Morphologically normal spermatozoa of patients with secretory oligo-astheno-teratozoospermia have an increased aneuploidy rate

Nunziatina Burrello[1], Giuseppe Arcidiacono[2], Enzo Vicari[1], Paola Asero[1], Daniela Di Benedetto[1], Adele De Palma[2], Rosa Romeo[3], Rosario D'Agata[1] and Aldo E.Calogero[1,4]

[1]Section of Endocrinology, Andrology and Internal Medicine, Department of Biomedical Sciences, and Master in Andrological and Human Reproduction Sciences, [3]Department of Human Anatomy, University of Catania and [2]Unit of Cardiology, Garibaldi Hospital, Catania, Italy

[4]To whom correspondence should be addressed at: Sezione di Endocrinologia, Andrologia e Medicina Interna, Dipartimento di Scienze Biomediche, Università di Catania, Ospedale Garibaldi, Piazza S. Maria di Gesù, 95123 Catania, Italy.
E-mail: acaloger@unict.it

BACKGROUND: Normal morphology is a major criterion for selecting spermatozoa to be injected. Given that teratozoospermia is one of the most critical parameters associated with sperm aneuploidy, the purpose of this study was to evaluate the aneuploidy rate of morphologically normal spermatozoa of patients with oligo-astheno-teratozoospermia (OAT). METHODS: Ten patients with secretory OAT and six age-matched normozoospermic men with a normal karyotype were enrolled. After assignment to normal or abnormal category, the location of each spermatozoon was recorded using an electronic microstage locator. Slides were then subjected to triple-colour fluorescence *in situ* hybridization for chromosomes X, Y and 12. RESULTS: OAT patients had a lower number of morphologically normal and abnormal spermatozoa carrying the X chromosome, compared with normozoospermic men. They also exhibited increased XY and XX disomy rates. Morphologically abnormal spermatozoa from normozoospermic men also had an increased XX disomy rate compared with normally shaped spermatozoa obtained from the same men. The total sperm aneuploidy rate of morphologically abnormal spermatozoa of normozoospermic men was 4.4-fold higher than that of spermatozoa with normal morphology. The total aneuploidy rates of spermatozoa with normal or abnormal head shape from OAT patients were similar to each other and to that of abnormally shaped spermatozoa from normozoospermic men, but they were higher than the rate found in normally shaped spermatozoa of normal men. CONCLUSIONS: Normally shaped spermatozoa of OAT patients have an increased aneuploidy rate.

Key words: chromosomes 12/multicolour fluorescence *in situ* hybridization/oligo-astheno-teratozoospermia/sex chromosomes/aneuploidy/morphology

Introduction

ICSI has revolutionized the treatment of severe male infertility (Palermo *et al.*, 1992). Soon after its introduction, this technique raised concerns about the risk of transmitting genetic diseases to the offspring, because a spermatozoon with an abnormally genetic constitution may be selected and used for oocyte injection (Meschede and Horst, 1997). The risk seems particularly elevated since an increasing body of literature has shown that patients with oligo-astheno-teratozoospermia (OAT), who more frequently require ICSI to overcome their infertile condition, have an increased sperm aneuploidy rate, despite a normal blood karyotype (for a review see Calogero *et al.*, 2003). In particular, an inverse relationship has been reported between the sperm aneuploidy rate and sperm

concentration (Pang *et al.*, 1999; Vegetti *et al.*, 2000; Calogero *et al.*, 2001) or, to a lower extent, sperm motility (Vegetti *et al.*, 2000). Spermatozoa with an abnormal morphology have also been shown to correlate negatively with sperm aneuploidy (Bernardini *et al.*, 1998; Ushijima *et al.*, 2000; Calogero *et al.*, 2001). Indeed, patients with teratozoospermia have an aneuploidy rate significantly higher than that of normal controls (Gole *et al.*, 2001). We have shown that teratozoospermia is the sperm parameter best associated with sperm aneuploidy. Indeed, a group of patients with isolated teratozoospermia had a sperm aneuploidy rate similar to that of a group of OAT patients (Calogero *et al.*, 2001). Furthermore, Harkonen *et al.* (2001) showed that patients with severe teratozoospermia (normal forms <10%) have a sperm

aneuploidy rate significantly higher than that found in patients with a less marked degree of teratozoospermia.

ICSI bypasses all natural sperm selection processes, since the embryologist chooses the spermatozoon to be injected into the oocyte. Only motile and morphologically normal spermatozoa will be selected. However, these criteria will not exclude the presence of an abnormal karyotype, since the altered intratesticular environment may disrupt the fine-tuned mechanisms of chromosome segregation during spermatogenesis. In this case, spermatozoa with a normal morphology may also be aneuploid, cancelling out the careful selection of a normally shaped spermatozoon aimed at reducing the risk of transmitting aneuploidy to the ICSI offspring. The present study was undertaken to assess the sperm aneuploidy rate of spermatozoa with normal and abnormal morphology. To accomplish this, we evaluated the sperm aneuploidy rate for chromosomes 12, X and Y in single spermatozoa identified according to their morphology in patients with secretory OAT. Six normozoospermic men were selected as a matched control group.

Materials and methods

Patient selection

Ten OAT patients, aged 29 (24–40) years (median and range), with a normal blood karyotype were recruited for this study. Semen samples were collected by masturbation after 4–5 days of abstinence, and the main sperm parameters are shown in Table I. As previously reported (Calogero et al., 2001), all patients fulfilled the following criteria as an expression of semen abnormality mainly of testicular origin: normal or reduced (≤ 12 ml) testicular volume, normal or increased FSH levels and negative andrological history for pathology of the excretory genital tract. The patients with normal testicular volume and serum FSH had to have no clinical or ultrasound signs of epididymal abnormalities. Patients with abnormal spermatozoon morphology of possible epididymal origin (cytoplasmic droplets, coiled tails or short tails) were excluded from the study. Six healthy men, aged 26 (22–37) years (median and range), with normal sperm density, motility and morphology (Table I) served as controls. The protocol was approved by the Institutional Review Board and an informed written consent was obtained from each man enrolled in this study. Sperm parameter assessment was performed on fresh seminal semen according to the World Health

Table I. Aneuploidy rates for chromosomes X, Y and 12 in morphologically normal and abnormal spermatozoa of normozoospermic men and patients with secretory oligo- astheno- and/or teratozoospermia (OAT).

ID	Chromosome X (%)	Chromosome Y (%)	XY disomy (%)	XX disomy (%)	YY disomy (%)	Sex chromosome nullisomy (%)	Chromosome 12 disomy (%)	Chromosome 12 nullisomy (%)	No. of spermatozoa scored
Morphologically normal spermatozoa from normozoospermic men									
PR	50.98	47.71	0	0.65	0.65	0	0	0	306
SG	50.52	48.79	0	0.35	0.35	0	0	0	289
CG	49.47	50	0	0.26	0.26	0	0	0	380
BM	49.48	50.17	0	0	0.35	0	0	0	289
BV	49.27	59.56	0.29	0.59	0.29	0	0	0	341
IM	49.08	50.51	0	0.2	0.2	0	0	0	489
Mean ± SEM	49.80 ± 0.31	49.46 ± 0.43	0.05 ± 0.05	0.34 ± 0.10	0.35 ± 0.06	0 ± 0	0 ± 0	0 ± 0	349 ± 31
Morphologically normal spermatozoa from OAT patients									
GS	47.77	45.54	0.89	1.34	1.34	1.79	1.34	0	224
CG	49.15	45.76	1.27	2.12	1.27	0	0.42	0	236
RM	48.96	49.55	0.30	0.60	0.60	0	0	0	335
GM	36.70	36.70	4.59	9.17	6.42	1.83	4.59	0	218
PS	48.63	49.54	0.30	0.61	0.91	0	0	0	329
AG	48.45	49.07	0	1.24	1.24	0	0	0	161
DSD	48.24	48.74	1.01	1.01	1.01	0	0	0	199
SM	64.71	33.33	0.65	0.65	0.33	0	0.33	0	306
AI	49.23	49.62	0.38	0.77	0	0	0	0	260
BG	48.72	50.00	0	1.28	0	0	0	0	78
Mean ± SEM	49.06 ± 2.11	45.79 ± 1.88	0.94 ± 0.43	1.88 ± 0.82	1.31 ± 0.59	0.36 ± 0.24	0.67 ± 0.46	0 ± 0	235 ± 25
Morphologically abnormal spermatozoa from normozoospermic men									
PR	49.70	47.90	0	1.20	1.20	0	0	0	334
SG	48.32	48.32	0	1.29	1.29	0.26	0.52	0	387
CG	48.37	48.69	0.33	1.63	0.33	0	0.65	0	306
BM	47.61	50.63	0.50	0.50	0.76	0	0	0	397
BV	47.35	51.09	0.62	0.62	0.31	0	0	0	321
IM	47.31	48.92	0.54	1.08	1.61	0	0.54	0	186
Mean ± SEM	48.11 ± 0.37	49.26 ± 0.53	0.33 ± 0.11	1.05 ± 0.17	0.92 ± 0.22	0.04 ± 0.04	0.29 ± 0.13	0 ± 0	322 ± 31
Morphologically abnormal spermatozoa from OAT patients									
GS	42.86	40.2	3.99	7.31	2.66	1	1.33	0.33	301
CG	45.57	38.36	2.30	6.89	4.59	0.33	1.31	0.66	305
RM	47.85	49.00	0.29	1.43	1.15	0	0.29	0	349
GM	36.65	38.74	1.57	8.12	10.21	3.66	1.05	0	382
PS	47.31	46.04	1.53	2.05	2.56	0	0.51	0	391
AG	48.81	48.57	0.71	1.19	0.48	0	0.24	0	420
DSD	47.71	51.20	0.44	0	0.44	0	0.22	0	459
SM	47.84	50.64	0.21	0.64	0.64	0	0	0	472
AI	48.27	50.12	0.69	0.46	0.46	0	0	0	433
BG	49.06	49.90	0.21	0.42	0.42	0	0	0	481
Mean ± SEM	46.19 ± 1.21	46.28 ± 1.63	1.19 ± 0.38	2.85 ± 1.02	2.36 ± 0.98	0.50 ± 0.37	0.50 ± 0.17	0.10 ± 0.07	399 ± 21

Organization criteria (World Health Organization, 1999). The initial sperm morphology assessment was performed using the Papanicolaou staining method at 40× magnification. A spermatozoon was considered morphologically normal if it had: a smooth oval head (length, 4.0–5.0 μm; width 2.5–3.5 μm), a well-defined acrosomal region comprising 40–70% of the head area, no defects of the neck and the midpiece; cytoplasmic droplets less than half the size of the normal head; and a straight, thin tail with a length of 45 μm.

FISH analysis

Following liquefaction, semen samples were washed three times in phosphate-buffered saline (PBS), pH 7.2, centrifuged at 650 g for 10 min at 20°C and the sediment was then fixed in methanol/acetic acid (3:1). The specimens were spread on slides and single sperm morphology carefully evaluated using the same criteria recommended by the World Health Organization (1999) at 40× magnification, but without Papanicolaou staining. The results of the morphological assessment in methanol/acetic acid-fixed spermatozoa were not significantly different from the initial evaluation (fixed-stained spermatozoa). The location of each spermatozoon was recorded using the electronic microstage locator with which the DMRXA2 fluorescent microscope used for this study is equipped (Leica Microsystems SpA, Milan, Italy). The images were recorded using the software Mic-Control (Leica, Milan) and the coordinates written down and retrieved after fluorescence *in situ* hybridization (FISH) using the same software. The slides were then washed in 2× standard saline citrate (SSC) solution and incubated for 5 min in 1 mol/l Tris buffer, pH 9.5, containing 25 mmol/l dithiothreitol (DTT), to achieve a suitable sperm head decondensation, as previously reported (Calogero *et al.*, 2001).

A triple-colour FISH was carried out on each patient and control, using α-centromeric probes for chromosomes 12, X and Y. Alpha centromeric probes were obtained from plasmids containing a specific ~6 kb DNA insert: pBR12 for chromosome 12, pDMX1 for chromosome X and pLAY5,5 for chromosome Y. *Escherichia coli* with the specific plasmid were grown in a Petri dish with LB medium containing 50 μg/ml ampicillin, and incubated overnight at 37°C. Subsequently, a colony was selected and amplified in 10 ml of LB medium containing 50 μg/ml ampicillin overnight at 37°C. The extraction of the plasmid was carried out by miniprep, using the alkaline lysis method, as previously reported (Sambrook *et al.*, 1989). Plasmids containing the 12 and X chromosome probes (DNA 1 μg) were labelled with Fluor X-dCTP, whereas plasmids containing the 12 and Y chromosome probes were labelled with Cy3-dUTP, using a nick-translation kit purchased from Pharmacia Biotech (Milan, Italy). The size of the fragments obtained was checked in a 1% agarose gel.

Each slide was denatured with a solution of 70% formamide/2× SSC (pH 7.5) at 80°C for 150 s. The slides were immersed in a 70, 90 and 100% ethanol series for 3 min each and dried by air. The probes, precipitated and denatured at 80°C for 8 min, were applied directly to the slides which were then covered with a coverslip and sealed with rubber cement. Hybridization occurred overnight in a dark humidified container at 37°C, after which the coverslip was removed and the slides were immersed in a post-hybridization wash of 50% formamide/2× SSC three times at 37°C for 5 min, 2× SSC three times at 42°C for 5 min and 2× SSC/0.1% Tween-20 once at room temperature for 5 min. The slides were then mounted in 4′,6-diamidino-2-phenylindole (DAPI) counterstain and antifade and stored in the dark at 4°C until microscope observation was carried out.

The slides were observed on a Leica DMRXA2 fluorescent microscope with the appropriate set of filters: single band DAPI,

fluorescein isothiocyanate (FITC) and Cy3. Previously identified intact spermatozoa bearing a similar degree of decondensation and clear hybridization signals were scored; disrupted or overlapping spermatozoa were excluded from analysis. Spermatozoa were regarded as abnormal if they presented two (or more) distinct hybridization signals for the same chromosome, each equal in intensity and size to the single signal found in normal monosomic nuclei. We considered only clear hybridization signals, similar in size, separated from each other by at least one signal domain and clearly positioned within the sperm head. Divided (split) signals were not scored as disomies. Spermatozoa were scored as nullisomic if they showed no signal for a given chromosome, whereas the signal of the other chromosome tested was present. Finally, a spermatozoon was considered diploid in cases where it manifested two signals for each tested chromosome and in cases where the tail as well as the normal oval shape of a sperm head were evident. No FISH signals in a spermatozoon head showing DAPI stain were considered a case of no hybridization. The hybridization efficiency was >99%.

Statistical analysis

Results are shown as median and range throughout the study, unless otherwise indicated. The data were analysed with the Mann–Whitney test. SPSS 9.0 for Windows was used for statistical calculation. A significant statistical difference was accepted when the *P*-value was <0.05.

Results

The main sperm parameters of normozoospermic men and secretory OAT patients are shown in Figure 1. Among OAT patients, six had oligozoospermia and all had astheno- and teratozoospermia.

A total of 2094 spermatozoa were classified as being morphologically normal in the six normozoospermic men enrolled (median = 324 spermatozoa; range = 289–489), whereas 1931 had an abnormal head shape (median = 328 spermatozoa; range = 186–397), including amorphous, round, micro- and enlarged heads. In the group of patients with OAT, a total of 2346 spermatozoa were found to be
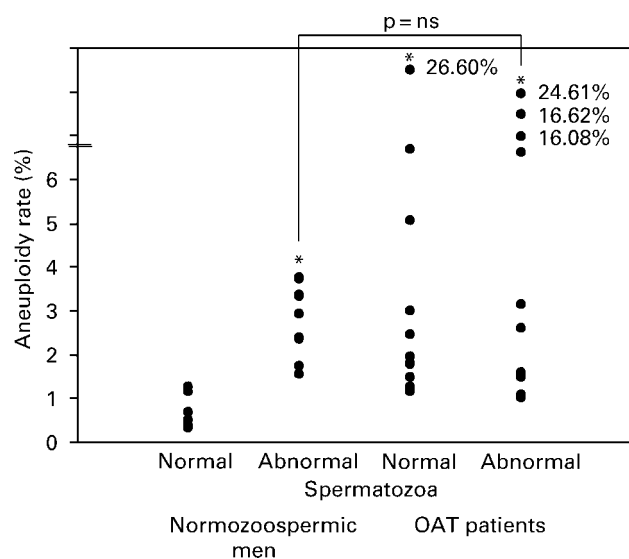
Figure 1. Individual sperm parameters of normozoospermic men and patients with oligo- astheno- and/or teratozoospermia (OAT). *P ≤ 0.001 versus normozoospermic men.

normally shaped (median = 230 spermatozoa; range 78–335), whereas 3993 had an abnormal head morphology (median = 406 spermatozoa; range 301–481). Because of the abnormal sperm parameters, we could not score a greater number of spermatozoa with a normal morphology among OAT patients. The coordinates of each spermatozoon categorized were recorded and it was retrieved after FISH to establish its chromosome complement.

Sperm aneuploidy rates for chromosomes 12, X and Y are reported in Table I. OAT patients had a slight, but significantly ($P < 0.05$), lower number of morphologically normal and abnormal spermatozoa carrying the X chromosome, compared with normozoospermic men. They also exhibited increased XY and XX disomy rates ($P < 0.01$), whereas YY disomy, sex chromosome nullisomy and chromosome 12 disomy and nullisomy rates were similar. Spermatozoa with an abnormal sperm head from normozoospermic men also had a significantly ($P < 0.05$) higher XX disomy rate compared with morphologically normal spermatozoa obtained from the same men.
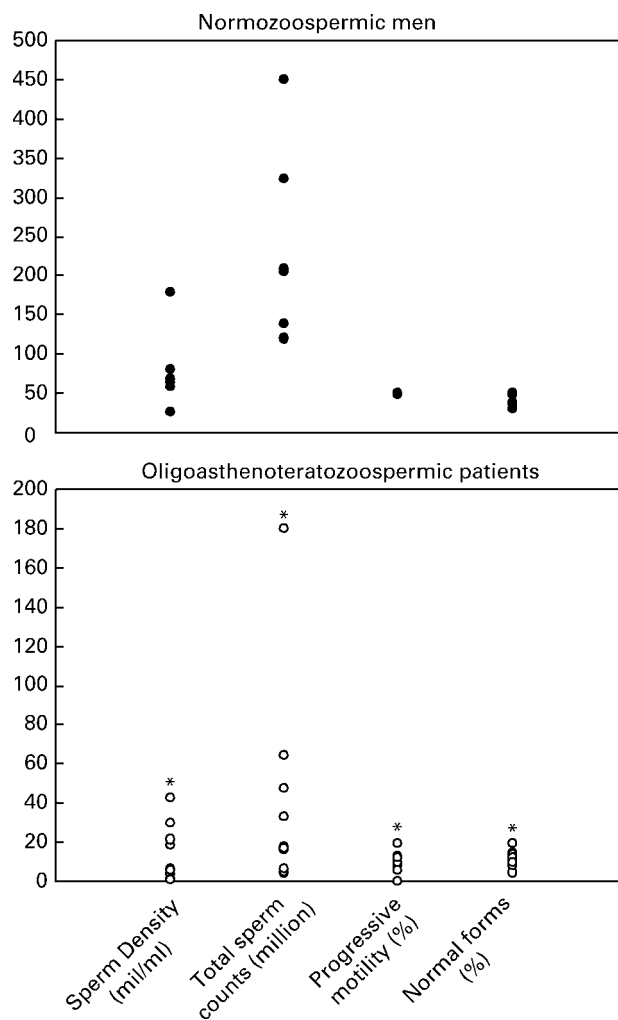
Figure 2. Individual total aneuploidy rate of spermatozoa with normal and abnormal morphology of normozoospermic men and patients with oligo- astheno- and/or teratozoospermia (OAT). *$P < 0.005$ versus morphologically normal spermatozoa from normozoospermic men. The y-axis is a combined scale for the x-axis parameters.

The total aneuploidy rates for chromosomes X, Y and 12 in spermatozoa with a normal or abnormal head from normozoospermic men and OAT patients is shown in Figure 2. Morphologically abnormal spermatozoa of normozoospermic men had an aneuploidy rate 4.4-fold higher than that found in spermatozoa with a normal morphology ($P < 0.001$). The total aneuploidy rates of spermatozoa with normal or abnormal head shape from OAT patients were similar to each other and to that of abnormally shaped spermatozoa from normozoospermic men, but they were significantly higher than the rate found in normally shaped spermatozoa of normal men ($P < 0.001$). In particular, normally and abnormally shaped spermatozoa from OAT patients had an aneuploidy rate 3.6- and 4.7-fold higher, respectively, than that found in normally shaped spermatozoa of normozoospermic men.

Discussion

The increased sperm aneuploidy rate in patients with secretory OAT has raised concern about the possible transmission of chromosomal abnormalities to the offspring generated through assisted reproductive techniques. The risk is particularly elevated since patients with severe spermatogenesis impairment, who more often undergo ICSI for the treatment of their infertility, have the greatest sperm aneuploidy rate and particularly those with the highest degree of teratozoospermia (Bernardini *et al.*, 1998; Ushijima *et al.*, 2000; Calogero *et al.*, 2001). Morphologically abnormal spermatozoa are not, however, selected for oocyte injection, apparently lowering the risk of generating an aneuploid embryo. Nevertheless, the aneuploidy rate of morphologically normal spermatozoa of patients with secretory OAT is not well known. For this reason, we evaluated the sperm aneuploidy rate of morphologically normal and abnormal spermatozoa of patients with secretory OAT or normozoospermia. Surprisingly, we found that morphologically normal spermatozoa of patients with secretory OAT have a sperm aneuploidy rate similar to that found in spermatozoa with head shape abnormalities. On the other hand, the aneuploidy rate of morphologically normal spermatozoa of normozoospermic men was significantly lower than that found in their spermatozoa with an abnormally shaped head. It can be postulated that the factors which cause OAT in these patients affect, at the same time, the fine-tuned mechanisms of chromosome segregation in all germ cells. Therefore, our data show that normal morphology does not indicate the selection of a chromosomally normal spermatozoon in patients with secretory OAT, suggesting a real increased risk for generating aneuploid offspring. Accordingly, the limited available data on ICSI fetal karyotypes reveal that, in comparison with a general neonatal population, there is a slight but significant increase in *de novo* sex chromosomal aneuploidy (0.6% instead of 0.2%) and structural autosomal abnormalities (0.4% instead of 0.07%) (Bonduelle *et al.*, 2002; Van Steirteghem *et al.*, 2002).

The results of the present study are in close agreement with those of Ryu *et al.* (2001). The authors studied the frequency of aneuploidy for chromosomes 18, X and Y in

morphologically normal spermatozoa of eight infertile patients having <4% normal forms according to Kruger's strict criteria. The aneuploidy rate of these spermatozoa was compared with that of normally shaped spermatozoa of six normal donors of proven fertility. The FISH analysis showed that the sperm aneuploidy rate of the infertile teratozoospermic patients was 3.3% (range 1.8–5.5%). This rate was nearly 3-fold greater than that found in morphologically normal spermatozoa of fertile men who had an aneuploidy rate of 1.3% (range 0–2.6%). The sex chromosome aneuploidy rate was 2-fold higher in the infertile group as compared with the control group. The authors did not show the aneuploidy rate of morphologically abnormal spermatozoa (Ryu *et al.*, 2001).

The increased aneuploidy rate found in normally shaped spermatozoa of OAT patients may also explain the lower success rate of ICSI in patients with OAT (for a review see Calogero *et al.*, 2003). However, the abnormal sperm parameters found in these patients may as well have a negative effect on the ICSI outcome. For instance, a recent study showed that ICSI clinical results correlate negatively with the degree of abnormal sperm morphology. Implantation and pregnancy rates were 9.6 and 20.2%, respectively, in patients with teratozoospermia, whereas they were 18.7 and 36.7% in patients with normal sperm morphology (De Vos *et al.*, 2003). However, we have shown recently that an increased sperm aneuploidy rate has a negative impact on ICSI outcome. Unselected patients undergoing ICSI were sorted into two groups according to their sperm aneuploidy rate: one with a normal and the other with an elevated sperm aneuploidy rate. Although fertilization and cleavage rates were similar between the two groups, patients with a normal sperm aneuploidy rate had higher pregnancy and implantation rates compared with patients who had an elevated aneuploidy rate, whereas the abortion rate was significantly higher in the latter. Other factors that may have a negative outcome on the clinical ICSI outcome, such as female partner age, abnormalities of conventional semen parameters or female factor of infertility, were similar in both groups (Burrello *et al.*, 2003).

In conclusion, the present study showed that morphologically normal spermatozoa of OAT patients carry an abnormal chromosomal constitution with the same frequency as that found in spermatozoa with an abnormal head shape. Therefore, the risk of selecting aneuploid spermatozoa to be used for oocyte injection is high in OAT patients. These findings further strengthen the needs for genetic counselling before carrying out ICSI, to decrease the risk of transmitting genetic disease to the offspring. This may be achieved, for example, by establishing methods to identity spermatozoa with a normal chromosomal complement (Cayli *et al.*, 2003).

References

Bernardini L, Borini A, Preti S, Conte N, Flamigni C, Capitanio GL and Venturini PL (1998) Study of aneuploidy in normal and abnormal germ cells from semen of fertile and infertile men. Hum Reprod 13,3406–3413.

Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A and Liebaers I (2002) Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 17,2600–2614.

Burrello N, Vicari E, Shin P, Agarwal A, De Palma A, Grazioso C, D'Agata R and Calogero AE (2003) Lower sperm aneuploidy frequency rates in ICSI programmes. Hum Reprod 18,1371–1376.

Calogero AE, De Palma A, Grazioso C, Barone N, Romeo R, Rappazzo G and D'Agata R (2001) Aneuploidy rate in spermatozoa of selected men with abnormal semen parameters. Hum Reprod 16,1172–1179.

Calogero AE, Burrello N, De Palma A, Barone N, D'Agata R and Vicari E (2003) Sperm aneuploidy in infertile man. Reprod Biomed Online 6, 310–317.

Cayli S, Jakab A, Ovari L, Delpiano E, Celik-Ozenci C, Sakkas D, Ward D and Huszar G (2003) Biochemical markers of sperm function: male fertility and sperm selection for ICSI. Reprod Biomed Online 7,462–468.

De Vos A, Van De Velde H, Hubertjoris MT, Verheyen G, Devroey P and Van Steirteghem A (2003) Influence of indiviual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. Fertil Steril 79,42–48.

Gole LA, Wong PF, Ng PL, Wang XQ, Ng SC and Bongso A (2001) Does sperm morphology play a significant role in increased sex chromosomal disomy? A comparison between patients with teratozoospermia and OAT by FISH. J Androl 22,759–763.

Harkonen K, Suominen J and Lahdetie J (2001) Aneuploidy in spermatozoa of infertile men with teratospermia. Int J Androl 24,197–205.

Meschede D and Horst J (1997) Genetic counselling for infertile male patients. Int J Androl 20,20–30.

Palermo G, Joris H, Devroey P and Van Steirteghem AC (1992) Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. Lancet 340,17–18.

Pang MG, Hegerman SF, Cuticchia AJ, Moon SY, Doncel GF, Acosta AA and Kearns WG (1999) Detection of aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y by fluorescent in-situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. Hum Reprod 14,1266–1273.

Ryu HM, Lin WW, Lamb DJ, Chuang W, Lipshultz LI and Bischoff FZ (2001) Increased chromosome X, Y, and 18 nondisjunction in sperm from infertile patients that were identified as normal by strict morphology: implication for intracytoplasmic sperm injection. Fertil Steril 76,879–883.

Sambrook J, Fritsh EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ushijima C, Kumasako Y, Kihaile PE, Hirotsuru K and Utsunomiya T (2000) Analysis of chromosomal abnormalities in human spermatozoa using multi-colour fluorescence in-situ hybridization. Hum Reprod 15, 1107–1111.

Van Steirteghem A, Bonduelle M, Devroey P and Liebaers I (2002) Follow-up of children born after ICSI. Hum Reprod Update 8,111–1116.

Vegetti W, Van Assche E, Frias A, Verheyen G, Bianchi MM, Bonduelle M, Liebaers I and Van Steirteghem A (2000) Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. Hum Reprod 15,351–365.

World Health Organization (1999) Laboratory Manual for the Examination of the Human Semen and Sperm-Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge.