Simultaneous Detection of Chromosomes X, Y, 13, 18, and 21 by Fluorescence In Situ Hybridization in Blastomeres Obtained from Preimplantation Embryos

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Purpose. Simultaneous fluorescence in situ hybridization (FISH) was used in a preimplantation genetic diagnosis program to determine which embryos were normal for chromosomes X, Y, 13, 18, and 21.

Methods: Single blastomeres were disrupted and attached to glass slides using acetic acid and ethanol. Using a ratio mixture of chromosome enumeration DNA probes in combination with locus-specific identifier DNA probes, FISH was performed for the identification of chromosomes X, Y, 13, 18, and 21.

Results: Fourteen couples enrolled in IVF produced 134 embryos for biopsy. Blastomeres subjected to five-color FISH revealed that 22% of embryos were normal for chromosomes X, Y, 13, 18, and 21. In addition, 52% were abnormal and no results could be detected for 25%. Twelve couples underwent embryo transfer, two couples did not receive embryos due to lack of any normal embryos, and three couples became pregnant.

Conclusions: The simultaneous detection of five-color FISH is a feasible method to detect aneuploidy in preimplantation embryos from women of advanced maternal age.

KEY WORDS: advanced maternal age; aneuploidy; fluorescence in situ hybridization; preimplantation genetic diagnosis.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) in conjunction with in vitro fertilization (IVF) is becoming increasingly popular as a tool for genetic diagnosis of preimplantation embryos. As the technique of embryo

biopsy and cell processing becomes more routine, along with completion of the human genome project, IVF will be able to help not only the infertile population but also couples who are at risk for particular genetic diseases. Currently, PGD is being used to screen embryos for aneuploidy and single-gene defects (1).

Many embryos produced in IVF laboratories are from couples in which the female partner is of advanced maternal age (35 and older). In this age group, there is a significant increase in the number of aneuploid embryos (2). The frequency of chromosomal aneuploidy in chromosomes X, Y, 13, 18, and 21 is much higher in preimplantation embryos than in clinically recognized pregnancies (2). In women of advanced maternal age, replacing embryos free from chromosomal aneuploidies should increase their chance of pregnancy, reduce spontaneous abortions, and bring chances of pregnancy up to the levels of younger women.

This paper describes the results of application of a fluorescence in situ hybridization (FISH) technique in an IVF laboratory in conjunction with embryo biopsy, using a cocktail of five-color probes for chromosomes X, Y, 13, 18, and 21.

MATERIALS AND METHODS

Embryo Biopsy and Fixation

Fourteen couples (average maternal age, 39.2 years; range, 35–45 years) enrolled in the IVF program at our clinic consented to embryo biopsy. The informed consent forms were approved by the Western Institutional Review Board, protocol 120. Following standard oocyte aspiration, oocytes were inseminated either by conventional means or by intracytoplasmic sperm injection (ICSI) as described by (3). On the morning

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of the third day of development, all suitably developing embryos underwent embryo biopsy. Each embryo held by a holding pipette, had an opening created in the zona pellucida (ZP), using a 10- to 12- μ m flat-ended hollow microneedle filled with acid Tyrode's medium. A 30- to 40- μ m biopsy needle prefilled with medium was placed next to the hole in the ZP, adjacent to a single blastomere containing a single nucleus. The nucleated blastomere was removed by gentle suction using a mouth pipette and released free from the embryo. The embryo was removed and the cell was allowed to reform its original shape.

Blastomeres were placed on a glass slide while constantly being observed using a stereomicroscope (Olympus SZ40) (4). Blastomere movement was accomplished by using manually pulled capillary glass pipettes 1-mm inner diameter and 0.69-mm outer diameter, being approximately double the size of the blastomere's diameter. Individual blastomeres were rinsed in a drop of hypotonic solution (1% sodium citrate containing 6 mg BSA/ml in ultrapure water). To aid in the disruption of the cytoplasm, the blastomere was then placed on a glass slide along with approximately 10 μ l of hypotonic solution. When the drop of hypotonic solution had almost dried, a drop of fixative (1:3 acetic acid:ethanol) was dropped onto the blastomere. When the blastomere was no longer seen under the dissecting scope a circle was drawn around the cell using a tungsten carbide pencil. The slide was allowed to dry and then observed using a phase-contrast 10 $\times$ objective. If no nucleus was present, a second blastomere was removed from the embryo and subjected to the same treatment described above. The glass slide containing the fixed nuclei was dehydrated in a series of ethanol, 70, 85, and 100%, and allowed to air-dry.

Fluorescent In Situ Hybridization

A 22 $\times$ 22-mm coverslip was placed on the back of the slide containing the fixed blastomere nuclei and outlined using a tungsten-carbide pencil. A male lymphocyte slide (Vysis, Naperville, IL) was used as a positive control in each assay. The slide was submerged in a 70% formamide (GIBCO BRL, Gaithersburg, MD) and 2 $\times$ saline-sodium citrate, pH 7.5, solution for 5 min at 72.5°C. Following denaturation the slide was placed on a 45°C hotplate for 2 min. Probes for chromosomes X, Y, 13, 18, and 21 were prepared by ratio mixing. This mixing allowed for five-color simultaneous detection of the aforementioned chromosomes. CEP alpha satellite DNA for chromo-

somes X (Spectrum Aqua) and LSI probes for 13 (Spectrum Green) and 21 (Spectrum Orange) were used in the ratio mix (Vysis, Inc.). A fourth (pink) and fifth (yellow) signal were generated using a 2:1 ratio of satellite III DNA for chromosome Y (Spectrum Aqua and Spectrum Orange) and a 1:1 ratio of CEP alpha satellite DNA for chromosome 18 (Spectrum Green and Spectrum Orange), respectively. A cocktail of X, Y, 13, 18, and 21 probes were dried in a speed vac and reconstituted in 14 μ l of LSI hybridization buffer and 7 μ l of water. Following reconstitution, probes were denatured immediately in a 72.5°C water bath and spun in an Eppendorf centrifuge. Ten microliters of probe mixture was added to each slide while on the hotplate. A 22 $\times$ 22-mm coverslip was placed over the target nuclei, being careful to avoid inclusions of any air bubbles. The coverslip was attached using rubber cement and the slides were placed in a humidified chamber for 4 hr at 38°C. After 4 hr, slides were washed to remove unbound probe at 73°C for 5 min in 0.4 $\times$ saline-sodium citrate buffer/0.3% Nonidet P-40, pH 7.5, followed by a 1-min wash in 2 $\times$ saline-sodium citrate/0.1% Nonidet P-40, pH 7.5, at room temperature. For nuclear localization, 10 μ l of DAPI II counterstain [4', 6-diamino-2-phenyl indole (DAPI II); Vysis, Inc.] was added to each slide and covered with a 22 $\times$ 22-mm coverslip.

Scoring criteria were performed as described by Munné *et al.* (5). Slides were viewed on an Olympus BX60 fluorescent microscope equipped with an imaging system containing Quips software (Vysis, Inc.). Using single-bandpass filter sets including aqua, rhodamine, FITC, and DAPI (Vysis, Inc.), a dual-bandpass filter set, FITC/rhodamine (Vysis, Inc.), and a triple-bandpass filter set, DAPI/FITC/rhodamine, (Vysis, Inc.), we were able to observe all five chromosomes individually or in combination.

RESULTS

Fourteen patients with an average age of 39.2 years contributed 134 embryos for biopsy. Five-color FISH analysis revealed that 22% were normal (Fig. 1) for chromosomes X, Y, 13, 18, and 21. In addition, 52% were abnormal while no results were detected on 25%. Twelve couples had enough embryos for transfer, while two couples had no transfer due to lack of normal embryos. Pregnancy was initiated in four couples, however, in one couple, a spontaneous abortion occurred during the fifth week, giving an overall delivered/viable pregnancy rate of 21.4%.

Fig. 1. Fluorescence in situ hybridization of a nucleus removed from a day 3 embryo. Hybridization of CEP alpha satellite DNA for chromosomes X (aqua) and 18 (yellow), satellite III DNA for chromosome Y (pink), and LSI probes for chromosomes 13 (green) and 21 (orange). *Top:* Two of each of the chromosomes are present, indicating a normal female embryo. *Bottom:* Nucleus obtained from a normal male embryo.

In the first couple who initiated a pregnancy, the female, 40 years old, had fibroids, multiple miscarriages (one being trisomy 15), and one child from

natural conception. On day 13 of her cycle she went to egg retrieval and 24 oocytes were collected, following conventional insemination; 7 oocytes fertilized nor-

mally and 7 embryos were biopsied on day 3. Results revealed that two embryos were diagnosed as normal, one was diagnosed as monosomy 21, one was haploid, one was binucleate, and two had no nucleus following fixation. In the embryos with no nucleus following fixation, the blastomere ruptured during the biopsy procedure of the first embryo and the second embryo was severely fragmented. It was decided to replace three embryos, the two normal and the one with a reading of monosomy 21. A pregnancy was initiated and amniocentesis revealed a normal 46,XX karyotype followed by the recent birth of a healthy 7 lb 8 oz female.

In the second couple, the female partner, 38 years old, had tubal factor and had been pregnant three times. She has had two ectopic pregnancies and one spontaneous abortion with no karyotype information. Fourteen eggs were retrieved and 11 mature eggs were inseminated by ICSI. On the following morning eight normally fertilized oocytes were observed, and eight embryos were subjected to embryo biopsy. FISH analysis revealed two normal embryos, three abnormal, and three with no results, of which no nucleus was found during fixation in two and the cytoplasm was over the nucleus in one. The two normal embryos, plus the two embryos with no nucleus found following fixation, were replaced. The two embryos in the no-result category were transferred based on their morphology. They both had even-sized blastomeres with no fragmentation. Amniocentesis karyotype was reported as normal.

In the third case, a 37-year-old female and her husband consented to embryo biopsy in their third IVF cycle. The woman had never been pregnant and their infertility history included endometriosis, adhesions, and cervical stenosis. Thirteen eggs were retrieved on the day of egg retrieval, of which 11 mature oocytes were subjected to the ICSI procedure, in which seven eggs fertilized. On day 3 of development, embryo biopsy was performed on seven embryos. Two embryos were established as abnormal by FISH and not transferred. One normal embryo was transferred, three with no nucleus from fixation and one diagnosed with monosomy 13. The no-nucleus embryos were replaced based on their morphology criteria. Amniocentesis was performed and revealed a normal karyotype.

One patient, 37 years old, first cycle, who had had two previous pregnancies (one birth) presented with stage III endometriosis and fibroids. Twelve eggs were retrieved and conventionally inseminated, and seven embryos were biopsied on day 3. Only one embryo

was fragmented and the blastomere ruptured on removal from the embryo. This embryo was diagnosed as abnormal. Two embryos were diagnosed as normal, three were abnormal, and one had no nucleus from fixation. The two normal embryos and the one with no results were transferred. Unfortunately, 5 weeks into her pregnancy a dilation and curettage had to be performed, and the chromosome analysis of the products of conception indicated a 46,XX normal female karyotype. However, fetal tissue could not be identified with certainty prior to culture of the products of conception. The results could be from maternal tissue.

DISCUSSION

As described by Munné *et al.* (2), the number of aneuploid human embryos is increased in women of advanced maternal age. Due to this increase, there is a decrease in the number of viable embryos that may actually implant, thus lowering the implantation rate in these women. The data generated by our IVF program are in agreement with this, because we see a decrease in pregnancy rates in women of advanced maternal age. In our PGD program, our 21.4% pregnancy rate is below our IVF pregnancy rate for women of advanced maternal age. This is most likely due to the low number of patients having embryo biopsy and may also be due to the difficult infertility histories of the women who enrolled for this research program.

In cases in which too few normal embryos are available for transfer, embryos diagnosed with monosomy 13, 18, and 21 can be transferred. Autosomal monosomies are virtually undetectable in clinically recognized pregnancies. However, monosomy 21 has an incidence of 1/1000 in karyotyped abortions (6), and because the FISH technical error is greater (13.3%) (2) than the chance of the embryo implanting and going to term, these embryos can be transferred. The case presented here demonstrates the feasibility of replacing a monosomy 21 embryo. However, this diagnosis could have been made in error, because signal overlap does occur in FISH technology, thus a normal embryo could have been replaced. The pregnancy and birth of a healthy 46,XX female demonstrate the feasibility of replacing a monosomy 21 embryo.

The infertility histories of couples who did not become pregnant following embryo biopsy include endometriosis, severe male factor, confirmed amniocentesis for trisomy 21, and fibroids. These patients may have some genetic abnormality that was not

detected by FISH for the chromosomes in our screening panel. It is worth mentioning that patients who are undergoing IVF with ICSI have chromosomal analysis prior to their procedure. In a study involving 150 couples with male factor, an unexpectedly high percentage of women (6%) and men (12%) had chromosomal abnormalities. These abnormalities included Robertsonian translocations, reciprocal translocations, inversions, and autosomal reciprocal translocations (7). Therefore, more genetic analysis may be warranted for patients in this category and for patients who have had conventional insemination and who did not have a pregnancy initiated following embryo biopsy.

Another reason for not becoming pregnant following embryo biopsy is the use of Tyrode's acid for zona drilling. Even though 11 normal babies have been born following embryo biopsy (8), it is still unclear if Tyrode's acid is detrimental to the embryo if too much is used during the drilling procedure. Uniform hole size is of great importance to embryo biopsy. If the hole is too small, there is a chance that twinning can occur. If the hole is too large, then the entire contents of the embryo may be lost during transfer. Recently, manipulation of the ZP by lasers (9) has proven to be an attractive method by which the ZP may be opened. However, lasers are expensive, and consequently not many laboratories are using them in their PGD programs yet.

Two couples did not have an embryo transfer. In the first couple, the wife, 38 years old, had been pregnant eight times and had had eight spontaneous abortions. During their second IVF cycle, 12 embryos were produced for biopsy. Seven were found to be abnormal and five contained no nucleus. In the second case, the wife, 39 years old, opted for embryo biopsy during their fifth IVF cycle. Embryo biopsy was performed on 10 embryos. Six abnormal embryos were diagnosed, and no results occurred in four embryos. No male factor was involved in either case. However, a high percentage of fragmentation (>10%) was present in embryos produced by both couples. It was decided, based on genetic analysis, the poor embryonic morphology, and the difficult embryo biopsy, that no embryos would be replaced in either of these two couples.

Based on the embryo biopsy results presented here, it appears that the most difficult embryos to biopsy are the ones with greater than 10% fragmentation. The quality of embryos produced in IVF laboratories varies greatly. A variety of chromosomal abnormalities was detected in thawed embryos obtained from four centers worldwide (10). Some embryos develop equal-sized

blastomeres with little or no cellular fragmentation, while others develop uneven-sized blastomeres with fragmentation. When fragmented embryos are placed in the uterus, these embryos have limited developmental potential to initiate a pregnancy (11,12). When fragmented embryos were compared to embryos having good morphology there was an increase in chromosomal abnormalities in the fragmented embryos (13–15). Examined histologically, blastomeres obtained from fragmented embryos appeared to be undergoing apoptosis as a result of programmed cell death (16). In the case of embryo biopsy, the most difficult to biopsy are embryos that have excessive fragmentation, which, in part, could be contributing to our 25% no-results category. Other contributing factors include loss of the blastomere during the fixation process and the absence of a detectable signal from a FISH analysis.

Mosaicism is the most commonly found abnormality in preimplantation embryos (17–20). This phenomenon causes speculation on the accuracy of embryo biopsy. Removing one cell and obtaining a normal reading does not allow us to know the rest of the embryo's chromosomal constitution. Therefore, removal of two cells would be better if possible. It has been shown in several studies that abnormal cells in the early embryo are diverted to the trophoctoderm and not the inner cell mass (21). It also has been reported in humans that blastocysts have a mix of mosaicism of diploid and polyploid cells (22).

In conclusion, we have demonstrated that simultaneous five-color FISH is a feasible method to detect chromosomes X, Y, 13, 18, and 21 in human preimplantation embryos. The next step in our preimplantation genetics program is to incorporate chromosome 16 into our screening panel. Benadiva *et al.* (23) have reported a high occurrence of monosomy 16 in preimplantation embryos derived from women of advanced maternal age, thus suggesting that chromosome 16 aneuploidy may be contributing to the failure of embryo implantation.

We cannot emphasize enough that proper counseling of patients is required prior to embryo biopsy and PGD. Patients need to be informed about the possibility of having no normal embryos for transfer or not having a biopsy due to unhealthy and/or difficult to biopsy embryos. Patients requesting PGD are strongly encouraged to have an amniocentesis performed to confirm PGD results. It is hoped that in the near-future, when probes are available for all 24 chromosomes, amniocentesis may not be necessary in IVF patients of advanced maternal age.

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