Journal of Andrology, Vol. 22, No. 5, September/October 2001

Does Sperm Morphology Play a Significant Role in Increased Sex Chromosomal Disomy? A Comparison Between Patients With Teratozoospermia and OAT by FISH

L. A. GOLE, P. F. WONG, P. L. NG, X. Q. WANG, S. C. NG, AND A. BONGSO

From the Department of Obstetrics and Gynecology, National University Hospital, Singapore.

ABSTRACT: Eight semen samples from men with teratozoospermia (T), along with samples from 3 men with normal fertility and 5 samples from men with oligoasthenoteratozoospermia (OAT) were analyzed for X and Y chromosomal anomalies with the use of fluorescently labeled centromeric probes. This study was undertaken to determine whether patients with abnormal sperm morphology (teratozoospermia) have increased or decreased incidence of a sex chromosomal anomaly. The sex chromosome disomy for the T samples was 0.36% and for the OAT group it was 0.61%, compared with baseline value for the normozoospermia group (0.09%).

Key words: Sperm morphology, sex chromosomal aneuploidy, fluorescence in-situ hybridization.

J Androl 2001;22:759–763

With the introduction of intracytoplasmic sperm injection (ICSI) procedures, abnormal parameters of spermatozoa are no longer a hindrance to artificial reproductive technologies. This procedure bypasses all natural sperm selection processes that may be weeding out abnormal sperm and hindering them from fertilizing an egg. At present, even in the absence of a spermatozoan, the injection of a round spermatid into an oocyte can result in a viable embryo (Heidenreich et al, 2000). This leads to a concern of increased chromosomal anomalies in babies conceived by ICSI. Many studies have been undertaken to determine aneuploidy in spermatozoa from patients with oligoasthenoteratozoospermia (OAT) undergoing ICSI. Most show that the incidence of major chromosomal abnormalities is significantly higher in these patients. There is an ongoing debate about the influence of sperm morphology on male infertility. In vitro fertilization (IVF) results emphasize that when sperm morphology is evaluated using strict criteria, this parameter is believed to have an excellent predictive value and shows a significant positive correlation with successful fertilization. With this aspect in mind, we have selected samples from men with teratozoospermia (T) and tested them for sex chromosomal anomalies. Because these samples are difficult to obtain, cryopreserved samples were used in a couple of instances. To rule out any false positive results arising from these procedures, normal semen samples were cryopreserved and tested prior to the use of test samples.

This project was undertaken with funds provided by the National Medical Research Council (aided project NMRC3690025).

Correspondence to: Dr Leena A. Gole, Department of Obstetrics and Gynecology, Faculty of Medicine, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074 (e-mail: obggolel@nus.edu.sg).

Received for publication May 22, 2000; accepted for publication February 26, 2001.

Materials and Methods

Selection Criteria for Semen Samples

Semen samples were collected from adult men undergoing standard semen analysis in the andrology laboratory at National University Hospital. The following criteria for abnormal semen were used in this study: OAT was defined as fewer than 10×10^6 spermatozoa/mL, 20% or less were motile, and 15% or less of spermatozoa had normal morphology. The T group was defined as less than 10% of spermatozoa having normal forms, whereas the density and motility parameters were within normal ranges. The criteria for the normozoospermia (N) group included a sperm density of more than 20×10^6/mL and a vitality of >75%. Motility was considered normal if >50% showed forward progression or >25% showed rapid progression. More than 30% were required to show normal morphology (World Health Organization, 1992).

Freezing and Thawing Procedure

Three semen samples of normal parameters (group N) were used as controls for the effects of cryopreservation. A 1-μL aliquot of each of these semen samples was cryopreserved (group 1-FT/N [ie, frozen-thawed/normal in Table 1]) for 24 hours to test the efficiency of the fluorescence in situ hybridization (FISH) technique on prefrozen sperm (Chernos et al, 1989). In short, 1 mL of sperm sample was mixed with 0.7 mL of Spermfreeze medium (Fertipro, Beernem, Belgium) at room temperature. The sample was allowed to stand for 10 minutes and was then loaded into cryotubes. The cryotubes were then kept just above liquid

Table 1. *Incidence of chromosome X and Y disomy in 13 patients as determined by fluorescence in situ hybridization*

Patient	X	Y	No Label	XX	YY	XY	Total	ABN (%)	HYB (%)	X:Y
Column	A	B	C	D	E	F	G(ΣA-F)	100*(D+E+F)/A+B	100*C/A+B	A/B
Normal—Group 1										
N1	1720	1452	113	3	1	3	3292	0.22	96.57	1.18
FT-N1	1845	1572	103	0	1	1	3522	0.06	97.07	1.17
N2	1906	1858	132	1	0	1	3898	0.05	96.61	1.03
FT-N2	2058	2000	125	0	0	0	4183	0.00	97.01	1.03
N3	1214	1196	72	0	1	1	2484	0.08	97.10	1.02
FT-N3	1613	1515	57	1	1	3	3190	0.16	98.21	1.06
Total	10 356	9593	602	5	4	9	20 569	0.09	97.09	1.08
	X + Y = 19 949			XX + XY + YY = 18			AV = 3428	Σ(A+B+D+E+F) = 19 967		
Teratozoospermia—Group 2										
T1	1898	1749	65	1	0	3	3716	0.11	98.25	1.09
T2	1993	1735	26	2	0	0	3756	0.05	99.31	1.15
T3	1952	1672	147	9	3	29	3812	1.13	96.14	1.17
FT-T4	1880	1799	96	2	1	3	3781	0.16	97.46	1.05
FT-T5	1729	1699	78	1	0	2	3509	0.09	97.78	1.02
FT-T6	1760	1671	62	1	0	7	3501	0.23	98.23	1.05
FT-T7	1878	1857	47	2	0	2	3786	0.11	98.76	1.01
T-8	2193	2181	88	2	4	34	4502	0.91	98.05	1.01
Total	15 283	14 363	609	20	8	80	30 363	0.36	98.00	1.07
	X + Y = 29 646			XX + XY + YY = 108			AV = 3795	Σ(A+B+D+E+F) = 29 754		
Oligoazooteratozoospermia—Group 3										
O1	1587	1506	108	3	0	0	3204	0.10	96.63	1.05
O2	2086	1753	53	1	0	5	3898	0.16	98.64	1.19
O3	2015	1839	63	0	1	8	3926	0.23	98.40	1.10
O4	745	693	29	6	1	0	1474	0.48	98.03	1.08
O5	1067	916	41	1	1	60	2086	3.03	98.05	1.01
Total	7500	6707	253	11	3	73	14 547	0.61	97.95	1.08
	X + Y = 14 207			XX + XY + YY = 87			AV = 2909	Σ(A+B+D+E+F) = 14 294		

N indicates fresh normozoospermic; T, teratozoospermia; OAT, oligoasthenoteratozoospermia; FT, frozen-thawed; 0 = no signal; ABN, Total sperm cells disomic for chromosomes X and Y; HYB, hybridization efficiency.

nitrogen for 15 minutes before being plunged into the liquid nitrogen in the freezing Dewar flask.

Treatment of Sperm for Fluorescence In Situ Hybridization

Five semen samples that met the predefined criterion of OAT and 8 semen samples in the T group were selected for FISH evaluation after routine analysis at the andrology laboratory of our hospital.

The semen samples were prewashed with phosphate-buffered saline (PBS) before being incubated in 280 mOsmol/kg (optimum in vitro osmolarity) of Earles balanced salt solution (EBSS; Gibco BRL, Gaithersburg, Md) medium in a 37°C water bath. To this, 10 mM dithiothreitol (DTT) was added and this was then incubated for an additional 30 minutes for the sperm decondensation action of DTT.

The sperm suspension was centrifuged at 1200 × *g* for 5 minutes, the resultant sperm pellet was washed twice with PBS, and then centrifuged again at 1200 × *g* for 5 minutes. The final sperm pellet was fixed in a freshly made 3:1 methanol:acetic acid fixative. Two slides were prepared for each treated sperm. One slide was used for acridine orange staining to evaluate the decondensation sufficiency (Tejada et al, 1984). Decondensation was considered to have occurred when all the sperm heads fluoresced orange or red under the green, single-bandpass filter (Vysis set 30-150291; Downers Grove, Ill). The spermatozoa in which chromatin had not decondensed fluoresced green. The second slide was used for FISH evaluation.

Fluorescence In Situ Hybridization

Vysis centromeric probes for chromosomes X, Y, and 18 were used. Slides with the fixed sperm suspension were denatured for 5 minutes in 70% formamide/2× saline-sodium citrate (SSC) at 74°C. After overnight hybridization, a stringent wash of 0.4× SSC/0.3% NP-40 for 1–3 seconds was followed by 2× SSC/0.1% NP-40 for 1 minute at room temperature. DAPI (4′,6′-diamidino-2-phenylindole) was used as a counterstain and the slide was viewed under a fluorescence microscope with appropriate filters. Applied Imaging software (Applied Imaging, Newcastle Upon Tyne, United Kingdom) was used for image capture and analysis (see Figure 1). An approximate total of 3000 spermatozoa were scored per slide. Each slide was viewed by 2 operators. Signals were counted as separate only if they were apart from each other by a distance equal to or more than the diameter of a single signal.

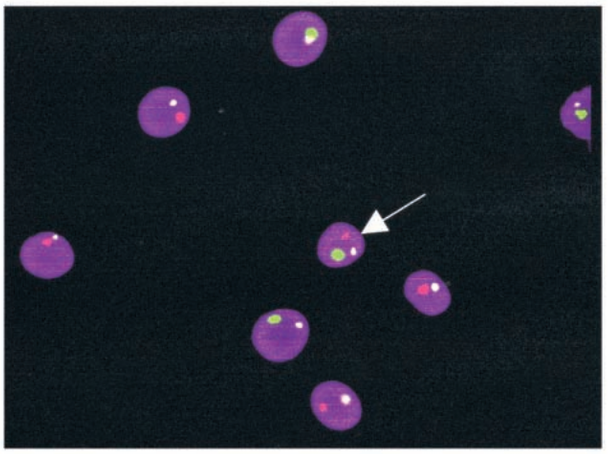

Figure 1. FISH on spermatozoa showing a signal each for chromosome X (spectrum green) and Y (spectrum orange), along with the internal control using chromosome 18 (spectrum aqua). The sperm in the center show sex chromosomal disomy.

Table 2. *Results of semen analysis of patients*

		Semen analysis		
Group	Patient code	Concentration	Motility	Morphology
N	N1	71.3	59	40
N	N2	50	61	42
N	N3	80.3	40.6	38
OAT	O1	0.6	0	19
OAT	O2	0.7	29	13
OAT	O3	1.6	6	19
OAT	O4	13.5	0	14
OAT	O5	1.7	18	5
T	T1	60.3	49	7
T	T2	79.7	40	4
T	T3	27.5	45	5
T	T4	27	50	5
T	T5	33	85	6
T	T6	73	56	10
T	T7	62	48	7
T	T8	62	41	10

N indicates normozoospermia; OAT, oligoasthenoteratozoospermia; T, teratozoospermia.

Statistical Analysis

Using the SPSS 8.0 statistical package for Windows (Chicago, Ill), the Pearson chi-square test for heterogeneity was applied on paired total scores of normal and abnormal sex chromosomal constitutions. A statistically significant difference was observed between the normal, OAT, and T groups, as well as between the T and OAT groups ($P < .001$).

Results

FISH was performed on 8 T semen samples, 4 of which were thawed from cryopreserved semen samples. Five OAT and 3 normal sperm samples were included in this study. The 3 normal samples were analyzed for FISH signals before and after freezing to evaluate the effects of the freezing and thawing procedures. No differences were observed in signal intensity, labeling efficiency, or signal splitting between the cryopreserved and fresh samples (Table 1, group 1). Table 2 shows the semen parameters of all patients in this study. Table 1 summarizes the frequencies of abnormal sex chromosome constitution (XX, YY, and XY) among the groups of normozoospermia (N; control), T, and OAT. Chromosome 18 was not analyzed because the hybridization efficiency of the probe was not as good as the X and Y (between 96% and 100%), at only 87%. Hence, it was used only as an internal control to confirm sex chromosomal disomies.

A total of 20 569, 30 363, and 14 547 cells were scored for groups N, T, and OAT, respectively, with an average of 3428, 3795, and 2909 cells scored per patient within each group, respectively. The average hybridization rates were 97%, 98%, and 97.9%, and the sex chromosome disomy rates for groups N, T, and OAT were 0.09%, 0.36%, and 0.61%, respectively.

Discussion

Infertile men, especially patients with OAT, are prime candidates for ICSI procedures in artificial reproductive technologies. Recent evidence suggests that these patients may be at an increased risk of transmitting numerical (predominantly sex chromosome) abnormalities to their offspring. In previous cytogenetic surveys of infertile men, an increased frequency of chromosomal abnormalities has been observed with sex chromosomal aneuploidies being more predominant (Rives et al, 1999). The incidence of major chromosome abnormality was significantly higher in patients with a sperm concentration of less than 5×10^6/mL, a total motile sperm count of less than 1×10^6/mL, raised serum follicle stimulating hormone and luteinizing hormone levels, low testosterone level, small testis volume, or a combination of these (Yoshida et al, 1997). The frequency of chromosomal abnormality is higher in men with azoospermia, approximately 15%, compared with 3% in men with oligozoospermia (Van Asche et al, 1996; Rivas et al, 1987). In the former group, sex chromosomal aneuploidies, especially 47,XXY, predominate. At most times, peripheral blood karyotypes appear to be normal, and men with oligospermia tend not to be routinely karyotyped. There is, however, a distinct possibility that very low levels of sex chromosomal mosaicism may go undetected (Peschka et al, 1999; Rives et al, 2000). On the other hand, despite having normal karyotypes, they may still have an elevated frequency of sex

Table 3. *Comparison with other studies for mean frequencies of percentage of disomy for sex chromosomes in sperm nuclei from fertile and infertile groups*

Study		XX	YY	XY
Bernardini et al, 1997	Fertile	0.15	0.25	0.45
	Infertile	0.36	0.27	0.7
Finkelstein et al, 1998	Fertile	0.06	0.06	0.1
	Infertile	very low	very low	0.9
Storeng et al, 1998	Fertile	0.03	0.24	0.47
	Infertile	0.28	0.28	0.9
Pang et al, 1999	Fertile			0.14
	Infertile	increased	increased	2.64
Rives et al, 2000	Fertile	0.22	0.17	0.35
	Infertile	0.21	0.24	0.54
Current	Fertile	0.02	0.019	0.04
	Infertile			
	(Teratozoospermia)	0.065	0.026	0.263
	(OAT)	0.075	0.02	0.501

OAT indicates oligoasthenoteratozoospermia.

chromosomal aneuploidy in their spermatozoa (Martin et al, 1996).

The FISH technique, which is both reliable and relatively easier to perform than karyotyping, has allowed comprehensive studies on numerical chromosome abnormalities in human spermatozoa (Downie et al, 1997; Lahdetie et al, 1997). A major problem with it, however, is availability of sperm samples for research studies from patients with sperm deficiencies. However, a small aliquot of a semen sample is usually frozen in most laboratories and use of this sample with the patient's consent is a good source of spermatozoa for analysis with FISH. We have used cryopreserved specimens in cases when fresh samples were not available. Studies carried out on normal sperm samples that were cryopreserved and then thawed showed that there was no difference in FISH signals, with reference to signal splitting or probe hybridization, when frozen-thawed samples have been used (Table 1, group 1).

Studies on chromosomal aneuploidies in spermatozoa between fertile and nonfertile men have been carried out by many groups (Table 3), showing definite differences in sex and autosomal aneuploidies between the fertile and nonfertile groups. Pang et al (1999) showed that a comparison of FISH labeling of 12 autosomes and the sex chromosomes of spermatozoa from 9 patients with OAT in an IVF program, with spermatozoa from 4 proven-fertile donors revealed significant differences. There was significant increase in total frequency of abnormal spermatozoa. Autosomal and sex chromosomal disomy was increased (0%–5.4% as opposed to 0.05%–0.2% for controls). Diploid spermatozoa were also increased (0.4%–9.6% as opposed to 0.04% for controls). Many previous studies on patients with OAT have revealed this higher incidence of aneuploidies (Bonduelle et al, 1998; McInnes et al, 1998; Moosani et al, 1995). However, few studies have evaluated chromosomal anomalies in only patients with T (ie, with a high percentage of morphologically abnormal spermatozoa but with normal count and motility). The ongoing debate about the influence of sperm morphology on male infertility is emphasized by IVF results, that when sperm morphology is evaluated using strict criteria, this parameter is believed to have an excellent predictive value and shows a significant positive correlation with successful fertilization (Green et al, 1999; Osawa et al, 1999; Szczygiel et al, 1999).

With this aspect in mind, those men with T and OAT in the infertile group were subdivided and analyzed for sex chromosomal aneuploidies. The rate of XY-bearing sperm was significantly higher in all 3 groups in our study. This suggests that nondisjunction at the first meoitic division is far greater than at the second meoitic division (Finkelstein et al, 1998; Storeng et al, 1998). Another observation was that the ratio of XX:YY-bearing sperm in the normal population is comparable, but in the infertile groups, the XX-bearing sperm were nearly 3 times greater than those in the YY group (see Table 3). Again, the frequencies of the YY sperm were constant in all 3 groups (~0.02%), whereas XX sperm were much higher in the infertile groups (~0.6%–0.7% compared with the N group, which was 0.02%). This would suggest that the X chromosome was more prone to disjunction in the second meiotic division than chromosome Y. Similar results were seen in studies by Bernadini (1997), Finkelstein (1998), and Storeng (1998). This is, however, in contrast to the studies of Rives et al (2000), in which YY disomy was increased as opposed to XX.

The total sex chromosome disomy (XX + YY + XY) for the N group was 0.09%, for the T group it was 0.36%, and for the OAT group it was 0.61%. However, XY disomic sperm are predominant in this study, with a percentage of 0.5% in the OAT group, which was considerably higher than the T group, which had 0.2% disomic sperm as opposed to 0.04% in the N group. The XY disomic frequencies are comparable to studies of Rives et al (2000) and only slightly

lower than those of Bernadini (1997), Finkelstein (1998), and Storeng et al (1998). The data obtained in this study suggest that the frequency of sex chromosome disomy for patients in both the T and OAT groups was statistically significantly different from that of the N group. The difference between the T and OAT groups is also statistically significant. However, interindividual differences within the infertile groups is also varied. A point to be noted is that patient 5, with a normal karyotype, had a very high percentage of morphologically abnormal sperm in addition to a low sperm concentration and motility compared with the other OAT patients in the same group, making it essential that more samples be further studied in both groups to come to any definite conclusions regarding evaluation of morphology as the most significant criteria for manifestations of chromosomal abnormalities.

A conclusion based on this study would be that the majority of sperm aneuploidies were due to nondisjunction at the first meiotic division than in the second, with the X chromosome being more prone to nondisjunction than the Y chromosome in meiosis II. Semen samples with all 3 characteristics of morphology, motility, and sperm count show a higher percentage of XY disomic spermatozoa compared with T patients. Clinically, studies with FISH on the spermatozoa of patients with low counts, reduced motility, or very abnormal morphology should be undertaken along with a routine peripheral karyotype of all potential ICSI fathers (Chandley et al, 1996). Routine peripheral blood karyotyping is often overlooked because it is usually normal; nonetheless, it is essential because it is quite possible that the increased frequency of sex chromosomal abnormalities in the gonads could be reflected as low-level sex chromosomal mosaicism in blood. Interphase FISH is an ideal method for scoring large numbers of cells for low level mosaicism, and this along with FISH on spermatozoa would be a good screen for infertile patients contemplating ICSI. This is because ICSI is the most significant therapeutic advancement in the treatment of male subfertility, and is being used in the treatment of a population of patients with a particularly higher risk of chromosomal aberrations.

References

Bernardini L, Martini E, Geraedts JPM, Hopman AHN, Lanteri S, Conte N, Capitanio GL. Comparison of gonosomal aneuploidy in spermatozoa of normal fertile men and those with severe male factor detected by in situ hybridisation. *Mol Hum Reprod.* 1997;3:431–438.

Bonduelle M, Aytoz A, Van Assche E, Devroey P, Liebaers I, Van Steirteghem A. Incidence of chromosomal aberrations in children born after assisted reproduction through intracytoplasmic sperm injection. *Hum Reprod.* 1998;13:781–782.

Chandley AC, Hargreave TB. Genetic anomaly and ICSI. *Hum Reprod.* 1996;11:930–932.

Chernos JE, Martin RH. A cytogenetic investigation of the effects of cryopreservation on human sperm. *Am J Hum Genet.* 1989;45:766–777.

Downie SE, Flaherty SP, Matthews CD. Detection of chromosomes and estimation of aneuploidy in human spermatozoa using fluorescence in situ hybridization. *Mol Hum Reprod.* 1997;3:585–598.

Green S, Fishel S. Morphology comparison of individually selected hyperactivated and non-hyperactivated human spermatozoa. *Hum Reprod.* 1999;14:123–130.

Finkelstein S, Mukamel V, Yavetz H, Paz G, Avivi L. Increased rate of non-disjunction in sex cells derived from low quality semen. *Hum Genet.* 1998;102:129–137.

Heidenreich A, Altmann P, Engelmann UH. Microsurgical vasovasostomy versus microsurgical epididymal sperm aspiration/testicular extraction of sperm combined with ICSI. A cost-benefit analysis. *Eur Urol.* 2000;37:609–614.

Lahdetie J, Saari N, Ajosenpaa-Saari M, Mykkanen J. Incidence of aneuploid spermatozoa among infertile men studied by multicolor fluorescence in situ hybridization. *Am J Med Genet.* 1997;71:115–121.

Martin RH. The risk of chromosomal abnormalities following ICSI. *Hum Reprod.* 1996;11:924–925.

McInnes B, Rademaker A, Greene CA, Ko E, Barclay L, Martin RH. Abnormalities for chromosomes 13 and 21 detected in spermatozoa from infertile men. *Hum Reprod.* 1998;13:2787–2790.

Moosani N, Cox DM, Pattinson HA, Rademake, AW, Carter MD, Martin RH. Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. *Fertil Steril.* 1995;64:811–817.

Osawa Y, Sueoka K, Iwata S, Shinohara M, Kobayashi N, Kuji N, Yoshimura Y. Assessment of the dominant abnormal form is useful for predicting the outcome of intracytoplasmic sperm injection in the case of severe teratozoospermia. *J Assist Reprod Genet.* 1999;16:436–442.

Pang MG, Hoegerman SF, Cuticchia AJ, Moon SY, Doncel GF, Acosta AA, Kearns WG. 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. *Hum Reprod.* 1999;14:1266–1273.

Peschka B, Leygraaf J, Van der Ven K, Montag M, Schartmann B, Schubert R, Van der Ven H, Schwanitz G. Types and frequency of chromosome aberrations in 781 couples undergoing intracytoplasmic sperm injection. *Hum Reprod.* 1999;14:2257–2263.

Rivas F, Garcia-Esquivel L, Diaz M, Rivera H, Cantu JM. Cytogenetic evaluation of 163 azoospermics. *J Genet Hum.* 1987;35:291–298.

Rives N, Joly G, Machy A, Simeon N, Leclerc P, Mace B. Assessment of sex chromosome aneuploidy in sperm nuclei from 47,XXY and 46,XY/47,XXY males: comparison with fertile and infertile males with normal karyotype. *Mol Hum Reprod.* 2000;6:107–112.

Rives N, Saint Clair A, Mazurier S, Sibert L, Simeon N, Joly G, Mace B. Relationship between clinical phenotype, semen parameters and aneuploidy frequency in sperm nuclei of 50 infertile males. *Hum Genet.* 1999;105:266–272.

Storeng RT, Plachot M, Theophile D, Mandelbaum J, Belaish-Allart J, Vekemans M. Incidence of sex chromosomal abnormalities in spermatozoa from patients entering an IVF or ICSI protocol. *Acta Obstet Gynecol Scand.* 1998;77:191–197.

Szczygiel M, Kurpisz M. Teratozoospermia and its effect on male fertility potential. *Andrologia.* 1999;31:63–75.

Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange fluorescence. *Fertil Steril.* 1984;42:87–91.

Van Assche E, Bonduelle M, Tournaye H, Joris H, Verheyen G, Devroey P, Van Steirteghem A, Liebaers I. Cytogenetics of infertile men. *Hum Reprod.* 1996;11(suppl 4):1–24.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* 3rd ed. Cambridge, United Kingdom: Cambridge University Press; 1993.

Yoshida A, Miura K, Shirai M. Cytogenetic survey of 1,007 infertile males. *Urol Int.* 1997;58:166–176.