An association between sex chromosomal aneuploidy in sperm and an abortus with 45,X of paternal origin: possible transmission of chromosomal abnormalities through ICSI

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BACKGROUND: Although it has been speculated that the increased de-novo chromosomal abnormalities in ICSI pregnancies may be associated with an increase of aneuploidy in sperm from infertile men, little direct evidence exists to support this claim. We studied sperm from an infertile man with an abortus from ICSI to determine if increased sex chromosomal aneuploidy in the sperm could have contributed to the karyotype of the abortus.

METHODS: The couple underwent ICSI due to severe oligozoospermia. Spontaneous aborted material was subjected to cytogenetic and molecular tests to ascertain the existence, type and origin of a chromosomal abnormality. Sperm from the man were analysed by multi-coloured fluorescent in-situ hybridization (FISH) with probes specific for chromosomes X, Y and 18.

RESULTS: At 8+ weeks after embryo replacement, the patient spontaneously miscarried. Both cytogenetic and comparative genomic hybridization analysis of aborted material showed a 45,X karyotype. Origin of the abnormality was established as a loss of the paternal X chromosome. FISH analysis of sperm revealed 19.6% (1990/10 164) nullisomy for a sex chromosome and 18.6% (1886/10 164) with XY disomy, which is significantly increased when compared to controls with 0.3% (58/20 429) and 0.1% (20/20 429) respectively (P < 0.0001).

CONCLUSIONS: This study indicates that the paternal origin of the 45,X abortus was likely the result of a high level of nullisomy in the sperm and provides evidence for the transmission of chromosomal abnormality from sperm to the conceptus through ICSI.

Key words: aneuploidy in sperm/FISH/ICSI/parental origin of chromosomal abnormality

Introduction

ICSI is the most effective assisted reproductive technique in the treatment of male factor infertility. The injection of a single sperm through the oocyte membrane, resulting in fertilization, has allowed men with severely compromised semen parameters to father their own biological children. However, the invasiveness of this technique raises concern about the risk of transmitting genetic abnormalities and ultimately increasing the rate of chromosomal abnormalities in the resulting pregnancies. A recent report of prenatal diagnoses done on 1586 fetuses conceived from ICSI (Bonduelle et al., 2002) found a significant increase in de-novo (non-inherited) chromosomal anomalies of 1.58% (P < 0.001), while only 0.45% of de-novo abnormalities are found in the normal population (Jacobs et al., 1992). The de-novo sex chromosomal anomalies alone represented 0.63% of the prenatally tested ICSI fetuses (Bonduelle et al., 2002) compared to 0.19% in the population (Jacobs et al., 1992).

It has been recognized that abnormal chromosome constitutions occur more frequently in infertile men (Chandley et al., 1979). In addition, men with a normal somatic karyotype may have chromosomal abnormalities limited to only the germ cells (Egozcue et al., 1983; Calogero et al., 2001). Several fluorescent in-situ hybridization (FISH) studies have indicated an increased frequency of sex chromosome abnormalities in men with severe oligoasthenoteratozoospermia (OAT) (Moosani et al., 1995; Bernardini et al., 1997; Pang et al., 1999) and in men with abnormal semen parameters (Colombo et al., 1999). Despite the existence of much data on the aneuploidy rate in sperm from FISH studies and for aneuploidy rate in ICSI outcomes, the correlation between the two remains speculative.

FISH studies that looked at the sex aneuploidy rate in sperm from men who fathered (by natural conception) children with Turner syndrome (Martinez-Pasarell et al., 1999) and Klinefelter syndrome (Eskenazi et al., 2002) have also shown...
were amplified using highly polymorphic X-linked microsatellite markers (androgen receptor: AR; fragile X mental retardation: FMR1) (described in Allen et al., 1992; Carrel and Willard, 1996; Hecimovic et al., 1997). Analyses of PCR products were done with an ABI Prism 310 Genetic Analyzer, with GeneScan Analysis Software version 3.1.2.

If paternal origin was concluded, sperm collected from the father would be analysed by multi-coloured FISH, with probes specific for chromosomes of interest.

### Ascertaining and processing of sperm

Semen samples were obtained from the treatment couple and two other normal healthy males (aged 35 and 40 years) of assumed normal fertility. Semen samples were washed in 1×Hanks’ buffer, and fixed in methanol:glacial acetic acid (3:1). Cell pellets were stored at −20°C until processed for FISH.

Fixed sperm were dropped onto pre-cleaned slides and washed twice in 2×SSC (saline sodium citrate). The slides were then incubated in 20 mmol/l dithiothreitol in 1 mol/l Tris–HCl buffer (pH 8), and adequate decondensation of sperm was achieved when the diameter of sperm heads increased to a level that would allow for efficient hybridization and visualization of FISH probes while still being able to detect the sperm tails. The slides were then washed in 2×SSC and phosphate-buffered saline and dehydrated in ethanol series (70–100%) and air-dried.

### Fluorescence in-situ hybridization

All sperm samples (one test and two controls) were processed with directly labelled DNA probes specific to alpha-satellite repeat clusters in the centromeric region of chromosomes 18 [CEP 18 (D18Z1) SpectrumAqua; Vysis Inc., USA] and X and Y (CEP X SpectrumGreen/CEP Y SpectrumOrange; Vysis Inc.). Denaturation, hybridization and detection procedures were as recommended by Vysis. Each specimen slide was denatured in 70% formamide/2×SSC at 75°C for 5 min, placed in an ethanol series (70–100%) and air-dried. The probe mixture was denatured at 73°C for 5 min and spread onto the denatured specimen slide. A coverglass was applied to the slide, sealed and allowed to hybridize overnight at 37°C. After hybridization, slides were washed in 0.4×SSC/0.3% NP-40 at 73°C for 2 min and 2×SSC/0.1% NP-40 at ambient temperature for 30 s and air-dried. Sperm were counterstained with DAPI II (Vysis Inc.) and assessed with an epifluorescent microscope (Nikon Elipse E600W) equipped with a triple bandpass filter (DAPI/FITC/Cy3), a dual band pass filter (FITC/Cy3), and single bandpass filters for Aqua, and fluorescein isothiocyanate (FITC) or cyanine (Cy3).

### Scoring criteria

Scoring of nuclei was done in an area of the slide where consistent hybridization was evident on initial screening of the slide. Only nuclei with intact morphology and long sperm tails were scored to select for mature sperm and to exclude any other cell type or artefact present on the slide. Any nuclei that have abnormal morphology would not be scored to avoid overlapping cells being scored as one. Two signals of the same colour were scored as two copies of the corresponding chromosome.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mother</th>
<th>Chorion</th>
<th>Father</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR (genotype)</td>
<td>(ab)</td>
<td>(b)</td>
<td>(c)</td>
<td>Paternal loss</td>
</tr>
<tr>
<td>FMR1 (genotype)</td>
<td>(ac)</td>
<td>(c)</td>
<td>(b)</td>
<td>Paternal loss</td>
</tr>
</tbody>
</table>

AR refers to the androgen receptor gene locus on the X chromosome. FMR1 refers to the fragile X mental retardation (CCG repeat) region on the X chromosome.

### Case report

#### Clinical information

The female partner (age 36 years) had no evidence of tubal, ovulatory or pelvic infertility factors. The male partner (age 41 years) with a 46,XY karyotype was found to have severe OAT (≤1×10⁶ sperm/ml). The couple underwent ICSI after only a few motile sperm were recovered in a concentrated sample. A standard luteal phase ‘long protocol’, of controlled ovarian stimulation using a GnRH agonist and recombinant FSH with intravaginal progesterone as luteal support, was undertaken in the female partner. Of the 15 oocytes retrieved, 12 metaphase II oocytes were used for ICSI, 11 of these survived after ICSI procedures, and nine of the 11 (82%) fertilized normally. All sperm, regardless of the level of motility, were of abnormal morphology. Scoring of nuclei was only done in an area of the slide where intact morphology and long sperm tails were present, and was associated with an extremely high rate of sex chromosomal aneuploidy found in the sperm of the father. The mechanisms of meiotic segregation of the sex chromosomes that could be involved in the origin of a sex aneuploid pregnancy, such as 45,X, will also be discussed.
Table II. Sex chromosomal aneuploidy of patient compared to normal controls

<table>
<thead>
<tr>
<th></th>
<th>Patient (%)</th>
<th>Controls (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sperm scored</td>
<td>10,164</td>
<td>20,429</td>
<td></td>
</tr>
<tr>
<td>Sex ratio in euploid sperm</td>
<td>1:0.8</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>Overall aneuploidy</td>
<td>4149 (40.82)</td>
<td>198 (0.97)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total sex chromosome aneuploidy</td>
<td>3969 (39.05)</td>
<td>160 (0.78)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sex nullisomy</td>
<td>1990 (19.58)</td>
<td>58 (0.28)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>XY disomy</td>
<td>1886 (18.56)</td>
<td>20 (0.10)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>XX, YY disomy</td>
<td>16 (0.16)</td>
<td>28 (0.14)</td>
<td>0.66</td>
</tr>
<tr>
<td>Disomy 18</td>
<td>47 (0.46)</td>
<td>52 (0.25)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diploidy</td>
<td>113 (1.11)</td>
<td>15 (0.07)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Complete nullisomy (no signals)</td>
<td>43 (0.42)</td>
<td>11 (0.05)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Euploid sperm refers to sperm with normal haploid represented with 18,X and 18,Y constitutions.
Total aneuploidy includes all chromosomal constitutions different from that of euploid sperm.
Sex nullisomy refers to sperm with one signal for chromosome 18 and no signals for either the X or Y chromosome in one sperm.
Diploidy refers to sperm with two signals for chromosome 18 and two signals for sex chromosomes.

when they were comparable in brightness and size and were separated from each other by a distance longer than the diameter of each signal. Nullisomy of any individual chromosome was considered when the sperm clearly contained at least one of the other chromosomal signals, while complete nullisomic sperm, absence of any signal, was scored as such. Two-sample z-tests of two proportions were used for the statistical analysis.

Results

Cytogenetic analysis of cultured chorion from the placenta resulted in a 45,X karyotype. CGH analysis of DNA extracted from the chorion also supported this result with a profile showing normal copy numbers for all autosomes, but a loss of one X chromosome.
The test of origin of the abnormality by PCR amplification with both the primers for AR and FMR1 gene loci of the DNA samples from the peripheral blood of both parents and chorion from the placenta indicated absence of the paternal X chromosome from the embryonic cell line (Table I). Thus, the 45,X karyotype was the result of the fertilization of the oocyte with a sperm lacking a sex chromosome.

Results of FISH analysis of sperm are summarized in Table II. The most striking increases were seen for sperm with one chromosome 18 signal but nullisomic for a sex chromosome (Figure 1) at 19.58% (1990/10,164) and sperm with one signal for each of the chromosomes 18, X and Y at 18.56% (1886/10,164), when compared to the same constitutions in the control group with 0.28% (58/20,429) and 0.10% (20/20,429) respectively (P < 0.0001). The total aneuploidy rate involving sex chromosomes was thus dramatically increased (39.05%) compared to controls (0.78%). When comparing the combined incidence of 18,XX and 18,YY constitutions in the test case (0.16%) against that in the control group (0.14%), no significant increase was found. The use of an internal control, namely the co-hybridization of an autosomal probe (chromosome 18) along with probes for the sex chromosomes, allows for the distinction between single chromosome aneuploidy (the number of chromosome 18 signals not matching the number of signals for sex chromosomes) and diploidy (two chromosome 18 signals accompanied by two sex chromosomes). The disomy rates for the sex chromosomes are as discussed above, while rate of disomy 18 in the test case is 0.46% and in the controls is 0.25%, which was not significant. A significant increase was seen in the incidence of diploidy in the test case at 1.11% when compared the control rate of 0.07% (P < 0.001). The use of the internal control also allows for a distinction between sex chromosome nullisomy as mentioned above and nullisomy for all chromosomes (0.42% in test versus 0.05% in controls: P < 0.001).

Discussion

The increase in prenatally detected chromosomal abnormalities in ICSI pregnancies, which frequently involve sex chromosomes (Bonduelle et al., 2002), has led to discussions concerning the origin of abnormality and the risk of men susceptible to aneuploidy in their sperm. Although some of the 45,X conceptions from ICSI are maternally derived (Lam et al., 2001), results from various studies have implicated the father as the origin in these types of abnormalities. For example, Van Opstal et al. (1997) found six gonosomal anomalies prenatally...
which were of paternal origin from 71 fetuses conceived from ICSI. However, paternal origin of abnormalities cannot be explained solely by the infertility of the father, as an estimated 83% of 45,X karyotypes and half of all 47,XXY cases in the normal population arise paternally (Jacobs et al., 1999). The high level of sex nullisomy (19%) in the sperm was most likely the reason for the 45,X conception, which would make this case a very good example of the transmission of chromosomal abnormality from sperm to the conceptus through ICSI. We have suggested some ideas about the mechanism for the abnormalities encountered in the spermatogenesis of our case, but clearly more conclusive studies need to be done. Confirming previous studies of ICSI males, we have encountered significantly increased sex chromosomal aneuploidy attributable to malsegregation during spermatogenesis. The mechanism of meiotic non-disjunction and the role it plays in spermatogenesis is yet to be understood completely, but with the use of novel molecular approaches applied to the level of single sperm, we may be able to answer some of these questions. Although it is not possible to assess the genetic constitution of a particular sperm before it is used for ICSI, leaving preimplantation genetic diagnosis (PGD) as the only means of obtaining any genetic information about the embryo before transfer, information provided by FISH may be helpful in counselling couples interested in pursuing assisted reproductive technology about their risk of transmitting chromosomal abnormalities of paternal origin. According to the findings from FISH analysis of the sperm in this couple, the risk of conceiving a child with chromosomal aneuploidy, particularly involving the sex chromosomes, in any future attempt by ICSI is significant, despite best efforts to minimize this by selecting motile sperm (sperm with normal morphology was not available in this patient). PGD should be considered if this couple was to pursue future attempts at conception by ICSI. To estimate the true risk of abnormal conceptions through ICSI, large prospective studies are clearly needed to generate accurate rates of chromosomal aneuploidy. This can only be achieved if patient selection criteria and methodologies are standardized, correlating cytogenetic information in the progeny and parental origin of chromosomal abnormalities to information from molecular and cytogenetic analyses of sperm.

Acknowledgements
This study was supported by grants from the Hospital for Sick Children Foundation (XG02-086) and Canadian Institutions of Health Research (MOP-53067).
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Submitted on July 28, 2003; accepted on September 18, 2003

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