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Application of fluorescence *in situ* hybridization (FISH) as a tool to aid cytogenetics in 1,409 fetal samples

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Summary

Aim: To evaluate the technical application of fluorescence *in situ* hybridization (FISH) as a support to classical cytogenetic in numerical chromosomal aneuploidies studies in samples of amniotic fluid, chorionic villus, and fetal loss. *Materials and Methods:* The authors performed cytogenetic analyses in 1,409 patients (678 amniocentesis, 512 chorionic villus samples, and 219 spontaneous abortions) during one year. FISH molecular study aided traditional cytogenetic in 90 cases. These cases were indicated based on the diagnostic hypothesis of each patient or when no cellular growth was obtained. The authors standardized the FISH in discoloured slides. *Results:* They had 85% positive FISH in amniotic fluid, 70% in chorionic villus, and 90% in abortion material using 13, 18, 21 X and Y centromeric probes. It showed 12% of altered FISH in amniotic fluid (100% trisomies), 10% in chorionic villus (50% trisomy and 50% X - monosomy), and 22% in abortion material (50% trisomy, 25% X-monosomy, and 25% triploidy). FISH and cytogenetic analysis confirmed the results. *Conclusion:* This technique revolutionized clinical and research applications of cytogenetics. In this particular paper, FISH was a valuable and reliable technique to promptly identify rapid detection of aneuploidies in interphase cells, metaphase spread and paraffin-embedded samples. It is hoped that, in the future, the economic viability of array CGH and FISH, with the decreasing cost of testing and their genomics advantages can be incorporated as routine and customized in the approach of prenatal diagnosis.

Key words: Cytogenetic; Fluorescence in situ hybridization; Prenatal diagnosis; Spontaneous abortions.

Introduction

Cytogenetic prenatal diagnosis is utilized to detect chromosomal abnormalities in the fetus and it has been considered a safe and reliable method, which has been recognized for more than 30 years, mainly for pregnant women at increased risk to chromosomal abnormality [1-5]. These chromosome abnormalities are responsible for more than dozens identifiable syndromes, being more common than the monogenic Mendelian disorder. It is estimated that they affect 0.7% of live births, 2% of pregnancies at an older age, and 50% of spontaneous abortions in the first trimester [1,5]. These data emphasize the importance of human karyotype study.

Chromosome banding techniques, particularly G-band, which have been available since the seventies, represented a considerable progress in the area of human cytogenetics. This method made it possible to identify all chromosome pairs and also the breaking point observed in most structural rearrangements [6-10].

Therefore, with the great advances in genetics, the first fetal medicine services appeared in the eighties in North American and European universities. In the nineties, in Brazil the genetics laboratories had began switching to this new area forming a multidisciplinary team made up of physicians (specialists in fetal medicine, ultrasonographers, obstetricians, pediatricians, neonatologists, geneticists, pathologists), biologists, psychologists, nurses, and others [11].

Consequently, fetal medicine commenced with a variety of different purposes and preventive, diagnostic or therapeutic actions aimed at protecting, assessing, and assisting fetal health. Patients referring to the Fetal Medicine services must comply with the following criteria: advanced maternal age (over 35 years old), family history of chromosome abnormalities, abnormalities detected by ultrasound, exposure to radiation and drugs, prenatal infections, and chromosome X-linked diseases [12].

Patients are referred to prenatal diagnosis for amniocentesis or biopsy of the chorionic villus, based on evidence of high fetal loss risk and the gestational week. Chorionic villus sampling is performed between the 9th and the 12th gestational weeks, early amniocentesis, between the 12th and the 14th gestational weeks, traditional amniocentesis between the 15th and 20th gestational weeks, and percutaneous umbilical cord blood sampling between the 24th and the 30th weeks [11].

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The FISH technique on human metaphase and interphase nuclei using DNA probes (sequences of nucleic acids labeled with fluorochromes that identify specific regions of complementary DNA in chromosomes fixed on slides) has become an indispensable tool for the study of physical human genomic cartography, as it has allowed a accurate regional chromosomal localization of single copy genes or DNA repeated sequences. Furthermore, the introduction of methods for marking non-isotopic DNA probes allowed this technique to be performed in any cytogenetic laboratory, since it does not require the use of radioactive material [13-15]. Thus, with FISH it was possible to establish an early diagnosis of the numerical chromosomal aneuploidies [13, 16-19] in those cases with indication of chromosome abnormalities and also in those inconclusive cytogenetic results due to inadequate cellular growth for the cytogenetic study.

In situ hybridization offers new and extraordinary possibilities for gene mapping. Currently, it is possible to map any gene or DNA sequence that has been cloned. An additional advantage of this technique is the fact that it can be used to detect alterations in the metaphase and prometaphase, as well as in the interphase nuclei, and in paraffin-embedded material [13].

The application of FISH as an aid to cytogenetics is the fundamental importance in order to obtain a fast diagnosis of the numerical chromosomal aberrations, in amniotic fluid, and chorionic villus [20-23], as well as in spontaneous abortions and fresh or paraffin-embedded samples [14, 20-24].

The purpose of this study was to evaluate the technical application of FISH as an aid to cytogenetics in the study of the numerical chromosomal aneuploidies in samples of amniotic fluid, chorionic villus, and fetal loss, mainly in patients with increased risk to abnormalities chromosomal.

Materials and Methods

The sample consisted for 1,409 patients from the "Hospital São Paulo" and "Centro Paulista de Medicina Fetal", indicated for cytogenetic study, during one-year period. The cytogenetic analysis was performed in the laboratory of the "Centro Paulista de Medicina Fetal". Ethic Committees in Research of UNIFESP/Hospital São Paulo by CEP number 0559/02.

From the total samples (1,409), 678 correspond to patients that were submitted to amniocentesis for cytogenetic study of the amniotic fluid; 512 corresponding to patients submitted to biopsy of the chorionic villus and 219 correspond to spontaneously aborted products. Invasive prenatal testing was performed for the following indications: advanced maternal age, fetal abnormalities on ultrasound scanning, abnormal triple test (alpha-fetoprotein, hCG, unconjugated estriol), previous fetal abnormality, and maternal anxiety (usually because family history of malformations or aneuploidies, only advanced maternal age)

The cytogenetic study was implemented in 100 cases by FISH molecular study, indicated on the basis of the diagnostic hypotheses of each patient, such as: family history of chromosome abnormalities, abnormalities detected by ultrasound, suggestive of chromosome mainly aberrations (like 21, 13 or 18 trisomy) and in those cases where no cellular growth was obtained or hydatidiform mole.

FISH Protocol

The FISH technique was developed based on the studies of Speleman *et al.* [17], Kuchinka *et al.* [25], Eiben et al. [26], Shulman *et al.* [23], and Jobanputra *et al.* [27, 28], and standardized with some modifications of the Cytocell kit.

Amniotic fluid: Amniocentesis was performed between the 14th and 24th gestational weeks. By using ultrasound guidance, an average of 18 to 20 ml samples of amniotic fluid were collected and sent to the laboratory for centrifugation the fluid in two 15 ml tubes at 1,500 rpm in an eight-tube centrifuge. The supernatant was partially rejected, leaving two ml in each tube, which were homogenized and precipitated. The material from one of the tubes was placed in a culture medium for karyotyping and the other was used for FISH. To the latter, a hypotonic solution of 0.8% sodium citrate was added and left for ten minutes in an oven at 37°C and then centrifuged. A five-ml methanol/acetic acid (3:1) fixing solution was added to the precipitate and the material centrifuged for five minutes. This fixation was repeated twice. From the third fixation, three ml were reserved for dropping on previously iced slides and then placed on a water-bath at 60°C. The slides were left in an oven at 60°C for two hours for the following FISH pretreatment.

Chorionic villus: Chorionic villus biopsy was performed between the 11th and the 13th gestational weeks. The material was aspirated with ultrasound guidance and sent to the laboratory, where the chorionic villus was dissected with the aid of a stereomicroscope in order to start the direct preparation of the material. 0.1 ml of colcemid was added for 50 minutes to the material previously placed in the culture flask in an amniomax medium. After this period, the medium was removed and three ml hypotonic 1% sodium citrate was added, and the material placed in an oven at 37°C for five minutes.

A drop of fixing solution of methanol/acetic acid (3:1) was added, and the mixture left for five minutes at room temperature. The supernatant was removed, three ml of fixing solution were added, and the mixture left for ten minutes. The fixing of the material to the slides was then started using a plate heated at 45°C. After fixation, the slides were placed in an oven at 60°C for two hours moving on to item "a" below for the pretreatment of the slides.

Previously stained slides for cytogenetic analysis: The slides were discoloured by three increasing ethanol concentrations (70%, 85%, and 90%), and afterwards pretreatment was started.

"a" - pretreatment of the slides: The slides were incubated in 2xSSC (sodium citrate and sodium chloride solution) for 30 minutes at 37°C, dehydrated by increasing ethanol concentrations (70%, 85%, and 95%) for two minutes and left to dry at room temperature. After this stage, they were incubated with 70%/2xSSC formamide from three to five minutes in a water bath at 65°C for DNA denaturation. Then they were passed through increasing chilled ethanol concentrations (70%, 85%,95%) for two minutes and were left to dry at room temperature. *Hybridization*: ten ml of the probe was placed on each slide using an Eppendorf tube, and were left in an oven at 37°C along with the sample slides and cover slips for two minutes; after the probe was applied onto the sample and covered with the cover slip.

The sides of the cover slip were sealed with rubber cement and left for five minutes in the dark at 72°C. After that the slides were placed in a humid chamber in an oven at 37°C from 16 to 18 hours. After hybridization, the rubber cement was removed with tweezers and the slides were incubated in 0.4xSSC pH 70,

Table 1. — *Samples analyzed by the FISH technique and the respective success percentages.*

Sample	Nº of patients	FISH positive Nº	(%)	FISH negative Nº	(%)
Amniotic fluid	50	44	88	6	12
Chorionic villus	30	21	70	9	30
Abortion material	20	18	90	2	10
Total	100	83	83%	17	17%

with Tween in a water-bath at 72°C for five minutes without shaking. The slides were incubated in 2xSSC pH 7.0 for five minutes with shaking (~120 rpm) and finally placed in a 1xPBD (phosphate buffered detergent) solution for two minutes at room temperature. Finally they were stained with 15 ml DAPI or propidium iodate, covered with cover slips and sealed with enamel. The slides were left in the dark for 30 minutes before being analyzed in afluorescence microscope, equipped with individual filters for FITC, DAPI, and PI. From 100 to 150 nuclei were examined and the digitalized images were examined through the Q-FISH software.

Results

For the FISH analysis, 100 samples were selected, represented by amniotic fluid, chorionic villus, and spontaneous abortions. 81% of success was obtained, with 86% normal and 14% positive for aneuploidies (Table 1).

Using centromeric probes of chromosomes 13, 21, 18, X, and Y, trisomies were found in 70% of FISH analyses of the various types of materials, followed by X monosomy in 20% and triploidy in 10%. Trisomy 21 was the most frequent in amniotic fluid and in the chorionic villus, while trisomy 13 was more frequent in spontaneous abortions, beyond triploidy and X monosomy (Table 2).

Figure 1A represents the result of FISH in amniocytes with the chromosome 13/21 probe and Figure 1B shows the result using the centromeric probes of chromosomes 18, X and Y, stained with DAPI. The indication for this analysis was the advanced maternal age (AMA = 40 years old) with the presence of the ultrasound marker (golf ball) and

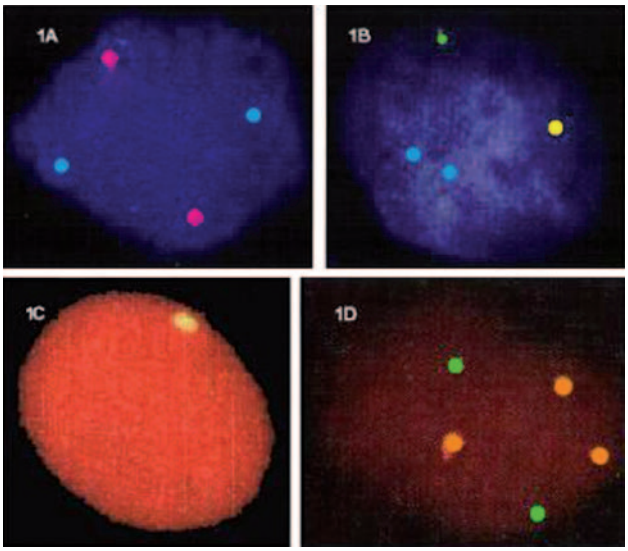

Figure 1. — A) FISH in amniocytes: visualization of the sign from the centromeric probes for chromosomes 13 and 21. Probe 13 •, probe 21•, nucleus stained with DAPI (×100). B) FISH in amniocytes: visualization of the sign of the centromeric probes for chromosomes 18, X, and Y. Probe 18 •, probe X •, probe Y •, nucleus stained with DAPI (×100). C) Visualization of the FISH sign in chorionic villus in interphase nucleus using the centromeric probes of X • and Y•. Nucleus stained with propidium iodate. X monosomy, one sign for X and none for Y (×100). D) Visualization of the FISH sign in prestained slides with interphase nucleus of abortion material. Probe 13•, probe 21• stained with propidium iodate. 13 Trisomy (×100).

TN= 3.4 mm, indicating risk of Down's syndrome. No cellular growth was obtained in the amniotic fluid and, consequently, the authors performed FISH for chromosomes 13/21, 18, X, and Y. The result was normal for all investigated chromosomes.

Figure 1C shows the results of FISH in chorionic villus of interphase nuclei with a single signal of the centromeric probe of chromosome X, stained with propidium iodate. In this case, the indication was an abnormality detected by ul-

Table 2. — *The distribution of the aneuploidies detected by FISH with the chromosomes probes (13, 21, 18, X, and Y), normal FISH and maternal age.*

Maternal age (years)	Nº of patients	No. of aneuploidies detected by FISH (100 patients) Trisomy 21	Trisomy 13	Trisomy 18	X monosomy	Triploidy	Normal FISH	TOTAL
≤ 20	52						2	2
21-25	236		2				4	6
26-30	327	1	1				19	21
31-35	355	1		1	1		30	33
36-40	315	3		1			12	16
41-45	109					1	4	5
46-48	15						1	1
Total	1409	5	3	2	1	1	71	83

trasound (cystic hygroma). One hundred interphase nuclei were counted and only one sign was visualized identifying X monosomy, confirmed by the cytogenetic study of the chorionic villus with karyotype 45,X.

Figure 1D shows the result of FISH in a slide containing abortion material. The material was placed in semidirect culture for 48 hours and the harvest and fixation was in seven slides. The slides were stained and once the existence of only interphase nuclei, and not metaphase was verified, the authors unstained the slides and performed FISH. In this case, they found three signs of chromosome 13 and two for 21, which led them to conclude that it was trisomy 13. This result was confirmed by the anatomopathological analysis.

In one of the samples of amniotic fluid that had inadequate cellular growth for karyotype analysis, the patient was with 25th gestational week and she presented ultrasound signs compatible with Patau syndrome (trisomy 13). In this case the FISH technique with probes for chromosomes 13 and 21 confirmed trisomy 13.

Discussion

In the last 30 years, the prenatal diagnosis with conventional cytogenetic analysis has been recognized as a safe and reliable method to determine chromosomal abnormality for couples with increased risk. However, in Brazil, lately FISH has played an important role in aiding cytogenetics in the identification of aneuploidies in prenatal diagnosis. The advent and development of new hybridization kits minimized many problems with the efficiency from FISH techniques in interphase nuclei [29-31].

Van Lijnschoten *et al.* [32], using centromeric probes of chromosomes 1, 16, 18, X, and Y in paraffin-embedded samples from abortions, concluded that FISH could be used in retrospective studies on this type of material, although the results with fresh material were better.

Jobanputra *et al.* [27, 28] reported 80% to 100% of success by applying FISH techniques in slides of amniotic fluid and chorionic villus, using centromeric probes of chromosomes 13, 18, 21, X, and Y. Of these results, 94% were normal, 6% altered and, of these, 97.3% were trisomy of chromosome 21. This study clearly shows the ability of FISH in detecting chromosome abnormalities in high risk pregnancies.

The present analysis showed that the results in samples of amniotic fluid, chorionic villus, and abortion material with respect to the success of the method, the results, and the importance of FISH to assist conventional cytogenetics in prenatal diagnosis were in accordance with Bryndorf *et al*., [33], Jobanputra *et al.* [28], Hsieh *et al.* [34], Moraes *et al.* [35], Tavokina *et al.* [36], Jovanovic *et al.*[37], and Braha *et al.* [38].

In amniotic fluid and chorionic villus the trisomy of chromosome 21 was the most frequent and in abortion material it was that of chromosome 13. These results were also confirmed by the literature [26, 28, 39], with the exception of the abortion material, since the centromeric probes of chromosomes 16, 22, and 15, were not included in the present analysis due to their the high cost.

For abortion material, also due to cost restrictions, Paradinas *et al.* [40] proposed FISH application only for the suspected cases of hydatidiform moles. In the present study, the authors realize the importance of FISH in paraffin material abortion, especially in cases where there was not cell growth (15%) and the results from the pathology suggested the presence of hydatidiform mole. To solve this paradigm the present authors will be studying these cases separately in the near future (Lewis *et al.*, 2013) [41].

Today, with the enormous advances with cytogenomics, the technique CGH array can be offered to specific cases in which there are morphological changes in ultrasound and normal karyotype. This technique enables the detection of gains and losses of small regions of genetic material that are not visualized by conventional cytogenetics [42-44]. Cytogenetics analyses using banding techniques can identify chromosome deletions and duplications in the range of 5–10 Mb, the higher resolution provided by microarrays can detect changes as small as 50–100 kb [23].

The application of molecular biology techniques has revolutionized the areas of science and technology, mainly in the field of fetal medicine, as a tool for cytogenetic study in the prenatal diagnosis of approximately 12% of the cases. This application has allowed the indication of safe results, mainly concerning the detection of numerical chromosomal aneuploidies through FISH for those cases with indication of chromosome abnormalities or where no cellular growth was obtained, thus avoiding a new harvesting of material.

It is hoped that in the future, the economic viability of array CGH and FISH, with the decreasing cost of testing and its advantages can become effective and the genetic and genomic data can be incorporated to customize the approach of prenatal diagnosis.

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