Preimplantation genetic diagnosis for aneuploidy screening in patients with unexplained recurrent miscarriages

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Objective: To determine the aneuploidy rate in embryos of women with idiopathic recurrent miscarriages and to evaluate whether preimplantation genetic diagnosis for aneuploidy screening could be a feasible approach to improve the possibility of successful pregnancy in these couples.

Design: Prospective cohort study.

Setting: Tertiary university referral center.

Patient(s): Women (n = 49) with recurrent idiopathic miscarriages.

Intervention(s): In vitro fertilization with preimplantation genetic diagnosis for aneuploidy screening.

Main Outcome Measure(s): Ongoing pregnancy rate (PR) and aneuploidy rate.

Result(s): The aneuploidy rate was, respectively, 43.85% and 66.95% in the younger and older group. The ongoing PR per cycle was 25.71% in the younger and 2.94% in the older patients.

Conclusion(s): There is no therapeutic evidence to prescribe IVF with or without preimplantation genetic diagnosis for aneuploidy screening for this heterogeneous group of patients. (Fertil Steril 2005;83:393–7. ©2005 by American Society for Reproductive Medicine.)

Key Words: Chromosomal aneuploidy, fluorescence in situ hybridization, preimplantation genetic diagnosis, recurrent miscarriages

Miscarriage is defined as the loss of a pregnancy before viability, which is according to the definition of the World Health Organization, the expulsion of an embryo or fetus weighing 500 g or less from its mother. This corresponds to a gestational age of 20–22 weeks, the irreducible age for fetal viability. Recurrent miscarriage is defined as the loss of three or more consecutive pregnancies (1). Some clinicians favor changing the definition of recurrent miscarriage to two or more consecutive losses, but the efficacy of commencing investigations after two losses has not been established. Furthermore, including patients with only two miscarriages dilutes this already heterogeneous group and renders literature reviews on this subject inconclusive.

Between 12% and 15% of clinically recognized pregnancies m miscarry (1). The expected chance of three consecutive pregnancy losses is therefore (15%)^3 = 0.34%. However, 1% of couples suffer from recurrent miscarriage (2). The fact that the observed frequency of recurrent miscarriage is significantly higher than that expected by chance alone implies that in some women there is a persistent underlying cause to account for their pregnancy losses.

Balasch et al. (3) suggested that IVF, by replacing several embryos into the uterus, may be a therapeutic approach for young women (mean age, 31 years) with unexplained recurrent miscarriages. They observed a 66.6% ongoing pregnancy rate (PR) in 12 patients (with a mean of 4.91 previous miscarriages). They hypothesized that by replacing three to four embryos into the uterus, it would be possible to potentiate the maternal recognition of pregnancy, by altering the maternal immune response. They believed, in addition, that the replacement of this high number of embryos would also increase the chance of becoming pregnant with a chromosomally normal embryo. However, Raziel et al. (4) found no advantage of IVF in older women (mean age, 38 years); 42 IVF cycles in 20 habitual aborters were retrospectively investigated: the pregnancy was 32% per cycle with a 50% miscarriage rate.

Recently some investigators (5–8) hypothesized that non-inheritable de novo abnormalities arising from random errors produced from gametogenesis through to embryonic development could be an important etiology in unexplained recurrent abortions. This was based on cytogenetic evaluations of first trimester spontaneous abortions, revealing an overall incidence of 50%–70% chromosomal abnormalities (9–13). Their results, in a limited group of patients younger than 37 years, indicated that a large proportion of the embryos were chromosomally abnormal and that preimplantation genetic diagnosis for aneuploidy screening (PGD-AS) could be a feasible approach to improve the possibility of successful pregnancy in these couples (with an already good prognosis).
We conducted a prospective cohort study in two groups of patients (women <37 years and women ≥37 years) to confirm these findings and to try to explain the conflicting results from Balasch and Raziel and their colleagues (3, 4).

MATERIALS AND METHODS

Study Population

Forty-nine patients aged between 26 and 44 years with a history of unexplained recurrent abortion were included in this study. All were screened according to the standard work-up for recurrent miscarriage, which comprises peripheral blood karyotyping in both partners, a pelvic ultrasound scan to assess ovarian morphology and uterine cavity, and screening for antiphospholipid antibodies (both lupus anticoagulant and anticardiolipin antibodies) performed on two occasions at least 6 weeks apart. Institutional Review Board approval was obtained. All patients gave their written consent before entering the study.

Ovarian Stimulation and Intracytoplasmic Sperm Injection Procedure

All female partners were superovulated using a GnRH analogue suppression protocol (short or long) (14) or a GnRH antagonist protocol (15) and hMG or recombinant FSH. Oocyte–cumulus complexes were recovered 36 hours after the administration of 10,000 IU of hCG. Then the surrounding cumulus and corona cells were removed and the nuclear maturation 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 (16, 17).

Assessment of Fertilization, Embryo Development, and Biopsy

Additional culture of injected oocytes was performed in 25-μL microdrops of culture medium under lightweight paraffin oil. Fertilization was confirmed after 16–18 hours 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 the 5-cell stage onward, two blastomeres per embryo were removed (18, 19).

Fluorescence In Situ Hybridization 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 (20, 21). Both blastomeres from the same embryo were fixed on the same slide in very close proximity. A two-round fluorescence in situ hybridization (FISH) procedure, as described previously (22), 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; DY3Z, SpectrumGold; LS13, SpectrumRed; D18Z1, SpectrumAqua; LS21 SpectrumGreen; Multivision PGT Probe Panel; Vysis Inc., Downers Grove, IL) was added to the nuclei, covered with a round coverslip (4 mm in diameter), denatured for 5 minutes at 75°C and left to hybridize for between 4 hours 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 minutes and 2 × SSC/0.1% Nonidet P40 for 60 seconds at room temperature. Antifade solution (Vecastain, Burlingame, CA) was added and fluorescence signals were evaluated. The nuclei were then examined using a Zeiss Axioskop fluorescence microscope (Zeiss; Oberkochen, Germany) with the appropriate filter sets. The FISH images were captured with a computerized system.

After the analysis of the first set of probes, the coverslips were gently removed and the slides rinsed in 1 × phosphate-buffered saline (PBS) at room temperature, denaturated in 0.0625 × SSC for 7 minutes at 75°C, and then dehydrated (70%, 90%, 100%, and 100% ethanol at −18°C, 60 seconds each). The second hybridization solution was prepared by mixing a probe for chromosome 16 (Satellite II DNA/D16Z3 probe, spectrum Orange, Vysis, Inc.) and a probe for chromosome 22 (LSI 22. 22q11.2, SpectrumGreen, Vysis, Inc.). The probes were denatured separately in a hot water bath at 75°C for 5 minutes. An aliquot (0.2 μL) of the probe solution was then added to the nucleus, covered with a round coverslip (4 mm in diameter), sealed with rubber cement and then hybridized overnight in a water bath at 37°C. Finally, the slides were washed for 2 minutes in 0.4 × SSC solution at 73°C and 2 × SSC/0.1% Nonidet P40 for 60 seconds at room temperature. The washed slides were then mounted with 6-diamino-2-phenylindole (DAPI) in antifade solution, and analyzed and the results interpreted by two independent observers.

Embryo Scoring and Selection for Transfer

The subsequent classification of embryos based on the biopsy result of two blastomeres was followed: [1] when both blastomeres had two copies of each chromosome analyzed, the embryo was classified as normal; [2] when both blastomeres had one or two chromosomes with an abnormal number of copies, the embryo was classified as aneuploid; [3] when both blastomeres had one, three, or more copies of each chromosome, the embryo was classified as haploid or polyploid; [4] when one blastomere was normal and the second blastomere had one or two chromosomes with an abnormal number of copies, the embryo was classified as mosaic; and [5] when at least one blastomere had more than two chromosomes with an abnormal number of copies, the embryo was classified as complex abnormal. Only chromosomally normal blastocysts were transferred on day 5.
Definition of End Point
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.

Statistical Analysis
The mean age at the time of oocyte retrieval of each cycle and the mean number of retrieved oocytes, metaphase II oocytes, normally fertilized oocytes, and the mean number of abnormal embryos of each cycle was first calculated per couple. In a second step the mean of all previous parameters of all the couples was calculated. The comparison of the variables was performed by means of two-way ANOVA, with Bonferroni t test to perform pairwise comparison of the two groups. P<.05 was considered statistically significant.

RESULTS
There were 49 patients (after screening) included in this study, divided (arbitrarily) in 25 patients younger than 37 years (35 cycles) and 24 women ≥37 years (34 cycles). The results are summarized in Tables 1, 2, and 3.

The younger patients were on average 32.48 ± 2.72 years old with a mean of 4.46 ± 2.10 previous miscarriages. The older ones were on average 40.15 ± 2.48 years old with a mean of 4.93 ± 2.41 previous miscarriages. After the retrieval of 13.84 ± 7.00 and 10.43 ± 5.92 oocytes, 11.70 ± 5.83 and 8.88 ± 5.14 metaphase II oocytes, and 8.69 ± 4.93 and 6.95 ± 3.89 normally fertilized oocytes, 240 and 173 embryo biopsies could be taken in each age group.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Characteristics of the patients, stimulation, and FISH results (values are means ± SD).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients &lt;37 years (35 cycles)</td>
<td>Patients ≥37 years (34 cycles)</td>
</tr>
<tr>
<td>Mean age (y)</td>
<td>32.48 ± 2.72 yrs</td>
</tr>
<tr>
<td>Mean no. of miscarriages</td>
<td>4.46 ± 2.10</td>
</tr>
<tr>
<td>Mean no. of COC</td>
<td>13.84 ± 7.00</td>
</tr>
<tr>
<td>Mean no. of MII</td>
<td>11.70 ± 5.83</td>
</tr>
<tr>
<td>Mean no. of 2PN</td>
<td>8.69 ± 4.93</td>
</tr>
<tr>
<td>No. embryos biopsied</td>
<td>240</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>% Normal</td>
<td>54.20% ± 21.74%</td>
</tr>
<tr>
<td>% Abnormal</td>
<td>43.85% ± 22.50%</td>
</tr>
<tr>
<td>% No diagnosis</td>
<td>1.94% ± 5.04%</td>
</tr>
</tbody>
</table>

Note: COC = combined oocyte complexes; MII = Metaphase II.


<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Chromosomal constitution of blastomeres derived from couples with idiopathic recurrent abortions (values are means ± SD).</th>
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<tbody>
<tr>
<td>&lt;37 years</td>
<td>≥37 years</td>
</tr>
<tr>
<td>% Normal</td>
<td>54.20 ± 21.74</td>
</tr>
<tr>
<td>% Abnormal</td>
<td>43.85 ± 22.50</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>39.91 ± 31.54</td>
</tr>
<tr>
<td>Complex</td>
<td>26.53 ± 28.95</td>
</tr>
<tr>
<td>Haploid/polyploid</td>
<td>5.03 ± 10.60</td>
</tr>
<tr>
<td>Mosaic</td>
<td>28.03 ± 33.33</td>
</tr>
<tr>
<td>% No diagnosis</td>
<td>1.94 ± 5.04</td>
</tr>
</tbody>
</table>

*P=.0032.

Sixty-three chromosomally normal embryos were transferred in 31 cycles of patients younger than 37 years, resulting in one biochemical and nine ongoing pregnancies (all singleton, except one twin pregnancy) with an implantation rate of 16.66% ± 30.32%. In the older age group, 37 chromosomally normal embryos were transferred in 18 cycles, resulting in two biochemical pregnancies, two miscarriages, and one ongoing pregnancy. There were four young patients who did not have transfer; one because of severe hyperstimulation syndrome (all embryos were frozen), one patient had only chromosomally abnormal embryos, and the embryos of two patients were of poor quality and not transferable. Sixteen older patients did not have embryo transfer, as 12 of them had only chromosomally abnormal embryos and four had no transfer because of poor embryo quality.

The overall percentage of chromosomal abnormal embryos in the younger and older group was, respectively, 43.85% ± 22.50% and 66.95% ± 29.27% (P = .0032). The classification of the abnormal embryos (in aneuploid, complex abnormal, haploid or polyploidy, and mosaic [see materials and methods]) in the two groups did not reveal any differences.

**DISCUSSION**

The aneuploidy rate in the group of young patients (43.85%) is similar to the results (41%–53%) of previous smaller studies (5–8). To our knowledge, our series is the largest study ever published, evaluating PGD-AS in patients with idiopathic recurrent miscarriages. A similar study (23) found even higher aneuploidy rates, especially in the younger patient group. The researchers, however, extended in this latter study the definition of recurrent aborters up to two miscarriages. It is therefore difficult to compare the two studies.

The PGD-AS does not seem to improve the PR in the group of young patients compared to our general IVF population. Although the aneuploidy rate is relatively high in this group (43.85%), PGD-AS did not improve the selection of the transferred embryos, resulting in a 29% ongoing PR per transfer. Without any treatment the expected live birth rate in this group of patients would have been >60% (24–26). For one (young) patient this PGD-AS cycle might have been diagnostic, as all her embryos were abnormal, making decisions (as oocyte donation) easier.

The pregnancy results in the older group were disappointing. It seems that these patients are near the end of their reproductive life. They first have several spontaneous pregnancies, sadly all resulting in miscarriages. They then have no pregnancies at all anymore, which makes them consult an assisted reproductive technology (ART) clinic sooner or later, in view of their older age. Half of these patients had no ET, as all their embryos were chromosomally abnormal; the other half had a very poor pregnancy outcome. Therefore, it seems that in these patients, PGD-AS does not bring any benefit, apart from being diagnostic and redirecting treatment to other options.

Our results confirm the data from both previously described articles (3, 4). The Spanish group achieved very good ongoing PRs by doing IVF in young patients; their results (58.3% ongoing PR) were even better than ours, probably due to the high number of embryos transferred (3 to 4), with a similar implantation rate (17.6%). The ongoing PR was, however, the same as the expected live birth rate, if no treatment would have been done. The Israeli group had the same disappointing IVF results as we had in our group of older patients. Therefore, it seems that the mean age of the patients with recurrent miscarriages predicts their future obstetric outcome.

Our PGD-AS results are based on seven chromosomes. It is possible that in the future with new techniques (comparative genomic hybridization, microarray), we will be able to screen for all chromosomes, which might influence our results as well. As there is a possibility that we missed some chromosomal abnormalities. The addition of more FISH probes decreases the efficiency of the procedure (27).

The PGD-AS might be beneficial in a subgroup of patients with recurrent miscarriages, who have proven aneuploidy concepti by cytotgenetic analysis. In our center, however, only a minority of patients have these results. A lot of fetal material is lost due to the unforeseen and dramatic circumstances and we believe that some clinicians find that the usefulness of cytogenetic investigation of fetal tissues is rather limited as it seldom influences clinical practice. The
The prognosis for future pregnancy is whenever possible. The young patients can be reassured that accurate basis for judging various treatment forms. Prognosis (30). At a scientific level it provides a more karyotypically abnormal fetuses have a good reproductive consequence pregnancy outcome as patients with may overcome these problems in the future. A cytogenetic treatment. The future obstetric outcome for the older patients remains grim, whatever treatment is done.

In conclusion, on the basis of our data there seems no therapeutic evidence to prescribe IVF with or without PGD-AS for this heterogeneous group of patients, but more sophisticated fetal cytogenetic testing should be performed, whenever possible. The young patients can be reassured that the prognosis for future pregnancy is >60% without any treatment. The future obstetric outcome for the older patients remains grim, whatever treatment is done.

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REFERENCES