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Incidence of Chromosomal Abnormalities from a Morphologically Normal Cohort of Embryos in Poor-Prognosis Patients

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Purpose: *Preimplantation genetic diagnosis of aneuploidy was performed on the embryos yielded by 70 poor-prognosis patients, with the aim of transferring those with a normal chromosomal complement, thus possibly increasing the chances of pregnancy.*

Methods: *Multicolor fluorescence in situ hybridization (FISH) was applied for the simultaneous detection of chromosomes X, Y, 13, 16, 18, and 21. Inclusion criteria were (1) a maternal age of 36 years or older (n = 33), (2) three or more previous in vitro fertilization cycles (n = 20), and (3) an altered karyotype (n = 17).*

Results: *A total of 412 embryos underwent FISH, resulting in 234 (57%) that were chromosomally abnormal. Euploid embryos were available for transfer in 59 patients, generating 19 pregnancies (32%), with an implantation rate of 19.9%.*

Conclusions: *High rates of chromosomally abnormal embryos in poor-prognosis patients can determine repeated in vitro fertilization failures when embryo selection is performed on the basis of morphological criteria alone. Hence, the FISH analysis could represent the prevailing approach for the identification of embryos possessing full potential for developing to term.*

KEY WORDS: aneuploidy; multicolor fluorescence in situ hybridization; multiple in vitro fertilization failures; poor-prognosis patients; preimplantation diagnosis.

INTRODUCTION

Numerical chromosomal abnormalities (CAs) in in vitro generated embryos have been postulated as the possible cause of failed implantation (1–5). Indeed, multiple in vitro fertilization (IVF) failures are reported in patients characterized with a poor prognosis for pregnancy, despite the transfer of embryos presenting a normal morphology and developmental rate. Therefore, the preimplantation genetic diagnosis (PGD) of aneuploidy was carried out on day 3 monospermic embryos yielded by these patients' category. The aim of this study was to verify the incidence of CAs and consequently identify embryos with a normal chromosomal complement to be transferred. The procedure entailed the implementation of the multicolor fluorescence in situ hybridization (FISH) technique for the simultaneous screening of chromosomes X, Y, 13, 16, 18, and 21, which are responsible of the most frequent numerical CAs in the human. Although mosaicism is certainly an obstacle, the use of multiple probes per single blastomere gives rise to valuable information about the chromosomal status of the analyzed cell (2,4,6).

In the present study, 70 couples with a poor prognosis for pregnancy voluntarily consented to undergo PGD of aneuploidy, in hopes of increasing their chance of pregnancy through the selection of embryos possessing the greatest potential for developing to term.

MATERIALS AND METHODS

Patients

From September 1996 to October 1997, 70 patients underwent an assisted conception cycle with PGD of aneuploidy. Inclusion criteria were (a) a maternal age of 36 years or older (group 1; $n = 33$), (b) three or more repeated IVF failures (group 2; $n = 20$), and (c) an altered karyotype in the peripheral blood (group 3; $n = 17$) due to gonosomal mosaicism ($n = 11$) or

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Robertsonian translocations ($n = 6$). Ovarian stimulation protocols and oocyte retrieval were carried out as described previously (7).

Assessment of Fertilization and Embryo Evaluation

Oocytes were analyzed for the presence of pronuclei and polar bodies at 16–18 hr postinsemination. Embryo evaluation was performed at regular intervals at 40 and 62 hr postinsemination by recording: number and appearance of blastomeres and nuclei and percentage of fragmentation. In all patients uterine transfers were performed and clinical pregnancies confirmed by ultrasonography. The implantation rate indicates the number of gestational sacs with a fetal heartbeat divided by the total number of embryos transferred.

Embryo Biopsy and Fluorescence In Situ Hybridization

Embryo biopsy was carried out on day 3 embryos that presented a regular morphology and cleavage rate. Each embryo was manipulated individually in HEPES-buffered medium. Breachings of approximately 25 μm were opened in the zona pellucida using acidic Tyrode's solution; fragments in the perivitelline space were gently removed during the procedure. Following fixation, the biopsied blastomeres were incubated with five DNA probes for the simultaneous detection of chromosomes X, Y, 13, 18, and 21 (8,9). More recently, a probe specific for chromosome 16 was added to the fluorescent mixture and this chromosome was also diagnosed in the embryos obtained by 56 of the 70 couples included in the study. Briefly, the probes were labeled as follows: chromosome X with Spectrum Aqua; chromosome Y with Spectrum Aqua, Orange, and Green; chromosome 13 with Spectrum Orange and Green; chromosome 16 with Spectrum Green; chromosome 18 with Spectrum Aqua and Orange; and chromosome 21 with Spectrum Orange. The hybridization solution was added to the fixed blastomeres and denatured at 78°C for 3 min. After 4 hr of incubation at 37°C in a moist chamber and washing in 0.4× SSC at 72.5°C for 2 min, diamidinophenylindole in antifade solution was added and the slide observed under a fluorescence microscope (Olympus BX 40) equipped with a triple-band pass filter (Aqua/Green/Orange, Vysis): the X chromosome signal appeared as blue, the Y as white, the 13 as orange, the 16 as green, the 18 as pink, and the 21 as red (Fig. 1).

Statistical Analysis

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

RESULTS

As depicted in Table I, a total of 490 embryos was generated; 412 of them exhibited a regular morphology and developmental rate and were selected for embryo biopsy. Following FISH analysis, 160 (39%) embryos were diagnosed as normal and 234 (57%) revealed an abnormal FISH complement, whereas in 18 embryos (4%) no result was obtained due to technical problems during the procedure. No normal embryos resulted in 11 patients' cohorts; in the remaining 59 couples, embryo transfer was performed, with the replacement of 2.6 ± 1.0 chromosomally normal embryos per patient. Nineteen (32%) clinical pregnancies resulted, of which 6 delivered 9 healthy babies, 10 are regularly ongoing, and 3 ended in abortion: 1 blighted ovum, 1 miscarriage, and 1 ectopic pregnancy (no information about these fetuses is available). All the pregnant patients were invited to undergo prenatal diagnosis to confirm the FISH results. Eight of the 10 ongoing pregnancies underwent amniocentesis; 2 of them are waiting for the results, whereas for the remaining 6, FISH results were confirmed.

The analysis of FISH results, illustrated in Table II, revealed comparable percentages of abnormalities in the three groups of patients included in the study (63% in group 1, 55% in group 2, and 58% in group 3). Therefore, the homogeneity of these results consented the analysis of the overall data without differentiating among the poor-prognosis indications.

Figure 2 shows that the distribution of chromosomally abnormal embryos was directly related to the num-

Table I. Clinical Outcome

No. of cycles	70
Total No. of embryos	490
No. of embryos FISHed	412
Normal (%)	160 (39)
Abnormal (%)	234 (57)
No result (%)	18 (4)
No. of cycles transferred	59 (84)
No. of embryos transferred	156
No. of clinical pregnancies (%)	19 (32)
On term	6
Ongoing	10
Miscarriages	3
Implantation rate (%)	19.9

Fig. 1. Fluorescence in situ hybridization of chromosomes X (blue), Y (white), 13 (orange), 16 (green), 18 (pink), and 21 (red) in a normal male blastomere.

ber of previous IVF attempts, when considering patients who had already had at least three cycles fail. Conversely, the incidence of aneuploidy on total abnormalities diminished significantly.

The correlation between CAs and embryo morphology is depicted in Fig. 3. Embryos at the seven- to eight-cell stage exhibited a highly decreased incidence of abnormalities (42%) compared with embryos with a slower cleavage rate (68% at the three- to four-cell stage, $P < 0.001$; 61% at the five- to six-cell stage, $P < 0.02$). In addition, the analysis of the embryos presenting nine cells or more at 62 hr postinsemination indicated that 76% of them were chromsomally abnormal.

Finally, all blastomeres from 101 of the FISH abnormal embryos were reanalyzed, resulting in an 8% error rate, with a misdiagnosis of 5% (4 embryos diagnosed as monosomic and 1 embryo as trisomic were normal).

Table II. Overall Outcome in Relation to FISH Indications

	Maternal age	Repeated IVF cycles	Altered karyotype
No. cycles	33	20	17
No. embryos FISH diagnosed	200	108	86
Abormal (%)	125 (63)	59 (55)	50 (58)
No. cycles transferred	27	17	15
Clinical pregnancies (%)	8 (30)	5 (29)	6 (40)
Implantation rate (%)	19.7	18.8	21.4

DISCUSSION

In this study a group of poor-prognosis patients underwent IVF and PGD with the hope of achieving a full-term pregnancy. The results obtained demonstrated that other factors besides maternal age have an effect on the incidence of aneuploid embryos. Indeed, comparable percentages of CAs were detected in the three groups of patients included in the study, whose indications were age, repeated IVF failures, and altered karyotype, respectively.

The analysis of the FISH data demonstrated that the percentage of chromosomally abnormal embryos was directly related to the number of failed IVF attempts. In addition, the incidence in terms of CAs was not due to aneuploidy, as in the case of advanced maternal age, but to other aberrations (mainly mosaicism) that point to disturbances in the mechanisms of mitotic divisions (2,4,10). These findings suggest that the failed implantation of morphologically normal embryos, even after repeatd attempts, could be due to the abnormal chromosomal complement in the majority of the embryos transferred. Therefore, the opportu-

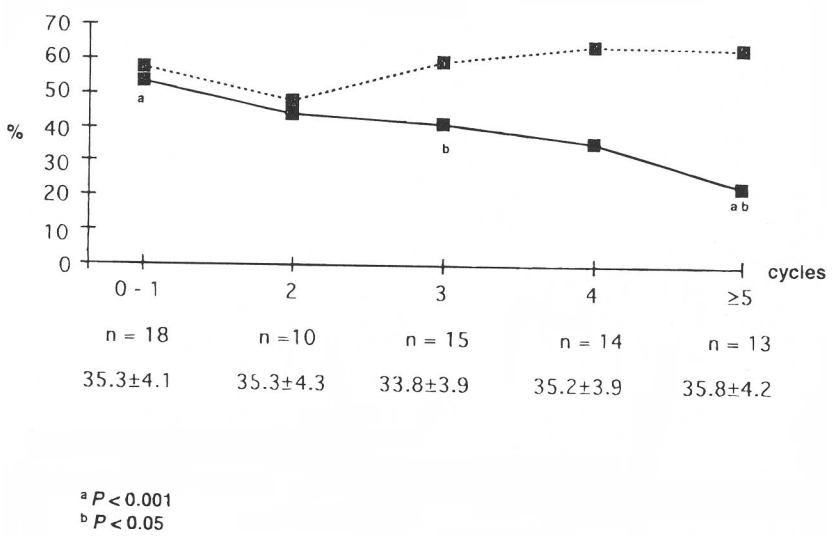

Fig. 2. Distribution of FISH abnormal embryos in relation to the number of previous IVF cycles. (-----) Chromosomally abnormal embryos; (——) aneuploidy over total abnormalities).

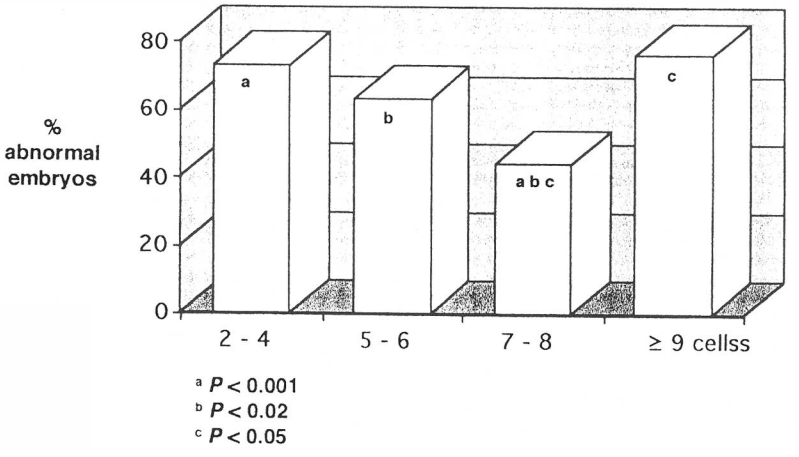

Fig. 3. Distribution of chromosomally abnormal embryos according to the cell number at 62 hr postinsemination.

nity of identifying embryos that possess full potential of developing to term is especially advantageous when there is the possibility of performing a selection within a wide cohort of embryos (11). Indeed, our data also indicate that the classical morphological criteria are not adequate for embryo selection in poor-prognosis cases. In fact, almost half of the embryos at the seven- to eight-cell stage are chromosomally abnormal, and the percentage is higher in embryos presenting a slower cleavage rate. Even more surprisingly, embryos presenting 9–12 cells at the observation performed 62 hr postinsemination exhibited the highest degreee of CAs observed (76%).

In conclusion, the repeated and often unexplained reproductive failure that characterizes the patients' categories included in this study are probably related to the generation of numerical CAs in their embryos, as a consequence of gemete aneuploidies or altered mitotic divisions. Thus, in view of the results obtained, PGD of aneuploidy deserves to be considered a valuable approach in offering couples the best chances of conceiving a healthy baby.

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