Brief Report

Preimplantation Genetic Diagnosis for a Couple with Recurrent Pregnancy Loss and Triploidy

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BACKGROUND: Triploidy may arise from fertilization of a mature haploid egg by two haploid sperm or by failure of meiotic divisions yielding a diploid gamete. We encountered a couple with habitual abortion, in which the last two fetuses were documented as viable triploid.

METHODS: To avoid dispermic penetration and development of abnormal preembryos, insemination was done by intracytoplasmic sperm injection (ICSI) followed by fluorescence in situ hybridization (FISH) of biopsied blastomeres.

RESULTS: Tests of the husband’s spermatozoa by FISH, revealed that only 2–3% of the sperm were disomic for chromosomes 16, 13, 21, X, and Y. No triple disomy was detected among chromosomes 16, 13 and 21, which makes it very unlikely that triploidy resulted from diploid spermatozoa. Following a controlled ovulation induction protocol, low quality oocytes with immature cumuli were revealed. After ICSI, five eggs became two pronuclei (2PN) zygotes and none of the other eggs developed a 3PN zygote. FISH was performed on chromosomes 16 and 21 in four preembryos developed to a 6–8 cell stage. Aneuploidy or mosaicism for each of these chromosomes was detected in one preembryo and later in two disaggregated blastocysts. FISH failed in one preembryo that became atretic after biopsy.

CONCLUSIONS: Although this case was unsuccessful in achieving embryo transfer and normal pregnancy, we detected many abnormal morphological features in the oocytes and chromosomal abnormalities in the cleaving preembryos. This protocol can be proposed to patients with recurrent pregnancy loss associated with chromosomal abnormalities in the fetus. Birth Defects Research (Part A) 67:946–950, 2003. © 2003 Wiley-Liss, Inc.

Key words: triploidy; fluorescence in situ hybridization (FISH); in vitro fertilization (IVF); intracytoplasmic sperm injection (ICSI); preimplantation genetic diagnosis (PGD)

INTRODUCTION

Human triploidy is a common sporadic abnormality that occurs in about 1–3% of clinically recognized pregnancies. Most triploid fetuses end as spontaneous or missed abortions. It is very rare to have triploid conceptuses reaching term. These fetuses usually end as severely abnormal liveborns (Hasegawa et al., 1999), and usually die soon after birth. When a large series of pregnancy losses were karyotyped, triploidy constituted approximately 5.4% (Priest et al., 1997) to 7% (Jacobs et al., 1982) of the detected karyotypes. Despite this common incidence among sporadic pregnancy losses, very few recurrences have been reported, even among couples with bona fide recurrent pregnancy losses. In a recent report, 6% of the karyotyped abortuses revealed three haploid sets of chromosomes (3n) in a group of women that were evaluated twice for recurrent abortions (Priest et al., 1997). When data of this study were combined with other reports in the literature, a total of eight recurrent triploids have been documented in 357 women who underwent chromosomal analysis of two consecutive spontaneous abortions. This constitutes a 2.2% recurrence rate (Priest et al., 1997). Only rarely has analysis been attempted on more than one product of conception from the same couple (Warburton et al., 1987). Out of 9493 karyotyped pregnancies, only 0.5% had repeated analysis (Priest et al., 1997). Therefore, as with other sporadic chromosomal abnormalities, the traditionally assigned recurrence risk for triploidy is not increased compared to the general population and is less than or equal to 1% in each future pregnancy.

Triploidy is most frequently derived from fertilization of a mature haploid egg by two haploid sperm cells (dispermy). Alternatively, triploidy can be caused by a failure of the first or second paternal or maternal meiotic divisions yield-
ing a diploid gamete. With recent access to preembryos, endoreduplication in a human zygote (Rosenbusch et al., 1997, 2002) and incorporation of the second polar body into a blastomere (Muller et al., 1993) have also been implicated. We report a couple who experienced two early miscarriages followed by two consecutive pregnancy terminations for triploidy. This couple was reluctant to try to conceive naturally and sought assisted reproductive technology (ART).

MATERIALS AND METHODS
Institutional Review Board and Patient Consent

The Institutional Review Board of the Faulkner Hospital approved the program to perform PGD on the human preembryos and also approved the patient consent forms. All couples, including the couple presented in this study, were informed in detail in several counseling meetings of the advantages, limitations, and risks of the treatment. In addition, the couples received detailed explanatory notes concerning the technique prior to providing signed consent to perform PGD on their preembryos.

Subjects

We were approached by a 30-year-old woman with a history of oligoovulation and her 29-year-old husband who had a history of hypertension. Both had normal peripheral blood karyotypes. The woman conceived twice with miscarriages occurring at seven and five weeks, respectively. The two conceptuses were not karyotyped. A third pregnancy was achieved within a year and was terminated at 17 weeks owing to severe fetal malformations; the karyotype was 69,XXX. Within a year the woman conceived again and an ultrasound examination again revealed that the fetus carried multiple abnormalities. Chorionic villi testing revealed a karyotype of 69,XXY. Despite severe malformations, this fetus continued to be viable well into the second trimester. The pregnancy was terminated at 17 weeks and the diagnosis of triploidy was confirmed. The placenta of neither triploid fetus showed any molar changes. During genetic counseling, the couple expressed the fear that they were at high risk for triploidy for any future pregnancy. Gamete donation was unacceptable to them owing to the difficulty to ascertain the “culprit” gamete. Other options, such as embryo donation or adoption, were also considered unacceptable.

Treatment Design

The treatment was designed based on the available information regarding the theoretical possible origins of triploidy. The planned protocol included sperm analysis (e.g., number, motility) with strict criteria for morphology (Kruger et al., 1988) and assessment for possible existing aneuploid sperm cells. The latter was done by fluorescence in situ hybridization (FISH) analysis of chromosomes X and Y or chromosomes 16, 13 and 21. For each of these two combinations 100 sperm nuclei were scored. Sperm that showed a single signal of each of these chromosomes were considered as probably haploid. This was followed by an in vitro fertilization protocol with ICSI and subsequent embryo biopsy and FISH analysis for chromosomes 16 and 21. The guiding reasoning was that ICSI would preclude triploidy as a result of dispermic penetration. The rationale behind blastomere biopsies and two-chromosome analysis was to safeguard against any other sources of abnormality, including oocyte meiotic failure or postzygotic occurrence of chromosome nondisjunction events. Chromosomes 16 and 21 were chosen because the rate of aneuploidy of both chromosomes is relatively high in aborted fetuses and miscarriages (Nicolaidis and Petersen, 1998; Fritz et al., 2001).

In Vivo Treatment and Oocyte Culture

Multiple follicular growth was induced with exogenous administration of follicle stimulating hormone (FSH), Metrodin (Serono, Randolph, MA), plus human menopausal gonadotropin (hMG), Pergonal (Serono) after pituitary suppression with Luprone (TAP Pharmaceuticals Inc.,Dearfield, IL). Ovarian follicular content was aspirated about 35 hr after a single intramuscular injection of 10,000 U hCG (Serono).

The retrieved cumulus oocyte complexes (COCs) were placed immediately in 1.5 ml of Human Tubal Fluid (HTF) medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% manufactured human serum (Irvine). Two COCs were placed in each organ-culture dish (Falcon, Oxnard, CA) and cultured at 37°C in 5% CO2 and 95% air. COC morphology was examined shortly after aspiration under an inverted light microscope (IM 2, Olympus, Japan). Maturation was graded according to the size of cumulus mass and the degree of dispersion of both corona and cumulus mass, as previously described (Bar-Ami et al., 1989). About 4 hr later, cumulus cells were removed by 1–2-min exposure to hyaluronidase, 100 IU/ml (Sigma, St. Louis, MO) and repeated pipetting using a glass pipette with a 130-μm internal diameter. The morphology of oocyte cytoplasm, first polar body (PBI), zona pellucida, size of perivitelline space, and presence of vesicles under the zona pellucida was examined, as previously described (Bar-Ami et al., 1989).

ICSI was carried out as described previously (Manor et al., 1994) utilizing a Humagen injecting pipette (Humagen, Charlottesville, VA) on 12 out of 14 eggs, excluding two degenerating eggs. Fertilization was checked on the next day and each of the eggs with two pronuclei (2PN) was transferred to a new and separate culture dish. Development of preembryos was examined every 24 hr and the culture medium was replaced every day with fresh culture medium. Embryos were graded according to the criteria of Hardy et al. (1995): grade A, even or uneven blastomeres with little fragmentation; grade B, even or uneven blastomeres with a little fragmentation; grade C, 25–50% of the blastomeres are fragmented; and grade D, more than 50% of the blastomeres are fragmented. In addition, the number of blastomeres, fragmentation, morphology of cytoplasm, and presence of nucleated or multinucleated blastomeres was also examined. Advanced preembryo growth and development was evaluated according to blastomere multiplication, formation of blastocyst cavity, and bulging of inner cell mass.

Sperm Fixation and FISH

Sperm preparation included several washes of the semen in HTF + 10% human serum albumin (HSA) (Irvine). Prior to fixation, sperm were washed in calcium- and magnesium-free phosphate buffered saline (PBS, Gibco, Grand Island, NY) containing 6 mM ethylene diamine tetra-acetic acid (EDTA) (Sigma Chemical Co., St. Louis, MO) and then
incubated 20 min in 2 mM dithiothreitol (DTT) in PBS. Sperm were washed in PBS and fixed in a 3:1 solution of methanol and acetic acid for 30 min at 4°C, then dropped on precleared microscope slides and air-dried overnight at room temperature.

The sperm on the microscope slides were further dehydrated through ascending ethanol concentrations to 100%. Some sperm nuclei were stained using CEP-X (spectrum orange) and CEP-Y (spectrum green) (Vysis, Downers Grove, IL). Other sperm nuclei were treated with Oncor probes (Oncor, Gaithersburg, MD) for chromosome 16 (fluorescein-labeled 16 alpha satellite) and for a combination of chromosomes 13 and 21 (Texas Red labeled 13/21 alpha satellite). FISH was done according to the manufacturer’s instructions. All slides were counterstained with a 1:100 dilution of diamin-o-2-phenylindol-dehydrochloride (DAPI) in Antifade (Oncor).

Preembryo Biopsy and FISH

On day three, when the preembryos were in the six-to-eight cell stage, two blastomeres were removed from each preembryo by micromanipulation using a fluid displacement biopsy technique (Pierce et al., 1997, 1998). In brief, embryos were placed in calcium- and magnesium-free PBS and medium was then injected under the zona pellucida to gently displace blastomeres through a previously made opening. If for any reason one of the biopsied blastomeres had degenerated, then a third blastomere was removed for FISH analysis. After washing in serum-, calcium-, and magnesium-free PBS (Gibco, Grand Island, NY), each blastomere was placed on a microscope slide in a 1.5-ml drop of lysis solution (0.1% Tween 20 and 0.01 N HCl in double distilled water) (Coonen et al., 1994) to cause cell breakage and spreading of the blastomere cytoplasm. Following this, clean nuclei are revealed. After partial drying, nuclei were fixed in methanol:acetic acid (3:1). The air-dried microscope slide was further dehydrated through an ascending ethanol series 50-100%.

The FISH probes for chromosome 16 (D16Z2 at centromere) and for chromosome 21 (LSI21 D21S259, D21S341, D21S342 at 21q22.13-q22.2) were purchased from Oncor and Vysis, respectively. The latter probe was specific for chromosome 21 and distinct from the probe detecting both chromosomes 13 and 21 used for FISH analysis of sperm. The chromosome 16 probe was biotin-labeled and its signal was used reagents from Oncor. The nuclei hybridization signal was counterstained with DAPI. No hybridization study was done on the nonfertilized oocytes due to their low quality at the time of retrieval, which became worse during the following 48 hr. Our practice protocol did not allow the study of nonfertilized oocytes in a shorter period for the possible occurrence of late fertilization.

Hybridization was observed using a Zeiss Axioskope microscope (Zeiss, Oberkochen, Germany), and images were captured and printed using an Applied Imaging CytoVision system (Applied Imaging Corp., Santa Clara, CA). Prior to FISH analysis on blastomeres, the protocol for staining chromosomes 16 and 21 was tested on 220 human lymphocytes. Of these, 211 (96%) nuclei showed two green (C16) and two red (C21) signals.

<table>
<thead>
<tr>
<th>Type of signal</th>
<th>Sex chromosomes</th>
<th>Chromosomes 16 + both 13/21</th>
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<tbody>
<tr>
<td>Haploid Signal</td>
<td>% Total</td>
<td>% Total</td>
</tr>
<tr>
<td>X</td>
<td>52</td>
<td>16 + 13/21d</td>
</tr>
<tr>
<td>Y</td>
<td>45</td>
<td>16 + 2–13/21f</td>
</tr>
<tr>
<td>Summary</td>
<td>97</td>
<td>Summary</td>
</tr>
<tr>
<td>Nonhaploid signal</td>
<td>2–Xb</td>
<td>2–16f</td>
</tr>
<tr>
<td>XY</td>
<td>2</td>
<td>2–16 + 4–13/21g</td>
</tr>
<tr>
<td>Summary</td>
<td>3</td>
<td>Summary</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disomy and/or aneuploidy</th>
<th>Disomy and/or diploidy</th>
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*No sperm having 2–Y were detected.
*Disomy for chromosome X
*Aneuploidy for the sex chromosomes.
*In 21 nuclei, the second signal was very weak.
*Double disomy would make it more likely as diploid sperm.
*Possible fragmented signal of chromosome 16.
*Such abnormality would be assumed to represent diploid sperm.

RESULTS

Sperm and Oocyte Parameters

The husband’s sperm parameters were within known normal limits in both fresh semen (volume = 4 ml; total sperm count = 76 × 10⁶; motility = 77%; and progression was good) and in sperm that were prepared for ICSI by the sperm rise technique (total sperm count = 28 × 10⁶, motility = 89%; and progression was excellent). Sperm evaluation by strict morphological criteria (Kruger et al., 1988) on two raw semen samples indicated that the percentage of normal forms was 4% and 6%. FISH analysis was done using different probe combinations of both X and Y chromosomes or chromosomes 16, 13 and 21. The X and Y probes provided bright fluorescence. The green signal for the Y chromosome was fragmented on most nuclei, where present, but could be scored as a single chromosome due to the close proximity of the fluorescent spots. The orange signal for the X chromosome was a single bright spot in most cases. Table 1 indicates that of the 100 sperm nuclei tested, 97 had either a single X or single Y chromosome signal, 1 had two X chromosome signals, and 2 had both X and Y chromosome signals.

The chromosome 16 probe with amplification provided strong signals with very low background staining. Of 100 sperm nuclei, 98 had one signal and 2 had two signals for chromosome 16. The probe recognizing both chromosomes 13 and 21 yielded one bright signal and one less intense signal on most sperm nuclei. The low signal intensity of one of these chromosomes was very likely the cause of the inability to clearly detect both chromosomes in 21% of the sperm (rather than aneuploidy); the score was 21 nuclei with 1 C16 and 1 clear C13/C21 (Table 1). Overall, this FISH analysis indicates that in 97–98% of the sperm tested, chromosomes 16, 13 and 21 were haploid (monosomic) and no triple disomy was noted in these autosomal chromosomes.

Preembryo Development and FISH Analysis

Of the 14 retrieved COCs, only 2 showed fully mature cumuli, 9 were partially mature, and 3 were intermediate.
After removing the surrounding cumulus mass and corona cells, various abnormal features were revealed. Five oocytes had grossly abnormal features such as vacuoles in the cytoplasm or dark and yellowish cytoplasm. Although all oocytes had emitted the first polar body (PBI), it was fragmented in each case.

Following ICSI, five eggs had 2PN and one egg had one pronuclei (1PN). A second polar body was emitted from each of these eggs. The 1PN egg and one of the 2PN eggs failed to cleave within 48 hr of sperm injection. The 1PN egg did not cleave, even after 120 hr incubation. The 2PN egg later cleaved to three uneven cells and remained so during 120 hr incubation. The four other 2PN eggs cleaved to two cells, all with fragments. Two had one nucleus per blastomere, one preembryo had a blastomere with three nuclei, and one preembryo had no detectable nuclei in either blastomere. At the four-cell stage all were graded as type C preembryos. These preembryos cleaved to six to eight cells by 72 hr postinsemination and therefore were selected for FISH analysis.

FISH signals were clearly detected only in one out of the four biopsied preembryos. The two biopsied cells each contained nuclei with two chromosome 16 signals and three chromosome 21 signals. This preembryo failed to cleave further and eventually started to degenerate. No hybridization signals were detected following FISH analysis on the remaining cells, probably due to poor quality of all available cells for hybridization and/or nuclear degeneration. Two of the other three preembryos developed to the blastocyst stage, but without a clear inner cell mass in either of them. In one of these preembryos, two nuclei had two chromosome 21 signals, but no chromosome 16 signals, while two other nuclei had no detectable hybridization signals. The other preembryo was found to be mosaic, with from one to four hybridization signals for either chromosome in the 19 nuclei examined. The fourth preembryo had no detectable nuclei at the two- or eight-cell stage and no nuclei or hybridization signals were detected following FISH. This preembryo stopped cleaving and eventually degenerated.

In summary, FISH analysis of chromosomes 16 and 21 revealed that none of the three diagnosable preembryos manifested simple trisomy for either of the tested chromosomes. However, aneuploidy or mosaicism for these chromosomes was detected in all three preembryos.

**DISCUSSION**

The basic assumption in the current attempt to avoid recurrent triploidy was that by using ICSI there was less possibility of obtaining 3PN zygotes due to dispermic penetration. Although the previous two abortuses of the current patient were found to be triploid, we did not detect 3PN zygotes. Observation of such tririsonucleated zygotes, if they occur, would most likely be a result of oocyte meiotic failure. In the present study, the use of ICSI prevented the possibility of dispermic penetration as a cause for abnormal preembryos, so we were unable to assess whether the eggs were able to produce the normal block to polyspermy. Previous studies indicated that double or triple disomy are equally useful to show the presence of diploid sperm (Guttenbach et al., 1997; Rademaker et al., 1997). In the present study, FISH analysis of sperm nuclei indicated no triple disomy for chromosomes 13, 16, and 21 (Table 1). Thus, diploid sperm penetration would be an unlikely cause of the past triploid fetuses.

The two past triploid abortuses of this patient were viable until removed at the second trimester and no indication of complete or partial hydatidiform mole development was noted in their records. Usually, such results are associated with fetuses developed from digenic oocytes, i.e., with an extra maternal chromosome complement as the result of meiotic division failure (Hoffert, 1998). Despite the high level of aneuploidy, the data collected did not uncover evidence of meiotic failure that would lead to the formation of a triploid embryo, such as failure to produce the first or second polar body. A recent study (Pergament et al., 2000) proposed that an error in maternal meiosis II led to the development of triploid preembryos in ICSI inseminated eggs of a woman who had two previous triploid conceptuses.

Our experience (Bar-Ami et al., 2001) is that the nuclear status of preembryos at the two- to three-cell stage is indicative of preembryo quality and developmental competence. In the general in vitro fertilization (IVF) preembryo population at least one multinucleated blastomere was detectable in about 50% of the two- to three-cell stage preembryos (Bar-Ami et al., 2001). This agrees with other recent studies, indicating that about 50% of the preembryos show nuclear abnormalities, defined as abnormal, mosaic, or chaotic (Carrera and Veiga, 1998; Munne et al., 1998). In the present study, examining preembryos at the two-cell stage revealed that one preembryo had one multinucleated blastomere and another preembryo had undetectable nuclei in both cells as well as in further developmental stages. This may be associated with the failure of these two preembryos to develop into blastocysts.

Recent studies indicated a significant increase in aneuploidy in preembryos obtained after IVF from patients with recurrent abortion compared to others without such a history (Simon et al., 1998; Stephenson et al., 2002; Rubio et al., 2003). Similarly, an increased rate of above 50% of aneuploid preembryos was noted in women with poor prognosis for full-term pregnancy (Gianaroli et al., 2000). This high incidence of aneuploidy was detected even in the morphologically normal population of preembryos of poor prognosis patients (Magli et al., 1998). Thus, some patients are more prone to have chromosomal abnormalities. This recent information, together with current knowledge regarding high chromosomal abnormality in IVF preembryos diagnosed as 2PN zygotes (Jamieson et al., 1994; Munne et al., 1998; Ruangvutilert et al., 2000), may indicate that the present couple has a higher tendency to produce preembryos with nuclear abnormalities. Preembryos with poor morphology have been associated with an increased tendency to produce aneuploid blastomeres (Edirisinghe et al., 1992; Pellestor et al., 1994; Laverge et al., 1997; Viville et al., 2000). In the present study, testing of the two chromosomes (16 and 21) by FISH indicated that none of the preembryos was normal and we found no evidence of triploid formation. This high aneuploidy in the successfully diagnosed preembryos could be anticipated as a consequence of the low quality of the eggs as determined by morphological criteria. In view of the fact that the two prior triploids were from natural pregnancies, it is possible that superovulation generated additional problems that superseded triploid formation and led to aneuploidy. This might be supported by current acceptance that high-de-
grees of aneuploidy and chaotic preembryos occur in IVF/ICSI preembryos.

As a result of the present study, we would like to propose its strategy for treatment of patients with recurrent abortion of chromosomal abnormal fetus who are reluctant to reattempt prenatal diagnosis after natural pregnancy. The present study program proposes an assisted reproduction technology (ART) strategy for achieving both a normal pregnancy in a controlled system and also acquiring some insight about the possible sources of the prior abortions and malformations.

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REFERENCES


