Detection of aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y by fluorescence in-situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection

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Recent evidence suggests that infertile males donating semen for intracytoplasmic sperm injection (ICSI) may be at an increased risk of transmitting numerical (predominantly sex chromosome) abnormalities to their offspring. The present study was designed to determine aneuploidy in spermatozoa from oligoasthenoteratozoospermic (OAT) patients undergoing ICSI. Aneuploidy frequencies of 12 autosome and the sex chromosomes were determined by fluorescence in-situ hybridization (FISH) on spermatozoa from fresh ejaculate of nine severe OAT patients and four proven fertile donors. FISH, using directly labelled (fluorochrome-dUTP) satellite or contig DNA probes specific for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X, and Y, was performed on decondensed spermatozoa. Per chromosome disomy frequencies for autosomes and sex chromosomes in OAT males were 0–5.4%. In contrast, the disomy frequencies in controls were 0.05–0.2%. The frequency of diploid spermatozoa in OAT patients was 0.4–9.6%; controls showed a mean of 0.04%. Using recently developed formulae, the total aneuploidy in our OAT patient population was estimated to be 33–74%. In contrast, estimates of mean total aneuploidy in the spermatozoa of controls ranged from 4.1 to 7.7%, depending upon method of calculation. Six series of ICSI were performed on five of the OAT patients. Four resulted in no establishment of pregnancy; the others failed to establish ongoing pregnancies. Our cytogenetic data show significantly elevated frequencies of diploidy, autosomal disomy and nullisomy, sex chromosome aneuploidy, and total aneuploidy in OAT patients, which may contribute to the patients’ infertility.

Key words: aneuploidy/FISH/ICSI/oligoasthenoteratozoospermia/spermatozoa
semen parameters (Van Steirteghem et al., 1993; Nagy et al., 1994). However, the miscarriage rate and the potential for aneuploid offspring remain unclear.

The possibility that aneuploid offspring may be relatively common following ICSI is suggested by the results of Tournaye et al. (1995) and cumulative data reported by Van Steirteghem et al. (1997), who reported on 1275 consecutive treatment cycles using ICSI performed in 919 couples with male factor infertility. All couples had at least one failed conventional IVF treatment cycle. Some males had semen parameters incompatible with standard IVF or were afflicted with excretery azoosperma, which required microsurgical epididymal sperm aspiration (MESA) or testicular sperm retrieval. The mean maternal age was 32 years. Of 491 fetuses diagnosed prenatally, 1% had sex chromosome aneuploidy (47,XXX; 47,XY) and an additional fetus had trisomy 20. An additional pregnancy yielded a newborn with Down’s syndrome. The expected collective frequency of 47,XXX, 47,XY and 47,XXY is ~3 in 1000 (0.3%) livebirths (Jacobs, 1992). The frequency of sex chromosome aneuploidy observed was, therefore, 3–4 times the frequency seen in newborns from the general population. In’t Veld et al. (1995) performed prenatal diagnoses for advanced maternal age on 12 patients whose pregnancies were established by ICSI in IVF clinics in Belgium and the Netherlands. Four out of 15 (27%) were diagnosed with chromosomal abnormalities. Three patients had a twin pregnancy. Cytogenetic results identified two XXY, one 45,X/46,X, dic(Y) (q11)/46,X,del(Y) (q11), and two with Turner syndrome. Subsequent to this report, In’t Veld (personal communication) suggested that this preliminary study may have been an atypical sample.

Thus, the genetic risks associated with ICSI remain unclear. To evaluate the cytogenetic make-up of spermatozoa from OAT males, several centres have reported aneuploidy in spermatozoa from infertile men. A preliminary study (Pang et al., 1994) was performed on 15 patients using FISH to determine aneuploidy for chromosomes 1, X and Y. OAT was defined by the criteria of both Kruger et al. (1986) for morphology and the World Health Organization (WHO, 1992) for concentration and motility. There were significant increases in aneuploidy for all chromosomes analysed in patients, compared with controls. Both sperm karyotypes and FISH analyses of spermatozoa from five infertile men with normal somatic karyotypes have been reported (Moosani et al., 1996). Two were teratozoospermic, two were oligozoospermic, and one was asthenozoospermic. FISH analyses showed a significant increase in the frequency of disomy for chromosome 1 in three teratozoospermic or oligozoospermic patients. One oligozoospermic patient showed a significant increase in the frequency of disomy for chromosome 12 versus controls. The frequency of XY disomy was significantly increased in four of five patients studied. Sperm karyotypes showed a significant increase in the frequency of numerical abnormalities relative to controls. Newberg et al. (1998) showed significantly higher frequencies of disomy for chromosomes 18, X and Y in spermatozoa from a male with moderate OAT and somatic cell mosaicism: 46,XY (90%)/45,X (10%). In’t Veld et al. performed molecular cytogenetic analysis on spermatozoa from a male with OAT (In’t Veld et al., 1997). FISH analysis showed de-novo chromosome abnormalities in nearly all sperm cells analysed. Fewer than 2% of cells were haploid, 40% were diploid, 24% were triploid, and 22% were aneuploid for the sex chromosomes. Lahdetie et al. (1997) analysed spermatozoa by FISH for chromosomes 1 and 7 from four males with severe OAT. Pooled data showed a significant increase (P < 0.05) in aneuploid and diploid spermatozoa from patients versus controls. They noted that this was primarily due to one patient with a high frequency of hyperhaploid spermatozoa. The incidence of diploid and disomic sperm nuclei in 45 infertile men has been reported on by Guttenbach et al. (1997). They determined aneuploidy for chromosomes 1, 7, 10, 17, X and Y as well as for diploidy by single- and double-target in-situ hybridization. They concluded that with the exception of two patients who exhibited significantly increased diploidy rates of 0.35 and 1.6%, neither disomy nor diploidy was increased in their group of infertile patients as compared to healthy, fertile males. All of the above studies reported disomy and nullisomy for the analysed chromosomes. Pang et al. optimized experimental strategies for sperm decondensation and hybridizations using normal controls and OAT samples (Pang et al., 1994, 1998). Nullisomy and disomy were scored and more nullisomic spermatozoa were found amongst spermatozoa from OAT males versus normal controls. This may reflect a difference in chromatin condensation and/or differences in sperm membrane integrity between OAT males and controls.

To understand more clearly the cytogenetic make-up of spermatozoa from OAT males undergoing ICSI, we evaluated spermatozoa from fresh ejaculates of nine severe OAT patients and four proven fertile donors for aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y. Aneuploidy frequencies in spermatozoa will be discussed, as well as fertilization and pregnancy results following ICSI.

Materials and methods

Sperm decondensation and sperm head swelling
Spermatozoa obtained by masturbation from four normal subjects and nine OAT patients were washed immediately after liquefaction and decondensed as previously described (Pang et al., 1998). Briefly, semen was rinsed in phosphate-buffered saline (PBS), pH 7, and incubated for 5 min at room temperature (RT). Following centrifugation, the pellet was mixed with 1 ml PBS containing 6 mM EDTA, pH 7, and incubated for 5 min at RT. Sperm head decondensation was accomplished using 2 mM dithiothreitol (DTT), pH 7 for 45 min in a 37°C incubator. Following centrifugation, the pellet was washed in PBS and re-isolated by centrifugation. The supernatant was discarded and the pellet was resuspended in fresh fixative (3:1 methanol:glacial acetic acid). Slide preparation was by the smear method and short-term slide storage was at 4°C. Long-term slide storage was at −20°C.

Normal semen donors
The four normal subjects used to determine control aneuploidy frequencies ranged in age from 29 to 33 years (mean 31.3 years). Their sperm counts were 79–160×10⁶/ml (mean 123×10⁶/ml). By strict criteria, 63–80% (mean 69.5%) of spermatozoa were motile.
and of normal morphology, was seen in 17–30% (mean 23.5%). The mean percentage of dead spermatozoa from controls was 10.3% (Table I). All donors abstained from sexual activities and alcohol consumption for 3 days. All have at least one child. An analysis of aneuploidy in these donors has been given in detail (Pang et al., 1998).

### OAT patients

The nine OAT patients were aged 25–39 years. Sperm counts were 2–15 × 10^6/ml. Motility ranged between 17.9 and 41.1% and 1–4.4% spermatozoa showed normal forms. The frequency of dead spermatozoa was 7.6–40.7% (Table I). All patients enrolled in this study were randomly selected. OAT was defined using the criteria of (Kruger et al., 1986) for morphology, and (WHO, 1992) for concentration and motility.

### FISH

Two-probe two-colour FISH was performed using probe sets for either chromosomes 4 and 6, 7 and 18, 8 and 13, 9 and 17, 10 and 21, or 11 and 12. Three-probe, three-colour FISH for chromosomes X, Y, and 18 was also performed. Probes used for FISH were from loci D4Z1, D6Z1, D7Z1, D8Z2, D9Z4, D10Z1, D11Z1, D12Z3, I3q22 contig (RB1), D17Z1, D18Z1, 21q22.1 contig (D21S259–D21S341–D21S342), DXZ1, and DYZ3. Alpha satellite and contig DNA probes were obtained from Vysis Inc (Downers Grove, IL, USA). Approximately 1000 spermatozoa per subject were scored with each autosome probe set and ~2000 spermatozoa per subject were scored using the X, Y, 18 probe set. Two-probe or three-probe FISH enabled differentiation of disomy due to non-disjunction from diploidy due to non-reduction. Simultaneous scoring of two autosomes also provided an internal control to differentiate nullisomy from lack of hybridization.

When two or three chromosomes were simultaneously probed with alpha satellite sequences, hybridizations were performed using 20 ng of each labelled probe in a hybridization mix of 60% formamide, 2× sodium chloride/sodium citrate (SSC), pH 7.0 [Total volume (TV) = 10 µl]. For probe sets consisting of one satellite sequence and one contig, a mixture of 20 ng of the satellite sequence and 60–100 ng of preannealed contig DNA in a hybridization mix of 60% formamide, 2× SSC, pH 7.0 (TV = 10 µl) was prepared. Hybridization mixes were added to prewarmed slides (42°C) and covered with 22 × 22 mm coverslips, which were sealed with rubber cement. Slides were denatured at 80°C for 5 min. All slides were hybridized in a moist chamber for 2–20 h at 37–42°C.

Stringency washes of 3 × 10 min in 50% formamide, 2× SSC, pH 7.0, followed by 10 min 2× SSC, pH 7.0 and by 10 min 2× SSC, 0.1% NP-40, pH 7.0, all at 37°C, were carried out. Transition to antifade was accomplished by a 5 min PBS (pH 7.0) wash at RT. Coverslips were added over 13 µl antifade either with (for two-colour FISH) or without (for three-colour FISH) 0.6 µg/ml 4,6-diamidino-2-phenylindole (DAPI) counterstain.

Microscopy was performed using a Nikon epi-fluorescent microscope equipped with a 20× PlanApo objective, a 40× 1.3 na fluorite objective, a 60× 1.3 na fluorite objective, and a 100× 1.4 na plan apochromatic objective, one beam splitter, and one emission filter. Multiple fluorescent signal detection was accomplished using a Lull filter wheel with six different excitation filters. Images were captured using a Photometrics (Tucson, AZ, USA) Series 200 cooled CCD camera (grade 2 chip) controlled by a Macintosh Quadra 800 computer using BDS Image and FISH analysis software.

### Scoring criteria

Nuclei were scored only if they were not over-decondensed, did not overlap and were intact with clearly defined borders. A spermatozoon was scored as disomic for a particular chromosome if it showed two signals for that chromosome and one each of the simultaneously probed chromosome(s). Consistent with the scoring criteria of Martin and Rademaker (Martin and Rademaker, 1995), two spots separated by less than the diameter of one domain were scored as a single signal. To more clearly understand the cytotegenic differences in spermatozoa between OAT patients and normal controls, nullisomy was also scored. The absence of signal for a single chromosome was scored as nullisomy for that chromosome. Spermatozoa showing signal for none of the chromosomes of a probe set were scored as such, but were not included in the calculation of nullisomy frequencies as this outcome may be an artefact resulting from unsuccessful hybridization due to inadequate nuclear decondensation. A cell was scored as diploid if there were two signals for both probed chromosome pairs.

### Statistical analyses

Data were analysed using the χ² and Fisher’s exact tests where appropriate.

### Results

#### Two-probe, two-colour FISH

Summary data on autosomal and sex chromosomal nullisomy, disomy and total aneuploidy (expressed as the sum of disomy and nullisomy) are presented in Table II. Disomy frequencies for autosomes and sex chromosomes in OAT males ranged between 0–5.4%. In contrast, the disomy frequencies in controls ranged from 0.05–0.20%. Nullisomy frequencies for autosomes and the sex chromosomes in OAT males showed a range between 0–7% while control nullisomy frequencies ranged from 0–0.75%. Aneuploidy defined as the sum of disomy and nullisomy, for autosomes and sex chromosomes in OAT males ranged between 1.1 and 7.5%. In contrast, aneuploidy in controls ranged from 0.20 to 0.43%. The frequency of diploid spermatozoa in OAT patients ranged from 0.4 to 9.6%. In contrast, controls showed a mean frequency of 0.04%.

#### Three-probe, three-colour FISH

Table III shows results of three-probe, three-colour studies of the sex chromosomes and chromosome 18. Nullisomy in the sex chromosomes of patients ranged between 0.39 and 2.54%. Controls showed a mean of 0.2%. Similarly, sex chromosome
Aneuploidy in OAT patients

Table II. Frequencies (as percentages) of numerical and autosomal abnormalities in controls and patients with oligoasthenoteratozoospermia (OAT)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Chromosome</th>
<th>Disomy</th>
<th>Nullisomy</th>
<th>Aneuploidy</th>
</tr>
</thead>
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<tr>
<td></td>
<td>4 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 X/Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>1.6 2.2 2.5 2.2 1.8 1.5 1.8 1.3 1.8 2.0 1.7 1.8 3.5</td>
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<tr>
<td>OB</td>
<td>0.9 1.0 0.7 0.7 1.0 1.4 1.1 0.0 1.2 0.9 2.3 0.6 1.9</td>
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<tr>
<td>OC</td>
<td>0.9 0.4 1.1 1.3 0.7 0.9 0.3 0.2 1.1 0.9 0.7 1.4 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>1.0 1.0 0.7 1.3 1.2 1.1 2.0 0.3 1.5 1.5 2.0 2.0 1.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>OE</td>
<td>1.4 0.8 3.0 1.2 1.2 1.3 0.5 0.6 1.6 1.4 0.4 1.8 3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF</td>
<td>1.5 1.3 0.8 0.9 1.1 1.3 0.6 0.5 1.3 1.2 2.5 1.7 3.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG</td>
<td>1.7 1.0 2.2 2.0 2.6 2.2 0.0 0.0 2.1 1.9 3.1 2.4 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>2.4 1.7 2.6 3.5 2.6 2.9 5.4 1.6 2.6 3.1 0.0 3.4 2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI</td>
<td>2.8 3.0 2.9 2.6 2.9 3.4 3.4 1.7 3.5 1.7 2.3 2.4 4.9</td>
<td></td>
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<tr>
<td>Control (mean)</td>
<td>0.15 0.13 0.31 0.23 0.20 0.20 0.13 0.05 0.20 0.20 0.20 0.11 0.18 0.15</td>
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</table>

Nullisomy

<table>
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<tr>
<th>Subject</th>
<th>Chromosome</th>
<th>Disomy</th>
<th>Nullisomy</th>
<th>Aneuploidy</th>
</tr>
</thead>
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<tr>
<td>OA</td>
<td>1.2 2.0 1.5 1.2 1.9 1.4 1.3 1.3 1.2 1.4 1.8 1.5 1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB</td>
<td>2.8 2.5 4.0 4.1 2.6 2.3 2.4 3.3 2.7 1.9 3.1 3.4 1.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OC</td>
<td>0.5 0.9 0.8 0.8 0.9 0.4 1.1 5.7 0.6 1.0 4.8 0.5 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>2.1 2.7 1.5 2.9 3.0 2.5 1.7 5.3 2.8 2.2 4.2 2.1 1.7</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OE</td>
<td>1.3 0.9 3.4 2.7 1.6 3.0 3.7 3.3 1.8 1.4 3.9 1.7 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF</td>
<td>1.8 2.0 1.7 2.0 1.7 2.1 2.5 0.0 1.6 2.2 2.1 2.0 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG</td>
<td>3.0 2.6 4.0 2.5 1.8 2.3 7.0 1.1 2.1 4.2 1.3 2.2 0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>1.9 1.8 0.0 3.1 2.5 2.5 1.6 0.6 3.6 2.9 5.5 3.5 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI</td>
<td>1.5 2.2 2.3 0.6 2.1 1.9 0.6 2.8 2.1 1.2 0.0 1.9 2.6</td>
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<td></td>
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</tr>
<tr>
<td>Control means</td>
<td>0.10 0.75 0.11 0.05 0.10 0.05 0.13 0.18 0.03 0.00 0.11 0.10 0.20</td>
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Aneuploidy (defined as disomy + nullisomy)

<table>
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<tr>
<th>Subject</th>
<th>Chromosome</th>
<th>Disomy</th>
<th>Nullisomy</th>
<th>Aneuploidy</th>
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<tbody>
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<td></td>
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<tr>
<td>OB</td>
<td>1.8 3.5 4.7 4.7 3.6 3.6 3.5 3.3 3.9 2.7 5.4 4.0 3.4</td>
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<tr>
<td>OC</td>
<td>1.4 1.3 1.9 2.0 1.6 1.3 1.5 6.0 1.7 1.8 5.5 2.0 3.6</td>
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<tr>
<td>OD</td>
<td>3.1 3.7 2.2 4.2 4.2 3.7 3.7 5.6 4.2 3.8 6.2 4.1 3.2</td>
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<tr>
<td>OG</td>
<td>4.7 3.6 6.2 4.6 4.5 4.6 7.0 1.1 4.2 6.1 4.4 4.7 2.6</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI</td>
<td>4.3 5.3 5.2 3.2 5.0 5.3 4.0 4.4 5.6 2.9 2.3 4.3 7.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control means</td>
<td>0.25 0.20 0.43 0.28 0.30 0.25 0.25 0.23 0.23 0.20 0.23 0.28 0.35</td>
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</table>

disomy in patients ranged from 1.36 to 4.87%, while 0.14% of control spermatozoa was disomic. In OAT patients, chromosome 18 nullisomy and disomy ranged between 1.37 and 3.85% and between 0.29 and 2.62% respectively. Corresponding means for control were 0.14 and 0.15%. The range of diploid spermatozoa in OAT patients was between 0.49 and 9.59%, while controls showed a mean of 0.15%.

Determination of total aneuploidy in OAT patients and controls

Frequencies of total aneuploidy were estimated using formulae developed by Hoegerman et al. (1999), by calculating the aneuploidy frequencies (per chromosome) as either the sum of disomy and nullisomy, twice the disomy frequency, or twice the nullisomy frequency. These calculations assume independence of non-disjunctional events within the same cell. The total aneuploidy in spermatozoa from nine OAT patients ranged between 33 and 74% (Table IV). In contrast, the total aneuploidy in control spermatozoa ranged between 4.1 and 7.7%.

Statistical analyses

Using \( \chi^2 \) analyses and Fisher’s exact test, we compared the frequencies of aneuploidy for individual chromosomes both within patients and between patients. Due to the heterogeneity of aneuploidy frequencies both within and between OAT patients, these data were not pooled. On the other hand, data from controls were homogeneous and were therefore pooled.

Frequencies of numerical autosomal abnormalities in OAT patients and controls

For 108 individual measurements of disomy frequencies (12 chromosomes times nine subjects) in OAT patients, 90 comparisons to control means showed a significant increase \((P < 0.05)\) in autosomal disomy versus controls. In controls, chromosome 7 showed a significant \((P < 0.05)\) excess of disomy over nullisomy, whereas in OAT patients, chromosomes 4, 7, 8, 11, 18, and 21 showed a significant \((P < 0.05)\) excess of nullisomy over disomy.

Comparisons of nullisomy versus disomy within individuals

One control showed a significant \((P < 0.05)\) excess of disomy over nullisomy. Patients OH and OI showed a significant \((P < 0.05)\) excess of disomy, while patients OB, OC, OD, OE, OF, and OG showed a significant \((P < 0.05)\) excess of nullisomy over disomy. Overall, in the OAT patients, nullisomy predominated over disomy.
<table>
<thead>
<tr>
<th>Signals present</th>
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<th>OC</th>
<th>OD</th>
<th>OE</th>
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</table>

*These cells, with both autosomal and sex chromosome abnormalities, are listed in both categories.

### Sex chromosomes

The frequencies of X and Y bearing spermatozoa did not differ from the expected one to one ratio in normal donors. No significant variations in XX disomy, YY disomy, XY disomy, and nullisomy frequencies were noted across control donors. In OAT patients, there was significant heterogeneity ($P < 0.05$) in the frequencies of XX disomy and nullisomy. There was a significant increase ($P < 0.05$) for all types of sex chromosome aneuploidy in all OAT patients, except for disomy XX in patient OB and sex chromosomal nullisomy in OF and OH. In OAT patients, XX, sex chromosomal nullisomy, YY or XY were equally likely to be found. Overall, sex chromosome aneuploidy was significantly increased ($P < 0.05$) in OAT patients versus controls.

### Comparisons of the frequency of no signal cells in OAT patients versus controls

For two- and three-probe FISH, the percentage of spermatozoa showing no signal from OAT patients ranged between 2.7 and 7.5%, whereas no signal for controls showed a mean of 0.04%.

### Results of assisted reproductive technologies using spermatozoa from OAT patients

Six series of ICSI were performed using spermatozoa from five of the OAT patients (Table V). For patient OC, there was no embryo transfer due to fertilization failure. For patients OD and OE, no pregnancies resulted after one (OE) or two (OD) series of embryo transfer. With patient OF, there was a preclinical spontaneous abortion and with OH, there was a first trimester spontaneous abortion. Thus, no live births resulted.

### Discussion

Over the past decade, several studies have provided valuable information on the cytogenetic make-up of spermatozoa from normal men. Using the human sperm–hamster egg fusion technique, the frequencies of numerical chromosome aberrations observed in 16 studies of male pronuclear chromosomes were summarized and weighted means calculated (Hoegerman et al., 1999). Depending upon the method used to estimate the mean aneuploidy frequency, that value ranged between 1.9 and 2.9%. Estimating aneuploidy as twice the
Aneuploidy in OAT patients

Table IV. Percentage of total aneuploidy and diploidy in patients with oligoasthenoteratozoospermia (OAT) and controls

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total aneuploidy</th>
<th>Diploidy</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>OA</td>
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<tr>
<td>OB</td>
<td>60</td>
<td>38</td>
</tr>
<tr>
<td>OC</td>
<td>43</td>
<td>33</td>
</tr>
<tr>
<td>OD</td>
<td>61</td>
<td>46</td>
</tr>
<tr>
<td>OE</td>
<td>58</td>
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<td>OG</td>
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<td>57</td>
</tr>
<tr>
<td>OH</td>
<td>69</td>
<td>71</td>
</tr>
<tr>
<td>OI</td>
<td>65</td>
<td>74</td>
</tr>
<tr>
<td>Controls</td>
<td>5.9</td>
<td>7.7</td>
</tr>
</tbody>
</table>

A = the sum of disomy and nullisomy; B = twice the disomy frequency; or C = twice the nullisomy frequency.

disomy frequency yields the lower value while estimating it as nullisomy plus disomy yields the higher. The latter is often considered an overestimate as some chromosome counts of 22 or less may represent artefactual loss of chromosomes and not true aneuploidy.

An alternative technique for aneuploidy determination is FISH which directly identifies both aneuploidy and polyploidy in spermatozoa (Kearns and Pearson, 1994a,b). The FISH technique obviates the need to fertilize hamster eggs and permits characterization of spermatozoa without a possible selection bias based upon fertilizing ability. Recent studies on spermatozoa from normal donors using two- or three-colour FISH have been reviewed (Hoegerman et al., 1999). Calculations based upon the mean disomy frequencies of the 18 studied autosomes and sex chromosomes yielded extrapolations that between 6.6 and 7.5% of spermatozoa from normal men are aneuploid. This suggests that aneuploidy identified by FISH in controls is roughly three times that found in the studies of pronuclear chromosomes. This discrepancy could result from the use of invalid FISH scoring criteria, non-independence of non-disjunctional events or aneuploidy associated reduction in fertilizing ability.

Combining data on pronuclear chromosomes and FISH analyses suggests that between 1.9 and 7.7% of spermatozoa from normal donors are aneuploid.

Several technical factors must be considered when determining the frequency of numerical chromosome abnormalities in spermatozoa. These include the scoring criterion, sample size, chromatin decondensation, and the specificity of the DNA probes for target chromosomes (Egozcue et al., 1997).

For each OAT patient in this study, estimates of total aneuploidy were calculated using the same equations developed by Hoegerman et al. (1999). This formula assumes that numerical chromosome abnormalities are randomly distributed among cells, i.e. that the presence of an abnormal copy number for one particular chromosome has no effect on the probability of an abnormal copy number for another chromosome and that the aneuploidy frequencies of unstudied chromosomes equal the mean aneuploidy frequency of all studied chromosomes. The calculations have strict statistical validity only if the per chromosome abnormality frequencies are homogeneous; a condition which holds for the control data but not for that of the OAT patients. The only accurate way to directly determine total aneuploidy in spermatozoa using FISH would be to simultaneously hybridize probes for all 24 chromosomes to a single sperm cell; an experimental condition currently not possible. Therefore, we have calculated the total aneuploidy in spermatozoa from the OAT males in order to make possible comparison to control means. Since patient-to-patient heterogeneity exists for all OAT patients, the OAT means should be considered as approximations. Using these equations, the total aneuploidy in spermatozoa from the nine OAT patients was estimated to range from 33 to 74%. The frequency of diploid spermatozoa ranged between 0.4 and 9.6%. In comparison, the total aneuploidy in control spermatozoa ranged from 4.1 to 7.7% with a mean of 0.04% diploid spermatozoa.

The factor(s) responsible for the heterogeneity present both within and between samples from OAT patients is obscure. Heterogeneity is unlikely to have resulted from variability in decondensation and hybridization protocols as controls and patient samples were processed contemporaneously. Biological heterogeneity in the OAT population might account for some of the cytogenetic variability seen between patients but would be unlikely to generate the heterogeneity seen between chromosomes within patients.

The current study showed a significant increase in autosomal and sex chromosomal aneuploidy in spermatozoa from OAT patients. The accurate determination of aneuploidy using FISH in spermatozoa requires optimal sperm head decondensation, FISH analysis, and scoring criterion. Previously, we optimized sperm head decondensation and FISH conditions using spermatozoa from normal controls and OAT patients (Pang et al., 1994, 1998). In those studies, an optimal sperm head decondensation protocol was developed by measuring the decondensed sperm head area, perimeter and degree of roundness. FISH analysis was optimized using multi-probe FISH conditions to eliminate spurious nullisomy or disomy due to incomplete or over-decondensed sperm heads. Scoring criterion was also considered. We employed a scoring criterion consistent with those of Martin and Rademaker (Martin and Rademaker, 1995). Their criterion requires that two fluorescent signals separated by less than one diameter of a signal be scored as a single chromosome. However, empirical evidence supporting this criterion is scant. Theoretically, the frequency of nullisomy should be equal to or greater than that of disomy.

Table V. Results of assisted reproductive techniques using spermatozoa from patients with oligoasthenoteratozoospermia (OAT)

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of mature oocytes</th>
<th>No. of fertilized oocytes</th>
<th>Procedure</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC</td>
<td>6</td>
<td>0</td>
<td>IVF</td>
<td>No embryo transfer</td>
</tr>
<tr>
<td>OD</td>
<td>18</td>
<td>11</td>
<td>ICSI</td>
<td>No pregnancy</td>
</tr>
<tr>
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<td>10</td>
<td>10</td>
<td>ICSI</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>OE-2nd</td>
<td>10</td>
<td>10</td>
<td>ICSI</td>
<td>No pregnancy</td>
</tr>
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<td>OF</td>
<td>8</td>
<td>8</td>
<td>ICSI</td>
<td>Preclinical abortion</td>
</tr>
<tr>
<td>OH</td>
<td>6</td>
<td>6</td>
<td>ICSI</td>
<td>First trimester loss</td>
</tr>
</tbody>
</table>

IVF = in-vitro fertilization; ICSI = intracytoplasmic sperm injection.
Equal frequencies would be expected if all aneuploidy arose through non-disjunction. An excess of nullisomy would be anticipated if nullisomy also arises by the loss of chromosomes following anaphase lag.

In this study, for controls, disomy was more common than nullisomy and there was a very low frequency (0.04%) of spermatozoa with no signal. In contrast, with the OAT patients, there was a significant excess of nullisomic spermatozoa and a markedly greater frequency of spermatozoa with no signal. The difference in the frequency of ‘no signal’ cells may reflect an inability to optimally decondense spermatozoa from OAT males and/or hybridization failure. Since normal controls and OAT samples were decondensed and hybridized contemporaneously, this is unlikely to have resulted from experimental variations. Sperm membranes or chromatid condensation in OAT males may differ in some way from controls. It is reasonable to hypothesize that the biological cause of teratozoospermia may introduce structural or physiological factors, which could alter sperm head structural stability. Another, albeit unlikely possibility is that some cells scored as disomic had cross-hybridization of probe to another centromere(s). We consider this unlikely, as frequently the two signals would differ in size, intensity or location within the nucleus. FISH conditions were first optimized by hybridization to metaphase chromosomes from several cell types to eliminate cross-hybridization artefact. There were sperm nuclei in OAT samples with very small or enlarged heads. Large, overly decondensed heads result in chromatin degradation with subsequent split-signal and aberrant disomy scoring. In contrast, unusually small sperm heads could be an experimental artefact associated with incomplete sperm head decondensation or a pathological entity associated with OAT. Spermatozoa with abnormally large or small heads were classified as unscorable and not included in the final analyses. It is unclear why, in the controls, disomy was more common than nullisomy, while the opposite was observed for the OAT patients. The only reasonable explanation for the excess of disomy over nullisomy in the controls is that some of the cells with two signals for a particular chromosome, as defined by the presence of split signals separated by a distance greater than the diameter of one fluorescent domain, may in fact be a single chromosome. However, the frequencies of aneuploid spermatozoa observed for these controls are consistent with previously reported studies (Hoegerman et al., 1999).

Analysis (Hoegerman et al., 1999) comparing aneuploidy frequencies observed with human–hamster fusions to those from FISH studies on spermatozoa from normal controls suggests that either the disomy frequencies scored using FISH are experimentally inflated or, alternatively, that aneuploid spermatozoa have a diminished fertilizing ability of hamster eggs. Aneuploidy estimates in spermatozoa based upon human–hamster fusions or FISH analysis have some uncertainties and therefore, should be considered as quantitative approximations and not absolute frequencies.

Collectively, our data show significant increases ($P < 0.05$) in the frequencies of diploidy, and both autosomal and sex chromosomal aneuploidy in spermatozoa from OAT patients versus controls. No successful pregnancies were achieved using these patients’ spermatozoa. The elevated levels of aneuploidy in spermatozoa from these males with severe OAT may be causally related to this lack of success.

The aneuploidy found in our OAT patients’ gametes does not show any consistent pattern of chromosome gain or loss with the exception of particularly elevated frequencies of sex chromosome aneuploidy. The observed ‘apparent randomness’ of autosomal aneuploidy suggests the possible operation of an aberrant meiotic process, possibly at the level of cell cycle checkpoint control or the attachment of kinetochores to microtubules (Nucklas, 1997).

The elevated frequencies of prenatally diagnosed sex chromosome aneuploidy reported by Tournaye et al. (Tournaye et al., 1995) are consistent with our data. Previously, Martin (Martin, 1996) suggested that there may be a risk of transmitting chromosomal abnormalities to offspring following ICSI. Rosenbusch et al. (1996) discussed the possibility of irregular chromosome segregation following ICSI. The elevated frequency of autosomal non-disjunction, which we observed, may not markedly impact the frequencies of autosomal abnormalities in newborns because all resulting autosomal monosomies and most trisomies are lethal and autosomal aneuploidy in eggs is common. It is unclear what the contribution of aneuploid spermatozoa is to the frequency of implantation and the incidence of ‘early’ spontaneous abortions containing aneuploid cells of paternal origin. However, the elevated frequencies of aneuploidy observed in the spermatozoa from this population of OAT males suggest they may be at increased risk for transmitting genetic abnormalities to their offspring. Further studies are required.

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References


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