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DETECTION OF ANEUPLOIDY IN HUMAN SPERMATOZOA USING FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

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Summary Fluorescence *in situ* hybridization (FISH) with chromosome specific alpha-satellite DNA probes was used to estimate the rates of aneuploidy of chromosomes 1, 17, 18, X and Y in human ejaculated sperm. Sperm samples were collected from six donors, and biotinylated DNA probes, D1Z5, D17Z1, D18Z1, DXZ1 and DYZ3 were hybridized to interphase sperm which had been pretreated with dithiothreitol to expand their nuclei. A minimum of 3,000 sperm per donor were analyzed. The hybridization efficiency was 99.68% for all the five probes. The frequencies of aneuploidy for chromosomes 1, 17 and 18 were 0.65%, 0.66%, and 0.61%, respectively. For XX- and YY-sperm the frequencies were 0.28% and 0.27%, respectively. To estimate the diploidy and disomy rates, a mixture of D17Z1 and D18Z1 were used as probes, and the frequency of diploid sperm was calculated to be 0.27%. After subtraction of the diploidy rate, the disomy rates for chromosomes 1, 17, and 18 were estimated to be 0.38%, 0.39% and 0.33%, respectively. The proportion of X- and Y-bearing sperm were 49.90% and 49.66%, consistent with an expected 1 : 1 ratio.

Key Words sperm, interphase, aneuploidy, chromosome, fluorescence *in situ* hybridization

INTRODUCTION

Numerical aberrations of human autosomes and sex chromosomes account for a significant proportion of chromosome abnormalities. Although the majority of aneuploidy arises during meiosis in the female, it also occurs in the male (Hassold and Sherman, 1993). Thus, it is important to characterize the occurrence of aneuploidy in the sperm. In 1978, Rudak *et al.* reported a new cytogenetic

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technique using human sperm/hamster egg interactions which first permitted a direct analysis of the chromosomal constitution in individual human sperm (Rudak *et al.*, 1978). In this way, human sperm chromosomes have been studied for both numerical and structural aberrations (Brandriff *et al.*, 1984; Martin *et al.*, 1982; Kamiguchi and Mikamo, 1986). However, this method is labor intensive and time consuming, and may reflect natural meiotic selection, since only fertilized eggs can be studied. Furthermore, it is difficult to evaluate the frequency of polyploidy in sperm because of the occurrence polyspermic fertilizations (Martin *et al.*, 1982). Over the past several years, the fluorescence *in situ* hybridization (FISH) technique has widely been used to identify chromosomal abnormalities in both metaphase and interphase cells. However, sperm obtained from ejaculation are too condensed to get an efficient probe hybridization (Joseph *et al.*, 1984).

In the present study, we utilized a method before hybridization to expand the sperm nuclei and improve detection of aneuploidy in ejaculated sperm. With this method, we studied the aneuploidy rates of chromosomes 1, 17, 18, X and Y in sperm from six normal men.

MATERIALS AND METHODS

Slide preparation. Semen samples were obtained from six healthy Japanese donors, aged 21 to 23 years. Prior to FISH, semen analysis (concentration, motility rate and volume) was performed according to the standard procedures (WHO, 1987), and all components were within the normal range. Samples were incubated at 37°C for 30 min, then diluted in Hank's balanced salt solution (Gibco, USA), and centrifuged at 2,000 rpm for 8 min. The pellet was fixed in fresh fixative (methanol : acetic acid, 3 : 1) at −20°C for 20 min. After two rinses with the fixative, sperm suspensions were dropped onto clean glass slides and air dried. the slides were stored at −20°C until pretreatment and denaturation for FISH.

Pretreatment of sperm before FISH. Slides were put into 0.6–1.5% dithiothreitol (DTT) (Sigma, USA) solutions at 37°C for 3–5 min, then the slides were rinsed with water and air dried.

Fluorescence in situ hybridization. Alpha-satellite DNA probes, D1Z5, D17Z1, D18Z1, DXZ1, and DYZ3 (Oncor, USA), which are specific for chromosomes 1, 17, 18, X, and Y, respectively, were used according to the manufacturer's protocol with minor modifications. To estimate the frequency of diploidy, a mixture of D17Z1 and D18Z1 probes (single color) were used. The hybridization solution consisted of 50% formamide, 5% dextran sulfate, and DNA probe (10 ng) were denatured at 70°C for 10 min prior to hybridization. Sperm slides were also denatured by immersion in 70% formamide, 2×SSC at 70°C for 5 min, then dehydrated in an ethanol series and air-dried. Hybridizations were performed at 37°C overnight. Post washings were carried out by immersion in 65% formamide and 2×SSC at 43°C for 1 to 3 min. Detection was achieved using alternating cycles

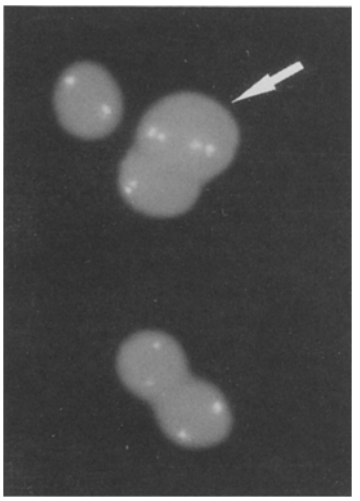

Fig. 1. Diploid sperm nucleus (arrow) showing two D17Z1-signals and two D18Z1-signals.

of fluorescein isothiocyanate (FITC)-conjugated avidin and anti-avidin antibody each at 37°C for 20 min, followed by one cycle of the FITC-conjugated avidin. After final avidin treatment, 0.25% propidium iodide was applied for counter stain. Then an anti-fade reagent (Oncor) was applied onto the slides, which were covered with cover slips and sealed with rubber cement.

Microscopic analysis. Hybridization signals on sperm nuclei were examined under a Nikon Optiphot microscope with a Nikon B2-A filter. The fluorescein-labeled hybridization signals appeared as yellow-green spots against the orange background of the propidium iodide-stained chromatins. At least 3,000 sperm were analyzed for each sample with each probe except Donor 2 for DYZ3. Sperm with two distinct signals were scored as hyperhaploid, which could indicate either disomy or diploidy. Sperm with four distinct signals using the alpha-satellite 17/18 probe cocktail were scored as diploid (Fig. 1). After subtracting the diploidy rate from the hyperhaploidy rate, the disomy rate was calculated from the residual. Because XY-bearing sperm were not observable with the single-color scoring system, total sex-chromosome disomy rates could not be obtained. The XX- or YY-bearing sperm frequencies were counted as the number of sperm with two signals among all sperm counted which would reflect combined frequencies for disomic and diploid sperm.

RESULTS

After pretreatment of sperm nuclei with DTT, sperm heads were expanded to 2 to 3 times as large as their original size. Of a total of 91,040 sperm, 55,000 were

examined for autosomes (chromosomes 1, 17, and 18) and 36,040 for sex-chromosomes. Based on the observation that 0.35%, ranging from 0.2% to 0.6%, of sperm lacked FISH signals, the hybridization efficiency was calculated to be greater than 99.65%. The mean frequencies of hyperhaploid sperm for chromosomes 1, 17, and

Table 1. Frequency of D1Z5, D17Z1 and D18Z1 FISH signals in sperm nuclei.

Donor	Probe	Number of sperm examined	Number of signals (%)		
			0	1	2
1	D1Z5	3,000	6 (0.20)	2,973 (99.10)	21 (0.70)
	D17Z1	3,000	9 (0.30)	2,967 (98.90)	24 (0.80)
	D18Z1	3,000	19 (0.60)	2,960 (98.67)	21 (0.70)
2	D1Z5	3,000	7 (0.23)	2,976 (99.20)	17 (0.56)
	D17Z1	3,000	8 (0.27)	2,975 (99.17)	17 (0.56)
	D18Z1	3,000	6 (0.20)	2,981 (99.37)	13 (0.43)
3	D1Z5	3,300	9 (0.27)	3,269 (99.06)	22 (0.66)
	D17Z1	3,300	8 (0.24)	3,272 (99.15)	20 (0.60)
	D18Z1	3,000	9 (0.30)	2,974 (99.13)	17 (0.57)
4	D1Z5	3,000	9 (0.30)	2,974 (99.13)	17 (0.57)
	D17Z1	3,000	11 (0.37)	2,972 (99.07)	17 (0.57)
	D18Z1	3,000	18 (0.60)	2,966 (99.87)	16 (0.53)
5	D1Z5	3,400	9 (0.26)	3,371 (99.15)	20 (0.59)
	D17Z1	3,000	8 (0.27)	2,974 (99.13)	18 (0.60)
	D18Z1	3,000	12 (0.40)	2,970 (99.00)	18 (0.60)
6	D1Z5	3,000	7 (0.23)	2,969 (98.97)	24 (0.80)
	D17Z1	3,000	10 (0.40)	2,965 (98.83)	25 (0.83)
	D18Z1	3,000	12 (0.32)	2,964 (98.80)	24 (0.80)
Total		55,000	177 (0.35)	54,472 (99.04)	351 (0.64)

Table 2. Frequency of DXZ3 and DYZ1 FISH signals in sperm nuclei.

Donor	Number of sperm examined	Number of signals for DXZ1 (%)			Number of sperm examined	Number of signals for DYZ3 (%)		
		0	1	2		0	1	2
1	3,000	1,484 (49.46)	1,507 (50.23)	9 (0.30)	3,000	1,501 (50.03)	1,492 (49.73)	7 (0.23)
2	3,000	1,497 (49.90)	1,495 (49.83)	8 (0.27)	2,940	1,486 (50.54)	1,445 (49.14)	9 (0.30)
3	3,000	1,516 (50.53)	1,475 (49.16)	9 (0.30)	3,000	1,503 (50.10)	1,487 (49.56)	10 (0.23)
4	3,100	1,552 (50.16)	1,541 (49.70)	7 (0.23)	3,100	1,499 (49.97)	1,496 (49.87)	5 (0.17)
5	3,000	1,488 (49.60)	1,503 (50.10)	9 (0.30)	3,000	1,497 (49.90)	1,496 (49.87)	7 (0.23)
6	3,000	1,478 (49.26)	1,513 (50.43)	9 (0.30)	3,000	1,496 (49.86)	1,494 (49.80)	10 (0.33)
Total	18,100	9,015 (49.82)	9,034 (49.90)	51 (0.28)	17,940	8,982 (50.06)	8,910 (49.66)	48 (0.27)

Table 3. Frequency of diploid sperm in 6 donors.

Donor	Number of sperm examined	Diploid sperm (%)
1	3,000	7 (0.23)
2	3,000	8 (0.27)
3	3,000	11 (0.37)
4	3,000	5 (0.17)
5	3,000	7 (0.23)
6	3,000	11 (0.37)
Total	18,000	49 (0.27)

Table 4. Frequency (%) of disomic sperm for chromosomes 1, 17 and 18.

Chromosome	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Mean
1	0.47	0.30	0.30	0.40	0.36	0.43	0.38
17	0.57	0.30	0.24	0.40	0.37	0.47	0.39
18	0.47	0.17	0.20	0.37	0.37	0.43	0.33

18 were 0.65%, 0.66%, and 0.61%, respectively (Table 1). The frequencies of X-bearing and Y-bearing sperm were 49.90% and 49.66%, respectively (Table 2). A ratio of X- to Y-bearing sperm was not significantly different from 1 : 1 ($p=0.345$). The mean frequency of XX-sperm was 0.28% and that of YY-sperm was 0.27% (Table 2). The mean frequency of diploid sperm was 0.27% (Table 3). The mean frequency of disomic sperm for chromosomes 1, 17, and 18 were 0.38%, 0.39%, and 0.33%, respectively (Table 4). Chi-square analysis was performed to compare disomic and diploid sperm rates among the donors and among the autosomes. There were no significant differences in the frequencies of disomy for chromosome 1 ($p=0.84$), chromosome 17 ($p=0.64$) and chromosome 18 ($p=0.48$) among the donors. Likewise, there was no significant difference in the frequency of diploidy rates ($p=0.62$) among the donors. In addition, there was no significant difference in the frequencies of disomy among the different autosomes studied ($p=0.74$).

DISCUSSION

The chromatin of the mammalian sperm is packed in a condensed and inactive state by intra- and inter-protamine disulfide bridges (Balhorn, 1982; Rodman *et al.*, 1982). To apply the FISH techniques successfully to human spermatozoa, it is critical to open the disulfide bridges to decondense sperm nuclear chromatin (Wyrobek *et al.*, 1990). Earlier studies did not use optimal pretreatment methods to enhance decondensation of the nuclei. Thus, the older methods permitted reduced access of probes to the nucleus and therefore lower hybridization efficiencies (Burns *et al.*, 1985; Joseph *et al.*, 1984). Lower hybridization efficiencies tended to result in under-estimation of disomy or diploidy rates. Our pretreatment method of sperm with DTT is simple and easy, compared with

other protocols (Robbins *et al.*, 1993; Holmes and Martin, 1993; Han *et al.*, 1992; Williams *et al.*, 1993). Previous investigators used DTT for longer treatment time and sometimes required subsequent treatment with a lithium salt. In this study, we used DTT at a relatively higher temperature (37°C) and consequently reduced the time of pretreatment to 5 min. Sperm heads were expanded to 2 to 3 times as large as their original size, with an increased hybridization efficiency of 99.65%. Signals were very clear and intense, and diffusion phenomenon was minimized.

With a single-color system, both disomic and diploid sperm nuclei produce two signals. A number of investigators tried to utilize large head size of sperm as a criterion to distinguish diploid sperm from disomic sperm (Coonen *et al.*, 1991; Han *et al.*, 1992). However, Carothers and Beatty (1975) showed that the cell size and morphology do not always correlate with DNA-based determinations of ploidy in sperm. Miharu *et al.* (1994) also observed a lack of correlation between cell size and ploidy in some cells by FISH, although a pretreatment to expand sperm heads may have contributed to the variability in cell size. In the present study, a probe cocktail for chromosomes 17 and 18 was used to identify diploid sperm and to estimate the diploidy and disomy rates. Although our system could not allow simultaneous examinations of both disomic and diploid sperm on the same slide, it is now available by means of two-color FISH, and no longer requires innovative or expensive techniques.

The disomy rate (0.38%) for chromosome 1 in this study is similar to that (0.41%) in the report of Guttenbach and Schmid (1991), while higher than those in the previous reports (Robbins *et al.*, 1993; Miharu *et al.*, 1994) (Table 5). The disomy rate (0.39%) for chromosome 17 in this study is comparable to those (0.33% and 0.31%) by Han *et al.* (1992) and by Guttenbach *et al.* (1994a), while higher than that (0.13%) in the report of Bischoff *et al.* (1994) (Table 5). The disomy rate (0.33%) obtained for chromosome 18 is comparable to those (0.36% and 0.25%) by Guttenbach *et al.* (1994b) and by Bischoff *et al.* (1994), while higher than that

Table 5. Summary of studies of disomy and diploidy frequencies (%) using FISH.

Study	Chromosomes			
	1	17	18	Diploid
Guttenbach and Schmid (1991)	0.41			
Han *et al.* (1992)		0.33		0.37
Williams *et al.* (1993)			0.08	0.34
Robbins *et al.* (1993)	0.14			
Bischoff *et al.* (1994)		0.13	0.25	
Guttenbach *et al.* (1994a)		0.31		
Guttenbach *et al.* (1994b)			0.36	
Miharu *et al.* (1994)	0.14			0.23
Griffin *et al.* (1995)			0.04	
This study	0.38	0.39	0.33	0.27

(0.08% and 0.04%) by Williams *et al.* (1993) and by Griffin *et al.* (1995) (Table 5). On the contrary, the diploidy rate in this study is comparable to those of other investigators (Han *et al.*, 1992; Williams *et al.*, 1993; Miharu *et al.*, 1994) (Table 5). The discrepancies in the disomy rates among investigators may have been due to different sample sizes and different scoring criteria of disomic sperm. Inclusion of sperm into the disomic sperm whose signals are close together by chance due to split signals or replicated chromosomes may lead to higher estimated rates of disomy. Since we did not simultaneously use X- and Y-specific probes, we could not analyze the XY-sperm rate. The rates of XX- and YY-sperm in this study reflect a combination of disomy resulting from meiosis II error and diploidy. Thus, the XX- and YY-sperm frequencies (0.28% and 0.27%) estimated in this study are higher than those reported previously (Wyrobek *et al.*, 1990; Robbins *et al.*, 1993; Griffin *et al.*, 1995) (Table 5). The rates of XX- and YY-diploid sperm are needed to calculate the true rates of disomy for sex chromosomes. Williams *et al.* (1993) reported that about 75% of diploid sperm were produced in meiosis II error. Using this datum, mean frequencies of XX- and YY-sperm in this study are calculated to be 0.18% and 0.15%, respectively. The disomy rates for autosomes in this study are generally higher than those estimated from conventional cytogenetic studies on hamster eggs (Martin, 1985; Brandriff *et al.*, 1985). The discrepancies of the disomy rates between FISH and karyotype studies may be due to the limited number of total sperm in the karyotype studies. Under 100 sperm could be studied per donor at one time in karyotype studies. With an approximate disomy frequency of one per 10,000 sperm, the sample size obtained by karyotype study is relatively small. Furthermore, the hamster egg may act as a barrier against some sperm with chromosomal abnormalities. In order to know an effect of infertility status on cytogenetic abnormality of sperm, Miharu *et al.* (1994) studied disomy frequencies both in infertile and fertile populations, and could not find any differences between the two populations. However, Wyrobek *et al.* (1994) did not find any differences in the frequencies of disomic sperm between FISH and karyotype studies using the hamster egg test. Further studies on all chromosomes with larger sample sizes are needed in this area. We could not find any differences in the disomy rates among the different autosomes studied in this paper ($p=0.748$). Although Williams *et al.* (1993) reported a significant increase of the XY-sperm rate by FISH experiments as well as karyotyping, no study on the comparison of the disomy rates among all chromosomes has yet been reported. Thus, further studies are also needed.

In summary we have utilized FISH to study aneuploidy rates in sperm with a simple pretreatment method which optimizes sperm head swelling and hybridization efficiency. This is the first report with Japanese male samples using large sample sizes and high hybridization efficiency. Multicolor FISH using mixtures of probes simultaneously should be done to resolve the true frequencies of disomic and diploid sperm.

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