FISH Preimplantation Diagnosis of Chromosome Aneuploidy in Recurrent Pregnancy Wastage

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Purpose: Our purpose was to detect aneuploidy for chromosomes 13, 16, 18, 21, 22, X, and Y in preimplantation embryos from patients with a history of unexplained recurrent miscarriage.

Methods: Three patients with a history of unexplained recurrent spontaneous abortion were included in this study. Embryos were biopsied at the eight-cell stage, individually fixed on slides, and processed for fluorescent in situ hybridization (FISH). A multiple FISH protocol for seven chromosomes pairs (13, 16, 18, 21, 22, X, and Y) has been developed.

Results: A total of 39 embryos was studied with the multiple FISH protocol developed. Successful analysis of the biopsied embryos was achieved within the time limits usually allowed in a preimplantation diagnosis program. Analysis of the blastomeres showed that 17 embryos were chromosomally normal for the probes used, 16 embryos were aneuploid, and in 6 embryos no informative results were obtained.

Conclusions: In the patients studied, a large proportion of embryos (41%) exhibited chromosomal abnormalities for the probes used. Preimplantation diagnosis to screen for chromosome abnormalities could be a feasible approach to improve the possibility of successful pregnancy in these couples.

KEY WORDS: aneuploidy; fluorescent in situ hybridization; miscarriage; preimplantation genetic diagnosis.

INTRODUCTION

Clinically, recurrent abortion has been defined as two or more consecutive miscarriages or more than three nonconsecutive pregnancy losses (1). The etiology of recurrent pregnancy loss remains obscure in most cases. Usually, the diagnostic workup in these couples includes an evaluation of risk factors (endocrine factors, uterine anomalies, immunologic factors, cytogenetic factors, etc.) (2). A possible therapeutic approach, based on the elimination of some of the risk factors, has been reported to improve pregnancy rates (3,4).

It is well-known that a majority of early pregnancy wastage is related to chromosomal abnormalities (trisomy, polyploidy, and X monosomy). More than 50% of first-trimester spontaneous abortions are chromosomally abnormal, and the incidence of cytogenetic abnormalities can exceed 80% in the periimplantation period (5–8). Overall, it seems clear that embryonic chromosome abnormalities could be an important etiologic factor in spontaneous abortion.

Furthermore, the fact that oocyte donation is used by several groups as an effective therapeutic approach for these patients supports the idea that, in some cases, the origin of abortions in these women may be related to “oocyte factors.”

Of the abnormal karyotypes observed in analyzed spontaneous abortions, the most common ones are trisomies (50%), mainly autosomal (chromosomes 16, 22, 21, 15, 14, 8, 18 and 13), and Turner syndrome (5–6%), and polyploidies are the second most common abnormalities detected (9).

Based on these data, we decided to initiate a prospective study, within our cytogenetic preimplantation genetic diagnosis (PGD) program, in patients with a history of unexplained recurrent miscarriage.

MATERIALS AND METHODS

This project has been approved by the Institutional Review Board on the Use of Human Subjects in

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Research at the Instituto Valenciano de Infertilidad and by the Ethical Committee on Animal and Human Experimentation of the Universitat Autònoma de Barcelona and complies with the Spanish Law of Assisted Reproductive Technologies (35/1988).

Preimplantation Embryos

Three patients with a history of unexplained recurrent spontaneous abortion, younger than 35 years of age, were included in this study. In all cases, exhaustive screening for repetitive miscarriage (endocrine tests, morphological examination, karyotyping, human leukocyte antigens, immunologic tests, etc.) was performed.

Ovarian stimulation, fertilization, and embryo culture were performed as standardized at the Instituto Valenciano de Infertilidad (2). Intracytoplasmic sperm injection (ICSI) was performed to ensure high fertilization rates and to avoid sperm attachment to the zona pellucida, which could interfere with embryo biopsy.

Embryos were biopsied at the eight-cell stage; one or two blastomeres were removed, individually fixed on slides as described by Tarkowski (10), and processed for fluorescent in situ hybridization (FISH).

Embryos were kept in a coculture system with autologous endometrial epithelial cells until transfer. Chromosomally normal embryos for the probes used which reached the blastocyst stage were selected for transfer.

FISH

We used probes, either repetitive or locus specific, directly labeled or indirectly labeled, to identify chromosomes 13, 16, 18, 21, 22, X, and Y. These were directly labeled probes for chromosome 13 (13q14, locus RB, LSI 13; Vysis, Downers Grove, IL), chromosome 18 (18p11-q11, locus D18Z1, CEP 18; Vysis), chromosome 21 (21q22.13-q22.2, loci D21S259, D21S341, D21S342, LSI 21; Vysis), chromosome 22 (22q11.2, locus bcr, LSI 22; Vysis), the X chromosome (Xp11-q11, locus DXZ1, CEP X; Vysis), and the Y chromosome (Yq12, locus DYZ3, VYSIS), and a digoxigenin-labeled probe for chromosome 16 (16p11.1-q11.1, locus D16Z2; Oncor, Gaithersburg, MD).

FISH was performed in two consecutive hybridization rounds following standard protocols and according to the manufacturer's instructions.

First Hybridization Round. In the first round, the probe mixture used was made of LSI 22 Spectrum Green, dual-color probe LSI 13 Spectrum Green/LSI

21 Spectrum Orange, LSI 13 Spectrum Orange, and the digoxigenin-labeled probe for chromosome 16. Hybridization was carried out for 6 hr at 37°C in a moist chamber.

Directly labeled probe evaluation was performed after standard washing of the slides and DAPI counterstaining.

Analyses were done using an Olympus BX60 epifluorescence microscope equipped with a triple-band pass filter for DAPI/Texas Red/FITC and single-band pass filters for FITC, Texas Red, and Aqua Blue. Images were either photographed or captured in a software format.

After slide evaluation for chromosome probes 13, 21, and 22, coverslips were removed in 1× PBD solution (Oncor). Amplification and detection of the digoxigenin-labeled probe for chromosome 16 were performed according to the manufacturer's instructions using fluorochrome-labeled antibodies. Slides were then counterstained with DAPI/antifade, covered with a coverslip, and reevaluated for chromosome 16 signals. Images were again photographed or captured in a software format.

Second Hybridization Round. First-round probe-signal elimination was done by washing the slide in 0.0625× SSC solution for 3 min at 73°C. Slides were subsequently dehydrated in alcohol solutions (70, 80, and 96%) and allowed to air-dry.

The second probe hybridization mixture used was made of CEP 18 Spectrum Green, CEP X Spectrum Orange, and CEP Y (satellite III) Spectrum Aqua. Ten microliters was added per slide and covered with a cover slide. Hybridization was carried out at 42°C, for 45–60 min in a dry chamber.

Directly labeled probe evaluation was performed after standard washing of the slides and DAPI counterstaining. The slides were again counterstained with DAPI/antifade, covered with a coverslip, and evaluated.

For evaluation of the second round of hybridization, the images obtained from the first round were used as controls for location of the fluorescent signals.

The total time elapsed from the beginning to the end of the diagnostic procedure was 12–14 hr.

RESULTS

By April 1997, a total of four treatment cycles from three patients had been performed. A total of 39 embryos suitable for blastomere biopsy was obtained, providing 48 blastomeres (Table I).

Table I. Patient Data and Preimplantation Diagnosis Results

Cycles	Age	Previous miscarriages	Biopsied embryos	Normal	Aneuploid	Noninformative	Embryo transfers	Pregnancy
1	34	4	5	2	2	1	1	No
2	32	5	9	6	3	0	2	No
3 ^a	34	6	10	3	5	2	2	No
4 ^a	35	6	15	6	6	3	4	Yes

^a Two cycles in the same patient.

Analysis of the blastomeres (Table I) revealed that 17 of the 39 embryos analyzed were chromosomally normal for the probes used, 16 embryos were aneuploid for one or more of the chromosomes analyzed, 3 embryos were noninformative for some of the probes used, and 3 embryos were noninformative as a result of biopsy characteristics (anucleated blastomere, large cytoplasmic debris, etc.).

Of the 16 abnormal embryos found, in 10 a single aneuploidy was detected (2 of them involving sex chromosome aneuploidies and 8 autosomal aneuploidies), multiple chromosome aneuploidies were observed in 4 embryos, and in 2 cases, haploid embryos (or embryos monosomic for all probes used) were detected. Detailed FISH results are given in Table II.

In the four cycles, chromosomally normal embryos for the probes used, which reached the morula or blastocyst stage, were selected for transfer. A total of nine embryos was transferred (Table I) and a single pregnancy was obtained. However, the fetus arrested its development at 8 weeks of gestation. Cytogenetic analysis of the abortive material revealed a normal karyotype (46, XY).

Table II. Summary of FISH Results

Normal	17
Aneuploid	
Haploid	2
Monosomy 13	2
Monosomy 16	1
Monosomy 18	1
Monosomy 21	1
Trisomy 13	1
Trisomy 16	1
Trisomy 21	1
XXY	1
XXX	1
Monosomy 16, monosomy X0	1
Monosomy 16, monosomy 21, monosomy 22	1
Nullisomy 16, monosomy 21, monosomy Y0	1
Monosomy 22, trisomy 16, trisomy 21. XYY	1
Noninformative	
For some of the probes used	3
Due to biopsy characteristics	3

DISCUSSION

PGD aneuploidy detection has been reported for up to five chromosomes (chromosomes 13, 18, 21, X, and Y), allowing for the screening of the most common aneuploidies at birth (11). In our study chromosomes 16 and 22 have been also evaluated to cover the most commonly detected abnormalities observed in spontaneous abortions (9).

Recently, Benadiva *et al.* (12) have evaluated the incidence of aneuploidy for chromosome 16 by reanalyzing nuclei for cells already studied with probes for chromosomes 13, 21, 18, X, and Y. In our PGD program, we have routinely incorporated a probe for chromosome 16 in patients with repeated abortions. The use of seven probes and of locus-specific probes requires a longer hybridization and screening time. However, using the protocol described in this work, results for the seven probes can be obtained in 12–14 hr. This allows transfer of the embryos within the time allowed in any PGD program, more specifically, in our PGD program, where transfer is carried out on day 5 or 6.

One of the limitations of the protocol is the evaluation of seven different probes in a single nucleus, because some colors must be used twice. However, probe-signal elimination, as described by Escudero *et al.* (13), can be successfully used to overcome the problem of simultaneous evaluation of more than five probes, thus allowing for a more accurate cytogenetic analysis in preimplantation diagnosis.

These preliminary results reflect that, in the patients studied, a large proportion of embryos [16/39 (41%)] exhibits chromosomal abnormalities. The aneuploidy figures observed in these embryos (41%) are close to the 37.2% reported by Munné *et al.* (14) in apparently normal embryos from older women (older than 40 years) and 10 times higher than the incidence of aneuploidies (4%) reported by the same author in embryos from women aged 20–34.

The limited data obtained in this study, where aneuploidies for all the probes used were observed, suggest

that these patients may be prone to the production of oocytes with multiple aneuploidies, maybe resulting from synaptic anomalies such as those described in infertile males (15) rather than resulting from single nondisjunction events. Possibly other probe combinations would provide similar results. In this sense, it has been suggested that the production of normal or aneuploid oocytes could be patient specific (16). Patients with multiple aneuploid oocytes may, therefore, be predisposed to recurrent failures. In fact, in recent cycles (unpublished data) we have observed two cases with almost-complete cohorts of aneuploid embryos.

Data from our series ("controls" from blastomeres after preimplantation diagnosis for sex-linked diseases /recurrent pregnancy loss) are still very limited and statistical comparisons of aneuploidy levels would be inaccurate to report. In this sense, a larger group of patients is under evaluation.

Overall, our results suggest that chromosome abnormalities could be an important etiologic factor responsible for the unexplained recurrent pregnancy losses observed in some of these patients. Thus, although we are aware that these are preliminary results and that the incidence of numeric abnormalities in embryos from patients with recurrent pregnancy loss needs further evaluation, preimplantation diagnosis to screen for the aneuploidies that are most common in spontaneous abortions could be a reliable approach to successful improve pregnancy rates and evolutive pregnancies in these couples.

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REFERENCES

1. Stirrat G. Recurrent miscarriage I. Definition and epidemiology. *Lancet* 1990;336:673-675
2. Berry CW, Brambati B, Eskes TKAB, Exalto N, Fox H, Geraedts JPM, Gerhart I, Gonzales Gomes F, Grudzinkas JG, Hustin J, Jouppila P, Lindblom, Mantoni M, Montenegro N, Nogales Fernandes F, O'Rahilly R, Pedersen JF, Peters PWJ, Regan L, Rushton DI, van Straaten HWM, Tarlatzis BC, Wells M: The Euro-team early pregnancy (ETEP) protocol for recurrent miscarriage. *Hum Reprod* 1995;10:1516-1520
3. Simón C, Gutierrez A, Vidal A, de los Santos MJ, Tarín JJ, Remohí J, Pellicer A: Outcome of patients with endometriosis in assisted reproduction: Results from in vitro fertilization and oocyte donation. *Hum Reprod* 1994;9:725-729
4. Balasch J, Creus M, Fábregues F, Cívico S, Carmona F, Martorell J, Vanrell JA: In vitro fertilization treatment for unexplained recurrent abortion: A pilot study. *Hum Reprod* 1996;11:1579-1582
5. Hassold T: Chromosome abnormalities in human reproductive wastage. *Trends Genet* 1978;2:105-110
6. Hasold TJ, Jacobs PA: Trisomy in man. *Annu Rev Genet* 1984;18:69-97
7. Brambati B: Fate of human pregnancies. In *Establishing a Successful Human Pregnancy*, RG Edwards (ed). New York, Raven Press, 1990, pp 269-281
8. Eiben B, Bartels I, Bähr-Porsch S, Borgmann S, Gatz G, Gellert G, Goebel R, Hammans W, Hentemann M, Osmers R, Rauskolb R, Hansmann I: Cytogenetic analysis of 750 spontaneous abortions with the direct-preparation method of chorionic and its implications for studying genetic causes of pregnancy wastage. *Am J Hum Genet* 1990;47:656-663
9. Egozcue J: Of course not. *Hum Reprod* 1996;11:2077-2078
10. Tarkowski AK: An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* 1966;5:394-400
11. Munné S, Lee A, Rosewaks Z, Grifo J, Cohen J: Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum Reprod* 1993;8:2185-2191
12. Benadiva CA, Kligman I, Munné S: Aneuploidy 16 in human embryos increases significantly with maternal age. *Fertil Steril* 1996;66:248-255
13. Escudero T, Coll MD, Fuster C, Egozcue J: Cytogenetic analysis using simultaneous and sequential fluorescence in situ hybridization. *Cancer Genet Cytogenet* 1998;100:111-113
14. Munné S, Alikani M, Tomkin G, Grifo J, Cohen J: Embryo morphology, developmental rates and maternal age are correlated with chromosome abnormalities. *Fertil Steril* 1995;64:382-391
15. Egozcue J, Templado C, Vidal F, Navarro J, Morer-Fargas F, Marina S: Meiotic studies in a series of 1100 infertile and sterile males. *Hum Genet* 1983;65:185-188
16. Plachot M: Oocyte-genetic aspects. In *Gametes. The Oocyte*, JG Grudzinkas, JL Yovich (eds). Cambridge, Cambridge University Press, 1995, pp 95-107