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Meiotic behaviour of the sex chromosomes in three patients with sex chromosome anomalies (47,XXY, mosaic 46,XY/47,XXY and 47,XYY) assessed by fluorescence in-situ hybridization*

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Meiotic studies using multicolour fluorescent in-situ hybridization (FISH) and chromosome painting were carried out in three patients with sex chromosome anomalies (47,XXY; 46,XY/47,XXY and 47,XYY). In the two patients with Klinefelter syndrome, although variable percentages of XXY cells (88.5 and 28.3%) could be found in the premeiotic stages, none of the abnormal cells entered meiosis, and all pachytenes were XY. However, the abnormal testicular environment of these patients probably resulted in meiotic I non-disjunction, and a certain proportion of post-reductional cells were XY (18.3 and 1.7%). The fact that none of the spermatozoa were XY also suggests the existence of an arrest at the secondary spermatocyte or the spermatid level. In the XYY patient, most (95.9%) premeiotic cells were XYY. The percentage of XYY pachytenes was 57.9%. The sex chromosomes were either in close proximity (XYY) or the X chromosome was separated from the two Ys (X + YY). A high proportion (42.1%) of post-reductional germ cells were XY. However, only 0.11% of spermatozoa were disomic for the sex chromosomes. In this case, the data suggest the existence of an arrest of the abnormal cells at the primary and the secondary spermatocyte or the spermatid level, giving rise to the continuous elimination of abnormal cells in the germ-cell line along spermatogenesis. The fact that the proportion of diploid spermatozoa was only increased in one of the three cases (XXY) is also suggestive of an arrest of the abnormal cell lines in these patients. The two apparently non-mosaic patients were, in fact, germ-cell mosaics. This suggests that the cytogenetic criteria used to define non-mosaic patients may be inadequate; thus, the risk of intracytoplasmic sperm injection in apparently non-mosaics may be lower than expected.

Key words: 47,XYY/Klinefelter syndrome/sex chromosome pairing/sex chromosome segregation/sperm aneuploidy

Introduction

Klinefelter syndrome (47,XXY) and the 47,XYY karyotype are the most common sex chromosome anomalies at birth in the male (Hecht and Hecht, 1987).

XXY males are usually azoospermic, while mosaic XY/XXY and XYY individuals produce variable numbers of spermatozoa, ranging from severe oligozoospermia to normozoospermia (Skakkebaek *et al.*, 1973; Sharara, 1999). As a result, these patients often attend infertility clinics, where the incidence of such chromosome abnormalities is very high (Egozcue *et al.*, 1983; de Braekeleer and Dao, 1991).

The development of intracytoplasmic sperm injection (ICSI) solved the fertility problems of many of these patients, because a single spermatozoon was sufficient to obtain one embryo, and a few spermatozoa often produced enough embryos to obtain a continuing pregnancy. However, it soon became evident that in ICSI couples the incidence of sex-chromosome abnormalities was slightly, but significantly increased, with respect to controls (Liebaers *et al.*, 1995; ESHRE Task Force, 1998). Since then, different studies have tried to relate this increase of sex-chromosome anomalies to the behaviour of the sex chromosomes during meiosis in XXY and XYY males. Although in general it has been shown that the proportion of sex chromosome disomies was increased in these patients, the mechanisms producing such anomalies are still under discussion (Egozcue *et al.*, 2000).

So far, most meiotic studies concluded that XXY cells were unable to enter meiosis (Kjessler, 1966; Luciani *et al.*, 1970; Dutrillaux *et al.*, 1971; Laurent *et al.*, 1973). However, other meiotic studies (Skakkebaek *et al.*, 1969; Vidal *et al.*, 1984) observed pachytenes without a sex vesicle and with 24 elements at prophase I, suggesting the presence of an XX bivalent plus

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a Y univalent. In XYY males, most meiotic studies indicated that the extra Y chromosome was lost in the premeiotic stages (Thompson *et al.*, 1967; Melnyk *et al.*, 1969; Evans *et al.*, 1970; Luciani *et al*., 1973; Chandley *et al*., 1976), but in some cases the presence of one X and two Y chromosomes has been detected during prophase I as an X univalent plus a YY bivalent (Hultén and Pearson, 1971; Speed *et al*., 1991; Blanco *et al*., 1997). Theoretically, in Klinefelter's this situation should produce an excess of XX and XY spermatozoa, and in the second an excess of XY and YY spermatozoa. However, the incidence and types of disomic spermatozoa in XXY and XYY individuals has been shown to be quite variable. The increase in the frequency of disomic spermatozoa could be interpreted as a result of the abnormal segregation of the abnormal cell line during meiosis (see references in Tables IV and V) or as the result of the effect of an abnormal testicular environment on the segregation of a normal cell line (Mroz *et al*., 1999; Egozcue *et al*., 2000). In addition, there are many cases of an increased proportion of diploid spermatozoa that is difficult to explain (Egozcue *et al*., 2000). Therefore to try to solve this problem the behaviour of the sex chromosome during meiosis in testicular biopsies from patients with sex chromosome anomalies was analysed.

In this paper we report the results of multicolour fluorescence in-situ hybridization (FISH) and chromosome painting in premeiotic cells, pachytenes (morphologically recognizable), post-reductional cells (secondary spermatocytes or spermatids) and spermatozoa from an XXY, a mosaic XY/XXY and an XYY male.

Materials and methods

Patients, sample collection and processing

XXY patient

This 30-year-old azoospermic sterile male had a lymphocyte karyotype 47,XXY in all the metaphases analysed. FSH was 19.6 IU/ml.

XY/XXY patient

This 29-year-old sterile patient had a severe oligozoospermia. FSH was 18.3 IU/ml. His lymphocyte karyotype revealed that 35% of cells were 46,XY and 65% were 47,XXY.

XYY patient

This 34-year-old sterile patient had an oligoasthenoteratozoospermia. FSH was 9.13 IU/ml. His lymphocyte karyotype was 47,XYY in all metaphases studied.

Testicular biopsies were obtained from the three patients under local anaesthesia and processed as usual (Evans *et al*., 1970). All patients gave their informed consent and the procedure was approved by the ethical committees of the centres involved, where diagnostic testicular biopsies are included in the protocol of study of human male infertility or sterility when indicated.

In the XYY patient, a semen sample could also be obtained. The sample was fixed in methanol:acetic acid (3:1) and processed for FISH analysis as usual (Vidal *et al*., 1993). In each patient we have assessed the sex chromosome constitution of three different types of cells: interphase germ cells (spermatogonia, spermatocytes and spermatids), meiotic figures (pachytenes) and spermatozoa. Interphase germ cells were clearly distinct from somatic cells because Sertoli nuclei are fusiform and other somatic cells are oval, while germ cell nuclei are round.

Prior to FISH, all samples were analysed under phase contrast, and coordinates of spermatozoa and meiotic figures considered adequate for analysis were noted. Images were captured by video colour camera (Sony 3CCD) and were saved into a computer using a video card (Matrox Comet 1.21). These images were stored and were used after FISH processing to facilitate the identification and location of each cell.

Fluorescence in-situ hybridization (FISH)

A triple colour FISH with centromeric DNA probes (Vysis Inc., Downers Grove, IL, USA) for chromosomes 18, X and Y was used to determine the sex chromosome constitution of interphase germ cells and spermatozoa. Prior to FISH, sperm nuclei were decondensed by slide incubation in a solution of 5 mmol/l dithiothreitol (DTT) and 1% Triton X-100 (Vidal *et al*., 1993).

In order to determine the sex chromosome composition and the relationship among the sex chromosomes at pachytene, two rounds of FISH using two different combinations of probes were used (Vysis Inc.). A triple colour FISH with centromeric DNA probes for chromosomes 18 (Spectrum aqua), X (Spectrum green) and Y (Spectrum orange) in the first round, and a whole chromosome painting for chromosome X (Spectrum green) plus a specific probe for the heterochromatic region of chromosome Y (Spectrum orange) in the second round. Sequential FISH was done according to a previously published protocol (Escudero *et al*., 1998).

Microscope evaluation

Analyses were done using an Olympus BX60 epifluorescence microscope equipped with specific filter sets for FITC, Texas Red, Aqua and a multiband pass filter for DAPI/Texas Red/FITC. Interphase germ cells were classified as haploid or diploid according to the number of hybridization signals for chromosome 18. That is, two signals indicated the presence of two chromosome complements while a single signal denoted the presence of only one complement. In the first case the cells were classified as spermatogonia or primary spermatocytes, in the second as secondary spermatocytes or spermatids.

Sperm analysis was done according to the criteria described previously by us (Blanco *et al*., 1996). The X:Y ratio and the incidence of anomalies for the sex chromosomes were determined for each patient.

Finally, although sex chromosome pairing can only be conclusively demonstrated by synaptonemal complex analysis (Solari and Rey Valzacchi, 1997), pachytene cells were classified taking into account the distribution of hybridization signals from the two rounds of FISH as follows. (i) Sex vesicle with an XY chromosome constitution: both rounds of FISH with one signal for the X- and one signal for the Y-chromosome in close contact (Figure 1A), or separated (X + Y). (ii) Sex vesicle with an X chromosome and a close relationship between the two Y chromosomes (X + YY): both rounds of FISH showing two signals in close contact plus an extra separated signal (Figure 1B). (iii) Sex vesicle with a close spatial relationship between the three sex chromosomes (XYY): both rounds of FISH showing three signals in close contact (Figure 1C).

Results

The number of cells analysed by FISH for each case was ~1000 (Table I). In the XXY and the XYY patient, premeiotic cells were seen much more frequently than post-reductional cells; the opposite occurred in the XY/XXY patient. Furthermore, a certain number of pachytenes was analysed in each case (Tables I and II).

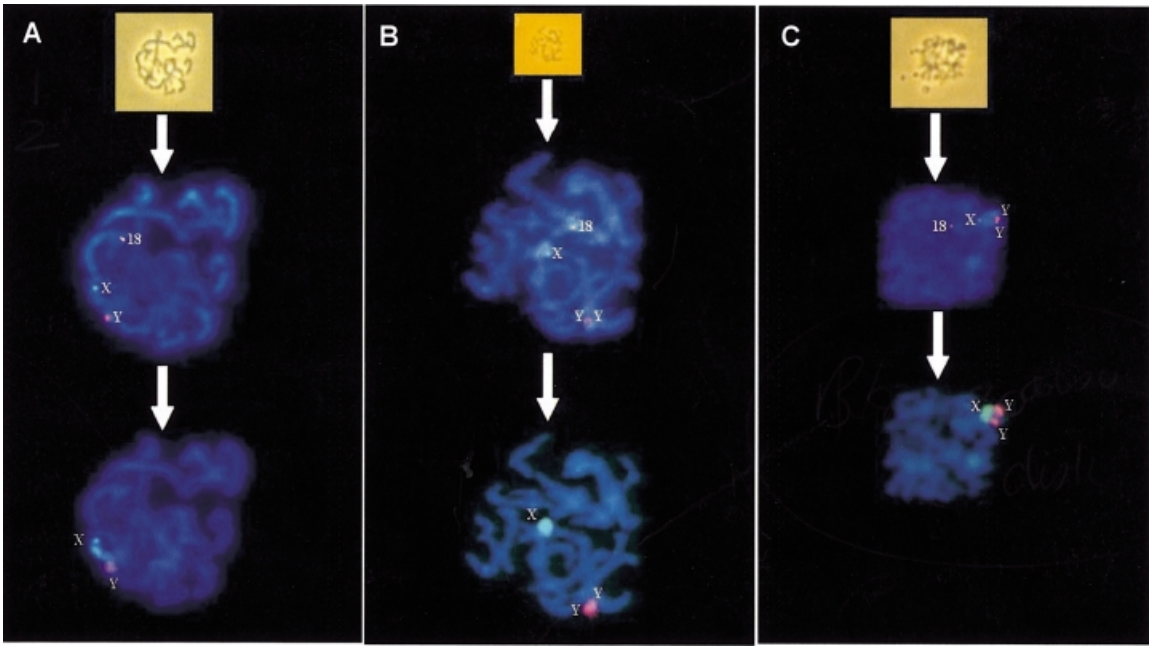

Figure 1. All figures, top: phase contrast; middle: centromeric probes; bottom: painting probes. (**A**) Pachytene from the XYY patient with a single signal for chromosomes 18 (synapsed centromeres), X and Y using multicolour FISH. Chromosome painting shows the presence of an X chromosome and a Y chromosome in the sex vesicle. (**B**) Pachytene from the XYY patient with one signal for chromosome 18 (synapsed centromeres) and for the X, and two signals for the Y chromosome by multicolour FISH. Chromosome painting shows the presence of an X chromosome separated from two Y chromosomes in close proximity (X + YY). (**C**) Pachytene from the XYY patient showing one signal for chromosomes 18 (synapsed centromeres) and for the X, and two signals for the Y chromosome using multicolour FISH. Chromosome painting shows the presence of three chromosomes in close proximity (XYY).

Table I. Chromosomal constitution of premeiotic cells (large, diploid), pachytenes and post-reductional cells (small, haploid)

	Premeiotic cells *n* (%)			Pachytenes *n* (%)			Post-reductional cells *n* (%)		
Patient	XXY	XYY	XY	XXY	XYY	XY	X	Y	XY
XXY	805 (88.5)	–	105 (11.5)	–	–	36 (94.7)	49 (40.8)	49 (40.8)	22 (18.3)
XY/XXY	167 (28.3)	–	423 (71.7)	–	–	37 (92.5)	368 (48.68)	375 (49.60)	13 (1.7)
XYY	–	883 (95.9)	38 (4.1)	–	33 (57.9)	19 (33.3)	4 (21.0)	7 (36.8)	8 (42.1)

Table II. Sex chromosome relationships at pachytene determined by sequential FISH

Pairing	XXY *n* (%)	XY/XXY *n* (%)	XYY *n* (%)
XY	36 (94.7)	37 (92.5)	13 (22.8)
XYY	–	–	20 (35.1)
X+YY	–	–	13 (22.8)
X+Y	–	1 (2.5)	6 (10.5)
Other	–	–	1 (1.7)
Ambiguous	2 (5.3)	2 (5)	4 (7.0)
Total	38	40	57

Table III. Sex chromosome constitution of spermatozoa determined by FISH

	XXY *n* (%)	XY/XXY *n* (%)	XYY *n* (%)
X-bearing	38 (52.05)	237 (42.86)	464 (50.93)
Y-bearing	32 (43.83)	278 (50.27)	415 (45.55)
Sex-chr. disomies	1 (1.37)	–	1 (0.11)
Diploid	1 (1.37)	–	4 (0.44)
Others	1 (1.37)	38 (5.60)	27 (2.96)
Total	73	553	911

FISH analysis of germ cells

All three patients were in fact germ-cell mosaics. Both the XXY patient and the XYY patient had a low proportion of XY cells (Table I). On the other hand, the XY/XXY mosaic, who in peripheral lymphocytes had a proportion of XY versus XXY cells of 15 and 65% respectively, showed an inversion of this proportion in pre-meiotic cells (28.3 XXY versus 71.7% XY; Table I). In the XYY patient, most (95.9%) pre-meiotic cells were XYY.

At the pachytene stage, which is morphologically identifiable (Figure 1), all figures analysed were XY in the XXY and in the mosaic XY/XXY patients, although in some cells of the mosaic patient (2.5%) the sex chromosomes were separated (X+Y) (Table II).

Table IV. Incidence (%) of sex chromosome anomalies in spermatozoa from patients with Klinefelter syndrome

	Karyotype	No. sperm	X:Y	XY	XX	YY	Diploid
Chevret *et al.* (1996)	47,XXY/46XY	27 097	53:44[a]	2.09[a]	0.11	0.003	0.33
Martini *et al.* (1996)[b]	47,XXY/46,XY	3500	47:43	1.3	0.5	0.7	–
Guttenbach *et al.* (1997)	47,XXY	2206	43:49[a]	1.36[a]	1.22[a]	0.09	0.23[a]
Foresta *et al.* (1998)	47,XXY	10 000	52:25[a]	15.58[a]	6.92	0.21	0.05
	47,XXY	10 000	56:29[a]	10.03[a]	3.34	0.09	0.03
Kruse *et al.* (1998)	47,XXY/48XXXY/46,XY	202	50:42[a]	5[a]	2[a]	–	–
Estop *et al.* (1998)[b]	47,XXY	24	21:29	25	–	–	4.2
Lin *et al.* (1999)[c]	47,XXY/46,XY	1701	47:50	0.41[a]	0.29[a]	0.06	1.70[a]
Rives *et al.* (2000)[c]	47,XXY	10 123	50:48	0.54[a]	0.45[a]	0.37[a]	0.23[a]
	47,XXY/46,XY	20 814	50:49	0.62[a]	0.24[a]	0.20	0.36[a]

[a]Significant differences versus controls.
[b]No statistical analysis.
[c]Autosome disomy was also increased.

In the XYY patient, one third of pachytenes were XY [of which about one third had the sex chromosomes separated (X+Y), while the other two thirds had the X and Y chromosomes in close proximity (XY) (Table I, Figure 1A)], and about two thirds of pachytenes were XYY (Tables I and II). The sex chromosomes were present as an X chromosome separated from two Ys in close proximity (X + YY) (Figure 1B), or as an XYY set (Figure 1C).

As is frequent in sterile patients with more or less severe oligozoospermia, no metaphase I figures were found in the biopsies studied.

In post-reductional germ cells, the proportion of sex chromosome anomalies increased or remained at high levels in all three patients (Table I). In the XXY patient, with no abnormal pachytenes, the incidence of XY post-reductional germ cells reached 18.3%, although over a low number of nuclei. In the XY/XXY patient, with no abnormal pachytenes, the frequency of XY post-reductional germ cells was 1.7% over a high number of nuclei. And in the XYY patient, in whom 57.9% of pachytenes were XYY, almost half of post-reductional cells (42.1%) were XY.

As is frequent in sterile patients with a more or less severe oligozoospermia, no metaphase II figures were found.

FISH sperm analysis

Analysis of the spermatozoa in the three patients showed a similar proportion of X-bearing and Y-bearing spermatozoa (Table III). An increase of sex chromosome disomies was only observed in the XXY patient, who also had an increased incidence of diploid spermatozoa. In the other two patients, the proportion of abnormal spermatozoa was within normal limits (Table III).

Discussion

Previously published results obtained from the analysis of decondensed sperm heads using multicolour FISH in XXY (Table IV) and XYY patients (Table V) indicated the presence in most cases of an increase in the proportion of XY spermatozoa and of XX spermatozoa in XXY males, and of XY, XX and YY spermatozoa in XYY males. The frequency of diploid spermatozoa was also increased in many cases (Tables IV and V). These data were interpreted as if the abnormal spermatozoa derived from the abnormal segregation of aneuploid cells entering meiosis, and often the data did not fit the expected proportions. For instance, in XXY males one would expect an increase in XY or XX spermatozoa, but usually only the proportion of XY spermatozoa was increased. In XYY males one would expect an increase in XY or YY spermatozoa and, in this case, the results better fit the expected sex-chromosome constitution of the spermatozoa (increased XY and YY) but not the expected proportions, because in general XY spermatozoa were found twice as frequently as YY spermatozoa.

In this work, we have not deduced the meiotic processes retrospectively from data obtained in spermatozoa, but analysed them in a prospective fashion from data obtained in spermatogenic cells from testicular biopsies.

Our data seem to confirm that in XXY males, the abnormal cells are unable to enter meiosis (Kjessler, 1966; Luciani *et al*., 1970; Dutrillaux *et al*., 1971; Laurent *et al*., 1973) because although a variable number of pre-meiotic cells have an XXY chromosome constitution, all pachytenes analysed were exclusively XY. The presence of a variable proportion of XY cells in post-reductional germ cells (secondary spermatocytes or spermatids) seems to confirm an earlier interpretation (Mroz *et al*., 1999), in the sense that a compromised testicular environment [e.g., an increase in FSH concentrations, which is common in these patients (Vendrell *et al*., 1999)] can induce non-disjunctional events, affecting mainly the sex chromosomes (Egozcue *et al*., 2000). Abnormal cells resulting from non-disjunction can again be arrested at meiosis II; this would explain the lower number of spermatozoa with sex chromosome anomalies found by us with respect to the figures observed in post-reductional germ cells (Figure 2). However, in another meiotic study carried out in Klinefelter patients (Foresta *et al*., 1999), the authors observed XXY germ cells, and came to the conclusion that spermatocytes with sex chromosome aneuploidies can complete the spermatogenic process. Anyway, both situations would lead to an increase of chromosomally abnormal spermatozoa, a phenomenon that could be related to the increased proportion of sex chromosome abnormalities found in ICSI children (Liebaers *et al*., 1995; ESHRE Task Force, 1998).

Table V. Incidence (%) of sex chromosome anomalies in spermatozoa from 47,XYY patients

	No. of spermatozoa	X:Y ratio	XY	XX	YY	Diploid
Han *et al.* (1994)	2006	46:47	0.25	0.30	0.40	2.6-3.35[a]
Mercier *et al.* (1996)	100 000	37:48[a]	9.37[a]	0.34	4.65[a]	0.11
Martini *et al.* (1996)[b]	3300	43:37	2.3[a]	2[a]	0.8[a]	–
	3500	45:35	5.4[a]	2.7[a]	2.3[a]	–
Blanco *et al.* (1997)	1974	50:45	0.30[a]	0.15	1.01[a]	0.30
Chevret *et al.* (1997)	24 315	43:55[a]	0.24	0.03	0.08[a]	0.23[a]
	10 827	43:56[a]	0.52	–	0.19[a]	0.13[a]

[a]Significant differences versus controls.
[b]No statistical analysis.

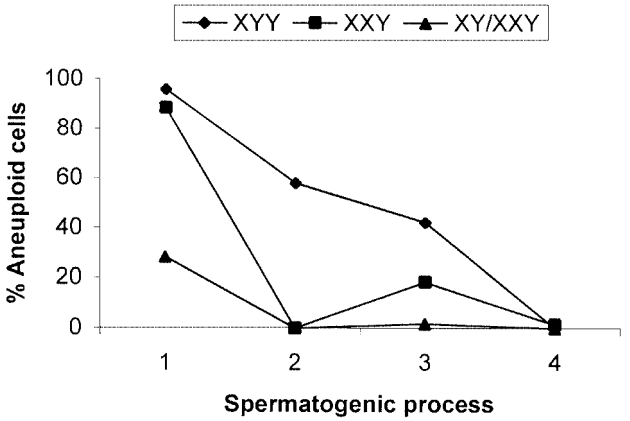

Figure 2. Percentage of XYY or XXY premeiotic cells (1), of XYY or XXY pachytenes (2), of XY post-reductional cells (3) and of spermatozoa (4).

On the other hand, in XYY males, although it was initially suggested that the extra Y chromosome was lost during the premeiotic stage (Thompson *et al*., 1967; Melnyk *et al*., 1969; Evans *et al*., 1970; Luciani *et al*., 1973; Chandley *et al*., 1976), there is increasing evidence that XYY cells can enter and, eventually, complete the meiotic process. It has been shown, both in mice and men, that cells with an XYY sex chromosome constitution can persist through meiosis (Burgoyne, 1979; Burgoyne and Biddle, 1980; Speed *et al.*, 1991; Blanco *et al*., 1997). The fact that apoptosis often affects aneuploid germ cells (Lin *et al*., 1997), giving rise to the continuous elimination of abnormal cells in the germ-cell line along spermatogenesis (Figure 2), does not necessarily preclude a variable number of them from becoming viable spermatozoa.

The elimination of abnormal germ cells is especially visible at pachytene (Figure 2) indicating the existence of a universal pachytene checkpoint (Roeder and Bailis, 2000) that may be mainly related to a saturation of pairing initiation sites. In this sense, it is possible that the only configuration compatible with the progress of meiosis would be the XYY trivalent (Rodriguez and Burgoyne, 2000). Other configurations, such as XY+Y, X+YY or X+Y+Y would be selectively eliminated.

The arrest of chromosomally abnormal cells during meiosis (Navarro *et al*., 1990) as well as their possibility of producing chromosomally abnormal spermatozoa (Arán *et al*., 1999; Pang *et al*., 1999) even in cases with extensive chromosome anomalies (Vendrell *et al*., 1999) resulting from synaptic errors (Egozcue *et al*., 2000) has been already well established. The presence of an increased frequency of diploid spermatozoa in some of these patients (Tables IV and V) has been interpreted as resulting from synaptic errors (Egozcue *et al*., 2000) that produce erratic chromosomes (Niklas, 1998). The presence of erratic chromosomes can delay anaphase I and give rise to an endomitotic process without cytokinesis, and produce diploid secondary spermatocytes or spermatids that will become diploid spermatozoa. This mechanism takes place during spermatogenesis, but not during oogenesis where the anaphase checkup seems to be more permissive (Eichenlaub-Ritter *et al*., 1999) and the process gives rise to aneuploidies.

In conclusion, the effect of chromosome abnormalities on the production of abnormal spermatozoa cannot be derived from sperm data, but must be analysed though the whole process of meiosis. Otherwise, erroneous interpretations can be reached, as shown by the results obtained in this work.

It is noteworthy that, although two of our patients were diagnosed as non-mosaic XXY or XYY males, both were germ-cell mosaics. Our data indicate that probably all Klinefelter males who produce spermatozoa in any numbers are XY/XXY mosaics, taking into account that, as shown, XXY cells are meiotically incompetent. As for XYYs, even non-mosaic cases could produce spermatozoa. These data are significant in the sense that they question the cytogenetic criteria for ruling out a mosaicism, based on the statistical significance of a given number of lymphocyte metaphases (Palermo *et al*., 1998).

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