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Concurrent use of flow cytometry and fluorescence in-situ hybridization techniques for detecting faulty meiosis in a human sperm sample

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Routine semen analysis in an infertile patient revealed severe teratospermia associated with malformation of head and tail in 100% of the sperm cells. Flow cytometry and fluorescence in-situ hybridization (FISH) were shown to supplement routine semen analysis by providing information on the sperm chromatin. Using flow cytometry, propidium iodide-stained spermatozoa from the same sperm sample were compared with a normal reference pool, and with human lymphocytes. The results point to a population of diploid sperm cells rather than to mature haploid spermatozoa. Numerical chromosomal abnormalities of the spermatozoa were subsequently evaluated using FISH. A total of 1000 sperm cells were scored for X and Y chromosomes, and an additional 1128 sperm cells for chromosome 18. Aneuploidy of chromosomes X and Y was revealed in 96.9% of the cells and of chromosome 18 in 90.3% of the cells. Non-disjunction of chromosome X and Y in meiosis I and II occurred in 54.8 and 2.7% of the sperm cells respectively. Non-disjunction in both meiosis I and II occurred in 39.4% of the sperm cells. A normal haploid pattern for chromosomes X and Y was observed in only 3.1%, and for chromosome 18 in 9.7%, of the cells. Using three colour FISH for the sex chromosomes and for chromosome 18, diploidy was demonstrated in 19.4% of 500 sperm cells and aneuploidy in virtually all sperm cells (99.2%). The use of flow cytometry and FISH in cases where genetic and developmental chromatin abnormalities are suspected is a valuable adjunct to other available techniques, and can guide the clinicians to decide which samples are unsuitable for intracytoplasmic injection.

Key words: flow cytometry/fluorescence in-situ hybridization/human spermatozoa/non-disjunction

Introduction

Spermiogram data is used routinely in the evaluation of the sperm sample quality. Conventional criteria of semen analysis, however, do not reflect sperm fertility potential, or sperm karyotype. Several reports, based on the comparison between cytogenetic parameters and the percentage of abnormal sperm morphology, indicate that there is no positive correlation between these parameters, suggesting that assessment of sperm morphology cannot be used as an indicator of chromosomal damage in human spermatozoa (Martin and Rademaker, 1988; Rosenbusch *et al.*, 1992).

Molecular biology techniques are now available for evaluating the chromosomal set. Among them, fluorescence in-situ hybridization (FISH) has been successfully applied to human interphase spermatozoa in order to establish aneuploidy rates (Han *et al.*, 1993; Martin *et al.*, 1993, 1996). Hybridization with several probes can distinguish between haploid cells, hyperhaploid cells (24 chromosomes) and diploid cells (46 chromosomes). Thus, the FISH technique provides an alternative approach to conventional cytogenetics methodology in which golden hamster oocytes, fertilized *in vitro* with human sperm cells, are used for chromosome analysis (Rudak *et al.*, 1978; Spriggs *et al.*, 1996). Use of specific DNA probes enables detection of aneuploidy in interphase sperm cells (Guttenbach and Schmid, 1990; Guttenbach and Schmid, 1991; Martin *et al.*, 1992; Holmes and Martin, 1993).

In this study, the sperm cells of an infertile patient with severe teratozoospermia and infertility who failed two in-vitro fertilization(IVF) treatment cycles by intracytoplasmic sperm injection (ICSI), were studied using sensitive, accurate and objective techniques, which revealed a series of sperm characteristics. The methods included chromosome evaluation by FISH, and assessment of sperm chromatin properties by flow cytometry (FC) using Acridine Orange (AO) and propidium iodide (PI) (Evenson *et al.*, 1986; Golan *et al.*, 1997).

Materials and methods

The patient attending our infertility clinic had no history of any physical illness or long-term medication affecting fertility, nor did he appear to have been exposed to environmental or occupational mutagens. He was 30 years old at the time, had been married for 4 years, had normal male characteristics and his karyotype determined from lymphocyte culture was 46XY. The hormonal concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin and testosterone were measured and found to be within the normal range. The hormones were measured by radioimmunoassay (Diagnostic Product Corporation, Los Angeles, CA, USA). The intra- and interassay coefficients of variation

of the radioimmunoassay were 6.5 and 8.4% respectively for FSH, and 7 and 8.5% respectively for LH. Intra- and interassay coefficients of variation for testosterone were 9 and 12% respectively. Four semen analyses were performed within a period of 16 months (4/1995–8/1996) according to World Health Organization criteria (WHO, 1992). The couple was referred for IVF due to long-standing infertility. Examination of the 28 year old woman was normal, except for the finding of bilateral tubal occlusion. The couple underwent two IVF treatments using our standard protocol of midluteal administration of gonadotrophin-releasing hormone (GnRH) analogue (Decapeptil; Ferring, Mälmö, Sweden) followed by ovarian stimulation with human menopausal gonadotrophin (HMG, Pergonal; Teva, Petach Tikva, Israel). A total of 14 metaphase II oocytes were treated by ICSI in both cycles. During sperm selection for ICSI, it was impossible to select spermatozoa with normal morphology, eventually a single-tailed spermatozoon, with head size suitable for the ICSI pipette (Cook, Queensland, Australia; i.e. <10 μm in diameter), were selected for injection. After 18 h, out of the 12 surviving oocytes, two developed into two-pronucleated zygotes (2PN), one developed into a single-pronucleated zygote (IPN) and two developed three pronuclei (3PN). By 48 h after ICSI, only the IPN zygote became a 2-cell embryo with $>25\%$ fragmentation, while the others remained arrested at the 1-cell stage. The 2PN and IPN embryos were transferred to the uterus during the first treatment cycle, but failed to implant.

The method for AO staining and FC of spermatozoa followed the two-step procedure of Evenson *et al.* (1986, 1990), and Golan *et al.* (1997). The procedure for staining spermatozoa with PI was as follows: spermatozoa which had been frozen in TNE (tris-hydroxymethyl-amino methane 0.01 M, NaCl 0.15 M, EDTA 1 mM pH 7.4)–glycerol were thawed, centrifuged, and resuspended in ice-cold TNE to ~10^6 spermatozoa/ml; PI (to 10 μg/ml final concentration) was added. After 10 min, the samples were analysed for red fluorescence by FC (BP 630 filter), and WINMDI data processing program (J.Trotter @ http://facs.scripps.edu) was used to produce histograms showing haploid and diploid regions.

FISH was carried out to determine the percentage of aneuploidy. Three colour FISH was used for the evaluation of diploid and hyperhaploid sperm cells, while two colour FISH analysis of the sex chromosomes enabled us to distinguish between non-disjunction in meiosis I and II and between three types of sex chromosomes disomies XY, XX and YY. One colour FISH was carried out for chromosome 18.

Using the three colour FISH only 500 sperm cells were scored because of the difficulty of distinguishing between a multitude of complex signals. However, 1000 and 1128 cells were evaluated for the two and one colour FISH respectively. The very high occurrence of aneuploidy allowed conclusions to be drawn in spite of the relatively small number of sperm cells, which is lower than the usual number used in aneuploidy analysis.

For the FISH procedure, the sperm suspension (0.5–1 ml) was washed 3–5 times in fresh methanol–glacial acetic acid (3:1 v/v) fixative by centrifugation (10 min at 500 *g*). Sperm suspension was then dropped onto clean microscopic slides and air-dried. To render the sperm chromatin accessible to DNA probes, slides were resuspended in dithiothreitol 10 mM (Sigma, St Louis, MO, USA) in 0.05 M Tris for 30 min at room temperature. The decondensation was checked under a phase-contrast microscope. Slides were incubated in 2× sodium chloride/sodium citrate (SSC) for 0.5 h, and washed for 2 min in a 70, 80 and 100% ethanol series.

For the three colour FISH, the X chromosome probe was labelled with green fluorochrome, the Y chromosome probe was labelled with orange fluorochrome, and the 18 chromosome probe was labelled with aqua fluorochrome. For the two colour FISH the X chromosome probe was labelled with green fluorochrome, and the Y chromosome probe was labelled with orange fluorochrome. For the one colour FISH the 18 chromosome was labelled with orange fluorochrome. The probes were purchased commercially (Vysis, Naperville, IL, USA). The hybridization target for chromosomes X and 18 was α-satellite repeat clusters in the centromeric region, and that for chromosome Y was satellite III DNA at YqIII.

FISH was performed according to the manufacturer's protocols for direct labelled probes. After hybridization slides were mounted with 10 μl of DAPI counterstain. Semen samples from normal donors of proven fertility were obtained from our sperm bank and were used as controls for all FISH analysis. Each case was analysed by two or three independent observers. Cells were scored for aneuploidy but not for nullisomy, since failure to detect a signal could be due to technical difficulties as well as non-disjunction.

Fluorescent microscopy was performed with a Zeiss Axiscope Microscope (Zeiss, Germany). A triple filter set (Vysis) for simultaneous detection of DAPI, Spectrum Orange and Spectrum Green were used to visualize red and green signals. A special aqua filter (Vysis) was used to visualize the blue-aqua signal. Several images were processed using Cytovision-automated system (Applied Imaging, Santa Clara, CA, USA). Each slide was scored by at least two observers.

Preliminary testing of metaphase preparations and FISH procedure with same probes, on lymphocytes derived from the patient showed no cross-hybridization for any of the probes. Peripheral blood from our patient and controls were processed by standard procedures to yield preparations for cytogenetic analysis and FISH procedure.

In the three colour FISH, a spermatozoon was considered to be disomic when two fluorescent signals were identified for either the sex chromosomes or the autosomes but not for both. A cell was scored as diploid if two signals were identified for both the sex chromosomes and the autosome (Griffin *et al.*, 1996). In the two and one colour FISH, a spermatozoon was considered to be disomic when two fluorescent domains of the same colour were clearly positioned within the sperm head comparable in brightness and size and at least one domain apart (Spriggs *et al.*1995; Martin *et al.*, 1995).

Results

The patients' semen was subjected to standard semen analysis according to WHO criteria. The mean ± SD results of semen analysis were as follows: volume: 1.62 ± 1.02 ml; sperm concentration: 10.3 ± 2.3× 10^6/ml; percentage motile cells: 23.7 ± 6.5%; and percentage normal forms: 0%. Hormonal concentrations for FSH, LH, testosterone and prolactin were 4.4 mIU/ml, 4.6 mIU/ml, 5 ng/ml and 5.3 ng/ml respectively, which is within normal range.

Teratozoospermia was found in repeated semen analysis. The majority of spermatozoa exhibited a combined malformation of head and tail; (Figure 1a,b). About 30% of the cells in the ejaculate were headless spermatozoa. The rest of the cells were of abnormal head structure: large, round, with or without acrosome. The abnormalities in tail structure could be identified by light microscopic investigation and included split or multiple tails.

AO staining FC showed that spermatozoa from the test sample exhibited extremely high red and green fluorescence values compared with those from a pooled normal sperm sample (Figure 2). Less than 1% of spermatozoa from the patients' semen fell in the zone characteristic of normal mature spermatozoa. Peaks of red fluorescence corresponding to

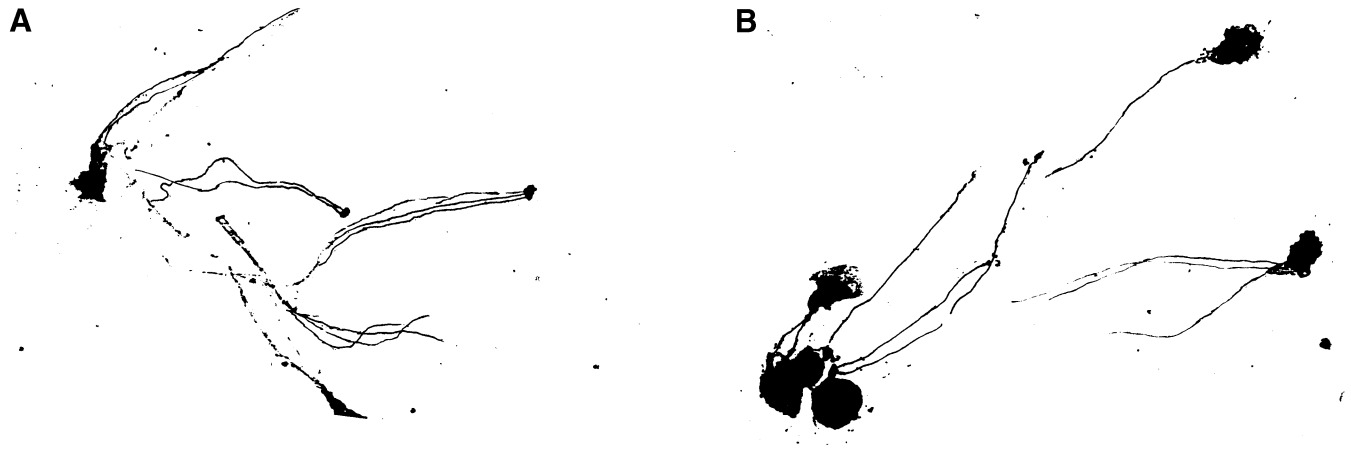

Figure 1. Light microscopy of (**a**) headless spermatozoa of the patient's sample with split and multiple tails, (**b**) large spermatozoa with abnormal head structure, missing and malformed acrosome. Some of the cells exhibit multiple tails (magnification ×1000).

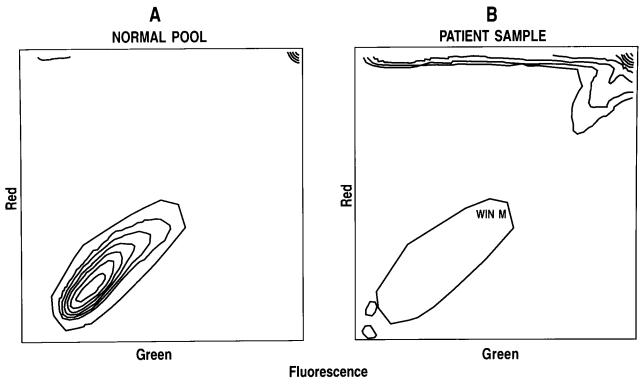

Figure 2. A scattergram of Acridine Orange-stained spermatozoa from (**a**) normal reference semen pool and (**b**) from patient sample. The samples were stained with Acridine Orange and analysed by flow cytometry.

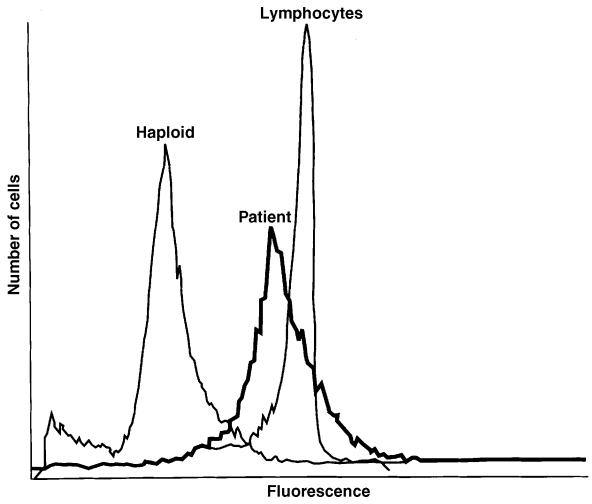

Figure 3. An overlay of histograms showing the red fluorescence intensities of spermatozoa from the reference pool (haploid), from human lymphocytes (diploid), and from the patient's spermatozoa (Patient). The sample was stained with propidium iodide and analysed by flow cytometry as described in the Material and methods section. The *x* axis represents fluorescence intensity values (in arbitrary units), and the *y* axis, the number of cells.

haploid and diploid cells were seen in histograms after PI staining FC (Figure 3). The staining zone of spermatozoa from the patient fell in the diploid region, with a fluorescence peak slightly lower than that of human lymphocytes.

In the two and one colour FISH, results were obtained by scoring separately 1000 sperm cells for X and Y chromosomes, and 1128 for chromosome 18. Aneuploidy frequency of chromosomes X and Y was revealed in 96.9% of the cells, and aneuploidy of chromosome 18 in 90.3%. Non-disjunction for chromosomes X and Y in meiosis I occurred in 54.8% of the cells, and in meiosis II in 2.7% of the cells. Only 3.1% of the cells had a normal pattern for the X and Y chromosomes (Table I). Disomy for chromosome 18 was observed in 45.6%, trisomy in 38.5%, and tetrasomy in 6.2% of the sperm cells. Only 9.7% of the cells had a normal monosomy pattern for the 18 chromosome (Table II).

As an additional control, aneuploidy in the patient's lymphocytes was determined by one and two colour FISH, using the same probes. Of the cells, 98.7% had X and Y signals in each cell, 0.43% had XXY signals and 0.87% had only X signal. In the one colour FISH, 93.3% of the lymphocytes cells had two 18 signals, 5.1% had only one signal and 1.6% of the cells had three 18 signals.

The results of three colour FISH in our patient's sperm cells indicate the virtual absence of normal haploid cells. Only 0.8% of the cells showed a normal X, Y and 18 chromosome complement. The rate of aneuploidy was 99.2% (Table III,

Table I. The occurrence of sex chromosome signals in sperm cells as pertaining to the case sample and normal controls

	Non-disjunction		Percentage sperm cells	
Chromosome signals	Meiosis I	Meiosis II	Patient	Controls (range)
X			1.9	47.2–52.6
Y			1.2	46.2–52.1
XY	+		54.8	0–0.5
XXYY	+	+	7.2	–
XXY	+	+	22.5	–
XYY	+	+	9.7	–
XX		+	2.6	0–0.2
YY		+	0.1	0–0.2

Table II. The frequency of chromosome 18 in 1128 sperm cells of case sample and normal control

	Patients (%) (n = 1128)	Controls (%) (n = 501; 513; 533) (range)
Monosomy	9.7	99.8–100
Disomy	45.6	0–0.2
Trisomy	38.5	0
Tetrasomy	6.2	0

Table III. Three colour fluorescence in-situ hybridization (FISH) for sex chromosomes and chromosome 18 in 500 sperm cells of patient and normal controls

Cells with X,Y and 18 signals	Patient (%)	Four controls (range)
X	0.4	0.19–0.57
Y	–	0.11–0.86
18	–	0–0.34
18,X	0.4	45.72–50.69
18,Y	0.4	47.07–52.28
18,XY	8.0	0.19–0.91
18,XX	0.8	0.51–0.9
18,18,XY	19.4	–
18,18,XXY	11.2	–
18,18,XXYY	14.2	–
18,18,18,XXY	10.2	–
XXYY	0.2	–
XY	0.6	–
Others[a]	34.2	–

[a]Others: 18XXY;18XYY;18XXYY;18,18,X; 18,18,XY;18,18,XYY; 18,18,18,XX; 18,18,18,XY; 18,18,18,XYY; 18,18,18,XYYY; 18,18,18,XXYY; 18,18,18,XXXYY; 18,18,18,XXYYY; 18,18,18,18,XY; 18,18,18,18,XXY;18,18,18,18,XXYY; 18,18,18,18.

Figure 4). Non-disjunction for chromosomes X and Y in both meiosis I and II occurred in 94.8% of the cells. The percentage of aneuploidy in sperm cells of our control fertile donors agrees with published data (Table III).

Discussion

Spermatogenesis consists of three phases: (i) multiplication of spermatogonia and production of primary spermatocytes; (ii) meiosis of spermatocytes to produce spermatids; and (iii) differentiation of spermatids to final spermatozoa. Faults in any of these processes will cause a decrease in sperm numbers or production of abnormal spermatozoa. In the case reported here, sperm numbers were lower in comparison with normal values (WHO, 1992). Meiosis was disturbed, as shown by FC and by FISH, and sperm morphology was abnormal both with regard to sperm heads and to tails, indicating defective spermatid development. Each of these phenomena will be discussed separately. Multiplication of spermatogonia is dependent upon hormonal stimulation by testosterone and by FSH. In the present case, these hormone values were normal. Likewise, the level of prolactin, which may adversely affect testicular function, was normal.

Meiosis occurs at the inner layer of the adluminal compartment of the germinal epithelium, under regulation of the Sertoli cells. In the present case meiosis was severely defective. FC of cells stained with PI or AO was used to determine ploidy and chromatin status of the spermatozoa. These dyes bind to DNA to produce fluorescence which was measured by the flow cytometer. The results clearly showed that the sperm cells were, in the main, diploid rather than haploid. The staining zone of spermatozoa from the patient fell in the diploid region, with a fluorescence peak, slightly lower than that of human lymphocytes suggesting that sperm DNA was bound to protamine rather than histone (Evenson 1986, 1990). Earlier, Holstein and Eckmann (1986a,b) and Holstein *et al.* (1988) reported that diploid spermatozoa originated from multinuclear primary spermatocytes and spermatids. Most binucleate spermatids degenerate in testicular tubules during spermatogenesis. However, some may develop to binucleate sperm cells, and reach the ejaculate. These reports were based on electron microscopic (EM) studies, and the authors point out that such cases are rare in young men and occur without any apparent cause. In spite of some efforts, we were not able to detect the presence of two nuclei using light microscopy in our study. In men aged >65 years this effect is more frequent, and is attributed to rest of spermatogenic activity in the germinal epithelium. Griffin *et al.* (1995) supported this observation by direct evidence of meiotic non-disjunction in the elderly human male. The authors concluded that the defect in meiosis observed in their cases occurred at the level of primary spermatocytes in zygotene or pachytene stage. In the present case, the meiotic process was essentially not operative. For further investigation, the sperm samples were subjected to FISH using probes for the three chromosomes X, Y, and 18. This allows for a more detailed understanding of the meiotic process than was possible by the EM procedures of Holstein and Eckmann.

In order to detect three types of sex chromosomes disomies (XX,YY and XY) two colour FISH study was applied (Goldman *et al.*, 1993; Han *et al.*,1993; Chevert *et al.*, 1995). The development of multicolour FISH permits the use of three probes concomitantly , thus enabling further distinction between disomy and diploidy in this interesting case.

Table I shows non-disjunction of sex chromosomes in the sperm cells from our patient using two colour FISH (in 96.9% of the cells), compared with 0–0.5% in the sperm cells derived from normal donors. In those sperm cells non-disjunction occurred during the first meiosis and continued through the second meiosis. Griffin *et al.* (1995) reported that meiosis I (XY) sex chromosome disomy was the most common disomy and occurred in 0.18% of the spermatozoa, meiosis II (YY or XX) sex chromosomes disomy was 0.03 and 0.02% respectively and disomy of chromosome 18 accounted 0.04%.

According to published data, frequencies of disomic chromosomes of sperm cells in normal people range between 0.07–0.18% for different combination of sex chromosomes totalling 0.42% (Spriggs *et al.*, 1995; Martin *et al.*, 1996). Our control results are similar to reported data but the patient's results with the two and three colour FISH point to essentially top frequency of aneuploidy (96.9 and 99.2% respectively).

Recently, Yurov *et al.* (1996) and In't Veld *et al.* (1997), described similar cases in which all the spermatozoa were chromosomally abnormal. Bernardini *et al.* (1997) compared the frequency of sex chromosome numerical abnormalities in sperm samples of normal men, men with unexplained infertility

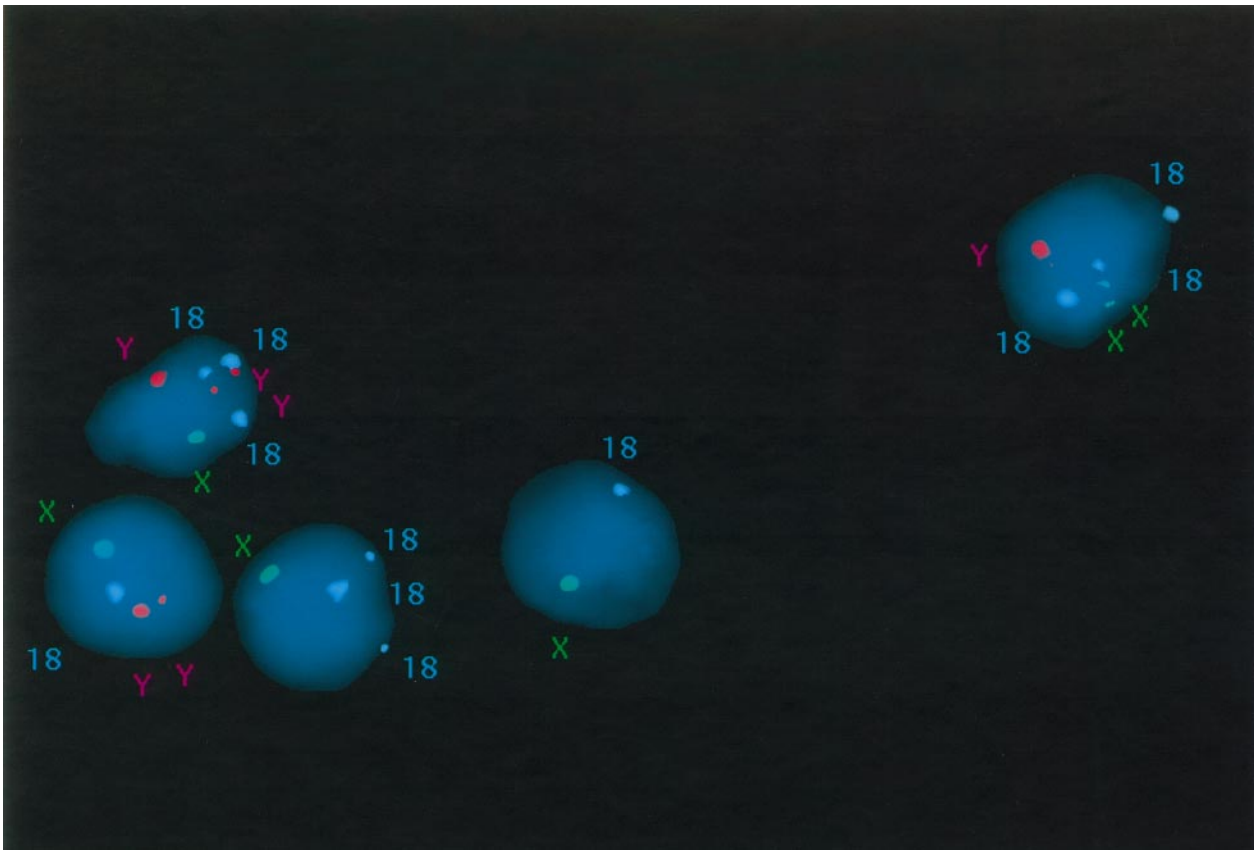

Figure 4. Example of sperm cells derived from the infertile patient demonstrating the distribution of the X, Y ,and 18 chromosomes in few cells, using three colour fluorescence in-situ hybridization (FISH) technique.

and men with severe male factor, using the experimental protocol of double target in-situ hybridization. They found a significantly higher value for abnormal chromosome constitution in the severe male factor group compared with the normal group (1.35 and 0.86% respectively). The authors concluded that even though this difference in value is relatively low, one has to consider the relative risk when ICSI is considered for a patient with oligoasthenoteratozoospermia (OAT).

A small number (0.8%) of cells with 18,18,18,XXXYY, 18,18,18,XXYYY and 18,18,18,X,Y,Y,Y chromosomes were observed (Table III); such cells might have been originated from aneuploid spermatogonia. Springgs *et al.* (1996) and Martin *et al.* (1995) mentioned YYY sperm cells in the samples they studied. However, because of their rare occurrence those cells were not included in any of the data calculations (no explanation of the origin of such cells was provided). The sperm cells showed abnormal morphology, indicating that the differentiation of round spermatids to mature spermatozoa was also defective. The morphological defects included those in head and tail structures. There are cases in which all spermatids develop into multinuclear cells, and this may be concomitant with malformation of structures such as acrosome and flagella, and formation of headless spermatozoa as in the present case. The reason for the serious disruption in spermatogenesis remains obscure ,as it is, actually, in most cases of primary testicular OAT.

The investigation of the patient's karyotype by conventional cytogenetics as well as by FISH of his lymphocytes with the same probes did not show evidence of obvious genetic abnormality, when compared with a normal fertile male, nor did his medical history indicate any genetic abnormality. The fact that the Sertoli cell plays a major role in regulating spermatogenesis, and that germinal cells receive most of their nutrients and metabolic signals by means of Sertoli cells, suggests a Sertoli cell involvement in OAT. Further research on spermatogenesis using such modern methods as FC and FISH may help to reveal further details of defects in spermatogenesis

The work reported here has shown how these techniques can be used to help define the nature of defects seen in human spermatogenesis, and can guide the clinicians in deciding which sperm samples are unlikely to be useful for ICSI.

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