Meiotic aneuploidy in the XXY mouse: evidence that a compromised testicular environment increases the incidence of meiotic errors

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Male mammals with two X chromosomes are sterile due to the loss of virtually all germ cells in the differentiating testis. The survival of rare germ cells, however, can give rise to patches of normal-appearing spermatogenesis in the adult testis. Intracytoplasmic sperm injection (ICSI) makes possible the establishment of a pregnancy using spermatozoa from severely oligozoospermic men and, indeed, has been successful using spermatozoa from human 47,XXY (Klinefelter syndrome) males. The risk of an abnormal pregnancy, however, may be significantly increased since several studies have demonstrated elevated levels of aneuploidy in spermatozoa from Klinefelter syndrome men. This has been suggested to reflect the consequences of meiotic segregation in XXY germ cells; however, it is also possible that it is a consequence of abnormalities in meiotic regulation in the XXY testis. We have addressed this question experimentally in the XXY male mouse. Analysis of testicular spermatozoa from XXY and control males demonstrates a significant increase in meiotic aneuploidy in the XXY mouse. Since previous studies have demonstrated that germ cells in the adult XXY testis are exclusively XY, the meiotic abnormalities observed must be attributable to segregation errors in XY germ cells. These findings have potential significance for ICSI pregnancies using spermatozoa from other types of male factor infertility patients, since they raise the possibility that increased meiotic errors are a generalized feature of the severely oligozoospermic testis.

Key words: aneuploidy/Klinefelter syndrome/meiosis/spermatozoa

Introduction

The 47,XXY condition is one of the most common chromosome abnormalities in humans, occurring in approximately 1 in 1000 live-born males (Perwein, 1984). The associated clinical disorder, Klinefelter syndrome (KS), is characterized by hypogonadism and sterility; in fact, KS is the leading genetic cause of male infertility in our species, accounting for over 3% of all such cases (reviewed in Van Assche et al., 1996).

Sterility in KS males, as in all male mammals with two X chromosomes, is due to germ cell loss during testicular development, so that virtually no germ cells are present in the adult XXY testis (Ferguson-Smith et al., 1957; Foss and Lewis, 1971; Gordon et al., 1972). Nevertheless, rare break-through patches of spermatogenesis are found in the testes of XXY males (Steinberger et al., 1965; Skakkebaek et al., 1969; Tournaye et al., 1996), and occasionally spermatozoa are present in the ejaculate (Ferguson-Smith et al., 1957; Foss and Lewis, 1971; Tournaye et al., 1996; Palmero et al., 1998). It is not clear how many KS adults have spermatogenically active testes, since the ejaculate of a KS male may exhibit azoospermia on one occasion and oligozoospermia on another. As a result, KS individuals who are, in fact, oligozoospermic may be misdiagnosed as azoospermic. Indeed, in one of the few confirmed cases of natural paternity by an apparently non-mosaic XXY male, the individual had been diagnosed previously as azoospermic (Terzoli et al., 1992); however, the pregnancy demonstrated that he was capable of some sperm production.

Sperm production in KS individuals indicates that rarely, germ cells are able to survive in an XXY testis. However, controversy exists about the genetic constitution of these surviving cells. Based on analyses of meiotic preparations from XXY individuals, it has been concluded (Skakkebaek et al., 1969; Vidal et al., 1984) that at least some of the germ cells had an XXY sex chromosome constitution. However, other reports have concluded that XXY germ cells are eliminated from the human testis (e.g. Rajendra et al., 1981) and that the rare surviving germ cells have an XY sex chromosome constitution.

Recently, chromosome-specific probes have been used in fluorescence in-situ hybridization (FISH) experiments to screen for aneuploidy in spermatozoa from XXY males. Using this approach, three groups have analyzed aneuploidy levels in ejaculated spermatozoa from four apparently non-mosaic XXY humans (Guttenbach et al., 1997b; Estop et al., 1998; Foresta et al., 1998) and, in all individuals, sex chromosome disomy levels were elevated (Table I). From this, the authors concluded that rare XXY germ cells not only survive in the adult testis, but are capable of completing meiosis. However, several aspects of the reported data are inconsistent with this interpretation. For example, if the XXY testis were populated exclusively by XXY germ cells, equal frequencies of disomic (XY or XX)
and euploid (X- or Y-bearing) spermatozoa might be expected. In fact, in each of the three studies, disomic spermatozoa comprised only a minority of all analysed spermatozoa. Furthermore, among the euploid spermatozoa, X-bearing cells should be significantly more common than Y-bearing cells. While this was the case for the two XXY individuals analysed by Foresta et al. (Foresta et al. 1998), the other two studies reported ratios of X- to Y-bearing spermatozoa not significantly different from 1:1 (Guttenbach et al., 1997b; Estop et al., 1998).

Therefore, while it seems clear that sex chromosome disomy is elevated in spermatozoa from XXY males, the reason for this effect is less obvious. Indeed, we have been interested in examining an alternative explanation for the excess of sex chromosome disomy in spermatozoa from XXY males. Specifically we postulate that, like the testis of normal males, the adult XXY testis is populated by XY spermatogonial cells; however, owing to the compromised testicular environment, these germ cells are particularly susceptible to meiotic abnormalities, including non-disjunction of the sex chromosomes.

To conduct a systematic analysis of this hypothesis, we have utilized a mouse model. The 41,XXY mouse shows a remarkably similar reproductive phenotype to human KS, with reduced testicular volume and extensive germ cell death, leading to oligozoospermia or azoospermia (Hunt and Eicher, 1991). Nevertheless, in the adult testis rare break-through patches of spermatogenesis are observed (Mroz et al., 1999). To determine the level of aneuploidy in spermatozoa from 41,XXY male mice, we performed a three-colour FISH analysis of the testicular spermatozoa from XXY cases and XY controls produced on the C57BL/6 background (Hunt and Eicher, 1991). Karyotypes were determined at autopsy from cytogenetic analysis of bone marrow preparations (Eicher and Washburn, 1978). For sperm preparations, adult testes were decapsulated and minced in 2.2% citrate. The suspension was allowed to settle in a 15-ml conical tube, the supernatant was spun at 800 r.p.m. for 5 min, resuspended in 2.2% citrate and spread onto clean slides. Air-dried slides were dehydrated for 5 min each in cold 70%, 80% and 100% ethanol, and stored desiccated at least overnight.

Three hybridization probes were used: pErS5 (Epplen et al., 1982), which hybridizes to the Y chromosome, four pooled subclones of a pericentric micr o mouse chromosome 8-specific repeat (Boyle and Ward, 1992), and DXWas70 (Disteche et al., 1987), which detects repeat sequences near the centromere of the X chromosome. A mixture of biotin-labelled (Boehringer Mannheim, Indianapolis, IN, USA) pErS5, digoxigenin and biotin-labelled (Boehringer Mannheim) chromosome 8 subclones, and digoxigenin-labelled (Boehringer

### Materials and methods

The mating scheme for the production of XXY male mice has been described previously (Hunt and Eicher, 1991). Testis size is significantly reduced and XXY males are sterile due to the perinatal loss of virtually all germ cells (Hunt et al., 1998). Nevertheless, in the adult testis rare break-through patches of spermatogenesis are observed (Mroz et al., 1999). To determine the level of aneuploidy in spermatozoa from 41,XXY male mice, we performed a three-colour FISH analysis of the testicular spermatozoa from XXY cases and XY controls produced on the C57BL/6 background (Hunt and Eicher, 1991). Karyotypes were determined at autopsy from cytogenetic analysis of bone marrow preparations (Eicher and Washburn, 1978). For sperm preparations, adult testes were decapsulated and minced in 2.2% citrate. The suspension was allowed to settle in a 15-ml conical tube, the supernatant was spun at 800 r.p.m. for 5 min, resuspended in 2.2% citrate and spread onto clean slides. Air-dried slides were dehydrated for 5 min each in cold 70%, 80% and 100% ethanol, and stored desiccated at least overnight.

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### Table I. Abnormal spermatozoa identified by FISH studies in four human 47,XXY males

<table>
<thead>
<tr>
<th>Study</th>
<th>Euploid</th>
<th>Disomic</th>
<th>Diploid</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23,X</td>
<td>23,Y</td>
<td>24,XY</td>
<td>24,XX</td>
<td>24,YY</td>
</tr>
<tr>
<td>Estop et al. (1998)</td>
<td>Case 5</td>
<td>7</td>
<td>(26.3)</td>
<td>(36.8)</td>
<td>(31.6)</td>
</tr>
<tr>
<td></td>
<td>Case 2</td>
<td>6</td>
<td>(52.8)</td>
<td>(25.0)</td>
<td>(14.8)</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>(n = 10)</td>
<td>(49.0)</td>
<td>(49.0)</td>
<td>(0.2)</td>
</tr>
<tr>
<td>Foresta et al. (1998)</td>
<td>Case 1</td>
<td>5600</td>
<td>2865</td>
<td>1003</td>
<td>334</td>
</tr>
<tr>
<td></td>
<td>Case 2</td>
<td>5187</td>
<td>2460</td>
<td>1458</td>
<td>692</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>(n = not stated)</td>
<td>(57.0)</td>
<td>(29.2)</td>
<td>(10.2)</td>
</tr>
<tr>
<td>Guttenbach et al. (1997b)</td>
<td>Case</td>
<td>5</td>
<td>(45.4)</td>
<td>(51.0)</td>
<td>(1.4)</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>(n not stated)</td>
<td>(49.0)</td>
<td>(49.0)</td>
<td>(0.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cells reported as nullisomic were excluded from consideration. Values in parentheses are percentages.
Mannheim) DXWas70, in Hybrizol VII (Oncor) was denatured, applied to the slide, and hybridized overnight. Slides were washed in 50% formamide/2× SSC at 39°C, 2× SSC at 37°C and held in PN buffer (0.1 M NaH2PO4, 0.1 M Na2HPO4, 0.05% Nonidet P-40, pH 8.2). Slides were blocked with PNM (5% non-fat dry milk in PN buffer, 0.02% sodium azide) and detected with rhodamine–avidin and fluorescein isothiocyanate (FITC)-labelled anti-digoxigenin (Boehringer Mannheim). Thus, signals specific to chromosomes Y, 8 and X were visualized as red, yellow and green fluorescent signals, respectively. After final washes in PN buffer, slides were mounted with a 50% glycerol/4X SSC solution containing 0.1 µg/ml p-phenylenediamine and 50 ng/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma).

Only cells with an attached tail or demonstrating the typical hook-shaped morphology of mouse spermatozoa were scored. Spermatozoa were analysed on Zeiss Axioshot epifluorescence microscopes using either a single band-pass filter (Chromatech) for simultaneous detection of red, green and yellow signals with blue total DNA background or with a dual band-pass filter (Omega) for the simultaneous detection of red, green and yellow signals and a single band-pass filter (Zeiss) for the detection of blue total DNA background. Spermatozoa were scored for the number of red, green and yellow signals present within the DAPI-stained sperm head. Disomy but not nullisomy was scored, since failure to detect a signal could be due to technical difficulties as well as to non-disjunction. Stringent criteria were applied before a sperm head was classified as disomic or diploid, i.e. the two signal domains had to be of equal intensity, separated from one another by at least one signal domain, regular in appearance, and clearly positioned within the sperm head. Statistical analysis was performed using standard goodness of fit tests.

**Results**

We were able to recover 1205 spermatids from eight adult XXY males. These cells were scored for the number of chromosome 8, X and Y signals, and the results compared with similar observations on 14 228 spermatids from five control XY males (Table II). Although slight male-to-male variation occurred, it was not significant and data from cases and controls were pooled for statistical analyses.

The overall level of disomy among spermatozoa from XXY mice was 1.16%, highly significantly elevated over the 0.16% value observed in the XY controls ($\chi^2 = 46.44; P < 0.001$). This difference was primarily due to an increase in XY disomy in the spermatozoa from XXY males as compared with XY controls ($\chi^2 = 58.03; P < 0.001$). However, we also observed a 3-fold increase in sex chromosome aneuploidy resulting from meiosis II errors (XX and YY disomy) and a 4-fold increase in the level of diploid spermatozoa in XXY mice as compared with XY control mice. Furthermore, XXY but not XY male mice occasionally produced hyperhaploid spermatozoa with more than two sex chromosomes; three such spermatozoa were identified (two 8XY and one 8XX), possibly representing successive MI and MII non-disjunctional errors or errors during the mitotic divisions immediately preceding meiotic entry. Considered separately, none of the abnormalities was markedly elevated in the spermatozoa of XXY mice; however, taken together the overall incidence of abnormal spermatozoa, excluding XY disomy, was 0.75% (9/1205), highly significantly elevated over the 0.15% (21/14 228) value observed in XY controls ($\chi^2 = 20.6, P < 0.001$).

In addition to analyses of aneuploidy and polyploidy, we examined the proportion of X- and Y-bearing cells among normal haploid spermatozoa. We found no evidence of deviations from a 1:1 ratio of X-bearing to Y-bearing spermatozoa among any of the individual males, nor were there any deviations in the pooled data from XXY mice compared with XY control mice.

**Discussion**

The results of the present study provide evidence for an increase in numerical abnormalities in the spermatozoa from XXY mice. The effect was largely attributable to an increase in XY disomy, but other numerical abnormalities were also elevated in the XXY males.

We can think of at least two explanations for an increase in numerical abnormalities in spermatozoa from XXY mice: (i) the XXY testis might be populated by XXY germ cells, resulting in the production of both normal and disomic spermatozoa; or (ii) the testis might be populated by XY germ cells but, owing to deficiencies in the XXY testicular environment, these germ cells might be susceptible to a variety of meiotic errors. As indicated in Table III, both explanations predict increases in XY and XX disomy, but they differ with regard to other expectations. In the former model the effects are attributable to segregation in XXY germ cells. Thus, only two types of chromosomally abnormal spermatozoa—XY and XX disomies—are expected, but the ‘sex ratio’ of euploid spermatozoa should be perturbed. Although the extent of this sex ratio skewing depends on the relative proportions of the several possible sex chromosome pairing configurations, virtually all configurations favour the generation of X-bearing spermatozoa. In contrast to the former model, the ‘testicular environment’ model assumes that it is the meiotic process—not the genetic composition of the germ cell—that is abnormal. Thus, this model predicts an increase in multiple categories of numerical abnormality, including autosomal disomy and diploidy, but predicts no alteration in the proportion of X- to Y-bearing spermatozoa among euploid spermatozoa.

Two aspects of the present results argue in favour of the ‘testicular environment’ model. First, we observed increases in multiple categories of numerical abnormality, including increases in XY, XX and YY disomy, in other types of hyperhaploidy, and in diploidy in spermatozoa from XXY males. The effect was most pronounced for XY disomy. However, this may simply reflect the increased susceptibility of the XXY bivalent to non-disjunction, e.g. FISH sperm studies of human male non-disjunction indicate that, even in chromosomally normal donors, XY disomy is significantly more common than autosomal disomy or XX or YY disomy (Downie et al., 1997; Egozcue et al., 1997; Hassold, 1998).

Second, we found no evidence for an increase in X-bearing spermatozoa among euploid spermatozoa, as would be expected if the progenitor germ cells had an XXY sex chromosome complement. These results are consistent with our previous studies of the adult XXY testis, in which we found that cells with an XXY sex chromosome complement were restricted to somatic tissues, with the rare surviving germ cells having an...
Table II. Abnormal spermatozoa identified in FISH studies of testicular spermatozoa from XXY and control XY mice

<table>
<thead>
<tr>
<th>Haploid</th>
<th>Disomic</th>
<th>Diploid</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>8X</td>
<td>8Y</td>
<td>8XY</td>
<td>8XX</td>
<td>8YY</td>
</tr>
<tr>
<td>XXY 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>83</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>53</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>39</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>93</td>
<td>85</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>261</td>
<td>237</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>617</td>
<td>568</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>(%)</td>
<td>(51.2)</td>
<td>(47.1)</td>
<td>(0.91)</td>
<td>(0.08)</td>
</tr>
</tbody>
</table>

XY 1 2703 2690 3 4 1 1 | 1 1 2 | 5406 |
| 2 987 1011 1 | 1 | 2000 |
| 3 1019 972 3 | 1 | 2000 |
| 4 2045 1949 3 | 1 | 4000 |
| 5 407 414 1 | 822 |
| Total 7161 | 7036 | 10 | 6 | 5 | 2 | 4 | 1 | 3 | 14228 |
| (%) | (50.3) | (49.5) | (0.07) | (0.04) | (0.04) | (0.01) | (0.03) | (0.01) | (0.02) |

Table III. Expected meiotic outcomes, assuming different causes of aneuploidy in spermatozoa from XXY males

<table>
<thead>
<tr>
<th>Cause of aneuploidy</th>
<th>Expected outcomes</th>
<th>Ratio of X:Y-bearing spermatozoa in euploid spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segregation of sex chromosomes in XXY germ cells</td>
<td>Unchanged</td>
<td>Increased</td>
</tr>
<tr>
<td>Increased risk of meiotic errors in XY germ cells due to deficiencies in testicular environment</td>
<td>Increased</td>
<td>Increased</td>
</tr>
</tbody>
</table>

For human XXY males, the situation is less clear. There is relatively little information on the genetic make-up of spermatozoa or germ cells in XXY males, and the available data are contradictory (Table I). For example, in FISH studies of ejaculated spermatozoa from an apparently non-mosaic XXY male, Guttenbach et al. (Guttenbach et al., 1997b) reported relatively modest and similar increases in XY and XX disomy, an increase in the only autosomal disomy (disomy 1) studied by comparison with previous laboratory studies of fertile males (Guttenbach et al., 1997a), and a 1:1 ratio of X- to Y-bearing cells among euploid spermatozoa, observations consistent with the ‘XXY germ cell’ model. In the only other FISH sperm study of an apparently non-mosaic XXY (Estop et al., 1998), only 19 cells were analysed, making any extrapolations difficult. Results of meiotic studies of testicular preparations from KS individuals have also been equivocal. In an analysis of diakinesis preparations from a mosaic XY/XXY individual (Rajendra, et al., 1981), no evidence was found for the presence of XXY germ cells, suggesting that they were eliminated from the meiotic process. This interpretation is consistent with earlier reports (e.g. Steinberger et al., 1965; Luciani and Stahl 1978). However, in an analysis of diakinesis in a non-mosaic KS individual (Skakkebaek et al. 1969), two cells with 24 elements (presumably representing the 22 autosomal bivalents, an X bivalent, and a univalent Y chromosome) were reported. Similarly, Vidal et al. (Vidal et al., 1984) reported finding occasional cells with 24 elements in synaptonemal complex preparations from an XY/XXY mosaic. Thus, the results of the testicular studies have provided both evidence for and against the presence of XXY germ cells in KS individuals.

The basis for these discrepancies remains unclear, and could derive from any of several factors, including technical variation...
among the studies, real inter-individual variation, or the small number of observations from XXY and XY/XXY individuals. Nevertheless, on balance, we think that the human data are more compatible with the ‘testicular environment’ model than with the ‘XXY germ cell’ model. This conclusion is based on the fact that, in several of the reports purported to support the ‘XXY germ cell’ model, the data can be interpreted otherwise. For example, the presence of 24 elements in a diakinesis preparation was interpreted as evidence of a 47,XXY germ cell (Skakkebaek et al., 1969); however, this might also represent a 46,XY cell in which the X and Y chromosomes had prematurely separated and, indeed, this seems a more likely explanation for the single example provided by Skakkebaek et al. (Skakkebaek et al., 1969). Additionally, the combined frequencies of XX or XY disomy in spermatozoa from the XXY males studied by Estop et al. (Estop et al., 1998) and Foresta et al. (Foresta et al., 1998) (i.e. the first three cases listed in Table I) were 37%, 22% and 14%, values much lower than the 50% anticipated from segregation of XXY germ cells. Similarly, in their analysis of an XY/XXY individual, Chevret et al. (Chevret et al., 1996) reported a highly significant increase in XY diploidy, an error presumably unrelated to malsegregation of XXY cells.

Thus, we conclude that, with the exception of the report of Foresta et al. (Foresta et al., 1998), there is little evidence that XXY germ cells are capable of completing meiosis. This interpretation is at variance with those of other investigators (e.g. Cozzi et al., 1994; Chevret et al., 1996; Guttenbach et al., 1997b; Estop et al., 1998; Foresta et al., 1998), and should be viewed with some caution; clearly additional studies of XXY males would be useful in resolving this discrepancy. Nevertheless, in the absence of any compelling evidence from humans, we think it reasonable to conclude that man and mouse behave similarly and that, in humans as in the mouse, the rare germ cells identified in the adult XXY testis have a normal XY sex chromosome complement.

If our interpretation is correct, it may have important implications for other types of male factor infertility. That is, the deficiencies in the XXY testicular environment may be shared by other forms of male infertility, implying a predisposition to aneuploidy for chromosomally normal, as well as KS, individuals. For example, elevated follicle stimulating hormone levels are a hallmark of infertility in chromosomally normal males (Check et al., 1995; Reyes-Fuentes et al., 1996; reviewed in Martin-du-Pan and Bischof, 1995), as well as a characteristic feature of KS (Schill et al., 1984). Indeed, plasma concentrations of gonadotrophins, androgens and prolactin are indistinguishable in males with Sertoli cell-only syndrome, hypospermatogenesis and meiotic arrest (Bar-On et al., 1995; Foresta et al., 1998). Thus, if the increase in meiotic errors in the XXY testis is a reflection of disturbances in the testicular endocrine environment, an increase in meiotic errors may be a generalized feature of male infertility. Consistent with this view, several studies have reported an increase in chromosome abnormalities—and, in particular, sex chromosome abnormalities—in infertile or subfertile males (Moosani et al., 1995; Bernardini et al., 1997). Moreover, there is contradictory evidence for an increase in chromosome abnormalities in pregnancies initiated through intracytoplasmic sperm injection (Feichtinger et al., 1995; In’t Veld et al., 1995; Bonduelle et al., 1996; Govaerts et al., 1996; Tarlatzis, 1996). Until the nature and causes of the increases in aneuploidy are better understood, it may well be prudent to screen all men attending infertility clinics for increased levels of sperm aneuploidy.

Acknowledgements

We thank Terry Ashley for the chromosome 8-specific probe and Sandy Narayanswami for the Y-chromosome-specific probe. This study was supported by the National Institutes of Health Research Grant R01 HD31866 to P.A.H. and a National Science Foundation Graduate Fellowship to K.M.

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Received on October 16, 1998; accepted on January 26, 1999