Constitution of semen samples from XYY and XXY males as analysed by in-situ hybridization*

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A brightfield microscopical in-situ hybridization (ISH) technique was applied to semen samples of two 47,XYY males, one 46,XY/47,XXY male with fertility problems, and two normal 46,XY men, who served as controls. The use of a standardized nuclear DNA decondensation method, together with double-target ISH and morphological staining, allowed an accurate study of the sex chromosomal content and morphology of spermatozoa. In the males carrying an extra sex chromosome, we detected X- and Y-bearing spermatozoa in a ratio which did not differ significantly from the 1:1 ratio found in normal males. Aneuploidy for the sex chromosomes was found in ~15% of the spermatozoa of both XYY males and in 3% of the XXY male. The most striking finding was the relatively low percentage of spermatozoa in these patients, with an average of 65% in the XYY males and 84% in the XXY male. The other cells represented immature germ cells (IGC), including spermatogonia and spermatocytes arrested at various stages of spermatogenesis. Apparently, in XYY or XXY men, these IGC are shed into the semen to an increased extent as compared to normal, fertile men. The sex chromosome constitution of these IGC was heterogeneous. However, the finding that the majority of spermatozoa in semen of 47,XYY and 47,XXY males carried a single sex chromosome strengthens the hypothesis that a 46,XY germ cell line must be present, apparently with a proliferative advantage over the 47,XYY or 47,XXY cells.

Key words: in-situ hybridization/sex chromosomes/spermatozoa/XYY males/XXY males

Introduction

The estimated incidence of chromosomal abnormalities in cells of subfertile men is 4–5% compared with 0.5–0.7% in the general population. It has been found that up to 11.9% of azoospermic and 4.4% of oligospermic patients carry a constitutional chromosomal abnormality (Harari et al., 1995). In a study performed on 496 infertile males an overall incidence of 7.1% constitutional abnormalities suggests that a selection criterion based on chromosome studies as part of the routine analysis of infertile men might be advisable (Retief et al., 1984). The most frequent anomalies found in subfertile males are sex chromosome aneuploidies, predominantly 47,XYY and 47,XXY.

Virtually all cases of XYY are due to paternal meiosis II non-disjunction. The frequency of XYY concepti and livebirths (0.1%) coincides with the observed frequency of YY spermatozoa in ejaculates of normal males (0.06–0.11%) (Rothwell, 1983; Bischoff et al., 1994). The first fluorescent quinacrine staining of testicular biopsies from XYY males was performed in 1971 by Hulten and Pearson. In 1988, Benet and Martin analyzed semen of one 47,XYY male by karyotyping and observed no increase in the percentage of spermatozoa with sex chromosome abnormalities. In 1994 fluorescence in-situ hybridization (FISH) was applied to spermatozoa of a 47,XYY patient (Han et al., 1994). The ratio of X- and Y-bearing spermatozoa appeared not to differ from the normal 1:1 ratio (Han et al., 1994; Benet and Martin, 1988). The offspring of 47,XYY men do not show an increased frequency of congenital abnormalities, or a higher frequency of 47,XXY or 47,XXX children (Stoll et al., 1979).

The 47,XXY karyotype is associated with Klinefelter's syndrome (KS), and is the most common form of male hypogonadism. It occurs in 1 out of 600 new-born males, and arises from maternal or paternal non-disjunction at meiosis. Jacobs et al. (1988) found no effect of parental age on the conception of 47,XXY males of paternal origin, but a very marked association between increasing maternal age and 47,XXY males of maternal origin. Although males with KS are usually sterile, patients with mosaic karyotypes may have tubules consisting of cells with a normal chromosomal constitution that produce spermatozoa. Cozzi et al. (1994) studied 543 spermatozoa karyotypes of an XY/XXY mosaic patient and found a significantly increased incidence (0.9%) of hyperhaploid 24,XY chromosome sets, in the absence of the expected corresponding hypohaploid cells. The results of Cozzi et al. (1994) support previous observations (Berthelsen et al., 1981; Vidal et al., 1984; Speed et al., 1991) which indicated that 47,XXY cells may be able to undergo meiosis.
and produce 24,XY spermatozoa. These results are supported by a recent study of Chevret et al. (1996), where FISH analysis was performed on semen of a 46,XY/47,XXY mosaic man, and a significantly increased frequency of hyperhaploid 24,XY spermatozoa was found, suggesting that a few 47,XXY germ cells might be able to produce mature spermatozoa.

This study was performed to investigate the morphology and chromosomal constitution of cells present in the semen of infertile men carrying an extra sex chromosome. The ISH procedure is based on an enzyme cytochemical detection procedure, and results in a stable and non-fading precipitate that can be analysed by brightfield microscopy. The use of Diff-Quik in combination with haematoxylin as a morphological stain allows the discrimination between morphologically normal and abnormal spermatozoa, as well as immature germ cells (IGC) (Martini et al., 1995b).

The combination of an efficient ISH method with an adequate morphological staining procedure for spermatozoa has been introduced recently (Martini et al., 1995a). Until now, most authors have not taken the morphological criteria into account when analysing aneuploidy in semen. As a result, abnormal spermatozoa (e.g. double heads with one tail) or overlapping spermatozoa could have been confused with somatic cells or diploid spermatozoa (Martin et al., 1993; Bischoff et al., 1994; Wyrobek et al., 1994). Therefore, in our opinion, a correct evaluation of the aneuploidy rate is hampered in assays where morphological parameters are not incorporated.

Materials and methods

Case histories

Case 1 concerns a 29 year old man, who was referred for intracytoplasmic spermatozoa injection (ICSI) because of oligoasthenospermia. Lymphocyte karyotyping was performed, and showed a 47,XXY constitution in all cells. Before an ICSI appointment was made, his wife conceived naturally.

Case 2 concerns a 33 year old man, who was referred for ICSI because of severe oligospermia. As part of the clinical work-up for ICSI, karyotyping was performed, and revealed a 47,XXY constitution in all cells.

Case 3 is a 45 year old man, who was referred for ICSI treatment because of a previous diagnosis of 46,XY/47,XXY mosaicism. After FISH analysis of 200 peripheral blood cells, five cells with an XXY constitution were detected.

Control samples were obtained from two men with a normal 46,XY karyotype.

Semen specimens were analysed using WHO (1993) criteria for concentration and motility, and Kruger's strict criteria for morphology analysis was applied (Kruger et al., 1988). The analysis showed the semen of the patients to have a concentration of 2×10^6/ml, 3.6×10^6/ml and 1.8×10^6/ml, and 1.8×10^6/ml, for cases 1–3 respectively. Motility percentages were 59, 50 and 1% respectively for the three cases, while the semen of the control men was within the normal value range.

All three patients and the two healthy volunteers gave written consent for the semen samples to be used for research purposes.

Preparation of spermatozoa nuclei

After routine semen analysis, spermatozoa from patients and controls were prepared for the ISH procedure as described below.

Sperm samples were washed three times in phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 280 g for 10 min, and then fixed in methanol/acidic acid (3:1). The specimens were stored at -20°C until slide preparation. Spermatozoa were cytospun on poly-L-lysine-coated slides and were kept at +4°C for 7–10 days. Then the slides were washed in 2X standard saline citrate (SSC) and incubated for 5 min in 1 M Tris buffer, pH 9.5, containing 25 mM dithiothreitol (DTT) (Martini et al., 1995a). After decondensation the slides were washed once in 2X SSC, once in PBS and finally dehydrated (Jones et al., 1987).

DNA probes and labelling procedures

For the detection of the centromeric region of the X-chromosome, we used the repetitive DNA probe pBamX5, 2 0 Kb (Oncor, Gaithersburg, MD) and for detection of the Y-chromosome, the repetitive DNA probe DYZ1, 3 4 kb (Cooke et al., 1982). The probes were labelled with biotin-11-dUTP (Enzo Diagnostic, New York) or fluorescein-12-dUTP (Boehringer, Mannheim, Germany) in a standard nick-translational reaction following the manufacturers' instructions.

ISH procedure

Double target ISH with the biotinylated centromeric probe for the X-chromosome and the fluorescein isothiocyanate (FITC)-labelled probe for the Y-chromosome was performed. The probes were used at a concentration of 0.4 ng/µl Hybridization and detection procedures were performed as described previously (Martini et al., 1995a). Briefly, after overnight hybridization, the biotinylated probe was detected with avidin-peroxidase (AV-PO, 1:50; DAKO A/S, Glostrup, Denmark), followed by the diaminobenzidine reaction (DAB, Sigma, St Louis, MO). The slides were then incubated for 10 min at room temperature in 0.01 N HCl to inactivate peroxidase activity. Subsequently, the fluoresceinated probe was detected with mouse anti-FITC antibodies (1 500, DAKO), and peroxidase-conjugated rabbit anti-mouse IgG (1:80; DAKO). After this last incubation step, the peroxidase-tetramethylbenzidine (TMB, Sigma) reaction was performed (Speel et al., 1994).

Counterstaining and embedding

The slides were first dehydrated, then briefly counterstained in haematoxylin, again dehydrated and finally counterstained with Diff-Quik (DADE AG, Dudingens, Switzerland) as described before (Martini et al., 1995b). We utilized exclusively the cytoplasmic staining step of the Diff-Quik staining procedure, which consists of Eosin G in phosphate buffer, pH 6.6 (1 22 g/l, solution 1). Embedding was accomplished with Entellan (Merck, Darmstadt, Germany), and slides were stored at +4°C until evaluation.

Microscopy

The ISH signals were evaluated with a standard Zeiss brightfield microscope. Microphotographs were made on a Zeiss Axioskop microscope, using a Kodak Color Gold 100 ASA film, in combination with blue and magenta filters.

Evaluation criteria

After ISH and haematoxylin/Diff-Quik staining, the slides were used to evaluate spermatozoa morphology and their sex chromosome constitution. Spermatozoa showing one head and one tail were defined as morphologically normal. Morphological evaluation according to Kruger's strict criteria was not possible after ISH, due to the exposure of the spermatozoa to DTT prior to ISH. Likewise, morphologically abnormal spermatozoa, characterized by two heads and one tail, or vice versa, or showing any other major morphological abnormality were recognized as abnormal, but could not be evaluated properly for their chromosome content (Martini et al., 1995a).
Table I. Constitution of semen samples of controls, XYY and XXY males

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>Control 2</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of cells scored</td>
<td>2820</td>
<td>2992</td>
<td>3300</td>
<td>3500</td>
<td>3800</td>
</tr>
<tr>
<td>ISH efficiency (%)</td>
<td>99.4</td>
<td>99.4</td>
<td>94.5</td>
<td>97.3</td>
<td>98.7</td>
</tr>
<tr>
<td>Morphologically normal spermatoza (%)</td>
<td>100</td>
<td>99.8</td>
<td>58.6</td>
<td>66.7</td>
<td>79</td>
</tr>
<tr>
<td>Morphologically abnormal spermatoza (%)</td>
<td>0</td>
<td>0.1</td>
<td>3.4</td>
<td>3.3</td>
<td>5.0</td>
</tr>
<tr>
<td>IGC (%)</td>
<td>0</td>
<td>0.1</td>
<td>3.4</td>
<td>3.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

ISH = in-situ hybridization; IGC = immature germ cells

Results

The results of ISH analysis of all samples are presented in Table I. The average ISH efficiency of all samples was 97.3%. X- and Y-bearing spermatozoa were present in the regular 1:1 ratios in all the samples analysed. The results of the routine analyses and ISH of control semen specimens were all within the normal range.

The most revealing finding in the patients' semen samples was that, besides mature spermatozoa, showing a normal morphology after ISH, a population of large, round cells was seen, which were interpreted as IGC (Figure 1b,c). These cells were present in a substantial proportion: in case 1, 38% of cells were classified as IGC, in case 2 we found 30%, and in case 3, 16% IGC. The percentage of IGC in the control semen samples was only 0.02%.

The ISH analyses for sex chromosome constitution in spermatozoa and IGC are given in Table II and examples of ISH staining results are depicted in Figure 1. In control samples 98.4% of spermatozoa carried either one X- or Y- chromosome and on average 0.6% were aneuploid. For the patients the frequency of aneuploid cells ranged from 2.5 to 11.1%, with the highest percentage in both the 47,XYY males (Figure 1d–g).

Morphologically abnormal spermatozoa were present in the patients at a level of 3–5% (Figure 1h,i), while in control samples only 0.13% of spermatozoa was classified as being morphologically abnormal.

Discussion

Based on ISH, combined with a morphological staining procedure, a distinction is possible between morphologically normal spermatozoa with a normal sex chromosome constitution on the one hand, and morphologically and/or chromosomally abnormal spermatozoa, somatic cells and IGC on the other (Martini et al., 1995a). We have applied this technique to the semen samples of three patients with sex chromosome abnormalities and two normal males in order to achieve a reliable estimation of the fraction of normal spermatozoa in the semen of these patients. For this purpose we have assessed the proportion of morphologically as well as chromosomally abnormal spermatozoa.

Summarizing our findings we can state that (i) the estimated risk of abnormal chromosomal complement in morphologically normal spermatozoa is 8.6% in XYY and 2.5% in XXY patients (depending on the percentage of mosaicism); (ii) the incidence of IGC in semen samples of patients with XYY and XXY mosaicism is higher than in normal controls; and (iii) further studies are needed to identify the stages of spermatogenesis, where chromosomally abnormal germ cells are segregated.

Several previous studies showed that 47,XYY males indeed present this sex chromosome constitution in all their somatic cells, but that a 46,XY/47,XYY mosaicism is found in testicular biopsies (Tettenborn et al., 1970; Berthelsen et al., 1981; Han et al., 1994). XYY cells can have difficulties in completing spermatogenesis, resulting in diminished spermatozoa production (Speed et al., 1991). The offspring of 47,XYY men is mostly 46,XY (Stoll et al., 1979), which is in agreement with the results obtained from karyotyped spermatozoa (Benet and Martin, 1988), spermatozoa analysed by means of FISH (Han et al., 1994) and also with the data of the present study, where no real shift in the X:Y ratio was apparent. In our study the evaluation of the sex chromosomal constitution of the spermatozoa in both XYY patients gave comparable results and revealed that the majority of cells was normal for their sex chromosome constitution. However, the data might indicate a slight shift in favour of the X-bearing spermatozoa, but our sample is too small to perform a reliable statistical analysis. FISH analysis on spermatozoa of a 47,XYY man did not show any major abnormality or aneuploidy (Han et al., 1994).

In contrast, in our study, high percentages of the IGC carried an XY, XYY, XX or YY constitution. This supports the hypothesis that the extra Y-chromosome is lost during spermatogenesis and that the 46,XY germ cell epithelium has a proliferative advantage as compared to the 47,XYY germ cell line (Berthelsen et al., 1981). Studies of spermatogenesis in XYY males confirm the tendency for the germ cells to lose the second Y chromosome, but also that some XYY cells can reach meiosis I. The degenerate category of pachytene and meiosis I spermatocytes seen in the study of Speed et al. (1991) represent germ cells with an X univalent or cells with an XY + Y genotype, where the presence of a Y univalent has disrupted cell progression. It is probable that spermatogonia...
Semen samples from XYY and XXY males as analysed by in-situ hybridization

Figure 1. Diff-Quik and haematoxylin-stained semen preparation to which double-target in-situ hybridization (ISH) was applied with an X-probe (brown) and a Y-probe (blue-green). (a) Control sample showing an XX-bearing spermatozoon. (b, c) Immature germ cells from an XYY male (b) and an XXY male (c) showing a XYY and XY constitution respectively. Morphologically normal spermatozoa from an XXY male (d, e) and an XYY male (f, g) showing a normal sex chromosome constitution and YY constitution respectively (h, i) Morphologically abnormal spermatozoa from the XXY patient. Magnification \( \times 1000 \).

and spermatocytes with a 47,XYY constitution are shed into the seminal fluid and detected as IGC in our study. Indeed, the ISH analysis on IGC confirm a high frequency of combined XY and XYY constitutions. Furthermore, in the study of Han et al. (1994), a high proportion of IGC and somatic cells were found, though not histologically or cytogenetically confirmed. It appears obvious that a distinction between different stages of germ cell development and somatic cells is important for future ISH studies on spermatogenesis and for semen diagnosis, and additional identification methods are thus needed (Wyrobek et al., 1994).

47,XXY men are usually azoospermic, but patients with an XXY/XY mosaicism may occasionally show seminiferous tubules, consisting of cells with a normal chromosomal constitution that produce spermatozoa (Harari et al., 1995). The XXY patient studied showed the XXY cell line in a very small proportion of cells in peripheral blood and the semen contained a lower percentage of IGC (16%) than the semen samples of the XYY patients. This lower percentage of IGC concurs with the explanation of Terzo et al. (1992), who found that KS patients are fertile when mosaicism is confined to testicular tissue. Studies in patients with a mosaic 46,XY/47,XXY constitution suggest that only normal XY-germ cells can complete meiosis (Luciani et al., 1978, Vidal et al., 1984).
Table II. Sex chromosome content of spermatozoa and immature germ cells (IGC) of control, XYY and XXY males as analysed by in-situ hybridization (ISH)

<table>
<thead>
<tr>
<th>Frequency mosaic</th>
<th>Control 1 XY</th>
<th>Control 2 XY</th>
<th>Case 1 XYY</th>
<th>Case 2 XYY</th>
<th>Case 3 XY/XXY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Sperm</td>
<td>% IGC</td>
<td>% Sperm</td>
<td>% IGC</td>
<td>% Sperm</td>
</tr>
<tr>
<td>X</td>
<td>51.3</td>
<td>0.0</td>
<td>49.0</td>
<td>0.0</td>
<td>43.5</td>
</tr>
<tr>
<td>Y</td>
<td>47.7</td>
<td>0.0</td>
<td>49.5</td>
<td>0.0</td>
<td>37.5</td>
</tr>
<tr>
<td>No signal</td>
<td>0.6</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
<td>8.2</td>
</tr>
<tr>
<td>XY</td>
<td>0.4</td>
<td>0.0</td>
<td>0.4</td>
<td>0.0</td>
<td>2.3</td>
</tr>
<tr>
<td>XYY</td>
<td>—</td>
<td>0.0</td>
<td>—</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>XXY</td>
<td>—</td>
<td>0.0</td>
<td>—</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>XX</td>
<td>0.4</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>YY</td>
<td>—</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>XYYY</td>
<td>—</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>XYY</td>
<td>0.4</td>
<td>0.0</td>
<td>0.7</td>
<td>0.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Only the chromosome constitution of apparently normal spermatozoa and IGC was evaluated. The morphologically abnormal spermatozoa in these preparations could not be analysed reliably for their chromosome content.

Cozzi et al. (1994) and Chevret et al. (1996), using spermatozoa karyotyping and triple-FISH on spermatozoa respectively, found a significantly increased incidence (0.9 and 2.09% respectively) of hyperhaploid 24,XY sets in the absence of the expected corresponding hypohaploidy. The results of these studies support the suggestion that some 47,XXY cells are able to go through meiosis and to form spermatozoa. This suggestion correlates with the relatively high frequency of XY-bearing spermatozoa (1.3%) found in the XXY patient, as compared to the percentage found in normal males in our study (0.4%), and by Chevret et al. (1996) (0.36%).

Our results show that an average of 80–90% of the morphologically normal spermatozoa of XYY and XXY males carry a normal sex chromosome constitution. Based on the results of the present study, the average estimated risk of morphologically normal spermatozoa having an abnormal sex chromosomal content is 8.6% in the XYY and 2.5% in XXY patients, including spermatozoa without sex chromosome. In this respect it should be kept in mind that our criteria to define morphologically normal cells in ISH/Diff-Quik/haematoxylin-stained samples, are not the same as those used in routine semen analyses. Whenever these patients apply for artificial reproductive techniques (ART) the relatively high percentage of aneuploidy spermatozoa should be kept in mind. These patients have to be evaluated individually and the risks of the ART procedure should be determined on the basis of the ISH results. It is our opinion that after fertilization pre-implantation diagnosis should be performed.

Furthermore, to study the relationship between morphological appearance and genetic/chromosomal constitution of spermatozoa, cytogenetic studies should also be performed. In this respect an additional approach would be to apply ISH analysis to testicular biopsies, in order to study cells with a XYY or XXY genotype and their difficulties in completing spermatogenesis. The possibility that germ cell loss in certain chromosomally abnormal situations could be explained by genetic imbalance should also be examined (Speed et al., 1991), and whether the stages of spermatogenesis where chromosomal abnormalities are introduced are more likely to be excluded. However, the number of such studies on ejaculates of 47,XXXY and 47,XXYY men is still limited and more studies of larger populations, should be performed.

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