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ORIGINAL INVESTIGATION

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Increased incidence of hyperhaploid 24,XY spermatozoa detected by three-colour FISH in a 46,XY/47,XXY male

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Abstract Meiotic segregation of gonosomes from a 46,XY/47,XXY male was analysed by a three-colour fluorescence in situ hybridisation (FISH) procedure. This method allows the identification of hyperhaploid spermatozoa (with 24 chromosomes), diploid spermatozoa (with 46 chromosomes) and their meiotic origin (meiosis I or II). Alpha satellite DNA probes specific for chromosomes X, Y and 1 were observed on 27,097 sperm nuclei. The proportions of X-and Y-bearing sperm were estimated to 52.78% and 43.88%, respectively. Disomy (24,XX, 24,YY, 24,X or Y,+1) and diploidy (46,XX, 46,YY, 46,XY) frequencies were close to those obtained from control sperm, whereas the frequency of hyperhaploid 24,XY spermatozoa (2.09%) was significantly increased compared with controls (0.36%). These results support the hypothesis that a few 47,XXY germ cells would be able to complete meiosis and to produce mature spermatozoa.

Introduction

The most frequent sex chromosome anomalies in human males are XXY (Klinefelter's syndrome) and XYY. The incidence at birth is close to 1 per 1,000 males for both XXY and XYY (Hecht and Hecht 1987). Despite this high rate, little is known about the meiotic behaviour of the extra gonosome. Klinefelter's syndrome is usually associated with azoospermia or oligozoospermia. Meiotic studies in subjects with mosaic constitutions 46,XY/47,XXY, suggest that only normal germ cells can complete meiosis (Kjessler 1966; Laurent et al. 1972).

Recently intracytoplasmic microinjection (ICSI) has successfully been applied in males with oligozoospermia (Van Steirteghem et al. 1993). Meiotic observations indicate that 46,XY/47,XXY patients would not have an increased risk of a chromosomally abnormal conceptus (Harari et al. 1995). However, Cozzi et al. (1994), analysing sperm karyotypes from an 46,XY/47,XXY male, have reported a significantly increased incidence of hyperhaploid 24,XY spermatozoa ($P < 0.001$). This result suggests that 47,XXY cells would be able to go through meiosis and to produce aneuploid spermatozoa. Such gametes may be considered as a risk for XXY progeny.

However, sperm karyotyping after heterospecific fertilization is labour intensive and cannot be performed when spermatogenesis is impaired. Recently fluorescence in situ hybridisation (FISH) has been successfully applied to human interphase spermatozoa to establish aneuploidy rates (Guttenbach and Schmid 1990; Coonen et al. 1991; Goldman et al. 1993). Cohybridisation with several probes allows differentiation between hyperhaploid cells (with 24 chromosomes) and diploid cells (with 46 chromosomes), and the meiotic origin of these events can also be determined. Two probes are required to analyse the meiotic segregation of autosomes, and three probes to analyse the meiotic segregation of gonosomes (Williams et al. 1993; Bischoff et al. 1994).

In the present study, 27,097 sperm cells from a 46,XY/47,XXY male were analysed by a three-colour FISH procedure, with specific probes for chromosomes X, Y and 1, in order to study the meiotic behaviour of the sex chromosomes

Materials and methods

The subject was 32 years old, with status ascertained after 2 years of infertility. The karyotype, determined from lymphocyte culture, was 46,XY/47,XXY with approximately 10% 47,XXY cells. Analysis of semen showed oligo-astheno-teratospermia and the sperm count was 9.6×10^6/ml. The mobility was 40% after a 1-h incubation. A semen sample was frozen and stored in liquid nitrogen.

Details of the hybridisation procedure have been described elsewhere (Chevret et al. 1995). Briefly, spermatozoa from fraction 90 of a Percoll gradient were treated with 10 m*M* dithiothreitol (DTT) in order to decondense sperm nuclei, dropped onto clean, dry slides, and fixed with ethanol-acetic acid (3:1). Alpha-satellite

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DNA probes specific for centromeres of chromosomes 1 and X, and a probe specific for a highly repeated sequence on the distal arm of Yq, were used. Probes were labelled by nick-translation with digoxigenin-11-dUTP (X probe) or biotin-16-dUTP (Y probe), or a mix of digoxigenin-11-dUTP/biotin-16-dUTP (1 probe).

After denaturation of probes (5 min at 75°C) and sperm nuclei (2 min at 80°C) the three probes were cohybridized for 20 h at 37°C in the hybridisation mix (0.5 × SSC, 0.5 × SSPE, 50% formamide, 10% dextran sulphate). The slides were washed in 50% formamide, 2 × SSC (three times for 5 min each), 2 × SSC (three times for 5 min each) and 0.1 × SSC (once for 3 min).

Biotinylated and/or digoxigenin-labelled probes were simultaneously detected with, respectively, avidin-fluorescein isothiocyanate (FITC) (diluted 1/300 in 4 × SSC, 5% skimmed milk) and anti-digoxigenin-rhodamine (diluted 1/200). The final incubation was followed by washing the slides in 4 × SSC and in phosphate buffered saline. After the final wash, slides were air-dried in the dark. Then, 10 µl of an antifade solution containing 20 ng/ml of the blue fluorescing dye DAPI (4', 6-diamidino-2-phenylindole) was applied to the slides.

The slides were examined on a Zeiss Axiophot equipped with a triple band pass filter FITC/rhodamine/DAPI. Only well-delineated spermatozoa were scored. Yellow, red and green spots, respectively, detected chromosomes 1, X and Y. Two signals of the same colour were scored as containing twice the corresponding centromere when they were clearly distinct and about the same intensity and size.

Results

A total of 27,097 sperm cells were scored from the 46,XY/47,XXY male (Table 1), and 142,040 spermatozoa were analysed from five control donors (Table 2). The X/Y ratio estimated from spermatozoa of the XY/XXY male, was different from the expected 1:1 proportion. Indeed, the rate of 23,X-bearing sperm cells was slightly increased compared with control sperm (according to Student's Test, $P < 0.05$), while frequency of 23,Y-bearing nuclei was in the same range as that obtained in controls ($P < 0.20$).

The disomy X rate was above the average ($P < 0.001$) and the disomy Y rate was lower ($P < 0.001$). The frequency of hyperhaploid spermatozoa 24,XY (2.09%) was significantly increased compared with controls ($P < 0.001$). The rate of diploid 46,XY sperm cells (0.28%), resulting from unaccomplished meiosis I, was higher than in the controls ($P < 0.001$). Absence of cytodiaresis during meiosis II should lead to diploid 46,XX or 46,YY sperm cells (meiosis II diploidy). The proportions of these diploid cells were 0.03% and 0.02%, respectively, roughly similar to those obtained from controls. The frequency of disomy 1 (0.18%) was in the same range as in controls. Tetraploid sperm cells were also observed at 0.003%. No such cells were found in controls.

In 8 sperm cells the fluorescence signal was ambiguous and in 160 spermatozoa (0.59%) only one yellow signal was observed. As this last observation could be due to technical artefacts, (including superposition of fluorescent signals) this rate was not considered as the nullisomy rate for the gonosomes.

Table 1 Overall results of X,Y and 1 probe labelling of sperm in a 46,XY/47,XXY man

Presumed karyotype	No. of spermatozoa	%
23,X	14,301	52.78
23,Y	11,890	43.88
24,XX	30	0.11
24,YY	1	0.003
24,XY	568	2.09
46,XY	75	0.28
46,XX	9	0.03
46,YY	6	0.02
24,X+1	38	0.14
24,Y+1	10	0.04
92,XXYY	1	0.003
22,-Xor-Y	160	0.59
Ambiguous	8	0.03
Total	27,097	100

Table 2 Meiotic segregation of chromosomes X, Y and 1: comparison between a 46,XY/47,XXY male and five control donors

Presumed karyotype	46,XY/47,XXY %	(%) Controls Mean ± SD
23,X	52.78	50.10 ± 3.39
23,Y	43.88	48.22 ± 2.23
24,XX	0.11	$0.04 \pm 3 \times 10^{-3}$
24,YY	0.003	$0.01 \pm 6 \times 10^{-5}$
24,XY	2.09	0.36 ± 0.08
46,XY	0.28	$0.09 \pm 7 \times 10^{-3}$
46,XX	0.03	$0.06 \pm 4 \times 10^{-3}$
46,YY	0.02	$0.005 \pm 2 \times 10^{-5}$
24,XorY+1	0.18	0.27 ± 0.03

Discussion

Meiotic studies of males with Klinefelter's syndrome (Ferguson-Smith et al. 1957; Futterweit 1967; Kaplan et al. 1963; Skakkebaek et al. 1969; Dutrillaux et al. 1971; Laurent et al. 1972; Rajendra et al. 1981; Vidal et al. 1984) have shown various degrees of spermatogenetic defect: absence of spermatogenesis, arrest of meiosis at primary spermatocyte or spermatid stages or foci of spermatogenesis in a few seminiferous tubules. It has been suggested that, in subjects with Klinefelter's mosaicism, 46,XY/47,XXY, spermatogenesis would be related to the presence of 46,XY cells among germ cells and other surrounding cells (Sakar and Marimathu 1983).

It has been assumed that only 46,XY germ cells would be able to complete meiosis (Luciani et al. 1970; Laurent et al. 1972). However, Skakkebaek et al. (1969) and Vidal et al. (1984) have proposed that a few 47,XXY germ cells can initiate the meiotic process.

In all meiotic observations the number of analysed cells was very low and only meiotic I events were studied. The direct cytogenetic analysis of spermatozoa, which are the final products of meiosis, would suitably complete the information on the meiotic behaviour of the extra gono-

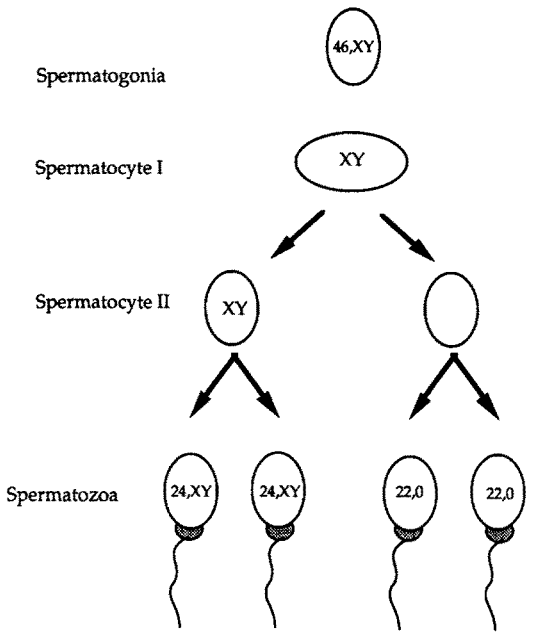
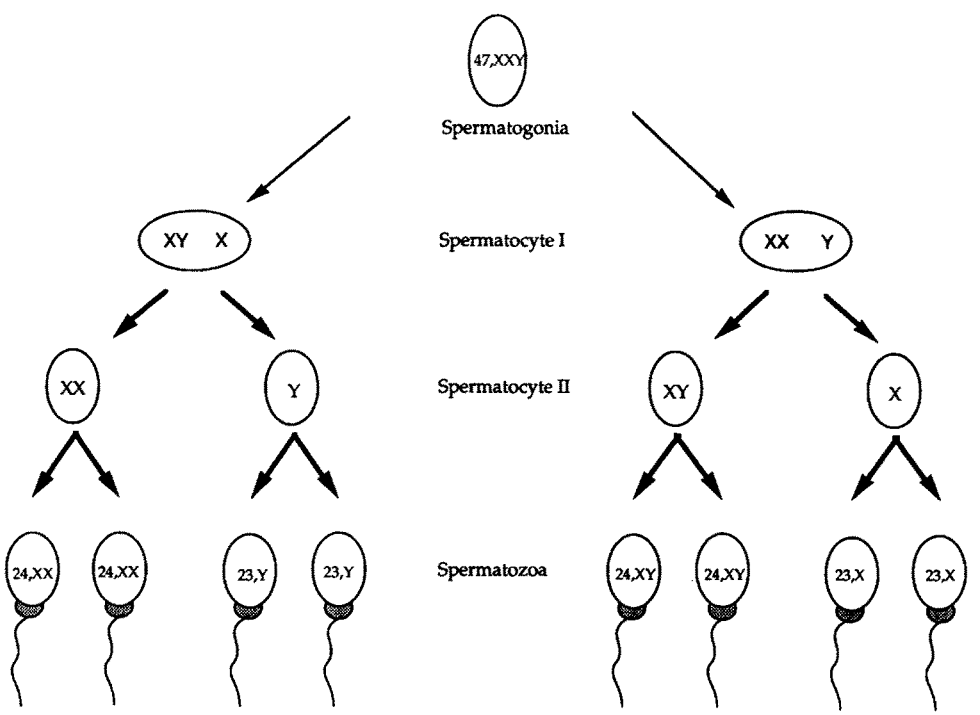


Fig. 1 Nondisjunction of sex chromosomes at metaphase I

some. Fertilization of hamster eggs has allowed the analysis of sperm complements in a subject with a mosaic Klinefelter's constitution (Cozzi et al. 1994). From 543 sperm complements a significantly increased incidence of hyperhaploid 24,XY spermatozoa ($P < 0.001$) was shown compared with compiled data of sperm karyotypes (Martin et al. 1991). Meiotic I nondisjunctions in a 46,XY germ cell line should result in the same proportions of 24,XY and 22,0 sperm cells (Fig. 1). However no increase in hypohaploid 22,0 spermatozoa was observed. These results, as well as the meiotic observations of Skakkebaek et al. (1969) and Vidal et al. (1984), suggest that 24,XY spermatozoa may be produced by regular meiosis of the 47,XXY germ cells. The present analysis of the meiotic segregation of chromosomes X and Y in another 46,XY/47,XXY male, by three-colour FISH has allowed the scoring of a large number of spermatozoa, and confirms the previous observations. Indeed the frequency of hyperhaploid 24,XY spermatozoa was six times higher than that observed in controls. These results support the hypothesis mentioned above that a few 47,XXY spermatocytes I should be able to go through meiosis and produce hyperhaploid 24,XY spermatozoa.

At the primary spermatocyte stage two of the three gonosomes would thus have to be paired. There are two theoretically possible pairings of sex chromosomes in bivalents during prophase I. In the first one, an XY sex vesicle is formed and the extra X chromosome is free. In the latter, the two homologous X chromosomes are synapsed and the Y remains unpaired. In the first situation, regular segregation of the sex chromosomes would produce 23,Y-bearing sperm cells and hyperhaploid 24,XX spermatozoa in the same proportions (Fig. 2, left). This model cannot explain our observations. Moreover no such pairing was observed in testicular biopsy material (Skakkebaek et al. 1969; Luciani et al. 1970; Vidal et al. 1984). In the second situation, the homologous sex chromosomes would be paired. Indeed this has been observed by Skakkebaek et al. (1969), screening testicular biopsy material from four Klinefelter patients. Two of five analysed cells in metaphase I presented 22 autosomal bivalents, an XX bivalent and a univalent Y; three contained an XY bivalent. Vidal et al. (1984), studying synaptonemal complexes in testicular biopsy material from both testes of a 46,XY/47,XXY

Fig.2 Regular segregation of sex bivalent and random distribution of the free sex chromosome during anaphase I



male, have reported 14 cells with a 23,XY constitution and 11 cells with no sex vesicle but with 24 chromosomal elements. They suggested that these 24 elements could correspond to 22 autosomal bivalents, an XX bivalent and a free Y chromosome. A regular meiosis in a 47,XXY spermatogonium with XX pairing and a univalent Y, should lead to 23,X- and 24,XY-bearing sperm cells in the same proportions (Fig. 2, right). Moreover it could explain the excess of hyperhaploid 24,XY spermatozoa reported by Cozzi et al. (1994) and observed in the present analysis. The preferential pairing of homologous sex chromosomes in spermatogonia with three gonosomes has previously been proposed by Hultén (1970), Tettenborn et al. (1970), Hultén and Pearson (1971), Berthelsen et al. (1981) and Speed et al. (1991) by meiotic I chromosome analysis of 47,XYY males.

According to these results, normal spermatogenesis in a male carrying an extra gonosome could be related to a particular pairing of sex chromosomes during prophase I. Following this hypothesis meiotic arrest could be the consequence of inaccurate pairing of the gonosomes. This impairment of spermatogenesis could lead to progressive testicular damage. Indeed, from observations in XYY mice (Burgoyne 1979; Tease 1990), it has been suggested that XY pairing associated with univalent Y would result in a high level of primary spermatocyte death, which would in turn lead to secondary damage. On the contrary preferential YY pairing and a free X chromosome would be associated with normal spermatogenesis. Germ cell behaviour during the meiotic process would then be linked to the ability to synapse of homologous sex chromosomes. Asynapsis, desynapsis and low chiasma counts would lead to early maturation arrest at the spermatocyte stage as suggested by Magid et al. (1990). In germ cells with three gonosomes, the homologous sex chromosomes would have to be paired in order to progress through meiosis and to produce spermatozoa, whereas a failure of such pairing would lead to arrest of spermatogenesis.

Fluorescence in situ hybridisation allows the indentification of large numbers of spermatozoa, even when spermatogenesis is impaired. It is thus a very informative method to study the meiotic behaviour of the three gonosomes in 47,XXY or 47,XYY males.

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