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SHORT COMMUNICATION

COIMBRA, PORTUGAL

Analysis of Human Spermatozoa by Fluorescence In Situ Hybridization with Preservation of the Head Morphology Is Possible by Avoiding a Decondensation Treatment

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***Purpose*:** Development of a hybridization technique for spermatozoa allowing the preservation of the head morphology.
***Methods*:** FISH analysis of fixed semen samples from oligoasthenoteratozoospermia (OAT) patients with a normal somatic karyotype attending the Cytogenetics and the IVF Laboratories of a University hospital for semen analysis. In situ hybridization with centromeric probes for chromosomes X, Y, and 18 and locus specific probe for chromosome 21.
***Results*:** More than 95% of the sperm heads showed clear hybridization signals and a conserved morphology including the visualization of the tail. Few cells with splitted signals were not considered.
***Conclusions*:** This is the first description of a simple and fast hybridization protocol for spermatozoa without a decondensation step, allowing preservation of the morphology of the sperm head that is particularly useful to correlate abnormal spermatozoa with specific chromosome aneuploidies. With this technique we were able to avoid troubles in interpretation of FISH spots that does not depend on the quality of nuclear decondensation, as it is the case in the previously described methods. Our goal was to demonstrate the efficiency of the method without loosing sperm head morphology. Further studies are needed to correlate the aneuploidy rates for specific chromosomes with sperm morphology.

KEY WORDS: Aneuploidy; FISH; male infertility; spermatozoa.

INTRODUCTION

Cytogenetic study of human gametes is a difficult task due to the high condensation of chromatin in the sperm head. Several authors have been publishing different protocols for in situ hybridization after various decondensation treatments (1–10). Infertile men often have severe morphological abnormalities of their sperm that correlate with the impairment of spermatogenesis and the low number of germ cells in the ejaculate. In individuals with a normal somatic karyotype some of the sperm cells may have aneuploidies that cannot be easily detected. In severe oligoasthenoteratozoospermia (OAT) patients the frequency of nondisjunction of autosomes and gonosomes increases as the sperm count decreases (1,2,11) and this increase in the aneuploidy rate in spermatozoa should not be underestimated. The incidence of aneuploidy rate in OAT patients may be relevant for couples undergoing assisted reproductive technologies, namely intracytoplasmic sperm injection (ICSI).

Karyotyping spermatozoa has only been possible after fusion with zona pellucida free hamster eggs, which is a very laborious procedure. Using FISH a much larger number of spermatozoa can be analyzed and even spermatozoa of infertile men that are not capable of fertilizing an egg can be scored. The slight increase in the aneuploidy rate that has been observed for several chromosomes in the sperm of infertile patients by FISH might led us to speculate a higher than expected total aneuploidy rate in spermatozoa from infertile men. However, not all chromosomes are evenly involved in the nondisjunction events. Reported protocols of decondensation are time consuming and laborious. After DNA decondensation the morphology of the sperm heads is partly distorted and the majority of the tails are lost which leads to problems in the distinction between abnormal sperm and somatic cells.

Here we describe a simplified protocol using high temperatures to improve the access of the probes to sperm DNA. This is a very simple and fast procedure that allows high hybridization efficiency with clear spots without loosing sperm head morphology.

MATERIALS AND METHODS

Patients

Samples were collected from patients with severe OAT and a normal somatic karyotype attending the Cytogenetics Laboratory and the IVF Laboratory of a University Hospital.

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Semen Preparation and Fixation

Semen was collected in modified Earle's medium and allowed to liquefy for 30 min. Evaluation of mobility and the general characteristics of the semen according to WHO criteria were recorded at first observation.

Semen samples were then centrifuged at 1500 rpm for 15 min in 40% Percoll. Supernatant was discarded and sperm washed in fresh Earle's medium and centrifuged again at 1500 rpm for 10 min. A total of 200 μL of IVF medium was then added to the pellet and the tube was kept at 37°C for 15 min to allow the migration of the motile spermatozoa. Slides for microscopic observation and FISH were prepared by spreading the total semen suspension and allowing them to dry at room temperature. Smears were fixed in absolute methanol for 2 min and stained by immersion in Giemsa for about 30 s. A final wash in tap water was done and slides were kept at −20°C until FISH procedure. For Patient 15 who had a 47, XYY karyotype we analyzed both the total semen suspension, the pellet, and the motile fraction recovered after a 40% Percoll gradient (however this patient was not included in the statistical analysis) (see Tables I and II).

Hybridization Technique

Slides were destained in Carnoy solution for 5 min and air-dried. Dehydration was performed through an ethanol series (70%, 80%, 100%). After air-drying, 10 μL of the hybridization mixture (CEP X green and CEP Y orange, Vysis) or (CEP 18 green and LSI 21 orange, Vysis) was applied to the slide under a coverslip and sealed with rubber cement. Codenaturation was done at 90°C for 10 min followed by hybridization overnight at 42°C, using a *Hybrite* system (Vysis).

Table II. FISH Results in 7 Men with a Normal Karyotype and Sperm Analysis

Patient	16	17	18	19	20	21	22
Sperm/mL	83	29	47	77	47	46	73
Normal morphology (%)/ overall motility	39/57	49/54	43/45	44/58	63/40	56/57	64/51
X	564	550	573	578	519	530	494
Y	401	442	408	368	468	461	498
XX	5	2	8	1	3	1	1
YY	13	0	5	0	1	0	3
XY	5	0	4	13	9	8	4
XXY	0	0	1	0	0	0	0
18 21	949	982	935	984	919	972	980
18 18	1	18	8	0	6	12	2
18 21 21	10	0	6	2	0	1	8
18 18 21	0	0	5	3	0	10	3

Slides were washed first in a solution of 0.4× saline sodium citrate (SSC) with 0.3% NP 40 at 72°C, for 90 s and then in a solution of 2 × SSC with 0.1% NP 40 at room temperature, for 10 s. Slides were allowed to dry at room temperature in the dark and counterstained with 10 μL DAPI 1/5 in antifade solution (Figs. 1–4).

Analysis

Slides were observed by two individuals using a fluorescence microscope (Olympus BX50) equipped with a triple band pass filter (DAPI, FITC, PI) and a dual band pass filter (TRITC, RHODAMINE).

Only spermatozoa with a conserved morphology and clear hybridization signals were scored. Overlapping spermatozoa were excluded and whenever two or more distinct hybridization signals for the same probe were present in the same sperm head the cell

Table I. FISH Results in 14 Patients with an Impaired Spermatogenesis and a Normal Karyotype and a 47, XYY Men

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15[b]	15a	15b
Sperm/mL	5.3	16.7	30.7	6.2	5	12.3	22	13	19.4	18.9	36.7	21	22	35	1		
Normal morphology (%)/ overall motility	30/43	29/42	37/34	61/47	42/52	0[a]/45	11/0	44/60	63/70	49/50	51/53	28/40	35/40	29/54	42/43		
X	530	468	544	468	109	593	455	432	535		580	496	517	490	246	452	245
Y	416	519	434	527	117	403	528	557	461		415	496	461	492	240	476	244
XX	1	1	3	2	10	1	2	2	1		10	0	4	3	*4*	*15*	*4*
YY	3	0	0	0	3	1	0	1	2		4	0	4	1	*7*	*22*	*2*
XY	60	12	17	3	18	2	15	8	1		18	8	14	13	*2*	*27*	*5*
18 21		873	310	987	57	931	601	975	510	417	365	973	963	949	0	652	
18 18		7	1	0	2	6	0	0	1	13	10	0	4	15	0	2	
21 21		3	0	0	0	0	0	2	0	0	22	3	5	5	0		
18 21 21		0	1	1	0	4	3	0	1	0	0	9	11	0	0	5	
18 18 21		0	2	10	0	0	1	0	0	7	0	0	0	0	*0*	*16*	
18 18 21 21		3	0	0	0	8	5	3	5	9	0	0	0	7	0	8	

[a] Globozoospermia.
[b] 47, XYY patient: 15a is the pellet and 15b is the total sperm.

Fig. 1. Spermatozoa with a normal head and two spots for chromosome 18 probe (CEP 18 orange)-arrow. The other visible sperm heads only have one spot for the 18 chromosome.

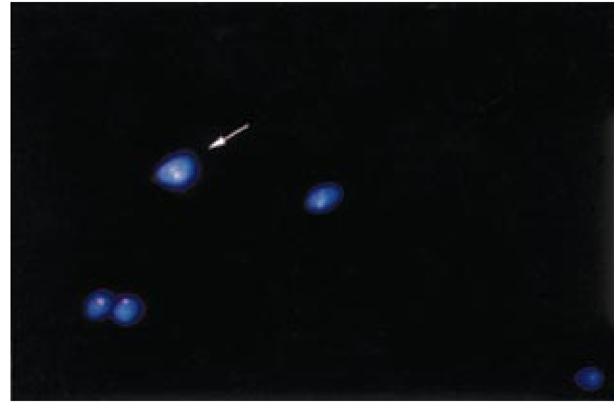

Fig. 3. Macrocephalous sperm with two spots for the 18 probe (CEP 18 orange) and one spot for the X probe (X-LSI Kallman green)-arrow. Notice the two normal heads in the bottom with an orange spot for 18 probe and the other head on the right with an X and an 18 spot.

was classified as aneuploid. Cells with splitted signals were not considered.

Statistical Analysis

All statistical analysis was performed using the Statistic 5.1 software. The Mann–Whitney test was used to study the following parameters: sperm/mL, normal morphology, overall motility, and motility Grade 3 and the chi-square test was used for the analysis of the nominal variables.

RESULTS AND DISCUSSION

We found a significative difference between Groups A (abnormal sperm analysis) and B (normal sperm count) in the concentration of sperm/mL ($p < 0.006$) and normal motility ($p < 0.04$), as we expected.

The aneuploidy rate for the heterochromosomes or for both the autosomes analyzed was not different in the two groups. However, the small number of cells analyzed per patient may have contributed to this. Other authors have reported that in infertile patients the gonosomes X and Y seem to be more prone to nondisjunction than the autosomes we analyzed (18 and 21). The higher incidence of XY disomy suggests that the majority of nondisjunction errors might occur at Meiosis I. Bernardini *et al.* (12) suggest that whenever the count of normal motile spermatozoa falls below 5 million (compared to 7–15 millionsin normal controls), an almost two-fold increase in

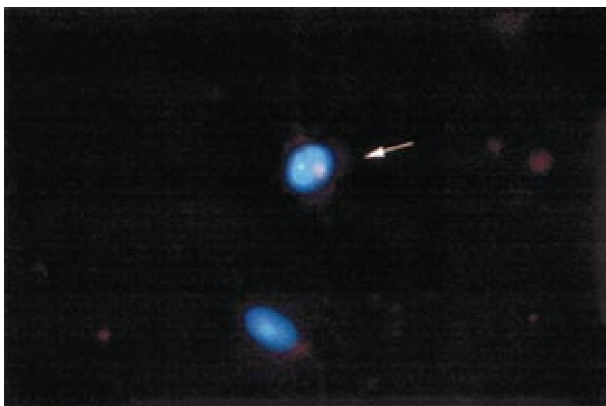

Fig. 2. Spermatozoa with a round head and one spot for each of the two autosomes analyzed (CEP 18 green and LSI 21 orange)-arrow.

Fig. 4. Notice the elongated head with an 18 spot (CEP 18 orange) and an X spot (X-LSI green)-arrow. The other visible head also shows similar FISH signals.

sperm disomy and diploidy should be expected for chromosomes X, Y, and 17 (total disomy rate of 2.34% versus 1.38% in controls). So far, aneuploidy and diploidy have only been related to sperm count and FSH concentrations (2,8) but not to sperm morphology.

A 13-fold increase in sex chromosome aneuploidies was observed in the somatic cells of infertile men with unexplained oligozoospermia compared to donors (4,13) and the results of FISH analysis and sperm karyotyping demonstrated a significantly increased frequency of aneuploidy in spermatozoa from infertile men, particularly of sex chromosomes. The overall data suggest that abnormalities in chromosome segregation and sperm morphology, differentiation, and maturation are probably associated phenomena (4,5). A pairing abnormality, confined to germ cells, may exist in infertile men, leading to meiotic arrest and aneuploidy during spermatogenesis. Our protocol of hybridization does not include a decondensation step, allowing a high efficiency of hybridization together with the preservation of sperm morphology that can be very useful to analyze the correlation between sperm morphology and aneuploidy on an individual cell basis. This technique avoids artificial estimates of disomy rates caused by decondensation procedures and swelling of the cells and does not depend on the quality of the decondensation to the efficiency of the hybridization and the interpretation of the results that is a limitation to FISH studies (2,8).

Our preliminary results do not confirm a high incidence of aneuploidy rates of sperm from patients with severely impaired spermatogenesis and a normal somatic karyotype.

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