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Frequency of aneuploidy in sperm from patients with extremely severe male factor infertility

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BACKGROUND: A protocol for the chromosomal analysis of sperm samples with a severely reduced number of sperm cells was designed. METHODS: A severe male factor condition was the main cause of infertility for 38 couples: 27 were oligoasthenoteratospermic (OAT) and 11 with non-obstructive azoospermia underwent testicular sperm extraction (TESE). A two-round fluorescence *in situ* hybridization (FISH) protocol was performed with probes specific for the chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22. The recording of the position of each sperm cell at the microscope allowed diagnosis of each spermatozoon for the nine tested chromosomes. RESULTS: A mean number of 122 ± 78.5 sperm were diagnosed per patient with an incidence of total abnormalities corresponding to 13.4%. χ^2-tests for the observed frequencies and goodness-of-fit test were highly significant in all cases. A significantly higher proportion of total aneuploidy was detected in 79% of the tested samples compared to the normal population. Testicular sperm were significantly more prone to aneuploidy than ejaculated sperm. CONCLUSIONS: The designed FISH protocol for the analysis of severe OAT and TESE sperm samples is reliable, implying that the studied sample is representative of the original population. In view of the high incidence of aneuploidy in most severe OAT and TESE sperm, the FISH analysis of pathological sperm samples can be routinely performed in order to estimate the chances of the paternal contribution to aneuploidy in the resulting embryos.

Key words: aneuploidy/disomy/fluorescence *in situ* hybridization/male factor infertility/preimplantation genetic diagnosis

Introduction

The unreliability of diagnosing male (in-)fertility by conventional sperm analyses gave impetus to investigating additional tests such as zona-free hamster oocyte test, mucus sperm penetration assay and the post-coital test. Unfortunately, these tests contributed poor information (Silber, 2000; Biagiotti *et al.*, 2002), while the study of the DNA array of male gametes revealed that alterations in infertile males with normal sperm analyses (Antonelli *et al.*, 2000; Saleh *et al.*, 2002) correlate with *in vitro* and *in vivo* fertilization outcomes (Burrello *et al.*, 2003). This novel approach is now regarded as a promising field of interest in the study of male infertility.

During the last decade, the application of multicolor fluorescence *in situ* hybridization (FISH) was proposed and enabled comprehensive studies on numerical chromosomal abnormalities in human sperm (reviewed by Egozcue *et al.*, 1997, 2003; Shi and Martin, 2000, 2001). FISH is a highly sensitive and specific technique that permits the analysis of large numbers of sperm by using chromosome-specific probes labelled with fluorochromes. The packaging of DNA arrays by disulphide bonds between adjacent protamines makes sperm nuclei very condensed and inaccessible to DNA probes. Therefore, a decondensation treatment with a reducing agent is necessary and is strictly related to the success of the technique (for reviews see Downie *et al.*, 1997; Egozcue *et al.*, 1997). The quality of the procedure affects the efficiency of hybridization and the definition of the fluorescent signals, and the possibility of distinguishing sperm heads from non-sperm cells.

This technique, through a combination of differently labelled fluorescent probes, opens up the possibility of evaluating the frequency of aneuploidy for two or three chromosomes in each round of FISH. The advantage of using multiprobes simultaneously resides in a more accurate estimation of aneuploidy, allowing differentiation between nullisomy, disomy and diploidy. However, due to the small size of the decondensed sperm head, the number of probes that can be used is restricted (generally no more than three). This limitation can be bypassed if sperm spreads are prepared on different slides and hybridization is performed with different combinations of probe. In this way, sperm samples can theoretically be diagnosed for any chromosome in the human complement. These characteristics allowed FISH to be included as a test for the study of infertile couples.

Unfortunately, severe oligozoospermic samples do not have sufficient numbers of sperm to be scored following this

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approach. In the most severe cases, including testicular samples, the low count numbers do not even permit the preparation of one slide, based on the assumption that the size of the sample affects the validity of the results. Consequently, a substantial proportion of infertile men are excluded from the possibility of having a FISH test done on their sperm due to the scarcity of cells available for analysis. The reports of higher frequency of chromosomal abnormalities in infertile males' sperm (Pang *et al.*, 1995; Egozcue *et al.*, 1997; Int't Veld *et al.*, 1997; Bernardini *et al.*, 2000) and the increased incidence of gonosome abnormalities in children conceived after ICSI (Bonduelle *et al.*, 2002) suggest that this proportion of male infertility could be the best candidates to benefit from FISH analysis on sperm. The frequency of high levels of aneuploidy is inversely correlated with sperm count and progressive motility in infertile men (Vendrell *et al.*, 1999; Vegetti *et al.*, 2000). It has been reported that up to 18% of men with severe oligoasthenoteratospermia (OAT) carry synaptic abnormalities originated at prophase I (Egozcue *et al.*, 2003). This predisposes to the production of aneuploid and diploid sperm and to the consequent risk of chromosomally abnormal conceptuses (Gianaroli *et al.*, 2000). The end result could be an increase in failed implantation and/or repeated abortions (Egozcue *et al.*, 2000; Bernardini *et al.*, 2004).

The aim of this study was to define a protocol for the chromosomal analysis of samples with a severely reduced number of sperm and to assess its reliability. 'Reliability' was defined as the studied sperm being representative of the original population.

Materials and methods

Patients

Thirty-eight infertile couples with a normal karyotype attended the S.I.S.Me.R. Reproductive Medicine Unit to undergo assisted conception cycles. A severe male factor condition was the main cause of infertility due to severe OAT ($n = 27$) or non-obstructive azoospermia with incomplete spermatogenetic arrest for which testicular sperm extraction (TESE) was performed ($n = 11$). Characteristics and history of the 38 couples and sperm parameters are detailed in Table I.

A control group of five normozoospermic patients was included in the study to evaluate the sensitivity of the technique. Control sperm samples were diluted to a final concentration of 0.5–0.1 $\times$ 10^6/ml and then treated as described for the study group.

Semen sample collection

The study was conducted between February 2002 and December 2003. Ejaculated sperm samples were analysed to evaluate concentration and motility according to World Health Organization (WHO) parameters (WHO, 1999), and sperm morphology was assessed, when possible, following strict criteria (Kruger *et al.*, 1988). OAT samples were fixed immediately after the collection with the exception of four samples, which had been previously stored in liquid nitrogen and were thawed and fixed. Sperm retrieved by TESE had been cryopreserved after the surgical intervention for subsequent use in assisted reproduction cycles (Gianaroli *et al.*, 1999); according to the patient's consent, two or three straws were thawed for FISH analysis.

Sperm nuclei preparation

Sperm samples were prepared as already described (Bernardini *et al.*, 2000). Briefly, the samples were washed three times in phosphate-buffered saline (PBS, pH 7.2) at 300*g* for 10 min. The pellet was resuspended in 1 ml of cold methanol:acetic acid (3:1) and eventually stored at -20°C until further processing. The fixed sperm were cytospun on poly-L-Lys-coated slides, washed in 2 × saline sodium citrate (SSC) with 0.3 mol/l NaCl, and decondensed in 1 mol/l Tris–HCl (pH 9.5) with 25 mmol/l dithiothreitol for 5 min at room temperature. After rinsing in 2 × SSC and PBS, the slides were dehydrated in increasing ethanol series (70, 96 and 100%) (Martini *et al.*, 1995).

Cytoplasmic staining

The cytoplasmic staining of Papanicolaou was used with some modifications (WHO, 1999). Slides were immersed in OG6 (Orange G solution, Merck, Germany) for 30 s and repeatedly washed in 95% ethanol; immersion in EA-50 (polychromatic solution EA-50, Merck, Germany) for 30 s followed by repeated washings in 95% and absolute ethanol were the final steps.

FISH on sperm

Multicolor FISH was used in a two-step protocol to diagnose each sperm cell for the chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22. The probe mixture used in the first round contained the probes specific for the chromosomes 13, 16, 18, 21 and 22 (Multivysion PB panel from Vysis; Vysis Inc., USA). A second round followed, with probes specific for chromosome X (CEP X alpha satellite, Xp11.1-q11.1), Y (CEP Y alpha satellite, Yp11.1-q11.1), 15 (CEP 15 satellite III, 15q11.2) and 17 (CEP 17 alpha satellite, 17p11.1-q11.1).

Sperm nuclei were denaturated at 75°C for 10 min in 70% formamide 2 × SSC followed by dehydration in the ethanol series; 3 μl of probes were denaturated at 75°C for 5 min and added to the slide. Hybridization was performed overnight in a humid chamber at 37°C followed by washings at 46°C in 2 × SSC/50% formamide for 10 min, in 2 × SSC for 10 min, in 2 × SSC/0.1% Nonidet P 40 for 5 min. The slides were then counterstained in antifade solution (Antifade II; Vysis) when using the first panel probes or with 4′,6-diamino-2-phenylindole (DAPI II; Vysis) for the second panel probes. Slides were observed using an Olympus BX40 fluorescence microscope at × 600 magnification equipped with a Ludl filter wheel with the following filter sets: dual band pass filters (Red/Green and Aqua/-Blue) and single band pass filters (Red, Green, Yellow, Aqua). Images were captured using a CCD PVCAM camera controlled by a MacIntosh computer and an image analysis software (Vysis Quips).

Sperm were scored according to previously described criteria (Blanco *et al.*, 1996). Briefly, they were diagnosed as abnormal if they presented two or more fluorescent signals for the same chromosome whose size and intensity were similar to those detected in normal nuclei; diploidy was defined by the presence of two signals for each of the studied chromosomes in the presence of the sperm tail and an oval head shape; sperm were defined as nullisomic when no fluorescent signals appeared. All signals were separated from each other by at least a single domain.

During the analysis at the fluorescence microscope, the position of each sperm cell was assessed by the coordinates defined by the graduated scale on the microscope table, allowing diagnosis of each spermatozoon for the nine chromosomes studied by the two round FISH.

Table I. History and sperm characteristics of the couples included in the study

Code	Age (years)		Sperm typology	Spontaneous no. of abortions	Total no. of IVF cycles	Total no. of transferred cycles	Total no. of transferred embryos	No. of clinical pregnancies	No. of abortions after IVF	Sperm parameters			
	Female	Male								Volume (ml)	Count ($\times 10^6$/ml)	Motility (%)	Normal forms (%)
OAT-1	33	40	OAT	0	0	0	0	0	0	3.5	0.2	0 + 0 + 0	4
OAT-2	40	40	OAT	0	8	8	13	2	2	3	0.7	2 + 5 + 10	6
OAT-3	38	38	OAT	1	1	0	0	0	0	1.6	1	20 + 30 + 10	9
OAT-4	35	38	OAT	0	3	5	8	0	0	1.2	0.0013	25 + 0 + 75	–
OAT-5	34	37	OAT	0	3	3	5	0	0	1.6	0.01	10 + 0 + 0	–
OAT-6	37	42	OAT	0	4	3	6	0	0	3.4	0.0003	0 + 20 + 20	–
OAT-7	33	34	OAT	0	4	4	7	0	0	2.3	0.14	20 + 10 + 10	–
OAT-8	40	36	OAT	0	3	2	4	0	0	0.4	0.05	0 + 10 + 20	–
OAT-9	36	38	OAT	0	3	3	7	1	0	4.3	0.95	10 + 20 + 0	7
OAT-10	33	36	OAT	0	3	3	8	0	0	3	0.1	6 + 14 + 30	–
OAT-11	41	40	OAT	2	1	1	3	0	0	5.2	0.2	10 + 20 + 10	–
OAT-12	35	35	OAT	0	2	1	1	0	0	1.7	0.34	0 + 5 + 5	–
OAT-13	40	44	OAT	0	0	0	0	0	0	2.1	0.0001	50 + 10 + 0	–
OAT-14	31	45	OAT	0	2	0	0	0	0	0.8	0.4	5 + 5 + 0	–
OAT-15	43	46	OAT	0	2	2	9	0	0	0.4	0.3	0 + 0 + 5	6
OAT-16	40	35	OAT	0	1	1	0	0	0	6.5	0.6	10 + 10 + 5	5
OAT-17	38	41	OAT	0	4	4	7	0	0	5	4.7	10 + 10 + 10	12
OAT-18	36	40	OAT	0	4	7	16	0	0	4	0.06	15 + 0 + 0	–
OAT-19	31	34	OAT	0	3	3	8	0	0	2.5	2	5 + 5 + 5	5
OAT-20	34	36	OAT	0	5	5	12	0	0	3.7	0.1	5 + 30 + 30	–
OAT-21	29	33	OAT	0	1	1	2	0	0	2	0.25	30 + 10 + 0	8
OAT-22	34	35	OAT	0	1	1	2	0	0	1.2	0.3	30 + 30 + 10	9
OAT-23	36	47	OAT	0	3	3	12	0	0	3	0.9	10 + 20 + 10	–
OAT-24	34	36	OAT	0	1	1	2	0	0	2	0.4	5 + 5 + 0	–
OAT-25	35	36	OAT	0	2	1	1	0	0	1.6	0.00001	0 + 0 + 0	–
OAT-26	26	36	OAT	0	1	0	0	0	0	0.4	0.06	5 + 20 + 5	
OAT-27	37	35	OAT	0	3	3	8	0	0	0.8	1.8	20 + 5 + 5	14
T-28	30	41	TESE	0	5	5	11	1	1	0.2	0.03	0 + 0 + 10	–
T-29	31	35	TESE	0	4	4	7	0	0	0.2	0.8	0 + 0 + 5	–
T-30	41	55	TESE	0	4	4	8	0	0	0.4	0.8	0 + 0 + 5	–
T-31	38	46	TESE	0	7	6	22	0	0	0.3	0.28	0 + 0 + 2	–
T-32	37	38	TESE	0	4	4	7	0	0	0.6	0.1	0 + 0 + 1	–
T-33	32	44	TESE	0	4	2	3	0	0	0.4	0.4	0 + 0 + 0	–
T-34	24	25	TESE	0	3	3	5	0	0	0.1	0.16	0 + 0 + 0	–
T-35	33	33	TESE	0	2	1	2	0	0	0.3	0.04	0 + 0 + 2	–
T-36	37	42	TESE	0	?	?	?	0	0	0.75	0.15	0 + 0 + 2	–
T-37	33	37	TESE	0	3	3	5	0	0	0.5	0.0005	0 + 0 + 20	–
T-38	38	49	TESE	0	2	0	0	0	0	0.3	0.01	0 + 0 + 0	–

OAT = oligoasthenoteratospermia; TESE = testicular sperm extraction.

FISH on embryos

Day 3 embryos with regular morphology and development were selected for embryo biopsy which was performed at 62–64 h after insemination according to a previously described protocol (Gianaroli *et al.*, 2002). Briefly, one nucleated blastomere was removed by mechanical opening of the zona pellucida. Fluorescent probes were used for the simultaneous detection of different chromosomes in successive rounds of FISH, including those implicated in the most frequent aneuploidies detected in spontaneous abortions and trisomic pregnancies (X, Y, 13, 15, 16, 18, 21, 22) (Munné *et al.*, 1998).

Data management and statistical analysis

Evaluation of the incidence of aneuploidy followed a conservative approach by doubling the incidence of disomy (Blanco *et al.*, 1996; Egozcue *et al.*, 1997).

The end-points of the current study were to ascertain whether: (1) a significant difference between the studied population and the theoretical sperm aneuploidy frequency exists (Williams *et al.*, 1993; Spriggs *et al.*, 1995; Egozcue *et al.*, 1997; Pang *et al.*, 1999); (2) the studied number of total, haploid and aneuploid sperm, as well as the frequency of aneuploidy for each chromosome, are representative of the original population; (3) aneuploidy within the same sperm cell is randomly or not randomly generated; (4) significant differences in terms of aneuploidy exist between ejaculated and testicular sperm.

Items 1 and 4 were investigated by χ^2-test for rare frequencies; item 2 was analysed by χ^2-test for observed frequencies ($\chi^2_{c\text{-}k\text{-}1}$); and item 3 was evaluated by goodness-of-fit test (χ^2_n) (Camussi *et al.*, 1995).

Results

The results derived from the control group are described in Table II. A haploid chromosomal complement was found in 98.5% of the 1117 analysed sperm cells, which is not different from the expected frequency in normospermic men. The goodness-of-fit test was highly significant in all cases, demonstrating that the studied sperm are representative of the corresponding samples.

In the study group, a total number of 4642 sperm cells was diagnosed accounting for an average of 122.0 ± 78.5 per patient (range 20–307). Of these, 4050 (87.25%) had a haploid chromosomal complement (106.6 ± 67.2 per patient) with a frequency of haploidy ranging from 71 to 98%. The number of sperm cells scored and the FISH results obtained for each patient are reported in Table III (ejaculated sperm) and Table IV (testicular sperm).

The cytoplasmic staining performed before sperm denaturation and hybridization enabled simultaneous visualization of the whole sperm shape, head and tail, and the fluorescent signals (Figure 1). This allowed us to distinguish sperm from non-sperm cells, provided that the decondensation method used maintained sperm morphology while ensuring efficient hybridization. As reported in Table III, 3749 sperm cells were diagnosed in the 27 OAT patients with a normal karyotype (138.9 ± 70.7, range 22–307) and the total rate of chromosomal haploidy was 88% (range 73–98%). Aneuploidy (expressed as the sum of disomy and nullisomy) was detected in 490 sperm (13%) and diploidy in 14 sperm (0.4%) accounting for 13.4% total abnormalities. The frequency of

Table II. Fluorescence *in situ* hybridization (FISH) results in normospermic samples

Code	No. of diagnosed sperm	No. (%) haploid	Aneuploidy (disomy + nullisomy), no. (%)								No. of sperm cells with double aneuploidies	No. (%) of disomies	No. (%) diploid	Goodness -of-fit test ($n \times 10^3$)	*P*
			XY	13	15	16	17	18	21	22					
N-1	126	124 (98)	1 (1.0)	0	0	0	0	0	1 (1.0)	0		1 (1.0)	0	156.1	<0.01
N-2	148	146 (99)	0	0	0	1 (1.0)	0	1 (1.0)	0	0		1 (1.0)	0	188.2	<0.01
N-3	397	392 (99)	0	1 (0.2)	0	1 (0.2)	0	1 (0.2)	2 (0.4)	1 (0.2)	1 for chromosomes 13, 21	4 (1.0)	0	132.6	<0.01
N-4	266	261 (98)	0	0	1 (0.2)	1 (0.2)	1 (0.2)	0	2 (0.4)	1 (0.2)	1 for chromosomes 17, 21	3 (1.1)	0	213.4	<0.01
N-5	180	177 (98)	0	0	0	0	0	0	2 (1.1)	2 (1.1)	1 for chromosomes 21, 22	2 (1.1)	0	222.3	<0.01
Total (%)	1117	1100 (98.5)	1 (0.1)	1 (0.1)	1 (0.1)	3 (0.3)	1 (0.1)	2 (0.2)	7 (0.6)	4 (0.4)		11 (1.0)	0		
χ^2_{c-k-1}		44.1	35.6	32.3	33.1	34.6	38.2	34.3	35.3	35.2					
P		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01					

Double aneuploidies are counted as a single event. Aneuploidy is expressed as the sum of disomy and nullisomy.

Table III. Fluorescence *in situ* hybridization (FISH) results in ejaculated sperm from oligoasthenoteratospermic (OAT) patients

Code	No. of diagnosed sperm	No. (%) haploid	Aneuploidy (disomy + nullisomy), no. (%)								No. of sperm cells with double aneuploidies	No. (%) of disomies	No. (%) diploid	Goodness -of-fit test $(n \times 10^3)$	*P*
			XY	13	15	16	17	18	21	22					
OAT-1	47	46 (98)	1 (2.1)	0	0	0	0	0	0	0		1 (2.1)*	0	481.1	<0.01
OAT-2	135	127 (94)	0	1 (0.8)	0	2 (1.5)	0	3 (2.2)	2 (1.5)	0	2 for chromosomes 16,18	3 (2.2)	2 (1.5)*	283.9	<0.01
OAT-3	165	151 (91)	0	3 (1.8)	0	4 (2.4)	0	3 (1.8)	2 (1.2)	3 (1.8)	1 for chromosomes 13, 21	5 (3.0)*	0	312.6	<0.01
OAT-4	134	108 (81)	0	4 (3.0)	6 (4.5)	1 (0.7)	6 (4.5)	0	6 (4.5)	4 (3.0)	1 for chromosomes 17, 21	7 (5.2)*	0	223.5	<0.01
OAT-5	131	111 (85)	0	7 (5.3)	0	0	0	0	3 (2.3)	5 (3.8)	2 for chromosomes 13, 21	10 (7.6)*	7 (5.3)*	267.3	<0.01
OAT-6	30	23 (77)	2 (6.7)	0	1 (3.3)	2 (6.7)	0	1 (3.3)	2 (6.7)	2 (6.7)	1 for chromosomes 15, 18 1 for chromosomes 16, 22 1 for chromosomes XY, 16	1 (3.3)*	0	254.3	<0.01
OAT-7	154	143 (93)	1 (0.6)	3 (1.9)	0	1 (0.6)	1 (0.6)	1 (0.6)	5 (3.2)	0	1 for chromosomess 16, 21	10 (6.5)*	0	271.2	<0.01
OAT-8	66	58 (88)	0	2 (3.0)	1 (1.5)	1 (1.5)	2 (3.0)	0	2 (3.0)	0		1 (1.5)	0	284.1	<0.01
OAT-9	201	175 (87)	4 (2.0)	2 (1.0)	4 (2.0)	4 (2.0)	6 (3.0)	4 (2.0)	5 (2.5)	4 (2.0)	3 for chromosomes 17, 21 1 for chromosomes 16, 18 1 for chromosomes 16, 18, XY 1 for chromosomes 15, 17	6 (3.0)*	0	775.6	<0.01
OAT-10	145	118 (81)	4 (2.8)	5 (3.4)	3 (2.1)	0	NA	1 (0.1)	6 (4.1)	8 (5.5)		16 (11)*	0	178.6	<0.01
OAT-11	105	80 (76)	5 (4.7)	5 (4.7)	3 (2.9)	4 (3.8)	NA	2 (1.9)	5 (4.7)	3 (2.9)	2 for chromosomes 16, 18	9 (8.6)*	0	298.3	<0.01
OAT-12	103	90 (87)	0	3 (2.9)	1 (0.1)	0	0	1 (0.1)	3 (2.9)	3 (2.9)		6 (5.8)*	2 (1.9)*	715.4	<0.01
OAT-13	22	20 (91)	0	1 (4.5)	2 (9.1)	0	0	0	0	0	1 for chromosomes 15, 17	2 (9.1)*	0	379.5	<0.01
OAT-14	220	192 (87)	2 (0.9)	2 (0.9)	5 (2.3)	4 (1.8)	8 (3.6)	8 (3.6)	1 (0.4)	4 (1.8)	3 for chromosomes 15, 17 2 for chromosomes 16, 18 1 for chromosomes X, 15	14 (6.4)*	0	771.0	<0.01
OAT-15	151	130 (86)	4 (2.6)	4 (2.6)	3 (2.0)	1 (0.7)	2 (1.3)	0	3 (2.0)	4 (2.6)		15 (9.9)*	0	195.6	<0.01
OAT-16	126	93 (74)	5 (4.0)	9 (7.1)	1 (0.8)	2 (1.6)	1 (0.8)	3 (2.4)	11 (8.7)	12 (9.5)	1 for chromosomes 13, 21 1 for chromosomes 13, 22 1 for chromosomes 16, 18 1 for chromosomes 17, 21 3 for chromosomes 21, 22 1 for chromosomes XY, 13 1 for chromosomes XY, 21 1 for chromosomes XY, 16, 18	15 (11.9)*	0	189.7	<0.01
OAT-17	307	298 (97)	2 (0.7)	2 (0.7)	1 (0.3)	0	1 (0.3)	0	2 (0.7)	1 (0.3)		6 (1.9)	0	171.8	<0.01

Table III. *Continued*

Code	No. of diagnosed sperm	No. (%) haploid	Aneuploidy (disomy + nullisomy), no. (%)								No. of sperm cells with double aneuploidies	No. (%) of disomies	No. (%) diploid	Goodness -of-fit test ($n \times 10^3$)	*P*
			XY	13	15	16	17	18	21	22					
OAT-18	115	110 (96)	0	3 (2.6)	0	0	0	0	0	2 (1.7)		4 (3.5)*	0	188.5	<0.01
OAT-19	306	266 (87)	2 (0.6)	5 (1.6)	7 (2.3)	9 (2.9)	4 (1.3)	9 (2.9)	5 (1.6)	2 (0.6)	1 for chromosomes 16, 15	22 (7.2)*	2 (0.6)*	408.7	<0.01
											1 for chromosomes 15, 17				
											1 for chromosomes 21, 17				
											1 for chromosomes 13, 21				
											1 for chromosomes 16, 18				
OAT-20	74	54 (73)	2 (2.7)	3 (4.1)	3 (4.1)	2 (2.7)	NA	1 (1.3)	6 (8.1)	6 (8.1)	1 for chromosomes 13, 15	9 (12.2)*	0	176.3	<0.01
											1 for chromosomes 13, 21				
											1 for chromosomes 21, 22				
OAT-21	134	127 (95)	0	2 (1.5)	0	0	3 (2.2)	2 (1.5)	0	0		3 (2.2)	0	233.0	<0.01
OAT-22	100	78 (78)	3 (3.0)	3 (3.0)	5 (5.0)	0	2 (2.0)	0	6 (6.0)	4 (4.0)	1 for chromosomes 13, 17	15 (15)*	0	282.3	<0.01
OAT-23	204	196 (96)	0	1 (0.5)	3 (1.5)	2 (1.0)	1 (0.5)	1 (0.5)	0	0		3 (1.5)	0	99.1	<0.01
OAT-24	232	221 (95)	4 (1.7)	2 (0.9)	0	0	NA	0	0	4 (1.7)		8 (3.4)*	1 (0.4)	886.3	<0.01
OAT-25	30	22 (73)	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)	NA	0	2 (6.7)	2 (6.7)		2 (6.7)*	0	98.2	<0.01
OAT-26	109	90 (83)	1 (0.9)	4 (3.7)	5 (4.6)	4 (3.7)	3 (2.8)	1 (0.9)	3 (2.7)	0	1 for chromosomes 13, 21	11 (10.1)*	0	2294.8	<0.01
											1 for chromosomes 13, 15				
OAT-27	203	170 (84)	3 (1.5)	5 (2.5)	4 (2.0)	5 (2.5)	3 (1.5)	0	4 (2.0)	13 (7.6)	1 for chromosomes 13, 21	13 (7.6)*	0	233.3	<0.01
											1 for chromosomes 13, 22				
											1 for chromosomes 15, 17				
											1 for chromosomes 21, 22				
Total (%)	3749	3297 (88)	46 (1.2)	82 (2.2)	59 (1.6)	48 (1.3)	43 ** (1.4)	41 (1.1)	85 (2.3)	86 (2.3)		217 (5.8)*	14 (0.4)		
χ^2_{c-k-1}	521	348	546	823	249	521	645	543	558	551					
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01					

Double aneuploidies are counted as a single event. Aneuploidy is expressed as the sum of disomy and nullisomy.
*Statistical significance for total aneuploidy and diploidy. To evaluate total aneuploidy, the observed disomies (reported in the table) were doubled. NA = not analysed.
**Analysed in 3163 sperm.

Table IV. Fluorescence *in situ* hybridization (FISH) results in testicular sperm

Code	No. of diagnosed sperm	No. (%) haploid	Aneuploidy (disomy + nullisomy), no. (%)								No. of sperm cells with double aneuploidies	No. (%) of disomies	No. (%) diploid	Goodness-of-fit test ($n \times 10^3$)	*P*
			XY	13	15	16	17	18	21	22					
T-28	70	50 (71)	1 (1.4)	3 (4.3)	6 (8.6)	0	4 (5.7)	0	3 (4.3)	2 (2.9)		11 (15.6)*	1 (1.4)*	132.9	<0.01
T-29	152	136 (89)	1 (0.7)	3 (2.0)	3 (2.0)	0	7 (4.6)	0	0	3 (2.0)	2 for chromosomes 15, 17	7 (4.6)*	1 (0.7)*	738.3	<0.01
T-30	70	61 (87)	1 (1.4)	1 (1.4)	2 (2.9)	0	0	1 (1.4)	4 (1.4)	0		7 (10.0)*	0	176.8	<0.01
T-31	50	47 (94)	1 (2.0)	0	0	0	0	0	0	0		0	2 (4.0)	399.6	<0.01
T-32	23	19 (83)	0	2 (8.7)	0	1 (4.3)	1 (4.3)	0	1 (4.3)	2 (8.7)	1 for chromosomes 13, 17 1 for chromosomes 16, 22 1 for chromosomes 21, 22	5 (21.7)*	0	176.6	<0.01
T-33	161	131 (81)	5 (3.1)	3 (1.9)	3 (1.9)	3 (1.9)	NA	3 (1.9)	8 (5.0)	9 (5.6)	3 for chromosomes 16, 18 1 for chromosomes Y, 21 1 for chromosomes XY, 22	11 (6.8)*	1 (0.6)*	231.6	<0.01
T-34	20	18 (90)	0	0	0	1 (5.0)	1 (5.0)	0	0	0		0	0	6991.0	<0.01
T-35	143	120 (84)	1 (0.7)	0	7 (6.0)	7 (6.0)	1 (0.7)	1 (0.7)	6 (4.2)	3 (2.1)	1 for chromosomes 15, 17 1 for chromosomes 16, 21, 22	9 (6.3)*	0	1646.8	<0.01
T-36	117	97 (83)	1 (0.8)	4 (3.4)	5 (4.3)	0	6 (5.1)	1 (0.8)	7 (6.0)	4 (3.4)	1 for chromosomes XY, 17 1 for chromosomes 18, 21 2 for chromosomes 15, 17 1 for chromosomes 17, 21, 22 1 for chromosomes 21, 22 1 for chromosomes 15, 22	3 (2.6)	0	3772.3	<0.01
T-37	31	26 (84)	1 (3.2)	1 (3.2)	0	0	NA	0	2 (6.4)	1 (3.2)	1 for chromosomes X, 21	4 (12.9)*	0	432.9	<0.01
T-38	56	48 (86)	0	1 (1.8)	3 (5.4)	2 (3.6)	1 (1.8)	0	2 (3.6)	0	1 for chromosomes 15, 21	4 (7.1)*	0	5127.2	<0.01
Total, no. (%)	893	753 (84)	12 (1.3)	18 (2.0)	29 (3.2)	14 (1.6)	21** (3.0)	6 (0.7)	33 (3.7)	24 (2.7)		61 (6.8)*	5 (0.6)		
χ^2_{c-k-1}	543	435	443	632	143	453	325	336	251	320					
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01					

Double aneuploidies are counted as a single event. Aneuploidy is expressed as the sum of disomy and nullisomy.
*Statistical significance for total aneuploidy and diploidy. To evaluate total aneuploidy, the observed disomies (reported in the table) were doubled. NA = not analysed.
**Analysed in 701 sperm.

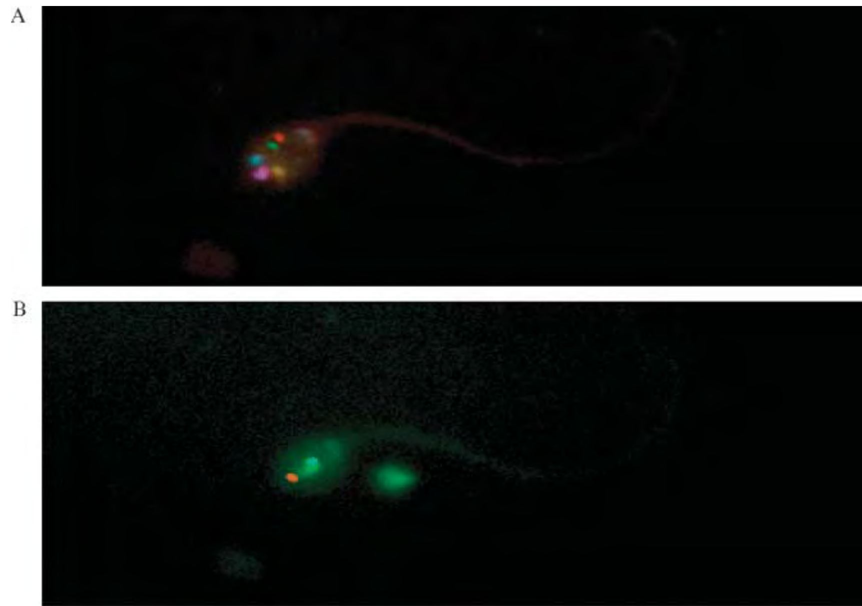

Figure 1. After recording the position of each sperm cell at the microscope, the spermatozoon is diagnosed for five chromosomes in the first round fluorescence *in situ* hybridization (FISH) (**A**): 13 (red), 16 (aqua), 18 (pink), 21 (green) and 22 (yellow). Four additional chromosomes are tested in the subsequent round hybridization (**B**): X (aqua), Y (white), 15 (orange) and 17 (green). This spermatozoon having one signal per probe is diagnosed as normal for the nine tested chromosomes.

aneuploidy for the analysed chromosomes was: 1.2% for chromosomes X and Y, 2.2% for chromosome 13, 1.6% for chromosome 15, 1.3% for chromosome 16, 1.4% for chromosome 17, 1.1% for chromosome 18, and 2.3% for chromosomes 21 and 22. χ^2-tests for the observed frequencies and goodness-of-it test were highly significant in all cases.

The results of FISH analysis on TESE sperm cells from 11 patients are shown in Table IV. A total of 893 cells was analysed and 753 were diagnosed as normal (84%, range 71–94%). The incidence of aneuploidy and diploidy was 17.6 and 0.6% respectively. The analysed chromosomes varied with the following frequency: 1.3% chromosomes X and Y, 2.0% chromosome 13, 3.2% chromosome 15, 1.6% chromosome 16, 3.0% chromosome 17, 0.7% chromosome 18, 3.7% chromosome 21 and 2.7% chromosome 22. As for OAT sperm, χ^2-tests for observed frequencies and goodness-of-fit test were highly significant.

The incidence of aneuploidy was evaluated following the conservative approach of doubling the incidence of disomy and yielded an overall value of 12.0%. After χ^2-test for rare frequencies, 30 samples (79%) showed a significantly higher proportion of total aneuploidy compared to the normal population: 22 out of 27 OAT (81%) and eight out of 11 TESE (73%). The incidence of diploidy was found to be significantly increased in seven samples, of which six were also abnormal for aneuploidy (Tables III and IV).

Figure 2 represents the incidence of total aneuploidy, evaluated as double disomy, in relation to the number of chromosomes with a significant variation. The highest values were detected for samples having between two and six chromosomes with a significant degree of aneuploidy (χ^2-test for rare distribution $= 83.6$, $P < 0.01$).

As presented in Figure 3, chromosomes 13, 21 and 22 showed the highest significant variations in 78, 61 and 61% of the tested samples, while chromosomes 16 and 18 had the lowest figures (15 and 22% respectively) (χ^2-test for rare distribution $= 71.4$, $P < 0.01$).

The aneuploidy rates of the different chromosomes, calculated as double disomy divided by the total number of tested cells, estimated the degree to which each chromosome was prone to meiotic errors (Table V). In OAT patients, the aneuploidy rate for chromosomes 13, 21, 22 and the gonosomes was significantly higher compared to the aneuploidy rate for chromosomes 15, 16, 17 and 18, and to the incidence reported in the normal population. In testicular sperm,

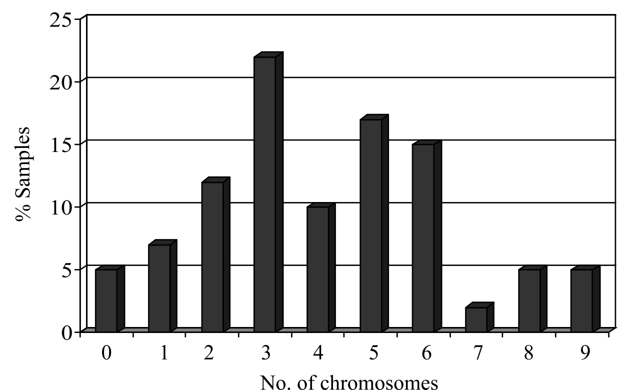

Figure 2. Incidence of total aneuploidy (evaluated as double disomy) in relation to the number of chromosomes with a significant variation compared to the expected frequency. The majority of samples presented two to six chromosomes with significant variations.

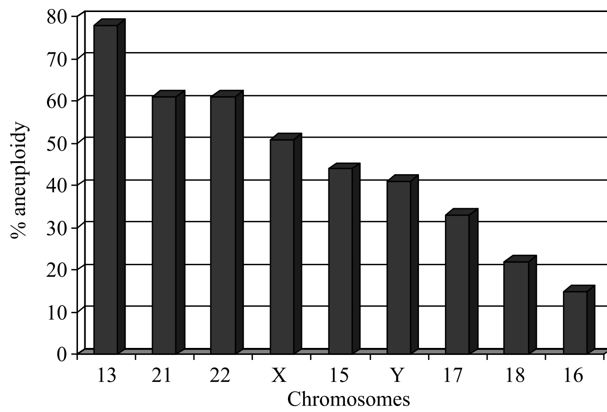

Figure 3. Incidence of total aneuploidy (evaluated as double disomy) for each of the tested chromosomes. Chromosomes 13, 21 and 22 showed the highest significant variations.

Table V. Incidence of total aneuploidy, evaluated as double disomy, per single chromosome

	OAT	TESE
XY	1.3* (50/3749)	1.3*(12/893)
13	3.1* (116/3749)	2.9*(26/893)
15	0.8* (30/3749)	2.7*(24/893)
16	0.4 (16/3749)	0
17	0.7 (22/3163)	0.9 (6/701)
18	0.7 (26/3749)	0.2 (2/893)
21	1.8* (68/3749)	3.3*(30/893)
22	2.8*(106/3749)	2.5* (22/893)
Total	11.6 (434//3749)*	13.7 (122/893)*

Oligoasthenoteratospermic (OAT) patients with a normal karyotype.
*$P < 0.001$ compared with the normal population.
TESE = testicular sperm extraction.

chromosomes 13, 15, 21, 22, X and Y showed significantly higher rates in comparison to chromosomes 16 and 18, as well as to the values characterized in the normal population. In testicular sperm, aneuploidy for chromosomes 15 and 21 22 proved to be significantly higher than in OAT sperm.

For 17 of the patients included in the study, the FISH results were related to the data derived from the chromosomal analysis in preimplantation embryos that was performed in 27 cycles (Table VI). The mean maternal age was 35.1 ± 3.8, with five women being aged ≥38 years. In all, 104 embryos were FISH diagnosed and 37 (38%) were chromosomally normal. Aneuploidy, intended as monosomy and trisomy, was detected in 31 embryos accounting for 48% of total abnormalities. In couples OAT-4 and T-35, maternal age 35 and 33 years respectively, the high frequency of aneuploidy for some chromosomes in sperm (nullisomy 21 in OAT-4, disomy 15 and nullisomy 16 in T-35) matched with the abnormalities detected in preimplantation embryos.

Discussion

Major concerns of all techniques studying male gametes are their standard landmarks and their representativeness of the original population (Silber, 2000). The absence of these qualifications led to the abandonment of the zona-free hamster oocyte test, the mucus sperm penetration assay and the post-coital test (Silber, 2000). Multicolour FISH was poorly studied in this regard, but it has been recognized that methodological and laboratory problems can represent a major bias of the technique (Rives *et al.*, 1999; Calogero *et al.*, 2001). The need to verify how the studied sperm sample was representative of the original population was felt to be particularly compelling in the current study, where nine chromosomes for each male gamete were studied in two different rounds of hybridization.

There are a few landmarks to ascertain the reliability of a FISH test. The most accepted in the literature is represented by the higher frequency of aneuploidy in OAT patients compared to normozoospermic (Williams *et al.*, 1993; Spriggs *et al.*, 1995; Egozcue *et al.*, 1997; Pang *et al.*, 1999). This external landmark (i.e. not pertinent to the test itself) has been chosen to check the reliability of the test presented here. Ejaculated sperm reach a higher maturation degree compared to those retrieved from the testis by TESE (Aboulghar *et al.*, 1997). Accordingly, they have a lower degree of aneuploidy; this constitutes the second external landmark of this study. Finally, internal landmarks were selected to assess the test reliability, by verifying: (i) whether an unselected population was studied; and (ii) whether the pathophysiological mechanism generating aneuploidy could be recognized in the studied sample.

The present data demonstrate that the designed FISH protocol for the analysis of severe OAT and testicular sperm is reliable, implying that the studied sample may be considered representative of the original population. The proportion of aneuploidy for each chromosome between the original and the examined population was determined, indicating that the preparation technique does not select aneuploidy, which is randomly represented in the studied sample.

According to the results reported in this study, significant differences between the observed and expected FISH frequencies exist, with testicular sperm being significantly more prone to aneuploidy than ejaculated sperm (Table III, IV). In addition, the simultaneous presence of aneuploidy events in the same gamete appeared to be not randomly distributed (Figure 2) confirming, by statistical analysis, that sperm aneuploidy derives from alterations of the meiotic spindle, in which each chromosome is allocated to a settled place (Egozcue *et al.*, 2000). These findings support the hypothesis that FISH analysis should be regarded as a marker of spermatogenesis, which influences sperm functions more positively than sperm count (Silber, 2000; Biagiotti *et al.*, 2002).

The introduction of the multi-probe FISH technique for sperm analysis has included the numerical study of chromosomes as a routine test for the screening of infertile couples. The results obtained in combination with epidemiological studies could assist the comprehension of paternally derived chromosome abnormalities. In view of the data regarding the karyotype of the children born after ICSI (Liebaers *et al.*, 1995; Bonduelle *et al.*, 2002), the paternal contribution to aneuploidy could be especially notable in cases of severe male factor infertility.

Table VI. Fluorescence *in situ* hybridization (FISH) results on sperm (S) and on embryos (E)
a. Oligoasthenoteratospermic patients

FISH on sperm	FISH on embryos	OAT-1		OAT-2		0AT-3		OAT-4		OAT-5		OAT-6		OAT-7		OAT-8		OAT-9	
		S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E
No. analysed	No. analysed	47	2	135	19	165	2	134	8	131	2	30	3	154	5	66	2	201	4
FISH normal (%)	FISH normal (%)	46	0	127 (94)	7 (37)	151 (91)	0 (0)	108 (81)	4 (50)	111 (85)	1 (50)	23 (77)	0	143 (93)	3 (60)	58 (88)	0 (0)	175 (87)	2 (50)
FISH abnormal (%)	FISH abnormal (%)	1	2	8 (6)	12 (63)	14 (9)	2 (100)	26 (19)	4 (50)	20 (15)	1 (50)	7 (23)	3 (100)	11 (7)	2 (40)	8 (12)	2 (100)	26 (13)	2 (50)
Aneuploid	Aneuploid	1	1	6	4	14	1	26	3	13	0	10	1	11	1	8	1		1
Nullisomy XY	Monosomy XY	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0
Disomy XY	Trisomy XY	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0
Nullisomy 13	Monosomy 13	0	0	0	0	1	0	1	0	2	0	0	0	0	0	1	0	1	1
Disomy 13	Trisomy 13	0	1	1	1	2	0	3	1	5	0	0	0	3	0	1	0	1	0
Nullisomy 15	Monosomy 15	0	1	0	1	0	0	6	0	0	0	1	0	0	0	1	0	3	0
Disomy 15	Trisomy 15	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
Nullisomy 16	Monosomy 16	0	0	2	1	4	0	0	0	0	0	2	0	0	0	1	0	4	0
Disomy 16	Trisomy 16	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0
Nullisomy 17	Monosomy 17	0	NA	0	NA	0	NA	6	NA	0	NA	0	0	0	NA	2	NA	NA	NA
Disomy 17	Trisomy 17	0	NA	0	NA	0	NA	0	NA	0	NA	0	0	1	NA	0	NA	NA	NA
Nullisomy 18	Monosomy 18	0	0	2	0	1	0	0	0	0	0	1	0	0	0	0	0	4	0
Disomy 18	Trisomy 18	0	0	1	0	2	0	0	1	0	0	0	0	1	0	0	1	0	0
Nullisomy 21	Monosomy 21	0	0	1	0	1	0	5	1	2	0	2	0	2	0	2	1	4	0
Disomy 21	Trisomy 21	0	0	1	1	1	0	1	0	1	0	0	0	3	0	0	0	1	0
Nullisomy 22	Monosomy 22	0	0	0	2	3	0	2	0	1	0	1	0	0	0	0	0	3	0
Disomy 22	Trisomy 22	0	0	0	0	0	0	2	0	4	0	1	0	0	1	0	0	1	0
Null	Haploid	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diploid	Polyploid	0	0	2	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0
Complex abnormality	Complex abnormality	0	0	0	6	0	1	0	1	0	1	0	2	0	1	0	1	0	1

b. Testicular sperm extraction patients

FISH on sperm	FISH on embryos	T-28		T-29		T-30		T-31		T-32		T-33		T-34		T-35	
		S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E
No. analyzed	No. analyzed	70	8	152	10	70	4	50	3	23	13	161	7	20	8	143	4
FISH normal (%)	FISH normal (%)	50 (71)	3	136 (89)	5 (50)	61 (87)	2 (50)	47 (94)	0	19 (83)	5	131 (81)	0	18	5	120 (84)	0 (0)
FISH abnormal (%)	FISH abnormal (%)	20 (29)	5	16 (11)	5 (50)	9 (13)	2 (50)	3 (6)	3 (100)	4 (17)	8	30 (19)	7	2	3	23 (16)	4 (100)
Aneuploid	Aneuploid	19	3	15	3	9	1	1	1	7	1	34	2	2	3	23	4
Nullisomy XY	Monosomy XY	1	0	0	0	0	0	1	0	0	0	2	0	0	1	1	0
Disomy XY	Trisomy XY	0	1	1	1	1	1	0	0	0	1	3	0	0	0	0	1
Nullisomy 13	Monosomy 13	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Disomy 13	Trisomy 13	2	0	3	0	1	0	0	0	2	0	2	0	0	0	0	0
Nullisomy 15	Monosomy 15	3	1	2	1	1	0	0	0	0	0	3	0	0	1	2	0
Disomy 15	Trisomy 15	3	0	1	0	1	1	0	1	0	0	0	1	0	0	5	1
Nullisomy 16	Monosomy 16	0	1	0	0	0	0	0	1	1	0	3	1	1	0	7	1
Disomy 16	Trisomy 16	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Nullisomy 17	Monosomy 17	3	NA	7	NA	0	NA	0	NA	0	0	NA	NA	1	NA	1	NA
Disomy 17	Trisomy 17	1	NA	0	NA	0	NA	0	NA	1	0	NA	NA	0	NA	0	NA
Nullisomy 18	Monosomy 18	0	0	0	1	0	0	0	0	0	0	3	1	0	0	1	1
Disomy 18	Trisomy 18	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Nullisomy 21	Monosomy 21	0	1	0	0	1	0	0	0	1	0	5	0	0	0	2	0
Disomy 21	Trisomy 21	3	0	0	1	3	0	0	0	0	0	3	0	0	0	4	0
Nullisomy 22	Monosomy 22	0	0	1	0	0	0	0	0	0	0	6	0	0	0	3	0
Disomy 22	Trisomy 22	2	0	2	0	0	0	0	0	2	1	3	0	0	0	0	0
Null	Haploid	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Diploid	Polyploid	1	0	2	1	0	0	2	0	0	0	1	0	0	0	0	0
Complex abnormality	Complex abnormality	0	2	0	1	0	1	0	2	0	6	0	5	0	0	0	0

Double aneuploidies are counted as single event.
NA = not analysed.

The study of aneuploidy in sperm from normozoospermic patients has demonstrated a mean autosomal disomy rate of 0.13%; this value for the gonosomes is higher, corresponding to 0.37% (Egozcue *et al.*, 2003). As a result, the total disomy frequency in a normal ejaculate should be ~3%, with total aneuploidy corresponding to 6%. This theoretical estimation is based on the assumption that each sperm cell only presents one aneuploid event, and that all chromosomes have the same rate of variation. Nevertheless, some chromosomes, such as chromosome 21 and the gonosomes, have a higher tendency to non-disjunction compared to the others (Blanco *et al.*, 1996; Martin *et al.*, 1996; Vegetti *et al.*, 2000; Rubio *et al.*, 2001; Carrell *et al.*, 2003; Egozcue *et al.*, 2003). Although this finding could represent a factor of reproductive risk, its clinical implications are considered almost irrelevant when compared to the maternal contribution to aneuploidy, as trisomic pregnancies, in 90% of cases, are maternal in origin (Koehler *et al.*, 1996). In this respect, it has been postulated that trisomies of paternal origin could have a lower survival potential compared to those generated during oogenesis, yielding an underestimation of their frequency at implantation (Hall, 1990). These data derive from the analysis of abortuses in the general population to which conceptuses generated by assisted reproduction give a small numerical contribution. Therefore, the situation could be different in cases of severe male factor infertility, including azoospermia, whose treatment by ICSI completely bypasses any mechanism of natural sperm selection.

There is increasing evidence that the outcome of ICSI is closely and positively related to the quality of spermatogenesis as well as to the degree of male gamete haploidy (Aboulghar *et al.*, 1997; De Croo *et al.*, 2000; Escudero *et al.*, 2003). One of the major problems in sperm FISH studies is represented by the low amount of available DNA (Harkonen *et al.*, 2001), for which a solution could be represented by the protocol described in this study. It has been suggested that sperm aneuploidies are provoked by an alteration of the meiotic spindle (Egozcue *et al.*, 2000) caused by a modification of the intracellular redox potential (Zini *et al.*, 2001). Estimating the degree of sperm aneuploidy associated with severe male factor infertility could be particularly interesting in view of the availability of therapies able to improve sperm count and euploidy by restoring the physiological sperm redox potential in idiopathic infertile males (Wong *et al.*, 2002; Cavallini *et al.*, 2004). As the chromosomal defects in gametes can be transmitted to the resulting embryos, an attempt was made to relate the FISH results generated by sperm analysis to those obtained on preimplantation embryos (Table VI). Although a higher number of cases will be necessary to draw valuable conclusions by controlling the dominant effect of female age, this approach seems to be promising in indicating correlations between sperm aneuploidy and the resulting embryos.

In conclusion, preimplantation genetic diagnosis for aneuploidy has been reported to have a prognostic role on subsequent attempts at assisted reproduction (Ferraretti *et al.*, 2004). For sperm, a threshold in the aneuploidy frequency could be proposed which may enable estimation of the reproductive possibilities for each infertile couple. This strategy, in the framework of an adequate therapeutic programme, represents the most advantageous situation in patients' management.

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