Investigation of Sex Chromosome Abnormalities in Teratozoospermia of Infertile Men using Fluorescence *in Situ* Hybridization

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Abstract

Fluorescence *in situ* hybridization is a fast and efficient method of investigating chromosomal abnormalities in human spermatozoa. In this study, we have established the frequency of sex chromosome disomy (XX, XY and YY) in teratozoospermia (98-100% abnormal morphology) of infertile men compared with normospermic men using double-probe FISH procedures. A total of 40000 sperms were scored in each group of semen for chromosome X and Y with overall hybridization efficiency of 97.00 per cent and 98.02 per cent in infertile men and normal men, respectively. It was found that the frequency of disomy XX, XY and YY was significantly higher in infertile men compared with normal men ($P<0.05$). It is concluded that the infertile men showed increased frequencies of sex chromomsomal abnormalities.

Key word : Sex Chromosome Abnormalities, Male Infertility

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At the chromosome level four types of abnormalities can be distingquished(1) structural aberration, numerical aberration, mosaicism and chimerism.

Human spermatozoa are haploid cells (n) which contain 22 autosomes and one sex chromosome, either X or Y. Disomy (hyperhaploidy) is the condition in which a spermatozoa has an extra chromosome (n+1). Disomy and nullisomy are collectively called aneuploidy. It can lead to infertility, mental retardation and behavioural abnormalities(2). Therefore, studying the incidence of

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aneuploidy in sperm is important. However, examination of sperm morphology alone has not proven reliable to determine the chromosomal constitution[3].

Since 1984, *in situ* hybridization has been used to study the composition of spermatozoa. Initially, the results obtained with isotopic probes[4] were limited due to conventional limitations. The recent development of fluorescence *in situ* hybridization, whereby a specific DNA probe is hybridized to complementary sequences on a target chromosome, then visualized using fluorescent haptens can be applied to detect aneuploidy in human spermatozoa using two or three probes simultaneously. This has been performed in several studies [5-12]. although variable methodology and scoring, have limited the specificity of results.

We have reported the determination of the ratio between X and Y bearing spermatozoa using double-label fluorescence *in situ* hybridization[13] and a few aneuploid sperm cells (XX, XY and YY) were identified. In this study, we established the incidence of sex chromosome disomy in teratozoospermia (98-100% abnormal morphology) of 20 infertile men using double-probe FISH.

MATERIAL AND METHOD

Sample recruitment

a) Infertile men

Twenty infertile patients were selected from men who came to our laboratory for semen analysis. The semen characteristics such as a volume, motility and concentration were normal according WHO criteria[14] whereas the range of abnormal morphology was between 98-100 per cent[14].

b) Normal men

Twenty normal healthy men with a history of having children were volunteers. All donors gave their informed consents prior to this study.

Sample collection and preparation

Fresh semen samples were collected from the two groups of subjects. After 30 minutes, each semen sample was analysed by a computer-assited semen analyzer (HTM, IVOS Version 10) using WHO criteria,[14] then washed twice by centrifugation at 320 g for 10 minutes, in phosphate-buffered saline (PBS), pH 7.3. The last washing was performed in 0.1 M Tris-HCl (pH 8.0) and cells were resuspended at a concentration of 50 X 10^6 sperm/ml, then smeared onto clean microscope slides. After being allowed to air-dry, they were dehydrated in an ethanol series.

Sperm decondensation

Sperm heads were decondensed by incubation in a decondensation solution 0.1 M Tris-HCl (pH 8.0) containing 0.01 M DTT and 0.01 M LIS (dithiothreitol and lithium salt) for 30 minutes and 1-3 h, respectively. The slides were rinsed in 2X SSC (pH 7.0), then in deionized water and finally air dried.

Preparation of DNA probes

The probe for the X chromosome was centromere-specific alpha satellite DNA (band p 11.1-q 11.1, locus DXZ 1) directly labeled with Spectrum Orange, whereas the Y chromosome probe was labeled with Spectrum Green and hybridized to satellite III (band Y q 12, locus DYZ 1). All probes were purchased commercially (Vysis Inc, USA).

Fluorescence in-situ hybridization (FISH)

After treatment with RNase A (100 µg/ml in 2X SSC pH 7.0) at 37°C for 60 minutes, the slides were washed in deionized water three times for 5 minutes, then dehydrated in an ethanol series (80%, 90% and 100% ethanol) and air dried. For the simultaneous denaturation of slides and probes, a hybridization mixture containing 7 µl of CEP hybridization buffer, 1 µl of CEP probe and 2 µl of deionized water was applied to each slide (10 µl), sealed under a coverslip with rubber cement, then denatured at 72°C for 10 minutes and hybridized overnight (16-18h) at 37°C in a moist chamber. The slides were then washed in 50 per cent deionized formamide in 2X SSC at 45°C for 5 minutes, followed by 3 washings at 60°C in 0.1 X SSC and air dried in darkness. Finally, the slides were covered with DAPI II (Vysis), then sealed with nail varnish and stored in the dark at-20°C.

Scoring criteria

Slides were coded and scored blindly by one observer (J.N) using an Olympus BX 50 microscope (Olympus, Tokyo, Japan) equiped with epi-fluorescence and a triple band-pass filter block (DAPI / FITC / Texas Red). This facilitated simultaneous observation of the green and red hybridization signals.

Table 1. Sex chromosome disomy estimates in spermatozoa of 20 normal men.

Donor no.	XX	YY	XY
1	0.05	0.05	0.10
2	-	0.05	0.05
3	0.05	0.05	0.20
4	0.05	0.05	0.20
5	-	0.05	0.05
6	0.05	0.05	0.10
7	0.05	0.05	0.10
8	-	0.05	0.15
9	0.05	-	0.15
10	0.05	-	0.15
11	0.05	0.05	0.05
12	-	-	0.20
13	0.05	0.05	0.15
14	-	0.04	0.08
15	0.05	-	0.10
16	-	0.04	0.08
17	0.05	0.05	0.15
18	0.04	-	0.09
19	0.05	0.05	0.10
20	-	0.05	0.15
$\bar{X}$	0.03	0.03	0.12

Table 2. Sex chromosome disomy estimates in spermatozoa of 20 infertile men.

Patient no.	XX	YY	XY
P.1	0.24	0.10	0.20
P.2	0.05	0.15	0.40
P.3	0.15	0.20	0.75
P.4	0.15	0.20	0.30
P.5	0.15	0.20	0.60
P.6	0.15	0.15	0.45
P.7	0.15	0.15	0.30
P.8	0.15	0.15	0.55
P.9	0.10	0.20	0.35
P.10	0.15	0.10	0.70
P.11	0.15	0.25	0.60
P.12	0.10	0.20	0.40
P.13	0.10	0.15	0.55
P.14	0.14	0.14	0.39
P.15	0.15	0.05	0.20
P.16	0.25	0.35	0.40
P.17	0.50	0.25	0.55
P.18	0.15	0.20	0.30
P.19	0.15	0.05	0.20
P.20	0.10	0.10	0.20
$\bar{X}$	0.16	0.17	0.42

The following scoring criteria were used :

a) Slides were scored only if the hybridization efficiency was ≥ 97 per cent.
b) Only nuclei with an attached tail were scored, not overlapping or clumped sperm.
c) Nuclei were not over decondensed or had indistinct boundaries.
d) Two signals were scored as disomic (XX, XY and YY) if they were of similar size.
e) Two-thousand spermatozoa were scored from each patient.

Statistical analysis

The frequency of sex chromosome disomy was established for each semen sample. Because in all cases the data were nonparametric, the Mann-Whitney U-test was used to compare data in between two groups. A $P < 0.05$ value was accepted as significant.

RESULTS

A total of 40,000 sperm were scored in each group of semen for chromosomes X and Y with an overall hybridization efficiency of 98.02 per cent and 97.00 in normal men and infertile men, respectively. Disomy XX, XY and YY as discerned per semen sample is presented in Table 1 and Table 2. It was found that there were differences between both groups of semen. Table 3 shows the frequency of disomy XX, XY and YY was significantly higher in infertile men compared to normal men ($P < 0.05$).

DISCUSSION

Since fluorescence *in situ* hybridization has been applied to study chromosomal abnormalities of cells in interphase it has become possible

Table 3. Frequencies of disomy in infertile men and normal men*.

Disomy for chromosome	Infertile men (n=20)	Normal men (n=20)
XX	0.16+	0.03
XY	0.42+	0.12
YY	0.17+	0.03

* Values are percentages.
\+ Statistically significant difference compared to normal men ($P < 0.05$)

to accurately determine the frequency for each chromosome. Therefore, studies of human spermatozoa eneuploidy have been reported by several researchers[4-6,8-12]. However, early reports used a single-probe[4], which does not allow distinguishing between diploid cells and disomic cells nor to identify nullisomy or a failure of probe hybridization. When the FISH procedures were modified to use double-and triple-probes, these problems could be overcome by using a control probe signal. Nevertheless, differences to most data remain because of differences in methodology, criteria applied and subjects studied. Therefore, it is difficult to compare these results.

In this study, we have established the frequency of sex chromosome disomy (XX, XY and YY) in morphologically abnormal spermatozoa (98-100%)[14] of infertile men compared with normospermic men using double-probe FISH procedures. The FISH method has produced clear signals and a ≥ 97 per cent hybridization efficiency with infertile men and ≥ 98 per cent with the control group. We found that the incidence of disomy XY was higher than that of XX and YY in both sample groups, which is not entirely unexpected as there is evidence to suggest that the sex chromosomes are more susceptible to first meiotic segregation errors [15]. In addition, in two infertile patients (P 16 and P 17) we found two additional sex chromosomes (XXYY) but only a few cells (1-2 per semen sample scored) were detected.

Comparison of the sex chromosome disomy in our normospermic men to the control group and those reported by others, showed the values to be similar[10-12,16]. In't Veld et al[17], presented a case report on infertile men with high levels of sex chromosome disomy and multiple abnormalities in semen with 100 per cent macrocephalic sperm heads, one to three tails per cell, absence of the acrosome cap, a sperm concentration of $15X10^6$/ml and a progressive motility of 20 per cent. However, they reported a much higher incidence of sex chromosome aneuploidy (22%) because their patients differed from ours and were more heterogeneous, whereas Moosani et al[18], reported that in infertile men the frequency of disomy XY was significantly higher than in normal men except for disomy XX and YY. However, this study was undertaken in only five patients.

In this study, the authors also examined sperm with multiple defects in quality of five infertile patients, P3, P5, P10, P11 and P17, that they showed a motilily of 25 per cent, 10 per cent, 7 per cent, 2 per cent and 3 per cent respectively, and abnormal morphology in 98-100 per cent. These spermatozoa showed a higher incidence of sex chromosome disomy (1.1%, 0.95%, 1.0% and 1.3% respectively) in the group. However, they comprised only 5 of the patients which was insufficient for estimation, so further studies of these semen characteristics are required to confirm the results. Nevertheless, our findings provide evidence of a higher incidence of sex chromosome disomy in spermatozoa of subfertile men as previously demonstrated by In't Veld et al[17]. In addition, the result of this study shows an increased possibility of inadventureously performing intracytoplasmic injection of sex chromosome disomic spermatozoa in treatment of male infertility due to abnormal sperm morphology.

This investigation confirms the usefulness of fluorescence *in situ* hybridization to study chromosome aberration in spermatozoa. Our data indicate that in infertile men with morphologically abnormal spermatozoa (98-100%) sex chromosome disomy was increased when compared with normal male spermatozoa. It is important to define the abnormal range of aneuploidy in human spermatozoa because this information provides a baseline for clinical studies. Therefore, our studies should be related to the incidence of chromosomally abnormal embryos which are both pre- and postimplantation. Ongoing studies in our laboratory are aimed at studying aneuploidy in embryos as a future genetic screening procedure.

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การตรวจหาความผิดปกติของโครโมโซมเพศ X และ Y ในอสุจิชายที่มีบุตรยากโดยวิธี Double labeled fluorescence *in situ* hybridization

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การตรวจหาความผิดปกติของโครโมโซมในอสุจิมนุษย์ด้วย fluorescence *in situ* hybridization (FISH) ถือว่าเป็นวิธีที่มีความถูกต้องแม่นยำและรวดเร็วที่สุดในปัจจุบัน การศึกษานี้ได้รายงานการตรวจพบความผิดปกติของโครโมโซมเพศที่เป็น disomy XX, XY และ YY ในอสุจิชายที่มีบุตรยากเปรียบเทียบกับอสุจิชายที่ปกติ โดยคัดเลือกตัวอย่างน้ำอสุจิจากผู้ชายที่ได้รับการรักษาปัญหาการมีบุตรยาก ผลการตรวจวิเคราะห์พบอสุจิที่มีรูปร่างผิดปกติ 98–100% (teratozoopermia) กลุ่มน้ำอสุจิที่ปกติได้จากผู้ชายที่มีบุตรแล้วอย่างน้อย 1 คน และคุณภาพอสุจิอยู่ในเกณฑ์มาตรฐานปกติ การตรวจสอบโดยใช้ double-probe FISH ในอสุจิทั้งสองกลุ่ม ๆ ละ 20 ราย ตรวจนับอสุจิจำนวน 40,000 ตัวในแต่ละกลุ่ม ผลพบว่าในอสุจิชายที่มีบุตรยากมีความผิดปกติของโครโมโซมที่เป็น disomy XX, XY และ YY สูงกว่าชายที่ปกติอย่างมีนัยสำคัญทางสถิติ (XX = 0.16 *vs* 0.03%, XY = 0.42 *vs* 0.12% และ YY = 0.17 *vs* 0.03%, $P < 0.05$)

คณะผู้วิจัยจึงสรุปว่าในน้ำอสุจิที่ตรวจวิเคราะห์แล้วพบตัวอสุจิมีรูปร่างผิดปกติสูง (98–100%) จะมีแนวโน้มของความผิดปกติของโครโมโซมเพศสูงกว่าน้ำอสุจิที่ปกติ

คำสำคัญ : โครโมโซมเพศผิดปกติ, ชายมีบุตรยาก

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