

Will preimplantation genetic diagnosis assist patients with a poor prognosis to achieve pregnancy?

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PGD (preimplantation genetic diagnosis) of aneuploidy for chromosomes X, Y, 13, 18 and 21 was carried out on 196 embryos from 36 infertile patients classified with a poor prognosis due to (i) maternal age, (ii) repeated in-vitro fertilization (IVF) failures and (iii) mosaic karyotype. The percentage of abnormal embryos was comparable in the three groups of patients: maternal age 63%, repeated IVF failure 57%, and mosaic karyotype 62%. The analysis of the overall data revealed an increased incidence of abnormal embryos in the older age categories (predominantly due to aneuploidy), even in embryos at the 7- to 8-cell stage. In addition, the percentage of chromosomally abnormal embryos was directly proportional to the number of IVF failures, where the increase in chromosomal abnormalities was not correlated to aneuploidy but to other aberrations such as mosaicism and polyploidy. Following PGD, 28 patients had at least one embryo transferred that appeared normal by fluorescent in-situ hybridization (FISH). Four clinical pregnancies resulted, with an implantation rate of 10% per normal embryo. In conclusion, the high rate of chromosomally abnormal embryos in poor prognosis patients may have been the cause of implantation failure in their previous IVF cycles. Therefore, the possibility of transferring embryos with a normal FISH complement could improve the chance of pregnancy in this category of patients.

Key words: aneuploidy/IVF failures/multicolour FISH/poor prognosis patients/preimplantation genetic diagnosis

Introduction

The preimplantation genetic diagnosis (PGD) of aneuploidy permits the investigation of the chromosomal status of embryos generated *in vitro*, thus allowing the selection of healthy embryos to be transferred. The procedure, which entails the removal of a blastomere from monospermic day 3 embryos, is rapid and has no effect on an embryo's potential to implant (Hardy *et al.*, 1990; Winston and Handyside, 1993). The expected advantages of PGD include (i) reducing the possibility of trisomic offspring, especially in women of advanced repro-

ductive age; (ii) reducing the chances of spontaneous abortions and (iii) increasing the chances of pregnancy by transferring chromosomally normal embryos which possess the full potency of developing to term.

The low implantation rates resulting from assisted reproductive techniques may be related to the high rates of chromosomal disorders which are routinely observed in oocytes obtained from stimulated cycles or during early cleavage events (Plachot and Mandelbaum, 1990; Pellestor, 1991; Delhanty and Handyside, 1995). This phenomenon, in combination with increased abortion rates, is especially evident in women aged >35 years. In fact, it has been recently demonstrated that aneuploidy in embryos generated *in vitro* increases notably with maternal age (Munné *et al.*, 1995a; Dailey *et al.*, 1996). Therefore, the reproductive failure that often occurs in women of advanced reproductive age could derive from a decline in the functional and structural qualities of the oocyte (Navot *et al.*, 1991). However, repeated idiopathic IVF failures are also reported in young patients, despite normal embryo morphology. In these cases, cytogenetic analysis of the embryos generated could provide additional information necessary for a better understanding of repeated unsuccessful attempts. Thus, the advantages and disadvantages of the PGD procedure and its routine application in selected patients are presently still open to question, notwithstanding the recent progress achieved in this field (Egozcue, 1996; Reubinoff and Shushan, 1996; Verlinsky and Kuliev, 1996).

In this study, PGD was performed on embryos generated *in vitro* from a group of patients with poor prognosis due to (i) advanced maternal age, (ii) repeated IVF failures and (iii) altered karyotype in peripheral blood (gonosomic mosaicism; these patients were included in the study since they were considered to be at high reproductive risk). PGD was carried out by using the multicolour fluorescent in-situ hybridization (FISH) technique that includes the simultaneous screening of five chromosomes per single blastomere, subsequently permitting the identification of the most frequent aneuploidies, haploidies and polyploidies present in the human (Delhanty *et al.*, 1993; Munné *et al.*, 1993, 1995a; Griffin *et al.*, 1994; Manor *et al.*, 1996). Despite the incidence of mosaicism reported in human embryos generated *in vitro*, the simultaneous application of multiple probes could represent a more reliable source of information on the status of the analysed blastomere (Munné *et al.*, 1993, 1995b; Harper and Delhanty, 1996). Consequently, the aim of the present study was to determine whether a high numerical rate of chromosomal abnormalities in embryos is present among patients with poor prognosis in IVF and possibly to increase their pregnancy rate

by transferring embryos that appeared normal by FISH (FISH-normal embryos).

Materials and methods

Patients

A total of 36 patients underwent an assisted conception cycle with PGD of aneuploidy. Both male and female partners were karyotyped before being admitted to our IVF programme. Inclusion criteria consisted of patients with (i) a maternal age ≥ 38 years ($n = 11$), (ii) ≥ 2 previous IVF failures ($n = 22$) and (iii) an altered karyotype (mosaics 46XX/45XO) detected in peripheral blood ($n = 3$). Induction of multiple follicular growth was carried out through the administration of gonadotrophin-releasing hormone analogue and exogenous gonadotrophins, as previously described (Ferraretti *et al.*, 1996). Ultrasound-guided oocyte retrieval was carried out ~36 h after human chorionic gonadotrophin administration. The recovered oocytes were preincubated in culture medium supplemented with 10% heat-inactivated maternal serum in a 5% CO₂ humidified gas atmosphere at 37°C. Prior to microinjection, cumulus and corona cell complexes were removed with 80 IU/ml hyaluronidase (type VIII; Sigma Chemicals, St. Louis, MO, USA) in HEPES-buffered Earle's medium using hand-drawn glass pipettes. Nuclear maturity and cytoplasmic morphology were then assessed.

Sperm evaluation and processing; insemination procedures

Semen samples were classified according to the criteria specified by the World Health Organization (WHO, 1992), with the exception of morphology, which was assessed on the basis of strict criteria (Kruger *et al.*, 1987). Seminal fluids were processed with conventional swim-up or the mini-Percoll technique, following previously described criteria (Fiorentino *et al.*, 1994). Insemination was carried out in microdroplets overlaid with oil, or with intracytoplasmic sperm injection (ICSI; Van Steirteghem *et al.*, 1993) in patients with a total motile sperm count of $\leq 0.5 \times 10^6$ ($n = 17$) or a previously failed fertilization attempt ($n = 4$).

Assessment of fertilization and embryo evaluation

Approximately 16 h after insemination, oocytes were scored for the presence of pronuclei and polar bodies. Embryo evaluation was performed at 40 and 62 h post-insemination. The number and morphology of blastomeres, and percentage of fragmentation were recorded. Clinical pregnancies were confirmed by ultrasound analysis. The implantation rate was calculated as the number of gestational sacs with fetal heart beat divided by the total number of embryos transferred.

Embryo biopsy and fluorescence in-situ hybridization

Embryo biopsy and blastomere fixation were performed on day 3 monospermic embryos as previously described (Munné *et al.*, 1993). For the multicolour FISH analysis, five DNA probes were used for the simultaneous detection of chromosomes X, Y, 13, 18 and 21 (Munné and Weier, 1997). Briefly, the probes were labelled as follows: chromosome Y with Spectrum Aqua (Vysis, Naperville, IL, USA), chromosome 18 in Spectrum Orange and Spectrum Aqua (1:1 mixture of probes; Vysis), chromosome X with Spectrum Orange and Spectrum Green (1:1 mixture of probes; Vysis), chromosome 13 with Spectrum Orange (Vysis) and chromosome 21 with biotin (Dr Weier, Lawrence Berkeley National Laboratory, UCSF, Berkeley, CA, USA), which was detected by fluorescein isothiocyanate-labelled avidin. The hybridization solution was made by adding 1 μ l of each probe, which were previously concentrated to 3 μ l, to 7 μ l of whole chromosome

Table I. Overall clinical outcome of preimplantation diagnosis in poor prognosis patients

No. of cycles	36
Mean \pm SD age (years)	34.8 \pm 5.6
Total no. of embryos	223
No. of embryos undergoing FISH	196
normal (%)	76 (39)
abnormal (%)	85 (43)
monosomic	24
trisomic	21
haploid	13
triploid	10
mosaics	17
anucleated (%)	23 (12)
damaged (%)	12 (6)
No. of cycles transferred	28
No. of embryos transferred	70
No. of clinical pregnancies (%)	4 (14)
Implantation rate (%)	10

FISH = fluorescent in-situ hybridization.

paint (WCP)-hybridization buffer (Vysis). The resulting 10 μ l of hybrid solution was added to the fixed blastomeres on a glass slide, covered with an 18 \times 18 mm coverslip and denatured at 78°C for 3 min. The slide was then left to hybridize for 4 h at 37°C in a dark moist chamber. After washing in 0.4 \times sodium chloride/sodium citrate at 72°C for 2 min, the fluorescence-labelled avidin followed by anti-avidin were added. Finally, the slides were counterstained in 4',6-diamidino-2-phenylindole in antifade solution (Vysis) and observed under a fluorescence microscope (Olympus BX60) equipped with a triple bandpass filter set for simultaneous observation of Spectrum Orange/Green/Aqua.

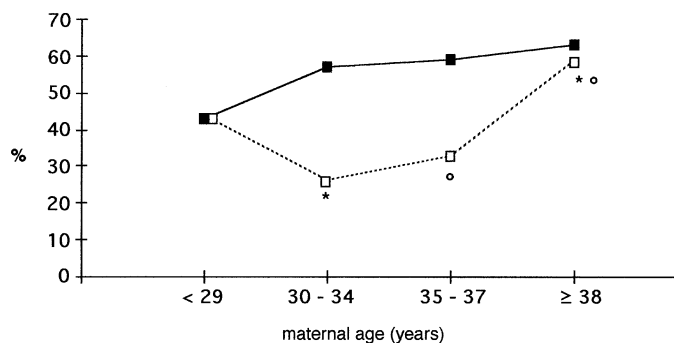
Statistical analysis

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

Results

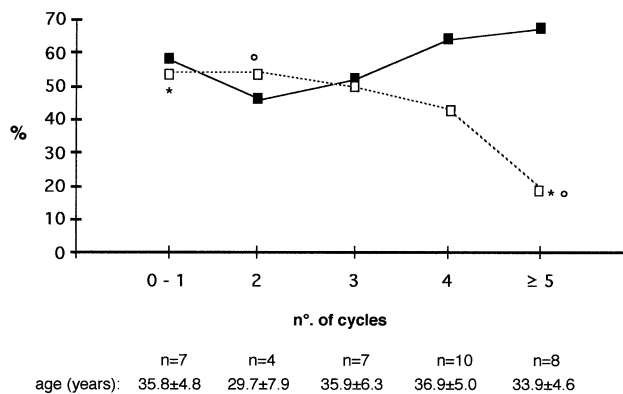
A total of 223 embryos were generated from 36 patients. Based on their morphological evaluation, 196 embryos were selected for FISH analysis. As indicated in Table I, 76 embryos presented a normal chromosomal complement (39%); 85 were diagnosed as abnormal (43%); no result was obtained for the remaining 35 embryos due to the absence of a nucleus in the analysed blastomere ($n = 23$, 12%) or damage occurring during the procedure ($n = 12$, 6%). In all, 28 patients had at least one FISH-normal embryo transferred. Four clinical pregnancies resulted, with an implantation rate of 10%. All blastomeres from FISH-abnormal embryos were analysed, resulting in: 45 aneuploidies, 13 haploidies and 10 polyploidies; in addition, eight mosaics and nine multinucleations with an overall abnormal complement were detected. The percentage of abnormal embryos following FISH analysis was comparable in the three groups of patients with poor prognosis indications (maternal age 63%, repeated IVF failure 57%, altered karyotype 62%). Therefore, due to the homogeneity of the results obtained in these three groups in terms of total chromosomal abnormalities, further data analysis was performed without differentiating between poor prognosis indications.

Figure 1 demonstrates the distribution of chromosomal abnormal embryos of all 36 patients in relation to age. An



* $\chi^2 = 9.17$ $P < 0.005$
 ° $\chi^2 = 5.09$ $P < 0.025$

Figure 1. Distribution of chromosomally abnormal embryos according to maternal age. —, total chromosomal abnormalities (CA); ----, aneuploidy over total CA.



n=7 n=4 n=7 n=10 n=8
 age (years): 35.8±4.8 29.7±7.9 35.9±6.3 36.9±5.0 33.9±4.6

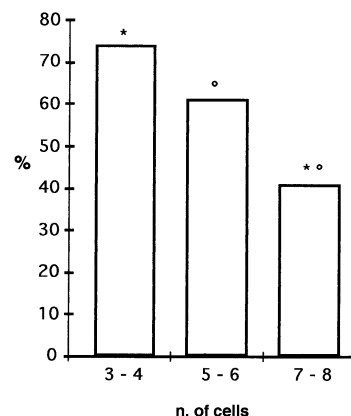
* $\chi^2 = 5.69$ $P < 0.02$
 ° $\chi^2 = 6.66$ $P < 0.01$

Figure 2. Distribution of chromosomally abnormal embryos in relation to the number of previous in-vitro fertilization cycles. —, total chromosomal abnormalities (CA); ----, aneuploidy over total CA.

increased incidence of abnormal embryos was observed in the older age categories when compared to the youngest, even though the differences were not statistically significant. However, when aneuploidy alone was considered, there was a significant increase proportional to age ($P < 0.005$), with the exception of the youngest category.

Additionally, results of the FISH technique were analysed in relation to the number of previous IVF cycles. Figure 2 shows that the number of failed IVF attempts was directly related to the percentage of abnormal embryos (excluding patients that were undergoing their first or second cycle), reaching up to 67% after five cycles. Conversely, the incidence of aneuploidy decreased.

The distribution of chromosomal abnormalities in relation to the cell number in the analysed embryos is depicted in Figure 3. Embryos at the 7- to 8-cell stage presented a significantly lower percentage of abnormalities (41%) when compared to embryos with a slower rate of division (74% at 3-4 cells, $P < 0.005$; 61% at 5-6 cells, $P < 0.025$). Embryos at the 7- to 8-cell stage were analysed in succession according to maternal age. The increase in terms of chromosomal



* $\chi^2 = 10.25$ $P < 0.005$
 ° $\chi^2 = 5.34$ $P < 0.025$

Figure 3. Distribution of chromosomally abnormal embryos according to the cell number on day 3.

Table II. Overall outcome of preimplantation diagnosis of intracytoplasmic sperm injection (ICSI) versus conventional in-vitro fertilization (IVF) embryos

	ICSI	IVF
No. of cycles	21	15
Mean ± SD age (years)	35.7 ± 5.7	33.6 ± 5.4
Total no. of embryos	118	93
7-8 cell (%)	33 (28)	32 (34)
No. of embryos undergoing FISH	117	79
No. of embryos diagnosed	114	70
No. of embryos FISH normal (%)	40 (35) ^a	36 (51) ^a
Chromosomal abnormalities		
monosomy	13	11
trisomy	13	8
haploidy	10	3
triploidy	6	4
mosaics	11	6
No. of anucleated blastomeres (%)	21 (18) ^b	2 (3) ^b
No. of cycles transferred	16	12
No. of embryos transferred	40	30
No. of clinical pregnancies (%)	2 (12)	2 (17)
Implantation rate (%)	7.5	13.3

^aSignificantly different: $\chi^2 = 5.08$, $P < 0.025$.

^bSignificantly different: $\chi^2 = 8.40$, $P < 0.005$.

FISH = fluorescent in-situ hybridization.

abnormalities was proportional to the patient's age and it was especially evident in women aged ≥ 38 years in comparison to the younger patients (65 versus 33% respectively; $P < 0.025$; data not shown).

Table II shows the outcome of the FISH analysis in embryos derived from ICSI compared to those from conventional IVF insemination. The IVF group yielded a significantly higher percentage of FISH-normal embryos (51%) when compared to the ICSI group (35%; $P < 0.025$). Analysis of the chromosomal abnormalities revealed that there was a similar distribution of aneuploidies between the two groups. Conversely, an increase in the number of embryos with anuclear blastomeres was observed in the ICSI group (18%) compared to the IVF group (3%; $P < 0.05$).

The overall results were also analysed in terms of sex and cleavage rate. The sex ratio of the embryos obtained was 45%

male and 55% female, and the data were comparable between the ICSI and IVF groups. Similarly, no correlation was observed between the sex of the embryo and the developmental rate (data not shown).

Discussion

In the unending efforts put forth to identify the underlying factors affecting infertility in poor prognosis patients, PGD has proven to be a powerful instrument in unravelling and successfully treating the problems that are confined to this group of patients. In this study, a group of poor prognosis patients underwent IVF and PGD for the transfer of chromosomally normal embryos, with the hope of achieving a full-term pregnancy.

The data generated in this study revealed that other factors besides age affected the incidence of numerical chromosomal disorders detected in embryos generated *in vitro*. In fact, similar percentages of chromosomal abnormalities were obtained following FISH analysis of embryos of patients with an age indication, younger patients with ≥ 2 IVF failures, or with an altered karyotype. Therefore, the homogeneity of the results obtained in the three groups in terms of chromosomal abnormalities permitted the analysis of the data obtained without differentiating between the poor prognosis indications.

As expected, the rate of chromosomal abnormalities increased with age. This was especially due to aneuploidy, where the incidence increased significantly along with the patient's age, thus confirming that non-disjunction events happen more frequently in oogenesis in older women (Figure 1). The unusually high percentage of chromosomal abnormalities observed in the youngest age category (43%) can certainly be associated with a poor prognosis condition, since according to data obtained in our centre, young patients without a poor prognosis yielded only 28% FISH-abnormal embryos; specifically, aneuploidy decreased to 5.4% (Munné *et al.*, 1995a).

Subsequently, data were analysed on the basis of previous IVF attempts and indicated that the percentage of chromosomally abnormal embryos was directly proportional to the number of IVF failures. An exception to the trend observed was represented by those patients who underwent their first or second cycle; this could have been attributed to a poor prognosis condition (altered karyotype, $n = 3$; maternal age, $n = 4$). Interestingly, the increase in chromosomal abnormalities was not due to aneuploidy, but to other aberrations as depicted in Figure 2. Since all the analysed embryos were derived from monospermic zygotes, the abnormalities could have arisen from several causes: diploid gametes, altered or blocked cytoplasmic division, or sperm centriolar defects (Artley *et al.*, 1992; Sathanathan *et al.*, 1996). In all cases, the mechanisms involved in cell division were not functioning properly, thus producing altered mitotic divisions. Indeed, it would be interesting to investigate the male contribution to the embryonic genome through assessment of the incidence of aneuploidy in the spermatozoa of the male partners.

The revelation of a high percentage of chromosomally abnormal embryos in patients with repeated unsuccessful IVF

attempts has finally provided an explanation for the failed implantation of morphologically good quality embryos even after repeated attempts. Indeed, confirmation of this hypothesis could be achieved by the repetition of FISH analysis in a subsequent cycle. In this way, if an individual patient again yielded an abnormal cohort of embryos, further IVF attempts could be discouraged.

As anticipated, the analysis of chromosomal abnormalities in relation to embryo morphology revealed that slow-cleaving embryos exhibit higher rates of chromosomal alterations (Figure 3). This is the genetic foundation supporting the already established correlation between embryo cleavage rate and pregnancies in IVF (Edwards, 1985; Munné *et al.*, 1995a; Gianaroli *et al.*, 1996), thus confirming the validity of the morphological criteria which determine the selection of embryos to be transferred. However, advanced maternal age still remains an important factor in the incidence of chromosomal abnormalities, even in embryos with normal morphology and development. In fact, one-third of the embryos being transferred in poor prognosis women younger than 37 years are abnormal; this incidence almost doubles in older patients. This would imply that morphological alterations would occur at later stages following genomic activation (Braude *et al.*, 1988). Therefore, morphological criteria alone are not sufficient for embryo selection in poor prognosis cases, as demonstrated by the low pregnancy rates characteristic of this patient category.

In addition to its diagnostic importance, PGD can be integrated into an assisted reproduction programme as a tool for increasing the chance of pregnancy in poor prognosis patients. Chromosomal analysis is especially advantageous when a large cohort is available, to detect the embryos with a normal chromosomal complement. Indeed, the four pregnancies reported in the present study occurred in patients who had a large cohort of good-quality embryos from which the selection of embryos for transfer was executed. In the absence of FISH results, the probability of transferring abnormal embryos is elevated, and this could have been the case in the previous unsuccessful cycles. On the other hand, the benefits of PGD in patients with a reduced number of embryos are limited to the detection of trisomic embryos. In this respect, PGD could be considered an alternative to the therapeutic termination of a pregnancy due to diagnosis of an abnormal fetus by routine pre-natal procedures. In fact, even with 10% error in PGD of aneuploidy, the chances of conceiving a trisomic fetus would be significantly reduced (Munné *et al.*, 1995a).

No increase in the percentage of gonosomal aneuploidies was found in embryos generated from patients with an altered karyotype. This indicates that no strict correlation occurs between mosaicism in maternal blood and in gametes. However, since this patient category is considered to have an increased reproductive risk compared to the normal population, PGD could represent a valuable option in such patients requiring IVF techniques.

One of the major drawbacks in the efficacy of PGD is mosaicism, due to the presence of different chromosomal complements in the same embryo (Harper and Delhanty, 1996). If a mosaic embryo is biopsied, misdiagnosis may result,

leading to the transfer of an embryo with abnormal cells. However, the implication of mosaicism in preimplantation embryos is still unknown, although it physiologically occurs in the trophectoderm of human blastocysts (Benkhalifa *et al.*, 1993). The relevance of mosaicism resides in the number of chromosomally abnormal cells: a recent finding revealed that mosaic embryos in which two-thirds of the cells were normal (analogous to what is present in thawed embryos) could normally develop to term (Munné *et al.*, 1995b).

The possibility of studying the chromosomal complement in preimplantation embryos also represents a valuable source of information regarding the different aspects of early processes in reproductive embryology. At the present time, ICSI represents the most successful choice for the treatment of severe male factor infertility. However, there is growing concern about the potential risk of genetic abnormalities associated with the ICSI population as opposed to conventional IVF with normal spermatozoa (Patrizio, 1995). It has been reported that a high frequency of constitutional chromosomal abnormalities is present in the severely infertile male population (Bourrouillou *et al.*, 1985); in addition, patients with severe asthenozoospermia may have centriolar defects, which can cause impaired cellular division (Sathananthan *et al.*, 1996). The results obtained from ICSI as compared with IVF techniques revealed a higher rate of chromosomal abnormalities in ICSI embryos compared to embryos generated with conventional insemination (Table II). No differences with respect to the distribution of chromosomal abnormalities were observed; indeed, there were similar aneuploidy percentages (including gonosomal aneuploidy) in the two groups. However, a higher incidence of anuclear blastomeres was observed following ICSI, implicating a disturbance in the processes regulating cell division. Furthermore, 30% of the 53 ICSI embryos diagnosed as chromosomally abnormal were haploid or polyploid. Since the analysed embryos derived from zygotes with two pronuclei and two polar bodies, it can be argued that these embryos either originated from nullisomic or diploid gametes, or from asynchrony between DNA synthesis and cytoplasmic division. Nonetheless, the effect of this increase in haploid and polyploid embryos is irrelevant since these abnormalities are invariably lethal. More data need to be collected to establish whether these results are valid or are inherent to the special category of patients considered in this report.

In conclusion, the present study indicates that patients with poor prognosis due to age, repeated IVF failures and altered karyotype generate a high percentage of chromosomally abnormal embryos, which if transferred could cause failed implantation. FISH analysis allows the identification of embryos which possess full potential for developing to term. Additional advantages of the implementation of PGD and IVF include the reduction of embryos being transferred (thus avoiding complications associated with multiple pregnancies) and cryopreserved. Therefore, when considering the negative outcome of the previous IVF cycles undertaken by 30 of the 36 patients studied, it is plausible that the chance of pregnancy is increased by PGD and also that the risk of carrying a trisomic fetus is minimized.

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