Article

Which patients with recurrent implantation failure after IVF benefit from PGD for aneuploidy screening?

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Abstract

Patients with recurrent IVF failure are defined as patients who are younger than 37 years and who had at least three consecutive unsuccessful IVF/intracytoplasmic sperm injection (ICSI) cycles with good quality embryos. These patients might be predisposed to chromosome errors in their embryos and therefore might benefit from preimplantation genetic diagnosis for aneuploidy screening (PGD-AS). This technique is, however, expensive and some normal embryos might be lost due to the error rate. The aim of this retrospective study was to define those patients who would benefit most from it. One hundred and twenty-one first PGD-AS cycles for recurrent IVF failure were analysed. The aneuploidy rate, 'no embryo transfer' rate, live birth rate per embryo transfer and implantation rate were respectively 48.3, 22.3, 29.7 and 19.5%. A multivariate logistic regression analysis gave us a predictive model demonstrating that to have a 90% probability of having an embryo transfer after PGD-AS, the patient should have at least 10 mature oocytes, eight normally fertilized oocytes and six embryos for biopsy. This study suggests that most patients with recurrent IVF failure may benefit from PGD-AS. Future studies, however, should more strictly define this heterogeneous group of patients, so that comparison is easier.

Keywords: *chromosomal aneuploidy, FISH, ICSI, IVF, PGD, recurrent IVF failure*

Introduction

Numerical chromosome errors are common in early human embryos (Harper *et al*., 1995; Munné *et al*., 1998a; Márquez *et al*., 2000) both *in vivo* and in IVF (Macklon *et al*., 2002). Most are lethal, probably causing embryos to die around the time of implantation. A young fertile couple trying to conceive has a 20–25% chance of being successful per monthly cycle. Many infertile couples fail to achieve a pregnancy after repeated transfers of morphologically good quality embryos produced by IVF treatment. These patients who have had three or more consecutive IVF cycles without clinical pregnancy are regarded as having recurrent implantation failure (RIF), and have a very low probability of achieving a pregnancy with further cycles. Repeated implantation failure is not only distressing for patients who require multiple cycles of treatment (Urman *et al*., 2005a), but also adds greatly to the cost of the procedure. These couples may be predisposed to a high aneuploidy rate in their embryos.

Several IVF centres offer these patients preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) using fluorescence in-situ hybridization (FISH) with specific probes for five to nine chromosomes to detect chromosome errors in the blastomeres biopsied from their embryos (Gianaroli *et al*., 1997, 1999; Magli *et al*., 1998; Kahraman *et al*., 2000; Voullaire *et al*., 2002; Munné *et al*., 2003, 2006; Pehlivan *et al*., 2003; Werlin *et al*., 2003; Wilton *et al*., 2003; Caglar *et al*., 2005). All centres demonstrated a high aneuploidy rate in the embryos of RIF patients and a higher than expected pregnancy rate after PGD-AS treatment in these poor prognosis patients, compared

with historical controls. At the Vrije Universiteit Brussel, since May 2000, PGD-AS for chromosomes X, Y, 13, 16, 18, 21, and 22 has been offered to patients with RIF who are younger than 37 years on the day of oocyte retrieval. In this way, an attempt is made to find an explanation for the multiple IVF failures of these patients and eventually improve their outcome by transferring chromosomally normal embryos. However, the heterogeneity of these patients is well known, as implantation failure can be caused by multiple factors (Urman *et al.*, 2005b). The first PGD-AS cycle of all RIF patients was reviewed, in order to restrict the number of PGD-AS cycles for RIF to those patients who would benefit most of it. This is especially important, as the cost of performing PGD-AS adds up to 25–50% of the cost associated with the IVF cycle and some normal embryos may not be transferred due to the technique (biopsy, fixation, hybridization, FISH signal interpretation).

Materials and methods

Study population

Patients, younger than 37 years at the time of oocyte retrieval and with at least three consecutive fresh IVF/intracytoplasmic sperm injection (ICSI) cycles with embryo transfer without clinical pregnancy, who had their first PGD-AS between May 2000 and December 2003 in the unit, were included in this retrospective analysis. All of them (and their partners) had a normal karyotype and a normal uterine cavity after hysteroscopic evaluation. Four patients had a previous biochemical pregnancy. Patients with an azoospermic partner were excluded. Only the data of patients with at least one biopsied embryo with a result were analysed. All patients gave their written consent before having PGD-AS.

Ovarian stimulation and ICSI procedure

All female partners underwent ovulation induction using a gonadotrophin-releasing hormone analogue suppression protocol (short or long) (Kolibianakis *et al.*, 2002) or a gonadotrophin-releasing hormone antagonist protocol (Kolibianakis *et al.*, 2003) and human menopausal gonadotrophins or recombinant FSH. Cumulus–oocyte complexes (COC) were recovered 36 h after the administration of 10,000 IU of human chorionic gonadotrophin (HCG). The surrounding cumulus and corona cells were then removed, and the nuclear maturity of the oocytes was assessed under an inverted microscope. Only metaphase II oocytes were injected with morphologically normal motile spermatozoa into the ooplasm. These procedures have been described previously (Van Steirteghem *et al.*, 1993; Joris *et al.*, 1998; Devroey and Van Steirteghem, 2004).

Assessment of fertilization, embryo development and biopsy

Further culture of injected oocytes was performed in 25 μl of culture medium under lightweight paraffin oil. Fertilization was confirmed after 16–18 h by the observation of two distinct pronuclei (2PN). Oocytes with 2PN were assessed on day 2 and day 3 after injection for embryonic development, and the embryos reaching at least the 5-cell stage on day 3 of development were biopsied. The selection criteria for embryo biopsy were similar to those used to decide whether an embryo was transferable on day 3 in the regular ICSI programme without PGD. Before biopsy, the blastomeres were checked for the presence of a nucleus. From the 6-cell stage onward, two blastomeres per embryo were removed (Van de Velde *et al.*, 2000; De Vos *et al.*, 2001).

FISH procedure

The individually biopsied blastomeres were spread onto a Superfrost Plus glass slide (Kindler GmbH, Freiburg, Germany) using 0.01 N HCl/0.1% Tween 20 solution (Coonen *et al.*, 1994; Staessen *et al.*, 1996). Both blastomeres from the same embryo were fixed on the same slide in very close proximity.

A two-round FISH procedure, as described previously (Staessen *et al.*, 2003), allowed us to detect the chromosomes X, Y, 13, 18, 21 (round 1) and 16, 22 (round 2).

In short, an aliquot (0.2 μl) of the probe solution (DXZ1, Spectrum Blue; DYZ3, Spectrum Gold; LSI13, Spectrum Red; D18Z1, Spectrum Aqua; LSI21 Spectrum Green; Multivision PGT Probe Panel; Vysis Inc., Downer's Grove, IL, USA) was added to the nuclei, covered with a round coverslip (4 mm diameter), denatured for 5 min at 75°C and left to hybridize for between 4 h and overnight at 37°C in a moist chamber. After washing in 0.4× standard saline citrate solution (SSC)/0.3% Nonidet P40 at 73°C for 5 min and 2× SSC/0.1% Nonidet P40 for 60 s at room temperature, antifade solution (Vectashield, Vector Labs, Brussels, Belgium) was added and fluorescence signals were evaluated. The nuclei were then examined using a Zeiss Axioskop fluorescence microscope with the appropriate filter sets. The FISH images were captured with a computerized system.

Following the analysis of the first set of probes, the cover slips were gently removed and the slides rinsed in 1 × phosphate-buffered saline at room temperature, denatured in 0.0625× SSC for 7 min at 75°C, and then dehydrated (70, 90, 100 and 100% ethanol at –18°C, 60 s each). The second hybridization solution was prepared by mixing a probe for chromosome 16 (Satellite II DNA/D16Z3 probe, Spectrum Orange; Vysis) and a probe for chromosome 22 (LSI 22, 22q11.2, Spectrum Green; Vysis). The probes were denatured separately in a hot water bath at 75°C for 5 min. An aliquot (0.2 μl) of the probe solution was then added to the nucleus, covered with a round coverslip (4 mm diameter), sealed with rubber cement and then hybridized overnight in a water bath at 37°C. Finally, the slides were washed for 2 min in 0.4× SSC solution at 73°C and 2× SSC/0.1% Nonidet P40 for 60 s at room temperature. The washed slides were then mounted with 4,6-diamidino-2-phenylindole in antifade solution and analysed. The FISH results were interpreted by two observers. Only chromosomally normal compacting stage embryos and blastocysts were transferred on day 5.

Definitions

A rise in serum HCG on two consecutive occasions from 11 days after transfer indicated pregnancy. An ongoing pregnancy was defined if at least one fetus with a positive heartbeat was revealed by vaginal ultrasound after 12 weeks of gestation. The implantation rate was defined as the number of viable fetuses,

as assessed by ultrasound at 7 weeks gestation, divided by the number of embryos transferred for each subject. Miscarriage was defined as pregnancy loss before 20 weeks of gestational age.

Statistical analysis

A multivariate logistic regression analysis was performed to view which factors (age, number of oocytes, number of metaphase II oocytes, number of normally fertilized oocytes and the cleavage rate) added most power to the chance of having an embryo transfer after PGD-AS. $P < 0.05$ was considered statistically significant.

Results

The data of the first PGD-AS cycle of 121 patients with recurrent IVF failure younger than 37 years on the day of oocyte retrieval were analysed. The overall results are summarized in **Table 1**.

Twenty-two patients had their previous IVF cycles in the authors' centre, 71 in one of eight Belgian IVF centres and 28 abroad.

The mean age of the patients and their mean number of previous treatment cycles was 32.4 years and 5.4 cycles respectively. A mean number of 14.5 COC was retrieved, of whom 12.3 oocytes were at the metaphase II stage. A mean number of 9.2 oocytes were normally fertilized. The mean cleavage rate (the mean number of biopsied embryos divided over the mean number of normally fertilized oocytes) as an indicator of the quality of embryo development was 76.7%. A mean number of 6.7 embryos (a total of 827 embryos) could be biopsied, resulting in a clear FISH result. In all, 0.7% of the embryos were lost during the biopsy procedure and 2.3% of the biopsied embryos did not show a clear FISH result. Most embryos (97%) had two cells biopsied. Eleven per cent of all embryos, where two blastomeres were analysed, had a discordant FISH result: one chromosomally normal blastomere and one abnormal; these embryos were considered as abnormal and not transferred. Two hundred and seven chromosomally normal embryos were transferred to 94 patients, resulting in five biochemical, two extrauterine, two miscarriages and 28 ongoing pregnancies (21 singleton, five twin and two triplet pregnancies) with an ongoing pregnancy and live birth rate per embryo transfer of 29.7%, an implantation rate of 19.5%, a miscarriage rate of 6.6% and a multiple pregnancy rate of 25.0%. Both patients with a triplet pregnancy had a non-selective reduction to two fetuses. Thirty-five healthy babies were born.

There were 27 patients (22.3%) who did not have an embryo transfer, because all their embryos were chromosomally abnormal (17 patients) or chromosomal normal but of poor quality and not transferable (10 patients). The overall percentage of chromosomally abnormal embryos was 48.3%.

A correlation analysis was performed between the embryo aneuploidy rate and the number of previous IVF/ICSI cycles; no statistically significant correlation was found (correlation coefficient of –0.048).

Table 1. Characteristics of the overall group of patients, stimulation and fluorescence in-situ hybridization results [values are means ± SD and (range)].

Characteristic	*121 patients*
Mean age (years)	32.4 ± 2.5 (25–36)
Mean no. of treatment cycles	5.4 ± 2.1 (3–12)
Mean no. of COC	14.5 ± 6.3 (2–37)
Mean no. of MII	12.3 ± 5.6 (2–31)
Mean no. of 2PN	9.2 ± 5.1 (1–28)
Mean cleavage rate (no. of biopsied embryos/no. of 2PN)%	76.7 ± 21.1 (16.6–100)
Mean no. of embryos biopsied with result [total no. embryos]	6.7 ± 4.3 (0–21) [827]
Mean no. of embryos transferred [total no. embryos]	1.7 ± 1.2 (0–5) [207]
Embryo transfer rate % [total no. patients]	22.3 [27][a]
Aneuploidy[b] rate %	48.3 ± 28.6
Clinical pregnancy rate/ET (%)	34.0
Ongoing pregnancy rate/ET (%)	29.7 (28)[c]
Live birth rate/ET (%)	29.7
Implantation rate (%)	19.5 ± 33.5
Miscarriage rate (%)	6.6
Multiple pregnancy rate (%)	23.3 (7)[c]

COC = cumulus–oocyte complex.
[a]For 17 of the 27 patients, all embryos were abnormal.
[b]Details of the types of abnormalities identified are already published (Staessen *et al.*, 2004).
[c]Pregnancy numbers are shown in parentheses.

Table 2. Literature review of studies on patients with recurrent IVF failure (RIF).

Study	*Definition of RIF*	*No. of cycles*	*No. of probes*	*No. of cells biopsied*	*Abnormality rate %*	*Clinical pregnancy rate/ET (%)*	*Implantation rate (%)*	*Miscarriage rate (%)*
Gianaroli *et al.* 1999	≥3 failed cycles and age not available	27	6/9	1	54.0	25.0	17.3	20.0
Kahraman *et al.* 2000	≥3 failed cycles and <36 years	23	5	1	43.2	30.4	N/A	N/A
Voullaire *et al.* 2002	≥3 failed cycles or ≥10 embryos transferred and <37 years	14	CGH	1	61.0	N/A	N/A	N/A
Pehlivan *et al.* 2003	≥3 failed cycles and < 37 years	32	7	2	65.4	40.7	24.6	9.1
Munné *et al.* 2003	≥2 failed cycles and ≥35 years	54	8/9/12	1	69.0	N/A	14.3	N/A
Platteau *et al.* 2005	≥3 failed cycles and <37 years	121	7	2	48.3	34.0	19.5	6.6

N/A: not available, ET = embryo transfer, CGH = comparative genomic hybridization.

A multivariate logistic regression analysis was performed on five factors that were thought to give most power to the chance of having an embryo transfer after PGD-AS (age, number of oocytes, number of metaphase II oocytes, number of normally fertilized oocytes and the cleavage rate). The probability for embryo transfer depended significantly on the number of metaphase II oocytes ($P < 0.001$), number of normally fertilized oocytes ($P < 0.001$) and cleavage rate ($P = 0.03$). With these three factors, a predictive model was made demonstrating that to have a 90% probability of having an embryo transfer after PGD-AS, the patient should have at least 10 mature oocytes, eight normally fertilized oocytes and six embryos for biopsy. None of these factors had statistically significant power to predict conception (positive HCG) when tested in a regression analysis. In relation to the analysed variables of age, number of oocytes, number of MII oocytes, number of fertilized oocytes and number of embryos, a regression analysis showed no statistically significant relationship between these parameters and a positive outcome (data not shown).

Discussion

The results after PGD-AS in this young (<37 years) group of patients who had had at least three consecutive failed fresh IVF/ICSI cycles with good quality embryos are encouraging: a live birth rate per embryo transfer of 29.7% and an implantation rate of 19.5%. It is, however, difficult to compare the present study, which is, so far as is known, the largest study ever published in this group of patients, with previously published results (**Table 2**). Different definitions have been used, including patients with only two failed cycles, patients who had 10 or more embryos transferred in the past (not specifying if these also included frozen cycles) in fewer than three cycles and patients older than 36 years, in this already heterogeneous group. A literature review about patients with recurrent IVF failures, comparing the used definitions of 'recurrent IVF failures', the number of PGD-AS cycles included, the number of FISH probes used, the number of biopsies performed per embryo, the abnormality rate, the clinical pregnancy rate, the implantation rate and miscarriage rate is summarized in **Table 2**.

Although the results are promising in these poor prognosis patients, it cannot be stated that this is due to the extra information obtained after PGD-AS. Only a prospective randomized controlled trial can clarify this situation. Such a trial is currently being conducted, but many patients who qualify to enter in the study refuse when they hear that there is a 50% chance of being allocated to the control group, as they are all convinced that PGD-AS will improve their chances (Urman *et al.*, 2005b). This is mainly based on information from Internet, newspapers and TV. They are rarely aware of the potential negative impact of PGD-AS on their embryos: a ±4% loss rate (personal observation, Catherine Staessen) due to the technique itself, a potential reduction (although never proven) of the embryo quality due to the biopsies, a high rate of mosaicism in day 3 embryos (Ziebe *et al.*, 2003), which might lead to false positive and false negative diagnoses (although the error rate should be low when 2 cells are analysed), and poor results after freezing of biopsied embryos (Joris *et al.*, 1999), although promising results have been reported recently (Jericho *et al.*, 2003). The error rate derived from the analysis of non-transferred embryos, especially in relation to 1- or 2-cell biopsy, would give an estimation of the rate of mosaicism and its effect on PGD, as well as of the utility of performing 2-cell biopsy. Such investigations were not performed in the current study due to the routine workload but have been performed previously (Staessen *et al.*, 2004). It would also be interesting to review retrospectively (blinded for the PGD-AS results) which blastocysts theoretically would have been transferred on morphology criteria only, without the extra information obtained from PGD-AS.

It was considered that this group of patients were predisposed to chromosome errors in their embryos and therefore would benefit from PGD-AS. However, there was no correlation between the aneuploidy rate and the number of previous IVF/ICSI cycles the patients performed. Neither was there a difference in the aneuploidy rates between the patients who fell pregnant after their first PGD-AS cycle and the ones who did not. It was expected that the patients who fell pregnant would have a higher rate of abnormal embryos and therefore would benefit from the extra information after PGD-AS for a better embryo selection at transfer. It seems, therefore, that in this large heterogeneous group of patients, endometrium, embryo quality problems and other unknown factors dilute the possible beneficial effect of PGD-AS.

From the prediction model based on the data from these 121 patients, it seems that only patients with at least 10 mature oocytes, eight normally fertilized oocytes and six embryos for biopsy on day 3 have a 90% chance (the same chance as ICSI patients have in general on the day of oocytes retrieval) to have an embryo transfer and therefore could increase their chances for pregnancy after PGD-AS. It could be that in this group of patients the advantages of PGD-AS might outweigh the disadvantages. A similar statement has been published previously by different authors (Munné *et al*., 2003). Seventy five per cent of all ongoing pregnancies also came from this group of patients.

It is clear that PGD-AS does not improve the chances of patients with poor embryo quality (10 patients in the present study) or patients with no more embryos than planned for embryo transfer, as nature will do the selection, avoiding any negative impacts from the screening. One could speculate that the poor embryo quality of those 10 patients might be due to the embryo biopsy; however, in the largest randomized controlled trial published at the moment (Staessen *et al*., 2004), there were as many patients in the control group as in the PGD-AS group (with biopsy) who had no blastocyst formation.

An additional benefit of PGD-AS is the information that all the embryos are abnormal and that the prognosis for a future pregnancy after IVF treatment is not good (Ferraretti *et al*., 2004). This information helped the 17 patients to decide to continue further treatment, to look for alternatives or to stop.

The abnormality rate in this study, as in other published studies (Gianaroli *et al*. 1999; Kahraman *et al*. 2000; Voullaire *et al*. 2002; Munné *et al*. 2003, 2006; Pehlivan *et al*. 2003), was higher than expected in this young group of patients; although it is impossible to quantify what the expected number would be as a 'normal' background control group cannot be easily defined. Comparing the different rates is, however, again difficult as each study used a different number of FISH probes (from 5 to 12) and even comparative genomic hybridization techniques. It is clear that the more chromosomes are evaluated the more abnormalities will be picked up (Voullaire *et al*., 2002). Seven probes were used, based on their more frequent representation in meiotically derived aneuploidy in live births and in spontaneously aborted fetuses. Using more probes might jeopardize the diagnosis, due to overlapping and difficult to interpret signals. Nor is it clear that the extra information gained by the addition of further probes for clinically less important chromosomes would compensate for these potential signal problems. In most studies, only one blastomere biopsy was performed per embryo, ignoring any form of mosaicism. The authors prefer to remove two blastomeres, where possible, per embryo, especially after the appearance of more and more publications of false-negative results after single biopsies (Munné *et al*. 1998b; Gianaroli *et al*. 2001; Verlinsky *et al*. 2004; Sermon *et al*. 2005).

Because of the very high multiple pregnancy rate (25.0%) in this study, it is suggested that only one or two screened embryos should be transferred, even though most of the patients had already had five unsuccessful IVF/ICSI cycles.

In conclusion, it was observed that, although the pregnancy results were very promising, the role of PGD-AS in poor implanters is not clear as yet. Patients who could benefit most of it are probably, good responders with at least 10 mature oocytes, eight normally fertilized oocytes and six embryos for biopsy on day 3. Future studies should more strictly define this heterogeneous group of patients, so that comparison is easier and be randomized with a control group.

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