, are also of fetal origin. This hypothesis is supported by our ongoing studies using PCR which indicate that a number of these erythroblasts with small dense nuclei are indeed of fetal origin.

Our data also provide a possible reason for this difference between putative fetal and maternal erythroblasts in the maternal circulation. Probably, this change in the nuclear phenotype may be triggered by the difference in oxygen tensions existing between the mother and fetus. In this regard, it is interesting to note that previous studies have shown that when fetal erythroblasts enter the maternal circulation, their nuclear DNA becomes

fragmented (Sekizawa et al., 2000), as detected by the TUNEL assay, and that this affect can be mimicked by exposing such cells to elevated oxygen concentrations (Kondo et al., 2002). The investigations of Hristoskova et al. (2003) have suggested that this process of nuclear cleavage is associated rather with terminal differentiation and subsequent enucleation, than with apoptosis, as these cells do not express other characteristic apoptotic markers. In our current analysis we have demonstrated that 24-hour culture under elevated oxygen conditions leads to a significant reduction in the size of fetal erythroblast nuclei, and that this significantly influences the efficacy of these erythroblasts to be analyzed by FISH.

As we have tested only a single 24-hour time point, it is currently unclear whether shorter periods of exposure also lead to similar nuclear condensation. This aspect, which remains to be explored, may help to explain why under certain conditions, such as immediate post-termination, fetal erythroblasts can be reliably detected by FISH analysis (Choolani et al., 2003).

Taken together, our data suggest that a large proportion of the erythroblasts in the maternal circulation cannot be reliably analyzed by FISH, and that it is probable that many of these cells are of fetal origin. It appears also that decrease in nucleus diameter is induced by the higher oxygen tension present in the maternal circulation.

Our data also hint at why fetal erythroblasts were detected with lower efficacies when using FACS-based procedures compared to MACS-based ones (Bianchi et al., 2002). In the studies using FACS, tight constraints were placed on enriching cells with well defined fetal erythroblast specific characteristics (expression of gamma globin), whereas in the studies using MACS, the cells were enriched only on the basis of CD-71 expression, and no attempt was made to determine whether the fetal cells being examined were indeed erythroblasts. It is therefore possible that in the latter instance fetal cells other than erythroblasts may have been examined.

Although we have examined exclusively the well defined mature erythroblasts in this study, it is possible that these data may be extendable to more immature fetal erythroblasts, such as those expressing fetal or embryonic globin molecules. The reason for this is that in most instances, the detection of such fetal cells by FISH has not been very reliable, except in samples obtained immediately after a termination of pregnancy or

an invasive procedure, such as chorionic villus sampling (CVS). It is therefore possible that under normal conditions these early fetal erythroblasts undergo nuclear changes once they enter the maternal circulation similar to that which we have seen in this study.
In summary, our data provide an explanation for the apparent lack of fetal erythroblasts in the maternal circulation, or the inability to detect them with high degree of sensitivity when using FISH for their analysis (Bianchi, 1999). This facet will have to be considered in future potential diagnostic approaches.

Alternative strategies may, hence, focus on the use of primed in situ-labelling (PRINS), as this method relies on smaller molecules than current FISH-probes for the detection of fetal specific sequences, which may be able to enter the dense fetal erythroblast nuclei more successfully.

3.2. Soybean Lectin-based enrichment

Although the efficiency of the erythroblast recovery by MACS that we have achieved in this study is comparable with data from other studies, it is clear that the SBA-lectin method offers a better recovery. The present study also serves to indicate that numerous erythroblasts are being lost during the MACS procedure. In this regard, it has previously been calculated, that cell loss due to centrifugation can be as high as 2-4% and that the most significant loss (almost 10-20%) occurred during the transfer of enriched cells onto microscope slides by cyto-centrifugation (Oosterwijk et al., 1998a). It is also likely that target cells are lost during the MACS procedure, in that they may be retained by the column or lost during the washing steps. Hence, it is possible, that the lack of such steps may contribute to the overall improved recovery by the SBA-lectin method.
Another apparent advantage of the SBA-lectin method is that the enriched cells appeared to have a better morphology, facilitating easier recognition and analysis, than cells which had been treated by the MACS protocol. This facet will have to be explored in a larger study.
A major drawback of all current enrichment methods, and especially that of the SBA-lectin method, is that the final enriched cell population is contaminated by a vast number of maternal cells. Although the recovery by the SBA-lectin method is greater than that

attained by the MACS method, the purity is significantly lower. This implies that a considerable effort still has to be made to locate potential fetal cells after the lectin enrichment procedure, which renders the analysis tedious and time consuming. Therefore it will be necessary to employ systems which permit the automated recognition and recovery of potential fetal cells, in order to obtain the optimal efficiency required for clinical applications. For this reason studies are ongoing which explore the use of systems facilitating automated recognition of rare events and their subsequent contact free isolation by laser pressure catapulting (Hahn et al., 2002).

In summary, our study indicates that the lectin-based method is more efficacious than MACS approach for the enrichment of erythroblasts from maternal blood, and that this method may be a promising alternative for future investigations concerned with non-invasive prenatal diagnosis.

3.3. XYY – FISH on whole blood

Krabchi et al. (2001) have shown 100% specificity for 12 male pregnancies in a non-blinded manner using hypotonic treatment combined with Carnoy`s fixation. Ten of these pregnancies were analyzed by conventional XY-FISH and two – by XY-chromosomal PRINS. Although these studies yielded important information concerning the number of circulating fetal cells in maternal blood, the results are possibly skewed by the fact that they were not conducted in a blinded manner concerning the sex of the fetus.

Using the same experimental set-up like Krabchi et al. (2001) but in a blinded manner we have obtained specificity 69.4% and sensitivity 52.4% in our previous study (Mergenthaler et al., 2005). The increase in specificity up to 89.5% in this study has been achieved by substituting the X-chromosomal probe with a second Y-chromosomal probe from a different region of the Y-chromosome (α- and III-satellite). However a slight reduction in sensitivity (down to 42.9%) was detected. Although this study did indicate that male fetal cells were present in the vast majority of pregnancies with male fetuses, we were anxious to improve upon the accuracy of our method. For this reason we have again used two similar Y chromosome-specific FISH probes (α- and III-satellite) but now in combination with an X chromosome-specific FISH probe (one-step two-color XYY-FISH). The present investigation indicated that circulatory male fetal cells can now be

detected with 75% specificity and 91% sensitivity, which is a considerable improvement over our previous results. The efficacy of this approach also becomes readily apparent in our parallel assessment of the same samples by conventional XY-FISH, where male fetal cells could be detected only with 50% specificity and 60% sensitivity which is comparable with data in our previous study (Mergenthaler et al., 2005).

The mathematical calculation of probability of labeling the fetal cells by XY-, YY-and XYY-probes combinations gives the values 0.81, 0.81 and 0.891, respectively (in the mathematical model we took equal FISH efficiency of 90% for X-, Y- (α-satellite) centromeric and Y (III-satellite) probes, as described by the manufacturer). Thus, the labeling of Y chromosome by two different probes (centromeric Yp11.1-q11.1 and q12 regions) by the same color in the one-step two-color XYY-FISH has advantage in detecting fetal Y-chromosome over other methods of labeling mentioned above. Theoretically, the increase in the number of Y chromosome probes labeled by the same color leads to an increase in the probability of Y-chromosome detection. In our present study we demonstrate the results which confirm this mathematical prediction.

Our data were also quite encouraging concerning the number of male fetal cells detected per 1 ml of maternal blood. The actual frequency of erythroblasts in the maternal circulation is difficult to assess, and it varies considerably according to the enrichment technique used and methods of evaluation. In an interstudy–comparison regarding fetal cell number the average concentration of 2 fetal cells per 1 ml of maternal blood found by Krabchi et al. (2001) matches approximately the concentration of 1.2 fetal cell equivalents per 1 ml of maternal blood found by Bianchi et al. (1997) using PCR. Our previous results (Mergenthaler et al., 2005) exceed these average cell numbers. In the present study we have enumerated from 8 to 16 and from 8 to 32 fetal cells per 1 ml of maternal blood by XY- and XYY FISH, respectively. It means that more male fetal cells were detected by the XYY-FISH approach in comparison with conventional XY-FISH.

The reasons for differences in the values between our study and studies of other researchers might be due to the much broader range of gestational age of our patients, due to the different manner of evaluation (blinded and non-blinded), due to the procedure-inherent factors influencing the FISH efficiency (Hromadnikova et al., 2002) and due to

the number of cases used for these studies which is non-sufficient for all mentioned above studies the statistically reliable result.

Akin to our previous study, male fetal cells could be detected in the majority of maternal blood samples examined. No male fetal cells could be detected in 3/10 (30%) of XY-FISH experiments and in 1/11 (9%) of XYY-FISH experiments of pregnancies with a male fetus. One of the reasons for this is that we used the amount 0.125 – 0.25 μl (1-2 slides) of maternal whole blood that does not seem to be sufficient to guarantee the presence of a reliable number of fetal cells.

Our study has revealed that the using double labeling of Y-chromosome (XY-Y-Qbiogene protocol) results in improved fetal cells recovery and enables a reliable prospective non-invasive fetal sex determination.

3.4. Spectral morphometric comparative analysis of erythroblasts from maternal and cord blood

Spectral morphometric analysis was used in the present study for the characterization of erythroblasts from maternal and cord blood on the basis of spectral nuclear and cytoplasm features.

In 1991, Haaf and Schmid (1991) stated that the existence of highly ordered organizational patterns in the cell nucleus probably could provide a structural framework for efficient processing of nuclear events. Therefore, in 1992, Sorensen hypothesized that the nuclear structure is a reflection of the metabolic state of the nucleus. In support of this hypothesis, many other researches suggest also that the specific nature of the compartmentalization could reflect the physiological state of a given cell (Popp et al., 1990; van Dekken et al., 1990; Vourc'h et al., 1993).

Zirbel et al. (1993) have proposed a model predicting that the surface of chromosome territories and a space formed between them provide a network-like three-dimensional nuclear compartment for gene expression, mRNA splicing, and transport, termed the interchromosome domain compartment.

During erythropoesis major nuclear components, such as DNA, RNA, histone and nonhistone proteins, inorganic materials, water and cytoplasmic content, for example, haemoglobin, as well as nucleo-cytoplasmic (N/C) ratio could undergo changes. These

changes may be different in erythroblasts in maternal blood and in cord blood and as a result, this could produce different spectral patterns in these cells.

On the basis of the present results, we assume that the circular patterns in erythroblasts from maternal blood and windmill patterns in erythroblasts from cord blood revealed by spectrally resolved imaging define a different three-dimensional compartmentalization of chromatin in the nucleus of these cells.

The symmetry observed in the nuclei may be maintained by electric forces. According to Zirbel et al. (1993), short-range and long-range electric forces resulting from charge distribution effects of chromosome territories and other nuclear components may be involved in the maintenance of the interchromosomal domain.

In our study we present the possibility of spectral morphometry for the disclosure of the distinctions of the erythroblasts. Although the MGG stained erythroblasts in maternal blood and in cord blood look similar, display similar properties, we have detected spectral distinctions between them.

In erythroblasts from maternal blood the reference spectra No.1 of the spectral library dominated in comparison with that in erythroblasts from cord blood. The reference spectra No.1 may be associated with high degree of condensation of the nuclei in maternal blood, whereas in the cord blood the nuclei are less condensed. These data are in good correlation with results of morphometric measurements and with results of FISH.

In erythroblasts from maternal blood the highly condensed chromatin was symmetrically condensed, whereas in the cord blood it shows windmill-like structure. In other study Rothman et al. (1997) have detected by spectral imaging the windmill-like structures of high symmetry in basophilic, polychromatic, and orthochromatic normoblast cells from bone marrow. They also have determined that apoptosis was associated with a gradual breakdown of the ordered arrays in the nuclei.

The reference spectra No.4 may be associated with accumulation of haemoglobin and other molecules or may be due to accumulation of degraded DNA in the cytoplasm of erythroblasts (Hendzel et al., 1998). We suppose that in erythroblasts from maternal blood the nucleus is more condensed and the DNA is accumulated in cytoplasm as a result of apoptosis.

The spectral imaging morphometry has advantages over the image analysis systems now in use. In addition to standard morphometric parameters, spectra (400-850 nm) are obtained for each pixel of an image, providing more information than conventional image analysis.

Today, the micromorphometry plays an increasing role in clinical diagnosis by providing clue derived from the shape and structure determinations of cell nuclei. Spectral imaging enhances the information obtained with light microscopy by providing multipixel spectra that otherwise are impossible to obtain from cytological specimens (Malik et al., 1996; Malik et al., 1997; Rothmann et al., 1997; Rothmann et al., 1998; Barshack et al., 1999; Greenspan et al., 2002). We propose that spectral morphometric analysis may serve as an additional diagnostic tool for detection of erythroblasts in maternal blood specimen.

3.5. Future directions

A long-term goal of all obstetricians and gynecologists is the development of simple, rapid, accurate, non-invasive tests for prenatal diagnosis. There is no doubt that fetal cells in maternal circulation do exist and that they can be used for the analysis of fetal aneuploidies and inherited Mendelian genetic disorders, but until now there exist some limitations for diagnostic applications. For example, the efficiency of FISH analysis on erythroblasts enriched from maternal blood is very low. In our study we have demonstrated that nuclear structure of erythroblasts is not suitable for FISH analysis. Our discovery that fetal erythroblasts in maternal circulation have small dense nuclei appears disappointing at present. However, more tests are needed to be done and a large number of samples are needed to be processed to confirm this and to understand more details on the nature and mechanisms of erythroblasts degradation in maternal blood.

In order to use the fetal cells in clinical applicability a significant amount of work have to be done concerning the choice of fetal cells for analysis, selection of specific fetal markers, as well as development of optimal enrichment procedures which have to be more effective and not so labour-intensive as current ones. It will also be important to determine more closely at what stage of pregnancy circulatory cells can be detected reliably, as in our current study we have examined a broad spectrum of gestational ages. Next important moment is to determine the frequency of fetal cells at early stages of

pregnancy at those stages when they may be useful as the basis for a non-invasive prenatal diagnosis during the first trimester. Furthermore, the fetal source (placental or haemopoietic) of fetal cells is also of considerable interest, as this may lead to the development of new tools permitting the efficacious isolation of fetal cells from maternal blood samples.

Therefore, the expansion of the molecular genetic diagnostic options, for example the primed *in situ* hybridisation (PRINS) analysis, needs to be optimized for use with fetal cells from maternal blood, or new techniques are to be developed.

The rapid PCR analysis of single fetal cells could be improved by the adaptation of laser micromanipulation protocols and by optimization of amplification procedures, for example applying the whole genome amplification procedures.

Finally, for clinical applicability, once all aspects of the non-invasive prenatal diagnostic technique have been optimized, these can be automated. The automated scanning technique (Metafer P; MetaSystems; Germany) can be combined with laser microdissection and pressure catapulting (LMPC; Germany), allowing automatic detection, isolation and collection of single fetal cells from maternal blood.

Such possibility has the promise of being a big step forward in developing protocols for non-invasive prenatal diagnosis.

4. Materials and Methods

Samples

The study was approved by the Cantonal Review Board of Basel and written informed consent was obtained in all instances. Cord blood samples were collected with EDTA immediately after birth from women with normal gestation who delivered normal infants. Maternal blood samples were collected with EDTA at 10 to 40 gestational weeks from women with normal singleton pregnancies. All samples were processed within 6 hours after blood sampling.

CD 71 Enrichment

We performed the CD 71 enrichment according to the protocol which had been established in our laboratory (Troeger et al., 1999a). In brief, the mononucleated cells were separated with a single density gradient using Histopaque 1083 (Sigma, Fluka Chemie GmbH, Buchs, Switzerland). After washing, for the positive selection, cells were incubated with anti-CD71 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach Germany) and separated using miniMACS separation columns following the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach Germany).

Slide Preparation and May-Grünwald-Giemsa (MGG) staining

The positively enriched cell fraction was transferred onto Superfrost PLUS glass slides (Mentzel-Gläser, Germany) by cytocenrifugation (Shandon, Frankfurt, Germany) in amount of 10^5 cells per one slide. The slides were stained differentially by May-Grünwald-Giemsa (MGG) (Sigma, St. Louis, MO, U.S.A.).

SBA galactose-specific lectin enrichment

The lectin enrichment method was performed as described previously (Kitagawa et al., 2002), except that a concentration of 100 µg/ml soybean agglutinin (Vector, Geneva, Switzerland) was used. In brief, for SBA enrichment the blood (7-8.5 ml) was diluted with phosphate-buffered saline (PBS) and the mixture was layered over 3 ml Histopaque 1077 (Sigma, Fluka Chemie GmbH, Buchs, Switzerland) that was adjusted to density 1.095 g/ml by adding sodium diatrizoate (Sigma, Fluka Chemie GmbH, Buchs, Switzerland). After centrifugation at 400g for 30 min at RT without break, the

mononuclear cells were collected and washed three times with PBS by centrifuging once at 750g for 10 min, and twice at 400g for 5 min at RT. The pellet was resuspended in 3 ml of panning solution (RPMI 1640 (Invitrogen AG, Basel, Switzerland) +5% FCS). The mixture of cells for negative enrichment was placed on dish coated with 5% inactivated FCS in panning solution and incubated at 37°C for 30 min. The attachment of cells was controlled under the microscope. After incubation, the suspension containing unattached cells was collected after several washes of the dish and washed by centrifugation at 400g for 5 min at 20°C. The suspension was diluted with SBA solution (100 µg/ml soybean agglutinin (SBA) (Vector, Geneva, Switzerland) in PBS-buffer) to a concentration of $2x10^6$ cells/ml. 1 ml of this suspension was put in each chamber of the slides (Nalge Nunc International, Millian, Geneva, Switzerland) coated with PV-MeA (Poly-[N-p-vinylbenzyl-O-a-D-galactopyramnosyl-(1-6)-D-gluconamide]) (NeTech, Kanagawa, Japan) at a concentration of 200 µg/ml and incubated at 15°C for 30 min while shaking the slides gently every 10 min.After incubation, the suspension containing unattached cells was decanted from the chamber slide, and the attached cells were washed with PBS buffer. 2 ml hypotonic solution (FCS/Water, 3:2) were added to every chamber and chamber slides were centrifuged at 17g for 5 min at 20°C. After centrifugation the medium was decanted from chamber and chambers were separated from the slides. The slides were centrifuged vertically at 68g for 10 min at 20°C and immediately dried with air for 30 min. The slides were incubated in a humid camera overnight at RT and then fixed in 95% mathanol for 20 seconds, in 50% methanol in 1/15 M phosphate-buffer for 20 seconds at RT and in 2% methanol in 1/15 M phosphate-buffer for 3 min at RT, washed briefly in Millipore water. Cells immobilized on the slides were MGG stained.

Light Microscopic Evaluation

Using a conventional microscope (Axioplan 2 imaging microscope system, Carl Zeiss, Zürich, Switzerland), every slide from cord blood and maternal blood after MGG staining was analyzed for cells content according to next criteria: a) optimal density of cells; b) undestroyed cellular morphology; c) good MGG staining. The cells with low nucleus-to-cytoplasm ratio, dense and small nucleus, and orthochromatic, non-granular cytoplasm were considered as erythroblasts.

For maternal blood slides total number of erythroblasts on the slide was accounted and position of every erythroblast was electronically marked using a digital automated location finder software (Axiovision 3.1, Carl Zeiss, Zürich, Switzerland) and MGG image for every cell was captured, using CDC camera (AxioCam, Carl Zeiss, Zürich, Switzerland).

Carnoy`s fixation on whole blood.

One milliliter of whole maternal peripheral EDTA-blood was processed within 6 hours after sampling. After washing with 8 ml RPMI 1640 (Invitrogen, Basel, Switzerland) by centrifugation at 1000 rpm for 8 min at RT the samples were incubated with 10 ml of pre-warmed 0.4% KCL for 20 minutes at 37°C, fixed several times with freshly prepared pre-cooled Carnoy`s fixative (methanol: glacial acetic acid, 3:1) and centrifuged at 1000 rpm for 8 min at RT and incubated overnight at –20°C. Then the cells were washed in 1% BSA/PBS, resuspended by pipetting. The slides were prepared by cytocentrifugation (8 slides per case).

Pretreatment of slides involved 0,005% pepsin digestion for 10 min and fixation for 10 min in 1% formaldehyde.

Immunocytochemistry (ICC)

The slides were fixed in 100% methanol for 10 min, 100% acetone for 10 min and in 10% formaldehyde for 10 min. Then they were incubated with 20% goat serum (Sigma) diluted in 1% BSA-PBS for 45 min followed by incubation with monoclonal mouse anti-human Glycophorin A (GPA) antibodies (1:2000 diluted in PBS) (Dako A/S, Denmark) for 10 min. Subsequent incubations were performed with biotinylated anti-mouse immunoglobulins (Dako A/S, Denmark), and with streptavidin (Dako A/S, Denmark), both were incubated for 10 min. Then the slides were stained by Vector blue alkaline phosphatase substrate (Kit III, Vector Laboratories, Burlingame, USA) for 15 min.

Fluorescence *In Situ* Hybridization (FISH)

Pretreatments:

Microwave – activated pretreatment: The slides were mounted on a small glass filled with 30 ml PBS and placed in the center of circling plate of commercially available microwave oven (Bosch, Germany). Activation was performed at 700 W for 40 sec.

Pepsin treatment: With examined different concentration, temperature and duration of time, the following optimal conditions were determined: 0.005% pepsin/water, for 30 sec at 37ºC (Table 2).

Proteinase K treatment: The concentrations 0.5 mg/ml and 1 mg/ml and time of treatment from 1 min to 30 min were examined.

HCL treatment: The concentrations 0.1 M and 0.2 M and time of treatment from 30 sec min to 20 min were examined.

Choice of Probes:

For the comparison of the efficiency of different direct labeled fluorescence DNA probes the MGG-stained slides were hybridized in different FISH-experiments with probes from Vysis and Qbiogene companies (Table 4; Figure5).

Vysis: two–color chromosome X/Y cocktail probe (CEP X spectrum green, alpha satellite, region centromeric Xp11.1-q11.1; CEP Y spectrum orange, alpha satellite, region centromeric Yp11.1-q11.1).

Qbiogene: two–color chromosome X/Y cocktail probe (X centromeric (DXZ1), alpha satellite, fluorescein and Y centromeric (DYZ3), alpha satellite, rhodamine).

Besides, we examined different combinations of Qbiogene probes:

Mixture of chromosome X centromeric (DXZ1), alpha satellite, rhodamine and chromosome 18 satellite probe, fluorescein, in proportion 1:1;

Mixture (and separately) of chromosome Y centromeric (DYZ3), alpha satellite probe, fluorescein and chromosome Ygh, classical satellite probe, rhodamine, in proportion 1:1;

Mixture of two-color chromosome X/Y cocktail probe (X centromeric fluorescein, Y centromeric rhodamine) and Ygh classical satellite probe, rhodamine; in proportion 1:1.

Hybridisation procedure:

The FISH procedure was performed according to the manufacturer's instructions with slight modifications.

Vysis probes hybridisation

For each slide, hybridization reaction was prepared by mixing 7 µl of CEP hybridization buffer (for repetitive sequence DNA probes) with 1 µl of probe and 2 µl of deionized water in Eppendorf tube for total 10 µl of hybridisation solution. The slides were dehydrated with 70%, 85%, and 96% ethanol, 2 min each, and then allowed to air dry.

After air-drying the slides, 10 µl of probe mixture were applied to slide and covered by coverslips. The slides were placed in HYBrite hot plate (Vysis). The probes and DNA were denatured at 72ºC for 2 min and then hybridized 37ºC for 16 hours.

The post-hybridization washes were made by transferring the slides to a bath of 0,4xSSC 0.3% Nonidet P-40 (NP-40) for 2 min at 72ºC and then were washed briefly in 2xSSC 0.1% NP-40 for 30 sec at RT. Then the slides were kept in the dark until air drying. Then for every slide DAPI/Antifade was applied and the slides were covered with glass coverslips.

Qbiogene hybridisation

The procedure was performed according to manufacturer`s protocol with slight modifications. The pretreatment of slides was made in 2XSSC, pH 7.0/0.5% Igepal (NP-40) at 37 C for 10 minutes. Then the slides were dehydrated with 70%, 80% and 95% Ethanol for 2 minutes each and then allowed to air dry. The probes were pre-warmed at RT and were applied in amounts of 10 µl to slides and covered by coverslips. The sample and probe were co-denatured in HYBrite hot plate (Vysis) at 80ºC for 2 min. The hybridization was at 37ºC overnight. The post-hybridization washing was performed in 0.5XSSC/0.1%SDS for 5 min at 37ºC without agitation. Then DAPI/Antifade was applied and the slides were covered with applied glass coverslips.

Fluorescence microscopy analysis

The FISH efficiencies for cord blood samples were determined by scoring the 100 cells from erythroblast population. All the erythroblasts that showed two clearly distinctive

hybridization signals were registered as correctly hybridized. Those erythroblasts that were recorded as XO, YO or OO were considered as a failure of the hybridization. All the signals observed with the triple band pass filter were confirmed with fluorochrome specific monochromatic filters (Carl Zeiss, Zürich, Switzerland).

After FISH the slides from maternal blood were rechecked for every recorded position of erythroblasts. The FISH result was evaluated according to the criteria, described above. Then Y signal screening for all slides was performed. The cells showed the representative signals were captured using CCD camera.

Morphometric analysis

The measurements were made on MGG images using the Zeiss Axiovision 3.1 measurement software (Carl Zeiss, Zürich, Switzerland). Every nucleus was measured along the longest axis and along shortest axis. The average of such two measurements for every nucleus was taken as "diameter" for further analysis. For every nucleus, the circumference of the nucleus was also measured. The N/C ratio was calculated from measurements of area of the nucleus and the cell

Terminal dUTP Nuclear End Labeling (TUNEL)

TUNEL staining was performed using a commercially available in situ cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. In brief, the cells were cytospinned on a glass slide, stained by the May-Grünwald-Gimsa (MGG) method and fixed with 4% paraformaldehyde/PBS solution at RT for 30 minutes. After washing with PBS the slides were permeabilized with 0.1% Triton X-100/0.1% sodium citrate for 5 minutes on ice and washed twice with PBS. Cells were then incubated with 50µl TUNEL mixture for 1h at 37º C in a humidified chamber. After washing twice, the slides were incubated with 50 µl Converter-AP for 30 minutes at RT, followed by incubation with the DAKO Fuchsin Substrate-Chromogen (DAKO) for 10 minutes at room temperature. As positive and negative controls, the samples were incubated with DNAase I and with label solution devoid of terminal deoxynucleotidyl transferase (TdT), respectively. The TUNEL staining result was checked by a light microscope (Zeiss Axioskop, Germany) for every recorded erythroblast.

Culture at different oxygen concentrations

Eight hundred microliters of cord blood were added immediately after delivery to 10 ml of PB-Max^{TM} (Gibco, Invitrogen AG, Basel, Switzerland) with 100 µl of Antibiotic/antimycotic solution.

Cells were then incubated for 24 hours under two different oxygen concentrations: 3% O_2 and 20% O_2 at 37^{o}C. To equilibrate the oxygen concentration in the medium, the medium was preincubated in the incubators for 2 hours at least. After 24 h of incubation the cells were transferred from the flask to 15 ml tubes and centrifuged at 1600 rpm x 10 min. The smears were made from fresh cord blood and from the same cases after cultivation. The slides were stained by MGG. The erythroblasts were then identified morphologically and counted. The relative number of erythroblasts was calculated as the number of erythroblasts per 100 nucleated cells.

Statistics

We used the Wilcoxon sign-rank test to analyze differences between the results for culture at 3% and 20% oxygen. P value (Asymp. Sig. (2-tailed)) less than 0.05 was considered as indicating the statistical significance.

To check the significance of difference in size between nuclei with XX FISH signals and without any signal and to analyze differences in distribution of spectra No.1 and spectra No.4 between cord blood erythroblast and the ones from maternal blood we used the Student's t-Test (2-tailed) assuming unequal variances, with a *p* value less than 0.05 indicating statistical significance. To check the significance of difference in size between nuclei with XX FISH signals and without any signal we applied ANOVA test also.

Laser Microdissection and Pressure Catapulting (LMPC) technology

MGG slides with and without special membrane from maternal blood cases which have demonstrated many erythroblasts and no FISH signals on them were subjected to laser microdissection and Pressure Catapulting (LMPC) (P.A.L.M. Microlaser Technologies, Bernried, Germany).

Single cell or pool of cells were collected in 15 µl of pro K solution/SDS (400ng/1µl) from every such case and frozen –20˚C overnight. After unfreezing the cells were centrifuged at 16000 rpm for 5 min and subjected to proteinase K (pro K) treatment at 56˚C for 8 hours with following inactivation pro K at 96˚C during 10 min. Thereafter the cells were centrifuged at 16000 rpm for 5 min and were tested by Real-Time PCR.

Real-Time Polymerase Chain Reaction (PCR)

Real-Time PCR specific for the SRY (sex-determining region Y-chromosome) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) genes or for 18 chromosome and 21 chromosome was carried out using a Perkin Elmer Applied Biosystems 7000 Sequence Detector (TaqMan, Applied Biosystems, Boston, USA). In brief, the 50 µl of amplification reaction mixture consisted of 300 nM of each primer and MGB labeled TaqMan probe, 25 µl TaqMan Universal PCR Master Mix (3.5 mM magnesium chloride, 100 mM dNTPs, 0.025 U AmpliTag Gold and 0.01 U Amp Erase (Perkin-Elmer, USA)). The following primers were used:

GAPDH forward primer 5`CCCCACACACATGCACTTACC3`

GAPDH reverse primer 5`CCTAGTCCCAGGCCTTTGATT3`

GAPDH MGB Probe 5`AAAGAGCTAGGAAGGACAGGCAACTTGGC3`

SRY forward primer 5`TCCTCAAAAGAAACCGTGCAT3`

SRY reverse primer 5`AGATTAATGGTTGCTAAGGACTGGAT3`

SRY MGB probe 5`CACCAGCAGTAACTCCCCACAACCTCTTT3`

18 chromosome forward primer 5`TGACAACCAAACGTGTGTTCTG3`

18 chromosome reverse primer 5`AGCAGCGACTTCTTTACCTTGATAA3`

18 chromosome MGB probe 5`GGTGTTTTGGAGGAGTT3`

21 chromosome forward primer 5`CCCAGGAAGGAAGTCTGTACCC3`

21 chromosome reverse primer 5`CCCTTGCTCATTGCGCTG3`

21 chromosome MGB probe 5`CTGGCTGAGCCATC3`

The following conditions were used for the PCR amplification: an initial incubation at 50ºC for 2 minutes and at 95ºC for 10 minutes, followed by 45 cycles of 60ºC for 1 minute and 95ºC for 15 seconds. In each real-time PCR analysis positive and negative controls were included.

Spectral Imaging

Fourier Transform Multipixel Spectrometry System for Microscopy. Spectral Imaging was performed using the SpectraCube SD-200 (Applied Spectral Imaging; Migdal HaEmek, Israel), (Malik et al., 1996), attached to a microscope (Axioscope 2 plus). Multipixel spectra were obtained from each cell in the range from 450 to 800 nm. A detailed description of the optical system is described by Malik et al., (1996) and Rothmann et al., (1997).

Library. The different spectra from normal erythroblast cells were analyzed. Five distinctive spectra: three from nuclei and two from cytoplasm, were collected into spectral library and a distinct pseudo color was assigned to each one of them. Reference spectrum No. 1 sampled from the condensed nuclei region was assigned red; reference spectrum No. 2 from the less condensed nuclei region, blue-violet; reference spectrum No. 3 from minimally condensed chromatin on the rim of the nuclei region, pink. Reference spectra No. 4 and No. 5 representing two distinct cytoplasm domains, were assigned green and blue, respectively.

Classification Analysis. To match each pixel in the analyzed image to the reference spectrum from library, so that spectral similarities were apparent, the classification algorithms were used. The criterion for this matching is minimal square error (MSE) mathematical function. The minimal square error algorithm measures the difference between the spectrum of each pixel composing an image and that of each reference spectrum. In such way, each pixel in the input image was identified with the most similar spectrum of the library; then, each pixel was displayed in an artificial color that represents a reference spectrum from library. The reconstructed image composed of artificial colors, reveals the degree of similarity between the library spectra and spectra of the image.

Standardization and normalization. In order to overcome differences in slides that resulted from the fact that the specimens were taken from different patients and were stained on distinct days and by a different batch of stain, the standard fixation and staining protocols were used. To eliminate the influence of light conditions during spectral capturing the same setup for microscopy was used. Variation in spectra may also result from differences in intensity. In order to neutralize the effect of intensity variations,

the classification was performed using normalized spectra and library. Normalization steps at classification were needed in order to treat uniformly the individual samples and to preserve a comparison consistent with the prelabeled spectral library.

Computational morphometric analysis. To provide objective quantitative data for cell characterization the region-of–interest being assessed was extracted from the pseudo-colored image and the area of this region in pixel was measured, then the percentage of area was calculated.

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6. Publications:

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