Advantages of Day 4 Embryo Transfer in Patients Undergoing Preimplantation Genetic Diagnosis of Aneuploidy

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Purpose: Following preimplantation genetic diagnosis of aneuploidy, embryo transfer was executed on day 4, with the aim of providing more time for expanding from six to nine the number of diagnosed chromosomes per single cell (Group 2; 45 cycles). The results obtained were compared to those derived from conventional day 3 transfer (Group 1; 71 cycles).

Methods: For multicolor fluorescence in situ hybridization analysis, two panels of probes were used: the first, specific for chromosomes XY, 13, 16, 18, and 21, was tested in all patients (Groups 1 and 2); the second was implemented only in Group 2 patients for the detection of chromosomes 14, 15, and 22.

Results: A total of 406 embryos underwent fluorescence in situ hybridization analysis in Group 1, and 236 in Group 2. Comparable percentages of both chromosomal abnormalities (61% and 62%) and pregnancy and implantation rates (36% and 24.5% in Group 1, 41% and 23.6% in Group 2) resulted, regardless of the higher mean age in Group 2.

Conclusions: The diagnosis of the nine chromosomes which are most frequently associated with aneuploidy in humans could have an immediate impact on the rate of spontaneous abortions. Additional advantages are represented by the more accurate morphological evaluation of euploid embryos; the advanced compaction, which means that embryos are less exposed to damage during the transfer procedure; and the possibility of performing a reanalysis in cases where a fluorescence in situ hybridization diagnosis is not obtained.

KEY WORDS: aneuploidy; FISH analysis; preimplantation genetic diagnosis; spontaneous abortions.

INTRODUCTION

Implantation of embryos in the endometrium is a fundamental aspect of mammalian reproduction

depending on a complex interplay of events where a synchronized interaction between the growing embryo and the uterus is imperative.

The low implantation rates resulting from assisted reproductive techniques have been related to many factors; among them, the high incidence of chromosomal disorders which originate during oogenesis and early cleavage events in stimulated cycles have been proposed to play a major role (1,2). This condition is especially notable in patients of advanced reproductive age, whose decline in terms of take-home baby rate is clearly related to an increased rate of aneuploidy, as demonstrated by the analysis of oocytes and embryos generated in vitro (3,4). Indeed, data from early spontaneous abortions suggest that a notable proportion of aneuploid embryos is lost very soon after transfer into the uterus even before a clinical pregnancy is recognized, possibly due to their abnormal constitution.

Based on this consideration, it is logical to hypothesize that the preimplantation genetic diagnosis (PGD) of aneuploidy could offer a dramatic advantage in terms of full-term pregnancy through the selection of embryos with a normal chromosomal status. For instance, some categories of patients diagnosed with a poor prognosis for pregnancy have been identified to have percentages of chromosomally abnormal embryos of between 55% and 63% (5). In these cases, PGD of aneuploidy could play a fundamental role in the selection of embryos endowed with the full potential of developing to term (6).

An interesting correlation has been shown between embryo morphology and chromosomal abnormalities. Actually, the lowest incidence of anomalies (42%) is exhibited in embryos which are at the seven- to eight cell stage at 62–64 hr after insemination (7). Hence, blockage of development in the case of an aberrant genome should occur at later stages, when embryos begin to rely completely on their own transcriptional machinery. Consequently, an extended period of cul-

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ture could permit more accurate selection based on the identification of those embryos capable of going through the fourth division, as an indicator of the ongoing genomic activity.

Therefore, we decided to delay the embryo transfer of biopsied embryos to day 4, with the following purposes: (a) improving the criteria for embryo selection on the basis of chromosomal analysis and morphological evaluation; (b) having more time to expand the number of analyzed chromosomes by performing two rounds of hybridization; and (c) decreasing the risk of damaging the embryos during the transfer procedure due to more advanced compaction.

In this sequential study the preliminary results obtained with day 4 transfer are presented and compared to those derived from the conventional schedule on day 3. The chromosomal analysis was carried out with the multicolor fluorescence in situ hybridization (FISH) technique for the simultaneous screening of chromosomes XY, 13, 16, 18, and 21. In addition, a second round of FISH analysis in the same blastomere to detect chromosomes 14, 15, and 22 was implemented in the group of patients who had the transfer programmed on day 4.

MATERIALS AND METHODS

Patients

A total of 86 patients classified as poor prognosis underwent 116 assisted conception cycles with PGD of aneuploidy between September 1996 and June 1998. Indications for chromosomal analysis were (a) maternal age ≥ 36 years (64 cycles), (b) three or more previously failed IVF attempts (26 cycles), and (c) an altered karyotype detected in peripheral blood, due to gonosomal mosaicism (17 cycles) or balanced translocations (9 cycles).

Ovarian stimulation was carried out by a desensitizing protocol using a long-acting gonadotropin releasing hormone analogue preparation in combination with human menopausal gonadotropins (8). Transvaginal oocyte retrieval was executed 36 hr after human chorionic gonadotropin administration; the collected oocytes were preincubated in Earle's medium supplemented with 10% heat-inactivated maternal serum at 37°C in a humidified gas phase of 5% CO₂ in air.

Fertilization Control and Embryo Evaluation

Oocytes were scored at 16–18 hr after insemination for evidence of normal fertilization. Embryo evaluation

was performed at 24 and 48 hr later; the number and morphology of nuclei and blastomeres and the degree of fragmentation were recorded. Following FISH analysis, chromosomally normal embryos were transferred to the uterus on day 3 in the late afternoon (Group 1; 71 cycles from September 1996 to November 1997) or day 4 in the morning (Group 2; 45 cycles from December 1997 to June 1998).

Clinical pregnancies were confirmed by ultrasound analysis for the presence of a fetal heartbeat. The implantation rate represented the number of gestational sacs with fetal heart activity divided by the total number of embryos transferred.

Blastomere Biopsy and FISH

Day 3 embryos with normal morphology were selected for FISH analysis. Each embryo was biopsied individually in HEPES-buffered medium. Zona pellucida was opened with acidic Tyrode's solution; the biopsied blastomere was placed in hypotonic solution, fixed on a glass slide, and dehydrated in ethanol (9).

For multicolor FISH analysis, two panels of probes were used: the first, specific for chromosomes XY, 13, 16, 18, and 21, was tested in all patients (Group 1 and Group 2), whereas the second, detecting chromosomes 14, 15, and 22, was implemented only in Group 2 patients.

The first panel included six probes for the simultaneous screening of chromosomes X (alpha satellite, DXZ1; Vysis Inc. Downers Grove, IL), Y (satellite III, DYZ1; Vysis), 13 (RB-1 locus, 13q14, expanding 440 kb; Vysis), 16 (alpha satellite; Vysis), 18 (alpha satellite; D18Z1, Vysis), and 21 (region 21q22.13–q22.2; Vysis) (10). These probes were labeled with Spectrum-Orange, Spectrum-Green, and/or Spectrum-Aqua (Vysis). They were combined in the following quantities (volumes refer to commercial stock solutions except where indicated with specific concentration values): 36 μ l Spectrum-Aqua CEP X, 16 μ l Spectrum-Aqua CEP Y, 12 μ l Spectrum-Green CEP Y, 30 μ l Spectrum-Orange LSI 13 (200 ng/ μ l), 24 μ l Spectrum-Green LSI 13 (200 ng/ μ l), 30 μ l Spectrum-Green LSI 16 (125 ng/ μ l), 22 μ l Spectrum-Aqua CEP 18, 14 μ l Spectrum-Orange CEP 18, and 30 μ l Spectrum-Orange LSI 21 (200 ng/ μ l). The probe mixture was concentrated with a Speed-Vac centrifugal evaporator (DyNA VAP, National Labnet Company) to a final volume of 90 μ l and added to 210 μ l of WCP hybridization buffer (Vysis). Ten microliters of the resulting hybridization solution was applied to the fixed blastomere on the glass slide and covered with an 18 $\times$ 18-mm coverslip. The slide was then placed for 3 min on a hot plate at

78°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for 4 hr. After washing at 72.5°C in 0.4 × saline-sodium citrate (SSC) for 2 min and counterstaining with 10 µl of DAPI in antifade solution (4', 6-diamino-2-phenyl indole; Oncor, Gaithersburg, MD), the slide was observed under a fluorescent microscope (Olympus B X 40) equipped with the triple-band pass filter set for simultaneous observation of the Spectrum-Orange, Spectrum-Green, and Spectrum-Aqua fluorescence: the X chromosome-specific signal appears as blue, the Y as white to yellow, the 13 as orange, the 16 as green, the 18 as pink, and the 21 as red. A 4',6-diamino-2-phenylindole filter (Olympus) was applied to find the nuclei.

After analysis of the first set of probes, the slide was washed in 1 × PBD (Oncor) to remove the coverslip and then dehydrated (70%, 85%, and 100% ethanol, 2 min each). For the second round of hybridization, a new protocol was applied using chromosome 14-, 15-, and 22- specific probes. This hybridization solution was prepared by mixing 2.5 µl of a probe for chromosome 14 (binding to the 14q11.2 region) labeled with Spectrum-Aqua, 1 µl of a probe for chromosome 15 (binding to the alpha satellite region) labeled with Spectrum-Orange, and 1 µl of a probe for chromosome 22 (binding to the 22q13.3) labeled with Spectrum-Green. The mixture was concentrated to 3 µl with a Speed-Vac centrifugal evaporator and mixed with 7 µl of WCP hybridization buffer (Vysis). Ten microliters of the second hybridization solution was applied to the slide. Denaturation was performed on a hot plate at 80° for 3 min, followed by 2 hr of hybridization at 37°C. After washing for 30 sec in 0.4 × standard saline citrate at 71°C, DAPI was applied and fluorescence analyzed.

The scoring criteria described by Munné and Weier (11) were followed without modification.

One blastomere was routinely biopsied from each embryo. The FISH signals were evaluated as follows: blastomeres with two specific signals for gonosomes and each chromosome were considered to be euploid; blastomeres which lack one or two signals, monosomic; blastomeres with one or two extra signals, trisomic; and blastomeres which exhibit monosomic or trisomic signals simultaneously, mosaics; blastomeres with one signal per chromosome were considered haploid, whereas three or more signals designated polyploid blastomeres.

Statistical Analysis

Data were evaluated by χ^2 analysis 2 × 2 contingency tables, applying Yates' correction.

RESULTS

A total of 780 embryos was generated, of which 663 were selected for blastomere biopsy on the basis of morphological appearance. The assessment of chromosomal status was performed on one blastomere in 640 embryos (96%), whereas for the remaining 23 embryos (4%) a second blastomere was analyzed. As shown in Table I, 249 embryos were diagnosed chromosomally normal after FISH analysis, 393 presented with numerical aberrations, and in 21 no result was achieved due to technical problems during the procedure. In all, embryo transfer was carried out in 91 cycles, with an average of 2.3 ± 1.3 euploid embryos. In the remaining 25 cycles, no FISH normal embryos were detected; therefore, embryo replacement was canceled. Thirty-four clinical pregnancies were initiated, yielding an implantation rate of 24.2%. Up to the present, 22 healthy babies were born from 15 pregnancies (8 singleton and 7 twin), and 16 are regularly ongoing beyond the 12th week of gestation. Three pregnancies ended in abortion: two early miscarriages and one ectopic. Pregnant patients were invited to undergo prenatal diagnosis to verify FISH results, which were all confirmed.

Table II illustrates the FISH and clinical results inherent to Groups 1 and 2. A total of 406 embryos was FISH diagnosed in Group 1 and 236 in Group 2, yielding comparable percentages of chromosomal abnormalities (61% and 62%, respectively). The two panels of probes contributed to the diagnosis of Group 2's embryos as follows: 119 embryos (82%) exhibited abnormalities for chromosomes XY, 13, 16, 18, or 21, whereas 27 (18%) were aneuploid for chromosome 14, 15, or 22. Pregnancy and implantation rates did not differ significantly between the two groups despite the higher mean maternal age in group 2.

All blastomeres from 146 abnormal embryos from Groups 1 and 2 were spread and analyzed in order to

Table I. PGD of Aneuploidy: Overall Outcome in Patients with a Poor Prognosis for Pregnancy

No. cycles	116
Age (M ± SD)	36.1 ± 4.2
No. embryos generated	780
No. FISH-analyzed embryos	663
Normal (%)	249 (38)
Abnormal (%)	393 (59)
No result (%)	21 (3)
No. embryos transferred (M ± SD)	211 (2.3 ± 1.3)
No. transferred cycles	91
No. clinical pregnancies (%)	34 (37)
Implantation rate (%)	24.2

Table II. FISH Results and Clinical Outcome in Groups 1 and 2

	Group 1	Group 2
No. cycles	71	45
Age (M $\pm$ SD)	35.4 $\pm$ 4.1*	37.2 $\pm$ 4.3*
No. diagnosed embryos	406	236
FISH normal (%)	159 (39)	90 (38)
FISH abnormal (%)	247 (61)	146 (62)
XY, 13, 16, 18, 21 (%)	247	119 (82)
14, 15, 22 (%)	—	27 (18)
No. transferred cycles	59	32
No. transferred embryos	2.4 $\pm$ 1.3	2.3 $\pm$ 0.8
No. clinical pregnancies (%)	21 (36)	13 (41)
Early abortions	2	0
Ectopic	1	0
Implantation rate (%)	24.5	23.6

* $P = 0.02$.

estimate the error rate inherent to the FISH analysis performed on one cell. As illustrated in Table III, 134 (92%) embryos had the diagnosis confirmed, whereas 8 (5%) were euploid and 4 (3%) were carriers of complex abnormalities.

DISCUSSION

PGD of aneuploidy is an exciting new advance in the field of reproductive medicine aimed to give patients with a poor prognosis a better chance of conceiving a healthy baby through the identification of embryos possessing a normal chromosomal assessment. At this time, the results obtained are promising and demonstrate that the possibility of selecting embryos on the basis of both morphology and chromosomal status correlates with an increased implantation potential. The advantage is especially evident when

the selection is carried out within a large cohort of embryos, thus leading to the transfer of two or three euploid embryos per patient (6).

The major limiting factors for the application of FISH analysis in preimplantation embryos are the time and number of cells available for the screening. Indeed, the genetic results need to be ready in a few hours in order to allow the embryo transfer to be executed in the fresh cycle, usually in the late afternoon of day 3. In addition, using a single cell for the genetic analysis implicates a 3% risk of not obtaining results due to technical problems and a 5% error rate (Table III). Consequently, the possibility of postponing the transfer to the following day could provide some additional time either to confirm unclear results or to amplify the screening range of FISH analysis. Therefore, starting from December 1997, we decided to program the embryo transfer of biopsied embryos on day 4; simultaneously, three additional probes were included, thus leading to the overall diagnosis of the nine chromosomes which are associated in humans with approximately 70% of the aneuploidies detected in spontaneous abortions.

The data generated in the present study reveal that pregnancy and implantation rates do not differ following transfer on day 3 or day 4, thus excluding any detrimental effect related to an extended culture period of biopsied embryos, as already anticipated in a previous report (12). Interestingly, the absence of miscarriages in Group 2 could be due, at least in part, to the contribution of information deriving from the screening of chromosomes 14, 15, and 22. As shown in Table II, the participation of these chromosomes to the total number of aneuploidies is substantial and their screening could represent a

Table III. Confirmation of FISH Results

	One-cell analysis	Re-analysis		
		Confirmation	Normal	Other anomalies
Monosomy (%)	46	38 (83)	6	2 mosaics
1 chromosome	31	28 (90)	3	—
2 chromosomes	13	10 (77)	3	—
3 chromosomes	2	0	0	2
Trisomy (%)	35	33 (94)	2	—
1 chromosome	27	25 (93)	2	—
2 chromosomes	6	6 (100)	—	—
3 chromosomes	2	2 (100)	—	—
Haploidy (%)	15	14 (93)	—	1 mosaic
Polyploidy (%)	15	14 (93)	—	1 mosaic
Mosaic (%)	35	35 (100)	—	—
Total (%)	146	134 (92)	8 (5)	4 (3)

notable improvement in the whole procedure. Surprisingly, the total rate of aneuploidies is not different between the two groups, suggesting a possible increase in complex errors involving more than one chromosome. Although more data need to be collected, the clinical trend is promising, especially when considering that patients in Group 2 have a higher mean age compared to patients in Group 1.

As an additional advantage, transfer on day 4 permits combining morphological evaluation and FISH results. Indeed, extension of the culture period beyond embryonic genome activation permits discrimination of embryos with a slower or arrested cleavage rate from those undergoing regular cell division, demonstrating viability, and consistently capable of implantation (13–15). In the case of biopsied embryos, this strategy could be especially important, as compaction has already taken place at this stage, and shedding of blastomeres through the hole in the zona pellucida during the transfer procedure is less likely. Moreover, the results obtained after thawing discourage the implementation of freezing on biopsied embryos and suggest extending the culture period of those with a normal chromosomal complement and selecting for transfer those presenting a higher cleavage rate (16).

Furthermore, in cases where either unclear results or no results are obtained after FISH on a single cell, delay of the transfer to day 4 provides the further possibility of either rehybridizing the same cell for reanalysis or biopsying a second cell and performing the chromosomal diagnosis de novo. In the current study, this approach made it possible to recognize three embryos as euploid, which had been diagnosed with no result at the first attempt. Hence, whenever a reanalysis needs to be carried out to obtain a diagnosis, a second blastomere can be removed and analyzed, with results available for the morning of day 4; this strategy is especially valuable in patients who do not have other euploid embryos for transfer.

Finally, from a practical point of view, day 4 transfer is more convenient for patients and clinical staff, since staying until late in the evening of day 3 is not required.

In conclusion, PGD of aneuploidy performed in two rounds makes feasible the diagnosis of nine chromosomes per single cell. According to our data, the delay of transfer to day 4 permits not only an increase in the diagnostic range by FISH, but also a more complete selection of viable embryos combined with the possibility of verifying and confirming the results obtained. The ultimate goal is to analyze a wider range of chro-

somes and give patients the best support in the achievement of a healthy, full-term pregnancy.

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