Preimplantation aneuploidy testing for infertile patients of advanced maternal age: a randomized prospective trial

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Objective: To assess the potential benefit of preimplantation aneuploidy testing on the outcome of in vitro fertilization (IVF) for women of advanced maternal age (AMA).

Design: Prospective randomized clinical trial.

Setting: Private IVF clinic.


Intervention(s): Fluorescent in situ hybridization (FISH) for chromosomes X, Y, 13, 15, 16, 17, 18, 21, and 22.

Main Outcome Measure(s): Preimplantation aneuploidy testing of biopsied blastomeres on day 3 of development.

Result(s): Fertilization and blastocyst developmental rates were similar for the test and control groups: 80% versus 77.4% and 49% versus 48.2%, respectively. The average number of embryos transferred was comparable at 2.2 for the test group and 2.7 for the control group. Implantation rates were also equivalent across the two groups: 37.3% in the control group versus 36.5% in the test group. Nevertheless, the spontaneous abortion rate was observed to be lower for the test group: 25.9% versus 32.26% in the control group. This resulted in an observed increase in delivery rates for the test group: 78% versus 67.74% in the control group.

Conclusion(s): Preimplantation aneuploidy testing does not appear to statistically significantly improve outcome parameters in infertile AMA patients; however, a trend toward a decrease in the spontaneous abortion rate with a subsequent higher delivery rate was observed. (Fertil Steril 2009;92:157–62. ©2009 by American Society for Reproductive Medicine.)

Key Words: Preimplantation aneuploidy testing, chromosomal aneuploidy, AMA

Preimplantation genetic diagnosis (PGD) is considered the earliest form of prenatal diagnosis performed on biopsied material from oocytes and/or embryos created during in vitro fertilization (IVF). Currently, preimplantation genetic screening (PGS) involves the screening for specific genetic abnormalities including chromosomal aneuploidies. Routinely up to 9 to 12 chromosomes can be analyzed on a single fixed nucleus by fluorescent in situ hybridization (FISH), in which DNA probes labeled with fluorochromes hybridize to their respective chromosomes, allowing for the identification of ploidy status. It has been demonstrated that increasing aneuploidy rates in oocytes and embryos are observed relative to increasing maternal age, defined as >35 years (1–3). In addition, implantation rates have been shown to decrease with advanced maternal age (AMA) (4). Hence, preimplantation aneuploidy testing is offered clinically as a method for increasing clinical pregnancy rates in AMA women seeking fertility treatment.

Although it is widely used, the benefit of preimplantation aneuploidy testing with regard to live-birth rates per cycle initiated has not been consistently demonstrated. Studies have demonstrated an improvement in implantation rates with preimplantation aneuploidy testing (5, 6), including more recently Stassen et al. (7) who also found a trend to improved implantation rates in a randomized, prospective trial using two-cell biopsy. The same group, comparing single-cell versus two-cell biopsy demonstrated a detrimental effect of two-cell biopsy; they suggested that if one-cell biopsy had been used in their study, implantation rates may have improved (8). Another recent study also failed to demonstrate an improvement in implantation rates (9), although that study has been criticized on methodology grounds (10–13).

It is also logical to assume that preimplantation aneuploidy testing would decrease spontaneous abortion rates. Yet this also remains an area of controversy; some studies have shown a benefit with preimplantation aneuploidy testing for patients with sporadic or recurrent miscarriage (14, 15), but others have not (16).

Our study conducted a prospective, randomized trial to evaluate the outcome of preimplantation aneuploidy testing in women of AMA undergoing infertility treatment.

MATERIALS AND METHODS

IVF Patients

From May 2002 to March 2004, 62 patients were enrolled in a prospective, randomized trial to evaluate the benefit of
preimplantation aneuploidy testing. Women included in the trial had to meet the following criteria: age ≥ 35 years, the presence of five or more embryos with ≥ 6 cells and ≤ 15% fragmentation on day 3, and fulfillment of the other requirements for entrance into the IVF program. This trial compared a control group, who underwent routine blastocyst transfer, with a test group who underwent day-3 embryo biopsy and FISH for chromosomes 13, 15, 16, 17, 18, 21, 22, X, and Y, followed by routine blastocyst transfer. End points included the rates of implantation, pregnancy, delivery, and spontaneous abortion, and the incidence of no transfer.

All patients were counseled regarding the scope, limitations, potential benefits, and risks of embryo biopsy and pre-implantation aneuploidy testing. Once they had given written informed consent, eligible couples were assigned randomly by a computer-generated random number table to blastocyst transfer alone or preimplantation aneuploidy testing plus blastocyst transfer. The study was approved by the Health-One Internal Review Board.

Human Embryo Culture

Media G1.2 and G2.2 containing 5 mg/mL of human serum albumin (17) were prepared weekly in the laboratory and were screened before use with a mouse embryo bioassay (18). Semen preparation was carried out by using a 50–70–95 discontinuous gradient of Pure Sperm (Nidacon, Gothenburg, Sweden). The resulting pellet was washed in fertilization medium (19) and stored in the incubator until insemination. We added 100,000/mL sperm to each oocyte. If intracytoplasmic sperm injection (ICSI) was performed, all oocytes were denuded by using hyaluronidase and drawn pipettes. Each mature oocyte was placed in a 6-μL droplet of phosphate-buffered saline supplemented with 15% fetal cord serum. The partner’s sperm was placed in a 6-μL droplet of polyvinylpyrrolidone (IVF Science Scandinavia, Gothenburg, Sweden). All droplets were overlaid with paraffin oil (BDH, Poole, Dorset, United Kingdom). We performed ICSI on a Nikon inverted microscope (Nikon, Melville, NY) with Narashige micromanipulators (Narashige, East Meadow, NY). Injected oocytes were then rinsed and placed in tubes of G1.2 until fertilization was assessed.

Assessment of fertilization took place 15 to 18 hours after insemination. Cumulus and corona cells were removed by dissection with 27-gauge disposable needles in an organ culture dish. All gamete and embryo manipulations occurred in a pediatric isolete designed to control humidity, temperature, and pH fluctuations. Embryos with two pronuclei were cultured in groups of three or four in medium G1.2 in pre-rinsed 5-mL Falcon tubes. Around noon on day 3, all embryos were transferred to medium G2.2 for a further 48 hours of culture (day 5 of development). On the morning of day 5, the percentage of blastocyst formation was determined, and each blastocyst was assigned a score by using the system of Gardner et al. (17). Blastocysts were transferred in medium G2.2 early in the afternoon; those that were not transferred were cryopreserved.

Embryo Biopsy and FISH

Typically, one cell per embryo was biopsied on day 3 of development, either by zona drilling using acidified Tyrode’s solution (n = 14), or laser ablation (n = 18; patients retrieved from November 2003 after the laser’s purchase; Hamilton Thorne Biosciences, Inc., Beverly, MA). Blastomeres were fixed individually following a protocol to minimize signal overlap and loss of micronuclei (20). Once fixed, cells/slides were sent by courier to the PGD lab (Repropogenetics LLC, Livingston, NJ, and Los Angeles, CA) for FISH analysis.

The FISH analysis consisted of two consecutive hybridizations following previously published protocols (21), but no more recent protocols using “no result rescue” to further decrease the error rate (22) because at the time of the study they were not available. The first hybridization was performed with probes for chromosomes 13, 16, 18, 21, and 22 (Multivision PB; Vysis, Downer’s Grove, IL). The second hybridization consisted of a home-made combination of probes for chromosomes X, Y, 15, and 17 (23). Scoring was performed by eye without the need of any software. A scoring criterion previously described elsewhere (24) was used for differentiating between close signals from two homologous chromosomes and two domains belonging to a split signal of a single chromosome.

Pregnancy Outcome

A positive pregnancy was defined as a concentration of human chorionic gonadotropin (hCG) of > 25 IU/L at least 9 days after embryo transfer. Implantation rate was measured by the presence of an intrauterine gestational sac with positive fetal heart activity at ultrasonography performed at least 6 weeks after embryo transfer. A spontaneous abortion was defined as loss of a pregnancy after the presence of a gestational sac had been visualized by ultrasound. Delivered pregnancy was defined as progression past the 24th week of gestation.

Statistical analysis of all outcome parameters was performed using either a two-tail unpaired t-test to compare means or the two-sided Fischer’s exact test with 95% confidence intervals (GraphPad Instat; GraphPad Software, La Jolla, CA).

RESULTS

A total of 32 patients were enrolled in the test group and 30 patients in the control group. There were no statistically significant differences between the test and control groups with regard to age, follicle-stimulating hormone (FSH) level, antral follicle count, or number of prior IVF attempts. In addition, the etiology of infertility was similar between the test and control groups (Table 1). The number of oocytes, oocyte maturity, and fertilization rate were also observed to be similar (Table 2). Embryo quality on day 3 of culture was comparable across the two groups as were the rates of blastocyst formation and the number of top quality blastocysts (see Table 2).

Figure 1 illustrates the preimplantation aneuploidy testing results for all embryos biopsied in the test group. For the
chromosomes tested, 28% of the embryos were analyzed as normal and 67% were abnormal. The most common chromosomal abnormality observed was monosomy (27%). This finding may be due to relevant meiotic errors originating in the oocyte or artifact related to suboptimal fixation and/or FISH procedures. This was followed by a trisomy rate of 14%, but no diagnostic result was obtained in 5% of embryos. The preimplantation aneuploidy testing results also indicated that one third (33.8%) of good-quality day-3 embryos were normal for the chromosomes tested, whereas on day 5, 60% of top-quality blastocysts were normal for the chromosomes tested, for 46.6% of blastocysts overall (see Table 2).

In the control group, 2.7 embryos were transferred compared with 2.2 in the test group (Table 3). Only one patient failed to undergo embryo transfer; that patient was in the test group and had no euploid embryos available for transfer. Implantation rates were similar in control and test groups at 37.3% and 36.5%, respectively (see Table 3). The spontaneous abortion rate appeared to be lower after preimplantation aneuploidy testing (25.9% vs. 32.26% in the control group; not statistically significant), hence resulting in a trend toward an increase in delivery rate for the test group (78% vs. 67.74% in the control group; see Table 3). There were no statistically significant differences in any of the outcomes measured ($P > 0.05$).

**DISCUSSION**

The clinical application of preimplantation aneuploidy testing is expanding rapidly around the globe. By 2004, several large referral centers had reported their experiences with approximately 5000 PGD cycles and 750 births (25). Although

<table>
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<tr>
<th><strong>TABLE 1</strong></th>
<th>Clinical parameters: patient characteristics and etiology of infertility.</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td><strong>Patient characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>38.3 ± 2.5</td>
</tr>
<tr>
<td>Number of prior IVF cycles</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>Mean FSH</td>
<td>6.9 ± 2.2</td>
</tr>
<tr>
<td>Mean antral follicle count</td>
<td>17.6 ± 6.8</td>
</tr>
<tr>
<td><strong>Etiology of infertility</strong></td>
<td></td>
</tr>
<tr>
<td>Tubal</td>
<td>0.0</td>
</tr>
<tr>
<td>Male factor</td>
<td>23.3%</td>
</tr>
<tr>
<td>Unexplained</td>
<td>10.0%</td>
</tr>
<tr>
<td>Advanced maternal age</td>
<td>23.3%</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>16.6%</td>
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<tr>
<td>Combined</td>
<td>26.6%</td>
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<th><strong>TABLE 2</strong></th>
<th>Embryology results.</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>19.8 (5.6)</td>
</tr>
<tr>
<td>Metaphase II oocytes</td>
<td>80%</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>77.4%</td>
</tr>
<tr>
<td>Good-quality day-3 embryos ($\geq 6$ cells, $\leq 20%$ fragmentation)</td>
<td>77.5%</td>
</tr>
<tr>
<td>Good quality day-3 normal by preimplantation aneuploidy testing</td>
<td>—</td>
</tr>
<tr>
<td>Day-5 blastocyst formation</td>
<td>48.2%</td>
</tr>
<tr>
<td>Day-5 blastocysts normal by preimplantation aneuploidy testing</td>
<td>—</td>
</tr>
<tr>
<td>Rate of perfect blastocyst formation</td>
<td>10.0%</td>
</tr>
<tr>
<td>Perfect blastocysts normal by preimplantation aneuploidy testing</td>
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preimplantation aneuploidy testing has been found to be beneficial for cases involving structural chromosomal rearrangements and single-gene defects, its value in AMA cases remains controversial.

Although the results of our randomized, prospective trial showed no statistically significant differences between the control and test groups, a tendency toward higher delivery rates and lower spontaneous abortion rates was observed. In a previous randomized, prospective control trial by Staessen et al. (7) that involved 400 patients, a greater number of embryos were transferred in the control group (2.8) compared with the study group (2.0), which lead to an increase that was not statistically significant in implantation rates in the study group (17.1% vs. 11.5%; \( P < .09 \)). In contrast, there was a nearly statistically significant increase in ongoing pregnancy rates in the control group (16.5%) as compared with the study group (10.4%; \( P < .06 \)). However, two cells per embryo were biopsied, which may have been detrimental for embryo survival (8, 26).

A more recent large randomized study by Mastenbroek et al. (9) reported a negative effect of preimplantation aneuploidy testing on pregnancy rates. These investigators documented that 20% of the embryos could not be analyzed; this compares with only 5% in our study. When they chose biopsied undiagnosed embryos for transfer, the implantation rate was considerably lower than for nonbiopsied embryos, which indicates that their biopsy procedure had a potentially detrimental effect on ongoing development (10–13). Other factors also may have contributed to the negative effect that they observed after preimplantation aneuploidy testing, including patient selection and the absence of screening for the clinically significant chromosomes 15 and 22 (10–13).

Other retrospective trials have found higher implantation rates with preimplantation aneuploidy testing and lower spontaneous abortion rates, with either no differences observed in ongoing pregnancy rates or a tendency toward higher ongoing pregnancies per cycle initiated (15, 27–30) and a significant reduction in trisomic conceptions (15). Taken together, the majority of studies indicate that preimplantation aneuploidy testing allows for similar ongoing pregnancy rates, while replacing fewer embryos and reducing the risk of spontaneous abortions. An average of two embryos was transferred in our reported test group, thereby reducing the risk of higher multiple gestations.

Screening embryos for aneuploidy as a means of embryo selection in AMA patients is theoretically very appealing because their incidence of chromosomal errors is known to be very high. It is thus puzzling that the beneficial effect of the technique is not higher. Several reasons for this have been proposed, including the limited number of chromosomes that are currently analyzed. Important chromosomal defects that lead to embryonic demise may therefore be missed. The use of comparative genomic hybridization or microarray technology, which is being tested clinically and is undergoing

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**TABLE 3**

<table>
<thead>
<tr>
<th>Results of embryo transfer.</th>
<th>Control group</th>
<th>Preimplantation aneuploidy test group</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>Incidence of no transfer</td>
<td>0</td>
<td>1 (3%)(^a)</td>
</tr>
<tr>
<td>Mean number of embryos transferred</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Pregnancy rate</td>
<td>76.6%</td>
<td>67.7%</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>37.3%</td>
<td>36.5%</td>
</tr>
<tr>
<td>Spontaneous abortion rate</td>
<td>32.26%</td>
<td>25.9%</td>
</tr>
<tr>
<td>Delivery rate</td>
<td>67.74%</td>
<td>78%</td>
</tr>
</tbody>
</table>

\(^a\) No normal embryos after preimplantation aneuploidy testing.

further optimization, will be able to screen for the full chromosomal complement of a single cell and could boost success rates. This could be particularly useful because the aneuploidy condition of the current FISH chromosomes are compatible with implantation.

In addition, mosaicism rates are known to be high in cleavage-stage human embryos (31), although the potential for error due to mosaicism may not be as high as the rates observed (32). However, it is feasible that the biopsy of a single cell may not represent the chromosomal compliment of the entire embryo, resulting in an intrinsic misdiagnosis rate from the analysis. A clinical error rate for PGD has been reported as approximately 2% by the European Society of Human Reproduction and Embryology (ESHRE), although this rate measures only abnormal babies born or miscarriages that have been karyotyped (33). Although some centers have reported error rates as low as 5% (22), others have reported rates as high as 50% (34). The occurrence of a misdiagnosis causing either a normal embryo to be wrongly discarded or the mistaken transfer of an aneuploid embryo could contribute to the lack of benefit seen with preimplantation aneuploidy testing. With lower rates of mosaicism observed in the human blastocyst on day 5 of embryonic development (35), blastocyst biopsy may be an alternative to embryo biopsy that can reduce the intrinsic misdiagnosis resulting from mosaicism.

Technically, the biopsy procedure itself may also negate some of the benefits of preimplantation aneuploidy testing. Indeed, embryo biopsy with either acid Tyrode’s or a laser has been shown to decrease cell numbers at the blastocyst stage compared with control embryos (36). Problems related to suboptimal fixation and/or FISH procedures may also contribute to a lack of benefit and the lower prevalence of trisomies to monosomies, emphasizing the importance of quality control and management in preimplantation aneuploidy testing.

Furthermore, the use of blastocyst culture as a method of selecting embryos for transfer in the control group may have minimized the selection advantage of preimplantation aneuploidy testing. High-quality blastocysts have been shown to have lower rates of chromosome abnormalities than embryos in general in this patient population (32, 37). Indeed, in our trial, selection of a top-quality blastocyst using morphologic criteria led to a 60% probability of transferring an embryo that was euploid. In addition, our study showed a correlation between embryo morphology and chromosomal aneuploidy on both day 3 and day 5. This is in agreement with several other published studies that have found a similar correlation during the stages of embryonic development (37–39). For example, in a study of 916 cycles, a statistically significantly higher incidence of chromosomal aneuploidies was observed in arrested, slow-cleaving, fast-cleaving, uneven, and fragmented embryos (39). Additionally, some patterns of pronuclear morphology have also been associated with a higher proportion of euploidy and a higher implantation rate (40). Thus, careful assessment of morphology alone can give some measure of selection against some chromosome abnormalities. All these factors contribute to the lower selection potential of preimplantation aneuploidy testing than previously anticipated, but they do not negate the observation that spontaneous abortions are reduced and fewer embryos need to be transferred.

In our prospective, randomized control trial, aneuploidy testing through embryo biopsy and FISH for a select number of chromosomes failed to significantly improve outcome in AMA patients. However, we observed equivalent implantation rates with or without preimplantation aneuploidy testing, so the biopsy technique had no obviously detrimental effect on blastocyst development rates. In addition, there was a trend toward decreased spontaneous abortion and increased delivery rates for patients in the test group.

Preimplantation aneuploidy testing requires a multidisciplinary approach. Extended embryo culture expertise, micro-manipulation skills for embryo biopsy, genetic counseling, and cytogenetics are all required. It has clear benefit in cases involving structural chromosomal defects and monogenic disease, including sex selection for X-linked disorders. Other potential benefits of preimplantation aneuploidy testing include the reduction in the number of embryos transferred and therefore the incidence of multiple births. In addition, preimplantation aneuploidy testing provides patients with diagnostic information about their embryos and their future potential for pregnancy, as the incidence of aneuploidy is similar from cycle to cycle (41). Hence, a patient whose cycle contains a high degree of chromosomal aneuploidy may choose to move on to other therapeutic options such as adoption or oocyte donation.

The future of preimplantation aneuploidy testing appears promising, with the upcoming opportunity to test all chromosomes using either comparative genomic hybridization or microarray technology (42, 43). In addition, experience is beginning to accumulate with blastocyst-stage biopsy (44), which has several advantages over cleavage-stage embryo biopsy. First, when embryos that arrest at the cleavage stage have been eliminated, there are fewer embryos to biopsy at the blastocyst stage. In addition, the rates of mosaicism (35) and lethal chromosomal abnormalities are also decreased (37). As the field of assisted reproductive technology moves toward single-embryo transfer, the requirement to select the “best” embryo for transfer will increase. Blastocyst biopsy combined with chromosomal aneuploidy testing of all 23 pairs of chromosomes may finally allow PGD to realize its true potential.

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