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A STUDY OF INDIVIDUAL CHROMOSOMES IN HUMAN EJACULATED SPERM BY FLUORESCENCE *IN SITU* HYBRIDIZATION

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ABSTRACT

The aims of the present study have been:

1. To determine the feasibility of detecting specific chromosomes in ejaculated human sperm using chromosome-specific DNA probes and fluorescence *in situ* hybridization (FISH).

2. To use this methodology to study specific aspects of male fertility including the sex ratio of X- and Y-bearing spermatozoa and the frequency of aneuploidy.

3. To apply the technology to discover whether there are any alterations in the sex ratio or the frequency of aneuploidy after manipulations designed to isolate motile sperm.

4. To examine the chromosome content of sperm of an XYY individual and of men with poor sperm morphology.

Preliminary experiments used a tritium-labelled X chromosome probe (TRX) to detect X-bearing human sperm by isotopic *in situ* hybridization. The cells were pretreated with SDS/DTT and a total of 6,197 sperm were scored from six normal donors. The frequencies of sperm labelled with the TRX probe, varied from 8.2 to 48.5%. Considering the variation in labelling efficiency it was concluded that sperm pretreatment with SDS and DTT was not satisfactory. Decondensation of sperm nuclei by sequential incubation in EDTA and DTT was found to be superior to SDS/DTT pretreatment and since isotopic *in situ* hybridization has a number of drawbacks (radioactive, lengthy autoradiography), FISH (fluorescence *in situ* hybridization) was introduced.

Initially the application of single label FISH to human sperm was assessed using biotinylated chromosome 17-specific (TR17) and X chromosome-specific (TRX) probes (Han et al., 1992). Semen samples from 13 healthy donors were evaluated. A labelling frequency of 96% was obtained using TR17 (14,095 cells scored), which included 95.4% haploid, 0.33% disomic, and 0.37% diploid. The labelling frequency of TRX was 48.2% and included 47.7% haploid, 0.29% disomic, and 0.20% diploid (13,396 cells scored). Regression analysis showed that there was a positive correlation ($r=0.83$, $P<0.001$) between the percentages of labelling by the autosomal (TR17) and the X-chromosome probes.

When only one sex chromosome probe is used, there will always be doubt as to whether the non-fluorescent cells are either nullisomic, contain the unlabelled sex chromosome or have failed to hybridize. Therefore a double label FISH technique was developed to enable the simultaneous detection of X- and Y-bearing sperm. The biotin-labelled TRX probe and a digoxigenin labelled Y chromosome-specific probe (HRY) were used in conjunction with fluorescein isothiocyanate (FITC) and tetramethethylrhodamine isothiocyanate (TRITC) fluorescence detection system respectively. Semen samples from 12 normal healthy donors were examined and 96% of the sperm (12,636 scored) were labelled with the TRX and HRY probes (Han et al. 1993). The overall proportion of X- to Y-bearing sperm was 47.9% to 47.2%. Chi-squared (χ^2) analysis showed that none of the individuals had ratios of X- and Y-bearing sperm significantly different from a 1:1 ratio. To my knowledge, this is the first study which conclusively confirms a 1:1 ratio of X:Y sperm in human ejaculates. Frequencies of 47.4% haploid X and 46.8% haploid Y sperm were found. In all, 0.46% were XX-sperm (0.28% disomic and 0.18% diploid) and 0.38% were YY-sperm (0.21% disomic and 0.17% diploid). In addition, 0.88% of cells contained X and Y chromosomes (0.21% haploid XY, 0.62% diploid XY and 0.05% tetraploid XXYY). The diploid XY and tetraploid XXYY-bearing cells were thought likely to be leukocytes or immature germ cells rather than mature sperm cells.

To assess the ratio of X- and Y-bearing sperm and the level of aneuploidy in motile spermatozoa, populations of motile sperm were isolated from ten donor semen samples using the swim-up procedure, and sperm from the neat semen (control) and swim-up fractions were then evaluated using the TRX/HRY probes and the double FISH methods. Overall 95% of sperm were labelled with the TRX and HRY DNA probes in both original semen and swim-up fractions. The proportions of the total X- and Y-bearing sperm were respectively 47.3% to 46.9% (original semen) and 48.4% to 47.1% (swim-up fraction); these were not significantly different from a 1:1 ratio. No changes were detected in the frequency of aneuploid spermatozoa in the neat ejaculates but the frequency of diploid cells was decreased after swim-up. Since the isolation of motile spermatozoa by swim-up is a routine procedure prior to intrauterine insemination (IUI), *in-vitro* fertilization (IVF) and gamete intrafallopian transfer (GIFT), the results of this study are an important demonstration that the sex ratio is not altered by this method of sperm preparation.

Investigation of sex chromosomes in sperm from 47,XYY men is of interest to study the fate of the extra Y chromosome during spermatogenesis. To date there is only one report on the ratio of X- and Y-bearing sperm in semen from an XYY individual using the zona free hamster oocyte method. In the present study, two semen samples and a swim-up fraction from a 47,XYY man were examined using biotin-labelled TR17 probe with single FISH procedure, and biotin-labelled TRX and a digoxigenin-labelled HRY probe with a double FISH technique. Ninety six per cent of the sperm were labelled with either the TRX or HRY probes in the original semen (2,006 cells scored), while 98% were labelled in the swim-up fraction (1,003 cells scored). The overall ratio of X- to Y-bearing sperm was 47% to 45.3% in the original samples, and 48.4% to 49.3% in the swim-up fraction. Neither of these ratios was as significantly different from a 1:1 ratio and the frequencies of haploid and disomic sperm were not significantly different from the previously examined normal donors. However, the frequencies of diploid cells (XY-2.7%, 17-2.6%) and tetraploid-17 cells (0.2%) were significantly higher than in normal semen. In the

swim-up fraction, none of the frequencies of haploidy and disomy was significantly different from the ten normal semen samples following swim-up isolation. The present results show that there is a 1:1 ratio of X to Y-bearing sperm in the semen of this XYY man and supports the hypotheses that the extra Y chromosome in XYY men is eliminated during spermatogenesis.

The FISH methodology was used to evaluate if there is an association between numerical chromosomal abnormalities (aneuploidy) and abnormal sperm morphology. Semen samples were obtained from four infertility patients whose sperm morphology was very poor (1-8% normal). The TR 17 probe was used to assess the incidence of autosomal aneuploidy, and TRX/HRY probes were used both to assess aneuploidy of sex chromosomes and to determine whether or not the X:Y ratio was altered in men with teratozoospermic semen. The results showed that the frequencies of haploidy and disomy for chromosomes 17, X and Y in these samples were within the normal range, and it thus appears that abnormal sperm morphology is probably not associated with higher levels of aneuploidy. Higher frequencies (3.3% - 4.2%) than normal (0.37% - 1.0%) of polyploid (including diploid) cells were observed in these semen samples ($p<0.00001$).

In conclusion, FISH using multiple chromosome-specific DNA probes is an efficient and practical means of sexing and studying aneuploidy in human sperm. It has several advantages over other techniques. Firstly, it is not radioactive, and is therefore safe and since FISH requires no exposure time, it is fast. Secondly, representative samples of the entire sperm population can be studied rather than only a selected subgroup of sperm such as those which can fuse with zona-free hamster eggs, the only alternative technique. Thirdly, the double labelling with X- and Y-specific probes identifies X- and Y-bearing sperm simultaneously and therefore the sex status of the sperm can be determined with certainty. In contrast, FISH procedures which use a single sex chromosome probe only identify either the X- or Y-bearing sperm and an assumption is made that the non-labelled cells carry

the other sex chromosome. In future, the double FISH method using X- and Y-specific probes will prove useful as an assay for example to test the validity of X and Y sperm separation procedures, and the development of multiple label FISH procedures using autosomal and sex chromosome probes will enable the accurate determination of aneuploidy levels in human sperm.