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

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DECLARATION

I declare that this thesis describes the results of research carried out in the Department of Genetics at The Queen Elizabeth Hospital and the Department of Obstetrics and Gynaecology of The University of Adelaide, at The Queen Elizabeth Hospital, South Australia. The research was carried out between June 1989 and May 1993, during the tenure of a Reproductive Medicine Postgraduate Scholarship from The University of Adelaide and Genetics Department at The Queen Elizabeth Hospital.

The results embodied in this thesis are my own work accomplished under the supervision of Prof. Colin D. Matthews, Dr. Judith H. Ford and Dr. Graham C. Webb, unless otherwise acknowledged in the text.

I give consent for this copy of my thesis, deposited in the Library of The University of Adelaide, to be photocopied or loaned.

Tie Lan Han

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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.

PUBLICATIONS

arising from experiments presented in this thesis

Articles:

Han TL, Webb GC, Flaherty SP, Correll A, Matthews CD, Ford JH (1992): Detection of chromosome 17- and X-bearing human spermatozoa using fluorescence *in situ* hybridization. Molec Reprod Dev 33: 189-194

Han TL, Ford JH, Webb GC, Flaherty SP, Correll A, Matthews CD (1993): Simultaneous detection of X- and Y-bearing human sperm by double fluorescence *in situ* hybridization. Molec Reprod Dev 34: 308-313

Ford JH, Han TL, Webb GC (1993): Dual-label fluorescence *in situ* hybridization detection of individual chromosomes in sperm using ejaculated semen. In Choo KHA (ed) "*In situ* hybridization protocol (Methods in Mol. Biol. series)" N.J. Human Inc. Press (in press)

Han TL, Flaherty SP, Ford JH, Matthews CD (1993): Detection of X- and Y-bearing human spermatozoa following motile sperm isolation by swim-up. Fertil. Steril. (submitted)

Han TL, Ford JH, Webb GC, Flaherty SP, Matthews CD (1993): Chromosomes of human ejaculated sperm in a 47,XYY male. J Med Genet (submitted)

Abstracts of papers presented at conferences:

Han TH, Ford JH, Webb GC, Flaherty SP, Correll A, Matthews CD (1992): Simultaneous detection of X- and Y-bearing human sperm by double fluorescence *in*

situ hybridization. Twenty Fourth Annual Scientific Conference, Australia Society for Reproductive Biology. September 28-30 1992) University of Adelaide. pp 94.

Han TL, Flaherty SP, Ford JH, Webb GC, Matthews CD (1992): Simultaneous detection of X- and Y-bearing human sperm using double fluorescence *in situ* hybridization (FISH). Proceedings of the 11th Annual Scientific Meeting, Fertility Society of Australia, 2nd-5th December, 1992. Adelaide, South Australia. pp 39FC.

ABBREVIATIONS AND SOME BASIC CONCENTRATIONS

bp . . . . . .base pair(s)

BrdU . . . . . .bromodeoxyuridine

cDNA . . . . . DNA complementary to RNA

DABCO . . . . . .1,4-diazo bicylo-(2,2,2) octane, anti-fade reagent

DAPI . . . . . .4,'6-diamidino-2-phenylindole

dNTP . . . . . 3' deoxynucleoside triphosphate

DTT . . . . . dithiothreitol

EDTA . . . . . ethylenediaminetetraacetic acid

FISH . . . . . .fluorescence *in situ* hybridization

FITC . . . . . .fluorescein isothiocyanate

FrdU . . . . . .fluorodeoxyuridine

Hepes-HTFM . . Hepes buffered human tubal fluid medium

ISH . . . . . .*in situ* hybridization

kb . . . . . .10^3 base pair(s)

LB Medium . . . Luria-Bertani Medium; 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl in 1 L deionized H_2O

PBS . . . . . .Phosphate buffered saline

SDS . . . . . .sodium dodecyl sulphate

SSC . . . . . .1 x SSC = 0.15 M sodium chloride and 0.015 M sodium citrate

SSCP . . . . . .10x = 0.12 M NaCl, 0.15 M Sodium Citrate, 0.1 M NaH_2PO_4

STE . . . . . .0.1 M NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA

$T_{10}E_1$ . . . . . .10 mM Tris-HCl, pH 7.5 - 1 mM EDTA

TAE . . . . . .1 x TAE = 0.04 M Tris-acetate, 0.002 M EDTA pH 8.0 $T_{10}E_1$ 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA

Tris-HCl . . . . . .Tris (hydrochloride) aminomethane

TRITC . . . . . .tetramethethylrhodamine isothiocyanate

Tween 20 . . . . . Polyoxyethylenesorbitan monolaurate

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CHAPTER ONE

GENERAL INTRODUCTION

Approximately 15% of marriages are barren and produce no offspring, and defects in the husband's reproductive system are responsible in about 50% of the cases (Wong et al., 1987). It has also recognized that the frequency of trisomy among human liveborn individuals is about 0.3%, among stillborns about 5%, and among spontaneous abortions about 25% (Hassold and Jacobs, 1984). Investigations of the reasons for aneuploidy have mainly been conducted on the maternal side (Risch et al., 1986; Hassold and Chiu, 1985) and there are very few data on any possible paternal contribution.

1.1 Chromosomes and Male Fertility

1.1.1 Meiotic chromosomes

The function of the adult male reproductive system is concerned with the production of the male sex cells or gametes, called spermatozoa, and their maturation, and transfer into the female genital tract. The success of this function depends on normal development and activity of the male sex organ or gonad - the testis.

In the testis of the adult male, a variety of cell types are found in the wall of the seminiferous tubule. Among these is the spermatogonium, which is a diploid cell, capable of mitotic division. However, it may enter the prophase stage of first meiosis, and once it does so, it is recognized as a primary spermatocyte. The last DNA synthesis occurs during interphase before the first meiotic division. Each primary spermatocyte is destined to complete both of the meiotic divisions and will

yield a tetrad of spermatids. This process reduces the number of chromosomes from the diploid number in man (46) to one-half this number (23). Each of the spermatids in turn will finally undergo transformations into a sperm without further significant changes in the genetic complement. In human, spermatogenesis takes about 64 days, starting with a spermatogonium and ending with the development of mature sperm (Figure 1.1, Alberts et al., 1983).

1.1.2 Chromosome studies in male infertility

Karyotypic studies in peripheral blood

In the last 25 years, many studies have addressed the role of somatic chromosome abnormalities in male infertility. Because different methods of ascertainment have been used in the different survey's reports, it is difficult to make a comparison of results. De Braekeleer and Dao (1991) reviewed these data, and divided them into three groups, those that studied all infertile males, those that studied only oligozoospermic men and those that studied only azoospermic men. Each group included about ten papers.

The incidence of chromosome abnormalities is much higher in subfertile and infertile males than in the general population. The most common somatic anomaly is Klinefelter syndrome. A total of 424 47,XXY males (4.6%) was found among the 9,207 men karyotyped in this review. This was 44 times higher than the frequency of 0.1% reported in newborn males. Klinefelter syndrome was mainly associated with azoospermia (11% of 1450 azoospermic men) but was also seen in 0.7% of 957 severely oligozoospermia men (sperm count $< 10 \times 10^6$/ml). Robertsonian translocations were seen in 0.8% (72 of 9207) of the males investigated for infertility, which is nine times higher than the frequency reported in newborn studies, and the probability of finding a reciprocal translocation is seven times higher in infertile men (0.6%) than in newborns (0.09%)

Spermatogonium

Primary Spermatocyte

Meiosis I

Secondary Spermatocyte

Meiosis II

Spermatid

Sperm

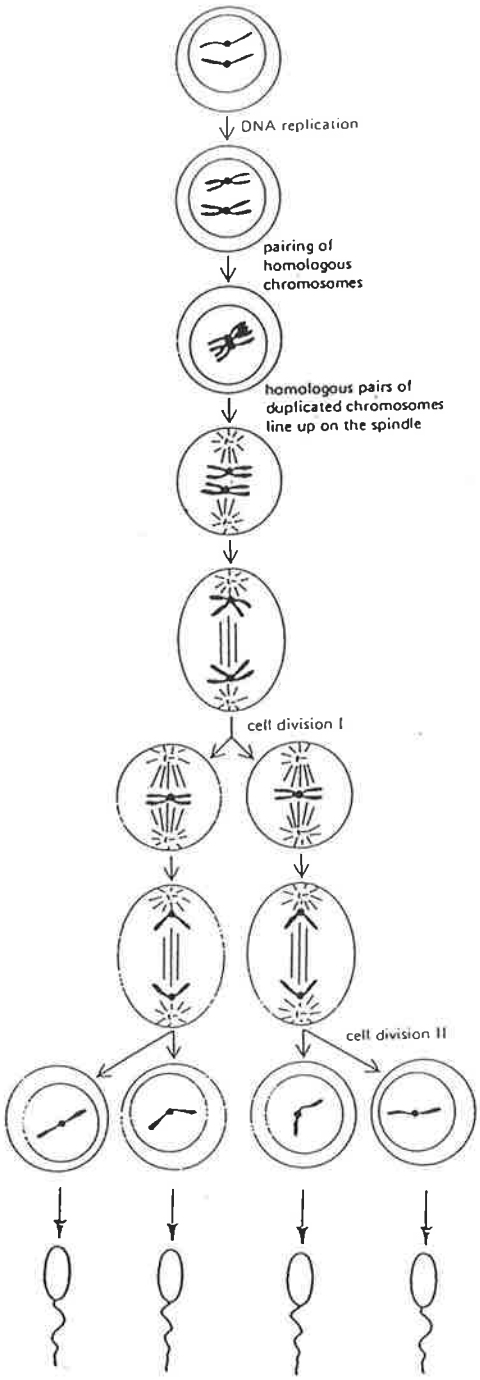


Figure 1.1*: Schematic diagram of the process of meiosis. Only one set of homologous chromosomes (homologs) is shown. The pairing of homologs is unique to meiosis; because each chromosome is duplicated and exists as attached sister chromatids before this pairing occurs. Two nuclear didvisions are required to produce the haploid gamates. Each diploid cell that enters meiosis therefore produces four haploid cells.

* taken from Alberts et al. (1983)

Meiotic studies in testicular biopsies

A summary of published reports on the incidence of meiotic chromosome abnormalities in infertile men is given in Table 1.1. The incidence of meiotic anomalies in males with fertility problems stands between 4.4% (Egozcue et al., 1983) and 17% (Hendry et al. 1976), with a mean of 7.9% (Table 1.1) (see De Braekeleer & Dao, 1991 for review). These abnormalities include: a low chiasma count, spermatogenic arrest (low ratio of MII/MI), the presence of univalents (an indication of desynapsis, asynapsis due to non-pairing of sex chromosomes or homologous autosomes in MI) bivalent fragmentation, asymmetrical bivalents and polyploidy.

Meiotic studies on ejaculated sperm

Templado et al. (1980) first reported their observations of meiosis in spermatocytes present in the ejaculated sperm from 180 men (azoospermia, severe oligozoospermia, polyzoospermia and teratozoospermia). From these studies, they identified one man with a reciprocal translocation and six oligochiasmatic males.

In 1983, Egozcue et al. studied 1,100 infertile and sterile males. Of these, testicular biopsies were studied from 599 men and the ejaculate from 501 men. They found comparable results of meiosis in ejaculated semen to those obtained in testicular biopsies. The percentage of cases with normal meiosis in ejaculated semen and testicular biopsies was different but this is thought to be due to bias in the selection of patients. The incidence of the meiotic abnormalities was 4.4% and the most common anomaly observed was meiotic arrest (3.7%).

There are problems in the interpretation of the results and in comparing the results from different studies. One of the main problems is that several different techniques have been used to study meiotic chromosomes, some using testicular

biopsy material (Evans et al, 1964; Meredith, 1969; Luciani et al, 1971), and some others using ejaculated semen samples. It is not clear whether or not the results obtained by different techniques and in different laboratories are in fact comparable. For example, Templado (1980) reported that their results were considered good in 5% of the cases, "readable" in 85%, and "unreadable" in 10%; other workers might set these criteria differently. Other problems include the presentation of the results and patient selection for the analysis. Considerable confusion still exists between the terms infertility, subfertility and sterility and there is some variation in definition arising from methods of referral, the analyses and treatments performed.

Nevertheless, it is clear that in about 7.9% of the patients (Table 1.1), meiotic chromosome anomalies contribute to infertility. Therefore, meiotic chromosome analysis is useful and should be performed, as part of the evaluation of male infertility, when no other cause can be found, but it is not a ideal technique for research.

Table 1.1: Meiotic chromosome abnormalities reported in testis from several series of infertile men*

References	Number of Men studied	Number with abnormalities	%
McIlree et al.(1966)	50	5	10.0
Hulten et al. (1974)	50	3	6.0
Chandley et al.(1976)	118	8	6.8
Ferguson-Smith (1976)	154	7	4.5
Hendry et al.(1976)	200	34	17.0
Micic et al.(1981)	90	5	5.6
Koulisher et al. (1982)	450	52	11.6
Egozcue et al. (1983)**	1,100	48	4.4
Total	**2,270**	**179**	**7.9**

* taken from De Braekeleer and Dao (1991)
** including 501 cases meiotic study within ejaculates.

1.2 Chromosome "bodies" and sperm sex selection

1.2.1 Chromosome "bodies"

Specific staining techniques have been used to differentially stain human chromosomes in sperm. Chromosome "bodies" have been recognized as chromosome 1 (Geraedts and Pearson, 1973), chromosome 9 (Bobrow et al., 1972) and Y (fluorescent bodies (Pearson and Bobrow, 1970; Sumner et al, 1971)) in human sperm. The quinacrine staining method for detecting fluorescent bodies in human sperm has been used to distinguish Y-bearing sperm.

Detection of fluorescent bodies in sperm is based on the assumption that quinacrine stains the distal part of the long arm of the Y chromosome. It is assumed that the fluorescence spots (F bodies) which can be seen in roughly 40% of the spermatozoa, represent Y bearing gametes (Barlow and Vosa. 1970). Sumner et al (1971) reported that 1.26% of sperm contain two fluorescent bodies and are therefore 24,YY. However, this seems to be high when one considers that if all chromosomes behaved in the same way, the overall level of aneuploidy in human spermatozoa would be around 40% (Hulten et al., 1985). Subsequent studies of sperm karyotypes using the "hamster" fertilization technique have also failed to confirm such high frequencies of 24,YY sperm (Martin et al., 1983).

Interestingly, a significant elevation ($P<0.0003$) of the frequency of "double fluorescent bodies" in 93 infertility males with varicocele has been recently reported by Bibbins et al. (1992). The cause of this increase is not known, but other abnormalities encountered with varicocele are thought to be associated with elevated intrascrotal temperatures (Robinson et al., 1968). Data associating aneuploidy with elevated body temperature are sparse; although, increased meiotic non-disjunction has been observed in mouse oocytes exposed to *in vitro* hyperthermic conditions (Baumgartner and Chrisman, 1981)

1.2.2 Sperm selection by sex; procedures and results

The quinacrine staining procedure has also been used to determine the ratio of X- to Y- bearing sperm to confirm previously reported methods of separating the two populations. In 1973, Ericsson and co-workers (1973) first reported that X- and Y-bearing human spermatozoa could be separated by a procedure consisting of centrifugation and migration of sperm through discontinuous albumin gradients (BSA, 6%, 10%, 20%). Samples containing up to 85% Y spermatozoa were recovered from the 20% bovine albumin fraction. A number of other laboratories (Dmowski et al., 1979; Beernink and Ericsson, 1982; Corson et al., 1984) confirmed this finding, and this patented method or its modifications are now used extensively in the U.S.A. for sex preselection. Beernink et al. (1993) collected clinical data from 65 centres and reported that 72% of 1,034 births were male after insemination with sperm selected by the albumin method. On the other hand, Evans et al. (1975) were unable to show any enrichment of Y-bearing sperm following the albumin separation using quinacrine staining. Karyotype studies (Brandriff et al, 1986a) used the human sperm/zona-free hamster oocyte, "hamster" system and examined 290 albumin-isolated sperm from six men. They found 57.2% X and 42.8% Y-bearing chromosome complements; a result which did not confirm enrichment for Y-bearing sperm.

Discontinuous Percoll gradients have also been used to separate X- and Y-bearing sperm (Kaneko et al. 1983; Iizuka et al., 1987; 1988). Iizuka et al. (1988) reported that a Percoll gradient consisting of 12 steps produced a 94% purification of X-bearing sperm (based on quinacrine staining) and they also reported that only female babies were born after using this enriched population of X sperm. However DNA methods have failed to confirm X and Y separation. Van Kooij and van Oost (1992) used a DNA probe (pDP34) which recognized distinguishable loci on both the X and Y chromosome, to examine sperm DNA after isolation of "X-bearing" sperm by Percoll centrifugation. Their results on the autoradiogram for Taq 1 digests of male

DNA showed bands of 14.6 kbp specific for the Y chromosome and either a band of 11.8 or 10.6 kbp for the X chromosome. They divided Percoll separated samples into three groups: (1) non-fractionated sperm (50% without F body), (2) the mid-portion of the gradient (58% without F body), and (3) the bottom fraction of the gradient (85% without F body). No differences in intensity between the X and Y-specific bands were discernible among the three groups and their results therefore argue against any selection for X-bearing spermatozoa by the Percoll method.

Check and co-workers (1989, 1993) reported a high percentage of male births following insemination with sperm isolated by a modified 'swim-up' procedure. These workers found that the proportion of sperm which possessed a F-body after quinacrine staining was greatly increased by the modified swim-up procedure. This result remains as equivocal as those above, although quinacrine staining has been used widely for detecting Y-bearing sperm for about 20 years, the validity and specificity of this technique has been questioned by chromosome complements and DNA analysis.

1.3 Karyotyping Sperm Chromosomes

The full complement of chromosomes from human spermatozoa can only be visualized after fertilization. Rudak et al. (1978) first demonstrated that human sperm chromosome complements can be analysed after penetration of zona-free hamster eggs. This method has enabled studies on the type and frequency of chromosomal abnormalities in human sperm and has therefore been used during the last 15 years.

1.3.1 Frequency of chromosome abnormalities in the sperm of normal men

Sperm karyotyping after fusion with hamster eggs has provided us with the first estimates of the frequency of sperm chromosome abnormalities in normal men. Results from three laboratories in three different countries showed that approximately 10% of human sperm have chromosome abnormalities, which included 0.9-5.2% aneuploidy and 3-13% structural abnormalities (see Table 1.2).

Martin et al (1987) examined the variation in the frequency and type of sperm chromosome abnormalities among 1,582 sperm from 30 normal men. The mean frequency of sperm chromosomal abnormalities in individual men was 10.4% with a range of 0-25%. For numerical abnormalities the mean was 4.7% with a range of 0-10% and for structural abnormalities in the mean was 6.2% with a range of 0-23%.

The frequency of hyperhaploid sperm complements in individual men in three large studies from Martin's laboratory demonstrated variable results (Table 1.2). They reported 2.4% hyperhaploid complements among 1,000 sperm from 30 men in 1983, 1.3% hyperhaploid complements among 1,582 sperm from 30 men in 1987, and 0.7% hyperhaploid complements among 6,821 sperm from 98 men in 1990.

Table 1.2: Frequency and type of chromosome abnormalities in sperm from normal men*

Author	Total No. of sperm	No. of donors	% Abnormality Number	hyperhaploid/ hypohaploid	structural	Total
Martin et al. (1983)	1,000	33	5.2	2.4/2.7	3.3	8.5
Martin et al. (1987)	1,582	30	4.7	1.3/3.4	6.2	10.4
Brandriff et al (1985)	2,468	11	1.7	0.7/0.9	7.7	9.4
Kamiguchi and Mikamo (1986)	1,091	4	0.9	0.5/0.5	13	13.9
Martin and Rademarker (1990)**	6,821	98	3.9	0.7/3.3	nd[1]	

nd[1] = not done

* taken from Martin RH (1988)

** not included in Martin RH (1988)

Chromosomes observed in aneuploid sperm

The analyses of human sperm chromosomes have demonstrated that all chromosome groups are represented among the aneuploid sperm, including disomy for chromosome 1, which has been reported in three sperm (Martin et al. 1983; Brandriff et al. 1985). Martin et al. (1987) reported on 1,582 sperm karyotypes and suggested that all chromosome groups have approximately the same frequency of non-disjunction with the exception of group G, in which there is a significant excess of hyperhaploid sperm.

1.3.2 Sperm Abnormalities

Martin and Rademaker (1987) using the zona-free hamster oocyte system studied sperm chromosome complements from 30 normal men, with five males in each of six age categories. They did not demonstrate a relationship between age and the frequency of numerical chromosomal abnormalities.

Exposure to mutagenic agents and sperm abnormalities

The hamster egg system was used by Martin et al (1986) to study the after effects of radiotherapy on the frequency of sperm chromosomal abnormalities in cancer patients. They found that the frequencies of both numerical and structural chromosomal abnormalities of sperm were significantly increased 36 months after radiotherapy. For individual men the frequency of sperm chromosome abnormalities ranged between 6-67% and a significant correlation between the dose of testicular radiation and the frequency of abnormalities was found.

To date sperm karyotyping after penetration of hamster eggs has been very labour-intensive and is not a practical test for screening reagents suspected of being mutagens, clastogens, or trisomogens. Additionally the method only examines a subgroup of sperm, ie those which can fuse and penetrate hamster eggs. When one considers that about 20 eggs are inseminated with 2-5 million sperm and only 20-30 sperm fuse and are karyotyped, then only a tiny subgroup of sperm is examined which may not be representative of the entire sperm population. Moreover, hamsters are not available in Australia due to quarantine restrictions.

1.4 *In situ* hybridization (ISH)

1.4.1 Isotopic ISH

Since its introduction in 1969 (Gall and Pardue, 1969), *in situ* hybridization (ISH) has formed an important bridge between cytogenetic and molecular analyses. ISH is a technique that detects DNA and RNA sequences in cytological preparations of sections of tissue (Nakamura, 1990). The nucleic acid probes can be labelled isotopically and detected by autoradiography. Nucleic acid sequences can be directly hybridized *in situ* to their complementary DNA within fixed chromosome preparations on glass slides. After autoradiography, a significant excess of silver grains may be scored within one region of a particular chromosome. This, together with improvements in the efficiency of hybridization and in the quality of chromosome banding, have made the technique of ISH sufficiently sensitive to permit the localization of single copy sequences of DNA. ISH is also an important complement to the use of deletion cell lines, somatic cell hybrids and linkage analysis, in the mapping of human DNA sequences (Buckle and Craig, 1986).

Probes are DNA or RNA segments used in various molecular hybridization techniques to detect target DNA or RNA. DNA probes include genomic probes, cDNA probes and oligonucleotide probes. Genomic DNA probes, which are derived directly from cellular or viral DNA and therefore contain both exons and introns, are most useful for the detection of target DNA. cDNA probes, derived from mRNA with the use of reverse transcriptase, contain only exonic sequences and are therefore excellent to target RNA. Synthetic oligonucleotide probes, usually 20-50 base pairs in length, may consist of introns and/or exons and may detect DNA or RNA. RNA probes , although somewhat complex to synthesize and label, are generally more sensitive, and produce less background than DNA probes (Strickler et al. 1990).

Probes are usually labelled by nick translation or random priming techniques. Isotope-labelled probes are more sensitive than non-radioactively labelled probes but require autoradiography for detection. The most common labels are ^{35}S and ^{3}H, each of which has advantages and disadvantages. Probes labelled with ^{35}S provide good sensitivity with average resolution and require three to ten days for development. Probes labelled with ^{3}H provide high sensitivity with higher resolution, but the development time of 14-90 days is impractical for most diagnostic purposes (Strickler et al., 1990).

1.4.2 Non-isotopic *in situ* hybridization (NISH)

Over the past decade, advances in molecular biology have made it possible both to generate probes from specific chromosomal regions and to modify chemically the probes for sensitive and specific non-isotopic detection. This has allowed NISH to be developed quickly and has applied a variety of applications in clinical diagnosis (cytogenetics, pathology, infectious diseases, cancer, and prenatal diagnosis) and research work (gene mapping, gene expression, genome evolution, microbiology and virology, mitosis/meiosis and somatic hybrid cell analysis) (Lichter and Ward, 1990).

Probes

The three types of probes used for NISH include α-satellite repeat sequences, libraries and cosmids with single copy DNA. Alpha-satellite repeat DNA is a heterogeneous family of randomly repeated DNA sequences based on a monomer repeat length of 171 base pairs and located in the centromeric regions of all human chromosomes. These sequences generate a brilliant hybridization signal but present some difficulties in clinical application because: (1) the chromosome specificity of the repetitive sequence probes is very sensitive to the conditions of hybridization; (2) the signal size reflects the size of each pericentromeric heteromorphism and thus

in some individuals chromosomes with small numbers of alpha-satellite repeats may be missed; and (3) acrocentric chromosomes share α-satellite repeat. This, together with their tendency to cluster around the nucleolar organizer region makes their identification difficult (Klinger et al., 1992; Willard and Waye, 1987).

Chromosome specific libraries are available for all human chromosomes eg. 21 library LL21 NS02 from The American Culture Collection (Lichter et al., 1988; Fuscoe et al, 1989). Because the probes from these libraries carry inserts from an entire chromosome, the hybridization signal covers the entire chromosome. These probe complexes have been named "chromosome paints". Such probe complexes have been used successfully in metaphase cells to identify translocated pieces of chromosomes or marker chromosomes in targeted cells. In interphase nuclei, chromosome paints generate a very large diffuse signal such that the edge of the signal can be difficult to delineate, and overlap of the domains is common. Again the nucleolar organization of the D (13,14,15) and G (21 22) group chromosomes often results in the close proximity of these chromosomes making identification difficult.

To overcome the limitations of both α-satellites and chromosome paints, probe complexes, comprised of cosmids which carry contiguous sequences of single copy DNA, have been isolated. These give superb spatial resolution in peripheral lymphocytes (Lichter et al. 1990) but weak signals in uncultured amniocytes (Klinger et al., unpublished data).

Labelling

Various methods can be used to label and detect nucleic acids non-isotopically. An enzymatic labelling method is preferred over the photochemical biotinylation procedure to incorporate labelling (Forster et al., 1985) for each of biotin (Langer et al., 1981; Lo et al., 1988; Weier et al., 1990), digoxigenin (Kessler et al., 1990), or

halogenated nucleotides (e.g. BrdU, FrdU). Probes are usually labelled by nick translation or random priming techniques, because of the high labelling efficiency. Biotin-labelled probes have good resolution and can be developed in several hours. However, other chemical modification schemes using acetylaminofluorene (AAF, Landegent et al., 1984) mercuration (Hopman et al., 1986a) or sulfonation (Verdlov et al., 1974) have been used successfully for sensitive non-radioactive detection of ISH probes.

Detection

The visualization of hybridized probes can be achieved in several ways including: (1) fluorochromes analyzed by fluorescence microscopy, (2) chemiluminescence detected by an emulsion overlay or directly by photon-counting devices, or (3) high-density coloured precipitates generated by enzymatic assays or the use of metallic compounds, for visualization by phase contrast, nomarski, or electron microscopy. All of the methods outlined above used indirect detection procedures (Lichter et al., 1991). When fluorochromes are coupled directly to the probe molecules (Dirks et al., 1990), direct microscope examination of the signal is feasible.

The sensitivity of each of the labelling/detection procedures reported has varied widely between different laboratories. This variability, at least in part, may reflect the quality of the reagents used or the degree to which experimental parameters were optimized. Biotin and digoxigenin labelling combined with fluorescent detection are currently the most widely used procedures because of the sensitivity of detection and the commercial availability of the reagents (Lichter et al., 1991).

1.4.3 Interphase cytogenetics and Multiple NISH

Next to their high sensitivity, one of the attractive features of NISH methodologies, is the ability to detect several nucleic acid target sequences simultaneously with

different fluorescent colours (Nederlof et al., 1989). Such multicolour NISH provide versatile tools for the detection of specific chromosome aberrations, not only in metaphases but also in interphase nuclei. For the analysis of interphase nuclei by NISH, the term "interphase cytogenetics" has been proposed (Cremer et al., 1986). A schematic illustration (Figure 1.2) of how genomic features on metaphase chromosomes can be detected in interphase nuclei by NISH was described by Lichter and Ward (1990).

Hopman et al (1986b; 1988) successfully developed a double hybridization procedure using non-radioactive methods. Herrington et al. (1989) used biotin and dioxigenin labelled probes and the double FISH method to detect human and virus nucleic acids simultaneously. Arnoldus et al. (1990) detected the Philadelphia chromosome in interphase nuclei using double FISH methods and achieved excellent results. The use of three different biotinylated probes, visualized with two fluorochromes in combination with one enzyme has also been described (Bresser and Evinger-Hodges, 1987). Nederlof et al.(1989) successfully reported three-colour FISH for simultaneous detection of three differently haptenized probes in interphase nuclei and metaphase chromosomes from human peripheral blood lymphocytes. They used three chromosome-specific (1, 15 and 18) repetitive probes labelled with either amino acetyl fluorine (AAF), mercury (CP), or biotin (bio) and detected them with FITC, TRITC and a new blue fluorochrome label, amino methyl coumarin acetic acid (AMCA). The emission as well as excitation spectra were well separated, and by using the filter combination given in Table 1.3, the three fluorochromes appeared with three distinct colours without significant overlap.

Later Nederlof et al (1990) described a multiple FISH procedure for detecting up to seven different probes simultaneously. This method is based on either single, double or triple hapteinization of probes and immunofluorescence detection using three different fluorochromes (Table 1.4).

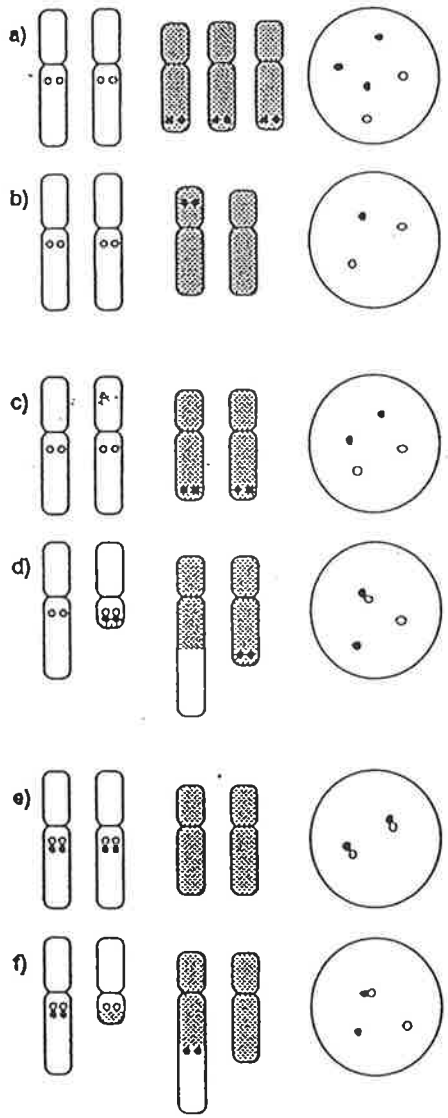
Figure 1.2*: Schematic illustration of how genomic features on metaphase chromosomes can be detected in interphase nuclei by non-isotopic *in situ* hybridization (NISH). In each lane, two metaphase chromosomes of a particular karyotype are shown as white and black circles. Signals in interphase nuclei are shown on the right side on each lane. Note the number or locations of signals.

a. Detection of trisomic chromosomal materials.

b. Detection of submicroscopic deletions or loss of a single gene.

c and d. Identification of a specific translocation that brings sequences of different chromosomes together. Compare normal case (c) and translocation (d).

e and f. Characterisation of a chromosomal break point or of break-point sequences. In the normal cell (e), two signal pairs are seen, whereas in the case of translocation (f), one signal pair and two separate signals are visible. Correspondingly, a single clone spanning the break point will give three versus the expected two singnals in cells with translocated chromosomes.



If the interphase cells are in G2-phase of the cell cycle, all signals are visible as doublets versus the singlets shown in this scheme for G1-phase nuclei.

* Taken from Lichter and Ward (1990).

Table 1.3: Filter specifications in the microscope*

	Excitation filters	DCM	Barrier filter
AMCA blue	LP340-SP380 UV	400	LP420
FITC green	LP450-SP490 blue	510	LP515
TRITC red	LP530-SP560 green	580	LP580

LP = long wave-pass filter;

SP = short-wave-pass filter;

DCM = chromatic beam splitter

* taken from Nederlof et al (1989)

Table 1.4: Scheme for multiple hybridization combining biotin, AAF, and chemiprobe in single-, double-, and triple-probe labelling*

	Colour signal		
Label	**Red TRITC**	**Green FITC**	**Blue AMCA**
bio	+	-	-
AAF	-	+	-
CP	-	-	+
bio/AAF	+	+	-
bio/CP	+	-	+
AAF/CP	-	+	+
bio/AAF/CP	+	+	+

* taken from Nederlof et al (1990)

TRITC, FITC, AMCA (see text)

1.5 FISH Studies on Human Sperm

Although the application of FISH to human sperm has undergone a recent, rapid development, it has been limited to only chromosome 1 and Y probes, and to the use of the single FISH method.

1.5.1 Structure and stability of nuclear chromatin in human spermatozoa

It is clear that the structure and genetic activity of the genome in eukaryotes is modulated by two distinct groups of chromosomal proteins which include the histones, and non-histone proteins (Balhorn, 1982). In contrast, mammalian sperm DNA is packaged in a very different manner. The DNA in these cells is associated with protamine (protein) and reconstituted DNA-protamine complexes. The sperm of humans contains three different protamines. Human sperm DNA is condensed into an almost crystalline state and, for all practical purposes is a biochemically inert and totally inactive genome (Balhorn 1982). The somatic histones are replaced by arginine-rich protamines during late spermatogenesis, and the nucleus condenses into a totally inert and inactive state. Disulphide bonds between adjacent protamines maintain the condensed, inert state of the nucleus.

Bedford et al (1973) introduced a method of decondensation of the nuclear chromatin of human ejaculated sperm with sodium dodecyl sulphate (SDS) containing dithiothreitol (DTT) as a reagent treatment. This method allowed detection of nuclear vacuoles in >90% of the spermatozoa. They reported that a technique of controlled nuclear decondensation provided a simple method by which the structural quality of sperm nuclei could be assessed. Joseph (1984) first attempted isotopic *in situ* hybridization on human ejaculated sperm but because of human sperm nuclei characteristics his experiment failed.

1.5.2 Previous reports of human sperm in FISH

While Joseph et al. (1984) were the first to apply isotopic ISH to study chromosome anomalies in human sperm, other reports soon followed. Following the report of Burns et al. (1985), the emphasis changed from isotopic ISH to FISH. At the time of commencing my studies with FISH in 1990, the only reports related to the FISH detection of chromosomes 1 and Y in human sperm. Burns et al. (1985) had obtained labelling frequencies of 35-66% using a Y-specific chromosome probe, and Coonen et al. (1991) had detected chromosome 1 in 40-90% of sperm from 32 donors. These results indicated that the major difficulty with sperm is to obtain sufficient decondensation of the sperm nuclei to allow entry of the probes without damaging the DNA. Other studies have examined the X:Y ratio in human semen using Y-specific probes. Guttenbach and Schmid (1990) reported 49.4% Y-bearing sperm and a disomic Y frequency of 0.27%, and Wyrobek et al. (1990) found that 50.1% of sperm contained a Y chromosome.

1.6 Studies in the present thesis

The aim of the present study was to develop a reliable method for simultaneously detecting two chromosomes in human sperm and to apply this method to the assessment of the ratio of X- and Y-bearing sperm in human semen; secondly, to determine the feasibility of detecting aneuploidy in human ejaculated sperm using FISH and probes specific for the chromosomes 17, X and Y.

The accurate sexing of sperm and the detection of aneuploidy in human ejaculates is important to an understanding of male fertility and some aspects of assisted reproduction. Research in this field has been limited because the validity of the F body detection method was questionable and because the "hamster" system is labour-intensive, time consuming and selective (only a subgroup of sperm, which

are capable of fusing with hamster oocytes, can be assessed) (Brandriff et al 1985; Martin et al., 1987). FISH, with chromosome specific DNA probes, may overcome these limitations and permit the entire population of sperm to be studied for sexing and aneuploidy by simple counting of the number of fluorescent spots. The order of the present work is as follows:

1. To develop reliable methods for detecting chromosomes in human sperm by single FISH.

2. To apply double labelled FISH methods to sperm in order to evaluate further any limitations of the single FISH methodology.

3. To determine the ratio of X- to Y-bearing sperm in human semen and to assess the frequency of aneuploidy.

4. To examine the ratio of X - and Y-bearing sperm in motile sperm following a commonly used method of motile sperm isolation.

5. To determine the X- and Y-bearing ratio of sperm from an XYY individual.

6. To assess any association between poor sperm morphology and aneuploidy.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Introduction

This section describes the general procedures used throughout this study. Specific methods that relate to specific aspects are detailed in each of the subsequent chapters.

2.2 Preparation of mitotic chromosome spreads

Mitotic chromosome spreads from human blood lymphocytes were used for controls. They were prepared with a modification of the method of Buckle and Craig (1986), as follows:

2.2.1 To prepare PHA-stimulated lymphocytes, 0.4 ml of whole blood was added to culture bottles containing 5 ml of medium (see below), and cultured for 72 hr at 37°C in a 5% CO_2 incubator.

Medium:

RPMI 1640 (with $NaHCO_3$ 0.5 g/L)

Hepes buffer 20 mM

Foetal calf serum 17% (v/v)

Glutamine 2 mM

Gentamycin 100 mg/L

PHA (phytohaemagglutinin, M form, GIBCO 10 ml lyophilized) 15 µl per ml of medium.

2.2.2 5-bromodeoxyuridine (5-BrdU) was added to attain a concentration of 200 µg/ml and the culture incubated for a further 16 hr. (After addition of 5-BrdU, the cells and slides must be protected from excessive light as they are U.V. sensitive.)

2.2.3 The cultured cells were centrifuged and washed twice with PBS and once with medium centrifuging at 175 g for 5 min after each wash. The cells were resuspended in fresh medium containing 10^{-5} M thymidine and cultured for a further 6 hr.

2.2.4 Colchicine at a concentration of 0.5 µg/ml of was added for a further 15 min incubation at 37°C. The cells were centrifuged as before. The supernatant was discarded and the cells were resuspended in 7 ml of hypotonic 0.075 M KCl solution at 37°C for 10 min.

2.2.5 2 ml of Carnoy's fixative (three parts methanol to one part of glacial acetic acid) was added to the pellet and the cells were mixed thoroughly and centrifuged. This step was repeated with fresh fixative two times.

2.2.6 Drops of fixative were added to the final pellet to form a milky suspension. Two drops of suspension were placed on each slide, and air dried.

2.3 Pretreatment of human ejaculated sperm for FISH

Sperm were pretreated to allow the chromatin to decondense allowing access to the DNA probe. Sperm decondensation was achieved with a modification of the method of West et al (1989)

2.3.1 The semen sample was mixed with PBS, containing 6 mM EDTA so as to obtain a final sperm concentration of 10-20 x 10^6 sperm per ml. The sperm was settled by centrifugation at 1000 rpm for 5 min. The supernatant was removed by suction.

2.3.2 The pellet was resuspended by pipetting, in PBS containing 2 mM DTT at the same sperm concentration as above and incubated at room temperature for 45 min with regular mixing.

2.3.3 The sample was re-centrifuged, the supernatant was removed and resuspended in 1 ml of PBS mixed and centrifuged again.

2.3.4 The supernatant was removed and 1 ml of Carnoy's fixative (see 2.2.5) was added drop by drop whilst vortexing. The sample was allowed to remain in the fixative at room temperature for half an hour before re-centrifuging. It was then resuspended in fresh fixative and re-centrifuged.

2.3.5 10-14 drops of fresh fixative were added to the sperm pellet to form a milky suspension. This suspension was dropped onto clean microscope slides and air-dried at room temperature.

2.4 DNA probes

2.4.1 Sources and description of the DNA probes

2.4.1.1 TR17 probe: a chromosome 17-specific probe, was a gift from Dr A K. H. Choo and has been described by Willard et al. (1986), Choo et al. (1987), and Jabs and Persico (1987). The insert sequence belongs to the alpha satellite DNA family

and was cloned into the pUC 9 plasmid. TR17 is 1.6 kbp long and recognizes a sequence with 3,000 copies located at the centromeric region of chromosome 17.

2.4.1.2 TRX probe: an X-chromosome specific probe, also a gift from Dr A. K. H. Choo and described in the same papers as TR17 (Willard et al., 1986; Choo et al., 1987; Jabs and Persico, 1987). The two probes belong to the same alpha satellite DNA family and the TRX probe was similarly cloned into the pUC 9 plasmid. TRX is 0.4 kbp long and recognizes 5,000 copies of a sequence located at the centromeric region of the X chromosome.

2.4.1.3 HRY probe: a Y-chromosome specific probe, was the gift of Drs K. Reed and K. Mattei who named it HRY after recloning from Amprobe RPN 1305X (Amersham) into pTZ 18u (Bio-Rad). The Y-sequences are identical to the HaeIII repeat in pHY2.1 orginally isolated by Cooke (1976) and described in detail by Cooke (Cooke 1976, Cooke et al. 1982). It is a 2.1 kb long sequence cloned into a $pGEM_2$ plasmid and it hybridizes to the heterochromatic region, Yq12, on the long arm of the Y-chromosome.

2.4.2 Transformation of Escherichia coli by recombinant plasmids

All solutions, materials, and glassware were sterilized by autoclaving, and all techniques required for C1 level of containment were practised.

2.4.2.1 Recovery of bacteria from Glycerol stocks

Escherichia coli (*E. coli*) JM 101 was a gift from Dr Michael Bowden at the Biochemistry Department at the University of Adelaide. It was maintained as a glycerol stock (1:1, 80% glycerol : bacteria in LB medium) at -70°C. A glycerol stock tube of *E.Coli* JM101 was removed from -70°C storage and placed on ice first. The

bacteria were then streaked on to a LB plate (LB medium containing 1.5% agar), and incubated at 37°C overnight.

2.4.2.2 Preparation of an overnight bacterial culture

A pure single colony of growth was isolated and placed into 10 ml of LB Medium in a 50 ml of tube, and incubated overnight at 37°C in a waterbath with agitation.

2.4.2.3 Preparation of competent cells

(1) 50 ml of LB Medium was dispensed into a sterile 250 ml flask, and prewarmed at 37°C for 20 min.

(2) 0.5 ml of the overnight bacterial culture was added to the 50 ml of LB Medium, and incubated at 37°C with agitation until OD_{600} was 0.3-0.6 (ie. in log phase).

(3) The bacteria were harvested by centrifugation 1,000 x g for 20 min at 4°C. The supernatant was carefully discarded.

(4) The pellet was very gently resuspended in 25 ml of ice-cold 0.1 M $MgCl_2$ by swirling the tube, and the cells centrifuged at 1000 x g for 20 min at 4°C. The supernatant was carefully discarded.

(5) The pellet was gently resuspended in 2.5 ml of cold 0.1 M $CaCl_2$ as in step (4), and the suspension was placed on ice for 1 hr. This suspension constituted the competent cells.

2.4.2.4 Transformation of competent cells

(1) 200 µl of competent cells was dispensed into 5 x 10 ml tubes, and placed on ice.

(2) 100 µl of 0.1 M Tris-HCL (pH 7.3) was added to each tube and on ice.

(3) 1.0 µl of 1 x $T_{10}E_1$ (10mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to tube 1 (negative control).

1.0 µl of control DNA (pUC 9 plasmid DNA) was added to tube 2 (positive control). 1.0 µl of diluted plasmid DNA at 2.5 ng/µl was added to tube 3. 1.0 µl and 5 µl of diluted plasmid DNA at 10 ng/µl were added to tubes 4 and 5 respectively. All tubes were placed on ice, mixed by gentle swirling, then allowed to stand for 30 min on ice.

(4) The tubes were placed in waterbath at 42°C for 2 min, then placed on ice for 30 min.

(5) The tubes were removed from ice and stood at room temperature for 10 min. One ml of LB medium was added to each tube and incubated at 37°C for 1 hr without agitation.

2.4.2.5 Selection of transformed cells

Agar plates were prepared with LB agar (LB Medium with 1-5% agar) containing 50 µg/ml of ampicillin. Aliquots of 100 µl, 200 µl and 300 µl of cell suspension from each tube were individually added onto each of the plates, and incubated overnight at 37°C. The control cells were plated onto a LB agar (without ampicillin) plate to check the efficiency of growth.

2.4.3 Confirmation of transformation by mini-preparation of the plasmid probes

Small-scale preparations of plasmid " mini-preps" were used to check that the correct plasmid probe had been prepared. A 1.5 ml culture could yield about 10 µg of plasmid DNA, enough for rapid restriction enzyme analysis and sometimes enough to be used as a probe for hybridization.

2.4.3.1 A single *E. coli* colony containing a recombinant plasmid was used to inoculate 5 µl of LB medium containing 5 mg/ml ampicillin. Cells were grown overnight at 37°C with vigorous agitation.

2.4.3.2 An aliquot of 1.5 ml of the culture was transferred to an Eppendorf tube and centrifuged for 2 min in a microfuge. The cell pellet was resuspended in 100 µl of Solution I (25 mM Tris-HCL, 10 mM EDTA, 15% sucrose) and stood at room temperature for 5 min.

2.4.3.3 Next, 200 µl of Solution II, (0.2 N NaOH, 1% SDS) was added. The tube was gently inverted twice and left on ice for 5 min.

2.4.3.4 Solution III (3 M sodium acetate pH 4.8) 150 µl was added, gently mixed and the tube left on ice for 5 min.

2.4.3.5 The mixture was centrifuged for 5 min; 450 µl of the supernatant was placed in a fresh tube and extracted once with equal volumes of phenol: chloroform/isamyl alcohol (the chloroform:isamyl alcohol ratio is 24:1 V/V).

2.4.3.6 After centrifugation to separate the phases, the upper phase was removed into a fresh tube, 2 volumes of ice cold 95% ethanol added to precipitate

the DNA. The precipitate was frozen at -20°C for 2 hr, centrifuged and washed with 70% ethanol to remove residual salts.

2.4.3.7 The pellet was dried under vacuum and dissolved in $T_{10}E_1$ (pH 8.0).

2.4.3.8 The transformed DNA was verified by restriction digestion as detailed in section 2.4.5

2.4.4 Large-scale plasmid preparation

2.4.4.1 Growth and amplification of plasmid culture

(1) A single colony of *E. coli* JM 101 was isolated and placed in 10 ml of LB medium containing 50 µg/ml ampicillin in a 50 ml tube, and incubated overnight at 37°C with agitation.

(2) A 25 ml of LB medium containing 50 µg/ml ampicillin in a 100 µl flask was prewarmed at 37°C for 30 min, prior to inoculation with 100 ml of the overnight culture. The flask was incubated at 37°C with agitation until OD_{600}=0.6, and then placed on ice.

(3) A 500 ml of LB medium containing 50 mg/ml ampicillin in a 1000 ml flask (prewarmed at 37°C for 30 min) was incubated with 25 ml of the log phase culture incubated at 37°C with agitation until OD_{600}=0.4.

(4) Choramphenicol (34 µg/ml in ethanol) 2.5 ml was added and the incubation continued overnight.

2.4.4.2 Isolation of plasmid DNA

(1) The overnight culture was dispensed into 2 x 250 ml centrifuge pots and cells were harvested by centrifugation at 1000 x g for 20 min at 4°C.

(2) The pellet in each pot was resuspended in 50 ml of ice cold STE (0.1 M NaCl, 10 mM Tris-Cl, pH 7.8, 1 mM EDTA) and transferred into two sterile 50 ml tubes, which were centrifuged at 1000 x g for 20 min at 4°C.

(3) Each pellet was resuspended in 2.5 ml of Solution I (50 mM glucose, 25 mM Tris-HCL pH 8.0, 10 mM EDTA) without lysozyme. To the pooled resuspension was added a 5 ml of solution I with lysozyme (final concentration 10 mg/ml), and placed at room temperature for 5 min.

(4) An aliquot of 20 ml of solution II, (0.2 N NaOH, 1% SDS) was added to the tube, mixed by inversion and placed on ice for 10 min.

(5) A 15 ml of solution III (3 M potassium acetate pH 4.8) was added, mixed and the suspension placed on ice for 10 min.

(6) The contents of the tube were dispensed into two Corex tubes, balanced, and centrifuged at 10,000 x g for 20 min at 4°C (in a J2-21M/ECentrifuge).

(7) The supernatant was carefully transferred to fresh Corex tubes and 0.6 volumes of isopropanol was added and the contents were then mixed and left at room temperature for 30 min.

(8) The tubes were centrifuged at 12,000 x g for 30 min at room temperature (in a J2-21M/E Centrifuge).

(9) All residual supernatant was removed carefully above the pellet.

(10) One ml of 70% ethanol was added to cover the pellet, and the tube was allowed to stand at room temperature until the precipitate settled.

(11) The liquid over the pellet was removed, and the pellet was dried under vacuum for 20 min.

(12) Each pellet was dissolved in 500 µl of $T_{10}E_1$ (pH 8.0), and the samples were pooled. Volume was adjusted to 2 x 7 ml with $T_{10}E_1$, stored at -20°C.

2.4.5 Restriction endonuclease digestion

2.4.5.1 A 10 µl of sample DNA (mini-preparation of plasmid DNA) was added to 7 µl of $T_{10}E_1$, 2 µl of 10 x B buffer, and 1 µl of *Hind III* (40 U/µl); and digested overnight at 37°C.

2.4.5.2 10 µl of the above digested sample DNA was added to 7 µl of $T_{10}E_1$, 2 µl of 10 x H buffer, and 1 µl of *EcoR* I (20 U/ml); and further digested for 2 hr at 37°C.

2.4.5.3 The sample DNA was then placed in a sterile Eppendorf tube, 1/10 of the sample volume (V) of 3 M sodium acetate (pH 5.2) was added to give a final concentration of

Ice cold 95% redistilled ethanol 2.5 volume was added, mixed, then the sample was placed at -20°C for 2 hr to precipitate the DNA.

2.4.5.4 The tube was then centrifuged at 10,000 x g for 15 min. The supernatant was discarded, and the precipitate dried under vacuum for 10 min.

2.4.5.5 The pellet resuspended in $T_{10}E_1$ (pH 8.0) and allowed to dissolve at 37°C for 1-2 hr, prior to storage at 4°C

2.4.6 Agarose gel electrophoresis

2.4.6.1 Gel preparation: A 8% of agarose gel in 1 x TAE (0.04 M Tris Acetate, 0.002 M EDTA pH 8.0) was prepared. A gel comb with 5 mm well slots was inserted in place 1 mm above the mould floor and gel mould was placed onto a level platform. The melted agarose was poured into the gel mould and allowed to set 1-2 hr. The gel buffer (1 x TAE) was poured into the gel tank to cover depth of 1 mm above wells.

2.4.6.2 Sample loading: Marker DNA, *EcoR* I digested SPP-1 bacteriophage DNA, 1 µl (100 ng/µl), and samples prepared with $T_{10}E_1$ and 6 x loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in H_2O) were slowly injected into slots.

2.4.6.3 Running the gel: The gel tank lid was closed and cathode and anode leads were connected to power supply, ensuring DNA was orientated to migrate to the anode or positive lead (red). Electrophoresis was performed at 65 V for 2 hr.

2.4.6.4 Detection of DNA: The gel was stained in ethidium bromide (2 µg/ml) and photographed using a Polaroid Type MP4 Camera fitted with a red filter under UV light (254 nm, using a Fotodyne Transilluminator).

2.5 *In situ* hybridization

A modification of the method of Arnoldus et al. (1990) was used in this section as outlined in steps 2.5.1 to 2.5.5. Methodology for the individual techniques of ISH, single and double FISH are outlined in chapters 3, 4 and 5 respectively.

2.5.1 RNase pretreatment of slides

The slides were treated with RNase A 100 µg/ml in 2 x SSC (0.3 M NaCl, 0.03 M NaCitrate) for 1 hr at 37°C (150-200 µl under coverslip), then washed 4 times in 2 x SSC, 2 min each at room temperature. The sides were dehydrated through an ethanol series (2 min each in 35%, 70%, 95% and 100% ethanol) and air dried.

2.5.2 Denaturation of cell slides (chromosome spreads and sperm preparations)

The slides were preheated to 70°C for 2 min and denatured in 70% deionized formamide in 2 x SSC (pH 7.0) for 2 min at 70°C, immediately cooled in 70% ethanol at -20°C for 2 min and then dehydrated in 80%, 95%, and 100% ethanol at room temperature and air dried.

2.5.3 Denaturation of probes

The hybridization mixture contained 10 µl of labelled probe (10 ng for tritium labelled, and 20-40 ng for biotin or digoxigenin labelled), 5 µl of salmon sperm carrier DNA (2 mg/ml), 10 µl of 10 x SSCP (1.2 M NaCl, 0.15 M sodium citrate, and 0.2 M sodium phosphate, pH 6.0), and 25 µl of deionized formamide with 20% dextran sulphate. The mix was denatured at 75°C (70°C for tritium work) 10 min, then cooled on ice for 10 min.

2.5.4 Hybridization

The hybridization mixture was applied to each slide (50 µl), sealed under a coverslip with rubber cement, and hybridized overnight (16-18 hr) at 37°C (42°C for tritium work) in a moist chamber.

2.5.5 Post washing for FISH

The slides were washed three times (5 min each) at 45°C in 50% deionized formamide in 2 x SSC, followed by 3 washes of 5 min each at 60°C in 0.1 x SSC.

CHAPTER THREE

DETECTION OF X-BEARING HUMAN SPERM BY *IN SITU* HYBRIDIZATION USING TRITIUM-LABELLED PROBE

3.1 Introduction

Since its introduction in 1969 (Gall and Pardue, 1969) the isotopic *in situ* hybridization technique has served as a powerful tool to localize specific RNA or DNA sequences to metaphase and interphase nuclei from many different cytological and histological specimens. The first report using isotopic *in situ* hybridization methods to estimate aneuploid levels for chromosomes 1 and Y in human *testicular* sperm was in 1984 (Joseph et al., 1984). Frequencies of 48% Y-bearing , 0.18% YY-bearing and 0.35% disomy 1 spermatozoa were found. These authors were unable to obtain results from human ejaculated sperm. West et al (1989) reported that 46.7% of ejaculated human sperm were labelled with a tritiated Y-chromosome specific probe; however no information on disomic Y was included.

No information is currently available relating to the frequency of X-bearing human sperm using *in situ* hybridization with a specific X-chromosome probe. In order to detect the frequency of X-bearing sperm in the ejaculate, I first adapted tritium labelled probes for *in situ* hybridization.

3.2 Methods

3.2.1 Subjects

The semen samples were produced by masturbation, allowed to liquefy at room temperature for at 10-30 minutes with gentle rocking and then analyzed according

to standard procedures (World Health Organization, 1987). The volume was measured using graduated, conical test tubes. To determine the sperm concentration, 100 µl aliquot of semen was diluted 1/10 or 1/20 with formalin diluent and the sperm were counted in a Neubauer haemocytometer. Sperm motility was assessed subjectively at a magnificantion of 400 X by trained Andrology technicians. The % progressive motility was calculated for 200 sperm. Sperm smears were fixed in 95% ethanol and stained with the modified Papanicolaou procedure. The frequency of various abnormalities were scored in 200 sperm from each sample using the 1987 WHO guidelines.

One semen sample was obtained from a healthy male who supplied semen at the Andrology laboratory in The Queen Elizabeth Hospital. The sample exhibited a concentration of 25 x 10^6 sperm/ml, 40% progressive motility and 46% normal morphology. The sample was divided into two aliquots for experiment 1. One aliquot was directly targeted by *in situ* hybridization, another was pretreated with SDS/DTT, then by hybridization.

For experiment 2, samples were obtained from six healthy donors who attended the IVF programme at The Queen Elizabeth Hospital. The samples exhibited a volume of > 2ml (range = 2-4), concentration of > 80 x 10^6 sperm/ml (range=80-240), > 50% progressive motility (range=50-60), and > 45% normal morphology.

3.2.2 Preparation of human ejaculated sperm

3.2.2.1 1 ml of semen was mixed with 9 ml of 1% SDS (sodium dodium dodecyl sulphate) in 0.5 M Tris-HCL (pH 9.0), containing DTT (Dithiothreitol) at a concentration of 2 mM/ml at room temperature (21°C-25°C) for 30 minutes.

3.2.2.2 The reaction was stopped by adding an equal volume of fixative (3:1::ethanol:acetic acid), and after 3 changes of fixative, two drops of sperm suspension were placed on each slide and air dried.

3.2.3 Labelling of the TRX and control probes with ^{3}H-dNTPs by nick-translation.

3.2.3.1 25 µl aliquots of each labelled nucleotide triphosphate ([1', 2', 2, 8-^{3}H] dATP, [1', 2', 5-^{3}H] dCTP, [methyl-1', 2'-^{3}H] dTTP: Amersham codes TRK 633, TRK 625, TRK 576 respectively) were added to an Eppendorf tube and dried down in a vacuum dessicator (about 2 hr).

3.2.3.2 The following were added to the dry tube:

- cold dGTP* 1.8 µl
- TRX or λDNA* control probe, 300 ng in H_2O or $T_{10}E_1$
- H_2O to 20.7 µl with probe volume
- enzyme*, mix, DNase\DNA pol I. 2.5 µl
- (Total 25 µl)
- (* Amersham nick kit N5500)

3.2.3.3 The nick mix was incubated at 15°C for 2 hr.

3.2.3.4 Then 15 units (3 µl) of DNA polymerase I was added and incubation continued for a further 30 minutes at 15°C.

3.2.3.5 The nick mix was layered onto a Sephadex G50 column (gel was washed 3 times with $T_{10}E_1$, pH 8.0, before the mix was added). The fractions were diluted with $T_{10}E_1$. The collection of fractions in each tube as follow:

tube 1	300 µl	tubes 13-17	300 µl
tubes 2-12	100 µl	tube 18	600 µl

3.2.3.6 2 µl of each fraction in 500 µl H_2O and 5 ml of water-miscible in a scintillate was counted for 1 min.

3.2.3.7 The tubes containing the labelled probe were determined, pooled and divided into convenient size lots (eg. 100 ng). The labelled probe was ethanol-precipitated with 1/10 vol. 3 M Na acetic (pH 5.2) and 2.75 vol. cooled 95% ethanol, and stored at -20°C.

3.2.3.8 The following formula was used to calculate the fraction incorporated with tritium and specific activity:

$$\text{Fraction} = \frac{\text{Total counts in probe}}{\text{Total counts in probe plus unincorporated bases}}$$

$$\text{Specific activity} = \frac{\text{Total microcurie} \times \text{fraction} \times 2.22 \times 10^6}{\text{Mass of probe of } ^3\text{H-dNTP in µg (DPM/µg)}}$$

(Using 1 microcurie = 2.22×10^6 DPM)

3.2.4 Stringency rinses

3.2.4.1 Rubber cement was removed and the coverslips were taken off, The slides were briefly rinsed in 50% deionized formamide/2 x SSC (pH 7.0) at room temperature.

3.2.4.2 The slides were washed with frequent agitation at 44°C for 3 min each in 3 changes of 50% deionized formamide/0.5 x SSC then 5 changes of 0.5 x SSC at 44°C for 3 min each.

3.2.5 Autoradiography and staining

3.2.5.1 Dipping slides. This section was performed in a darkroom under safelight.

(1) 15 ml of 1% glycerol was preheated at 46°C in a 50 ml tube.

(2) Bulk emulsion (Ilford L4) was warmed at room temperature for one hour before opening (under safelight). Shreds of emulsion were placed in the warm glycerol solution until the volume reached the 30 ml mark, melted and mixed until it looked homogeneous and coated the tube evenly.

(3) The slides were preheated for 5 seconds at 46°C and dipped back-to-back in the diluted emulsion for 5 seconds at 46°C, then removed slowly, and drained briefly.

(4) The slides were collected on a level rack, which was chilled to 10-15°C for at least 2 minutes to set the emulsion.

(5) The slides were dried in a stream of filtered air for 30 minutes in a light-tight ventilated box.

(6) The slides were packed into a light-tight black box (clay-Adams) and exposed for 7 days to 90 days at 4°C.

3.2.5.2 Developing and fixing slides

(1) The slide box was allowed to warm to room temperature for 1 hour. Slides were developed for 5 min in a solution of D19 (Kodak) developer diluted 1:1 with distiled water, at 20°C, shielded from the safelight (Ilford 904), without agitation.

(2) The slides were rinsed briefly in H_2O containing 5% of developer, and then fixed in Ilford Hypam fixer with hardener for 5 min with frequent agitation.

(3) The slides were rinsed in 5 changes of H_2O (2 min each).

3.2.5.3 Staining

(1) The slides were sensitized in 10 μg/ml Hoescht 33258 (Sigma; B2883) in 2 x SSC for 30 min at room temperature, then rinsed twice in 2 x SSC.

(2) The slides were laid in a flat container, just covered with 2 x SSC and exposed to long wavelength UV light (Blacklite-blue, 15 watt, 350 nm, warm-up 30 min) for 1 hour at a distance of 10 cm.

(3) The slides were stained for 20 min in Giemsa stain (Gurr's R 66, Improved; BDH) diluted to 10% in pH 6.8 phosphate buffer for 20 min, rinsed in the same buffer and air dried and a coverslip mounted.

3.2.6 Analysis of grain distribution

Silver grains were scored under pale blue light in order to avoid confusion with spots of stain. Only silver grains which were lying directly over, or clearly touching the chromosomes and nuclei of the sperm heads were scored.

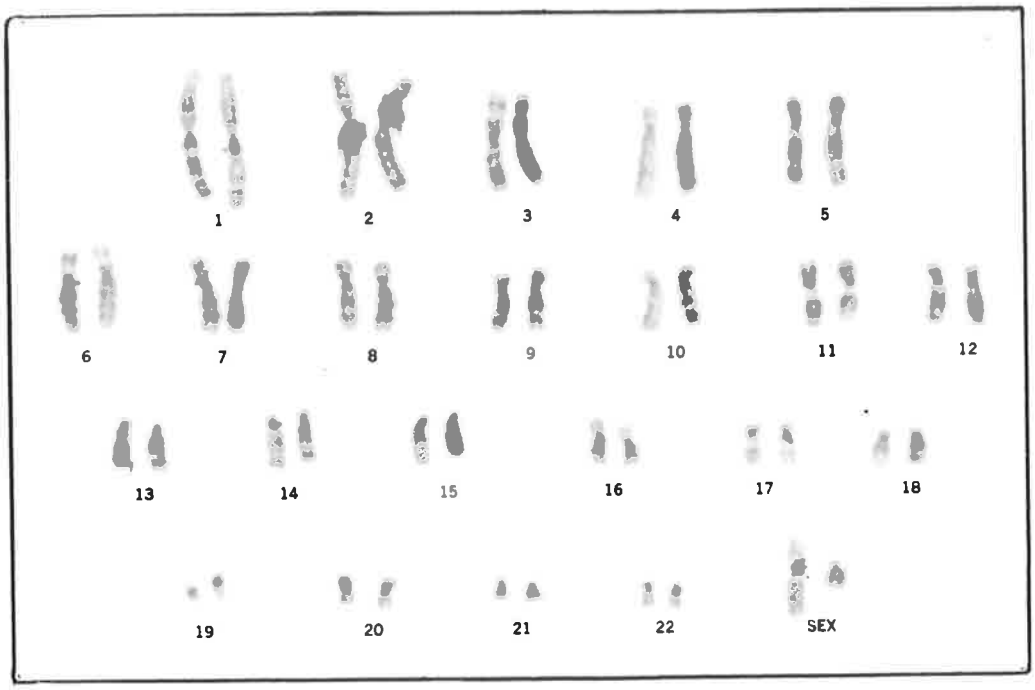
3.3 Results

The control slides of mitotic chromosomes showed labelling of the X chromosome with the ^{3}H-TRX probe (Figures 3.1 and 3.2). There was no labelling in the mitotic chromosome preparations with the ^{3}H-λDNA probe hybridization.

In the first *in situ* experiment with sperm from one donor pretreated with SDS/DTT and probed with ^{3}H-TRX, a total of 984 sperm were scored and the number of silver grains were also counted for each sperm. The frequencies of sperm labelled with varying numbers of grains (Table 3.1) showed two peaks, one at zero and one around 10 grains (Figure 3.3). Four hundred and seventy nine sperms (48.7%) of the sperm labelled with the TRX-probe had one "hot spot" (Figure 3.4), and two (0.2%) of the sperm had two "hot spots" (Figure 3.5). Overall 48.9% (481) of sperm were considered to be X-bearing and 51.1% (503) of the sperm were not labelled, and the majority of these were presumed to be Y-bearing. A small proportion might be nullisomic for a sex chromosome. With no pretreatment only 1.1% (six) of the 514 sperm scored were labelled and 98.9% (508) of the sperm were not labelled (Table 3.2).

In a second experiment, devised to repeat the first and to show variation between individuals, a total of 6,197 of sperm were scored from six normal healthy donors, and the frequencies of sperm labelled with the TRX-probe after pretreatment with SDS/DTT showed varying levels (from 8.2%-48.5%, Table 3.3). A total of 1,846 of sperm were labelled with the TRX-probe, 1,841 with one "hot spot" and five (0.08%) with two "hot spots", 4,351 of sperm were not labelled with the TRX-probe (from 51.5%-91.8%, Table 3.3).

The experiments using ^{3}H were not pursued exhaustively because it was regarded as more profitable to switch to non-insotopic methods.



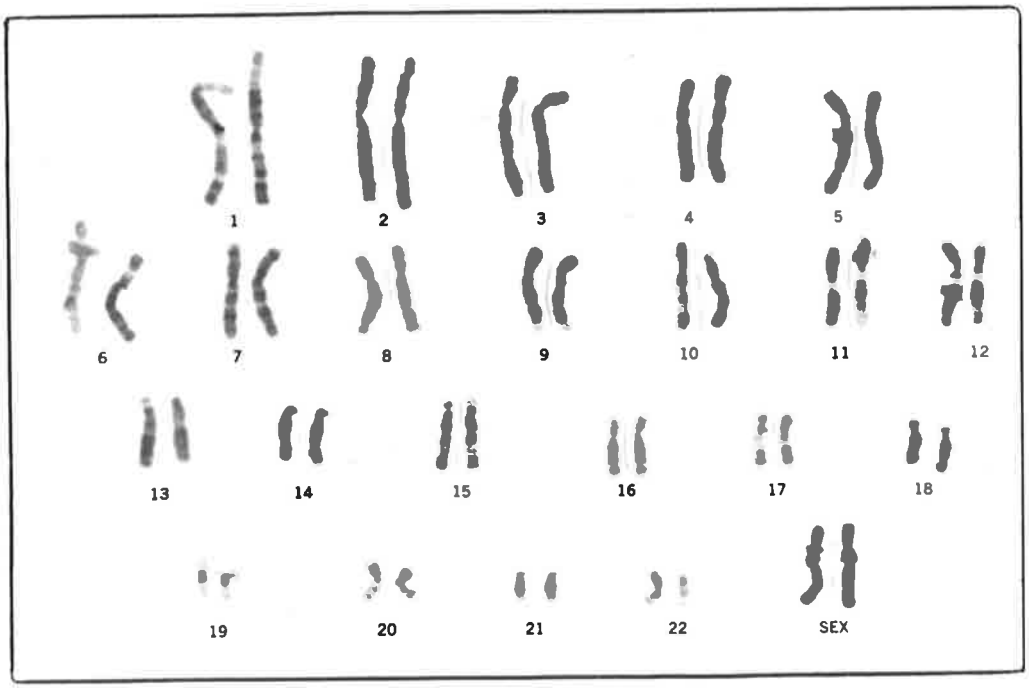


Figure 3.1 (top): **^{3}H-TRX labelled with the centromere of one X chromosome in a male lymphocyte metaphase.**

Figure 3.2 (bottom): **^{3}H-TRX labelled with the centromere of two X chromosomes in a female lymphocyte metaphase**

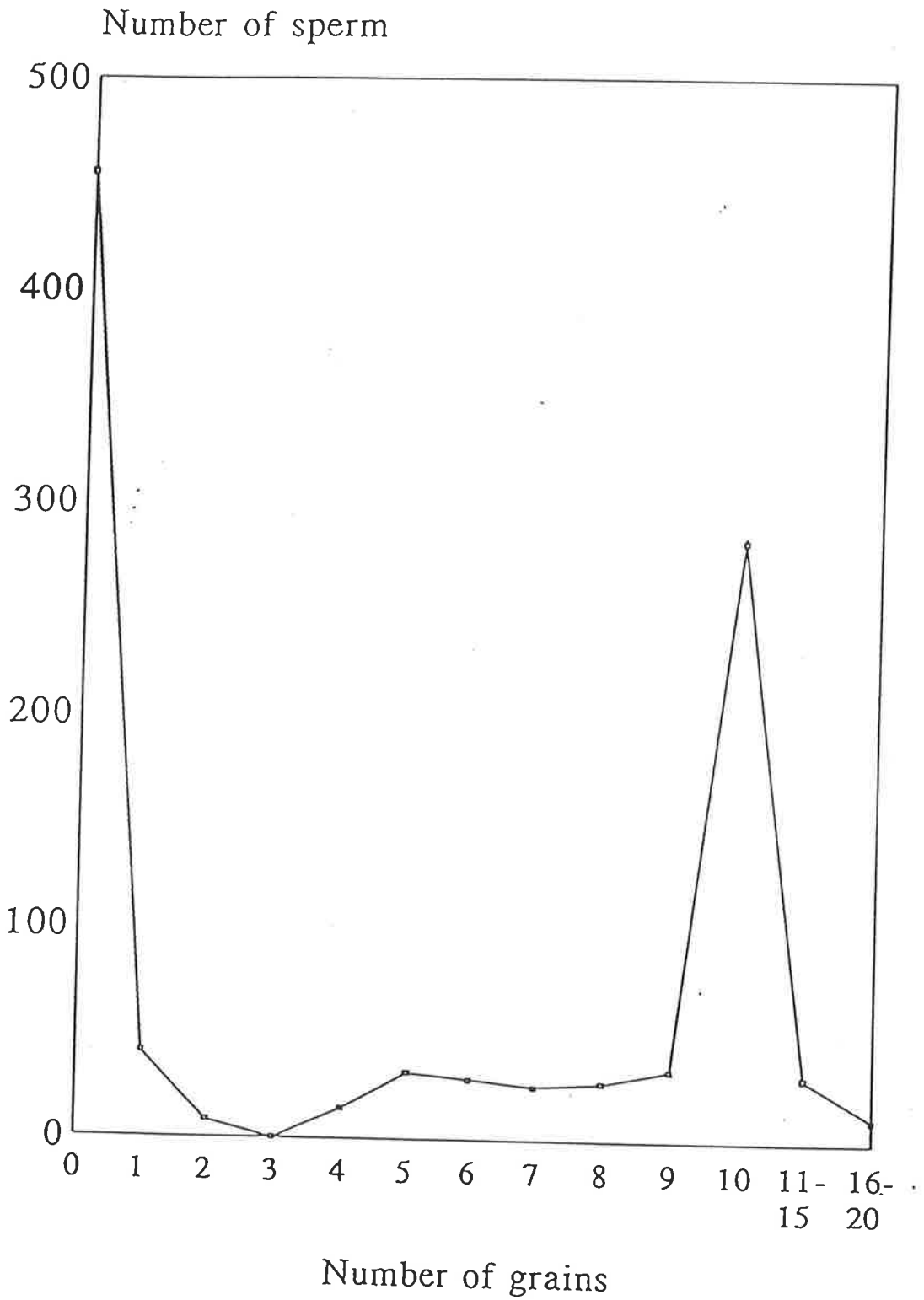
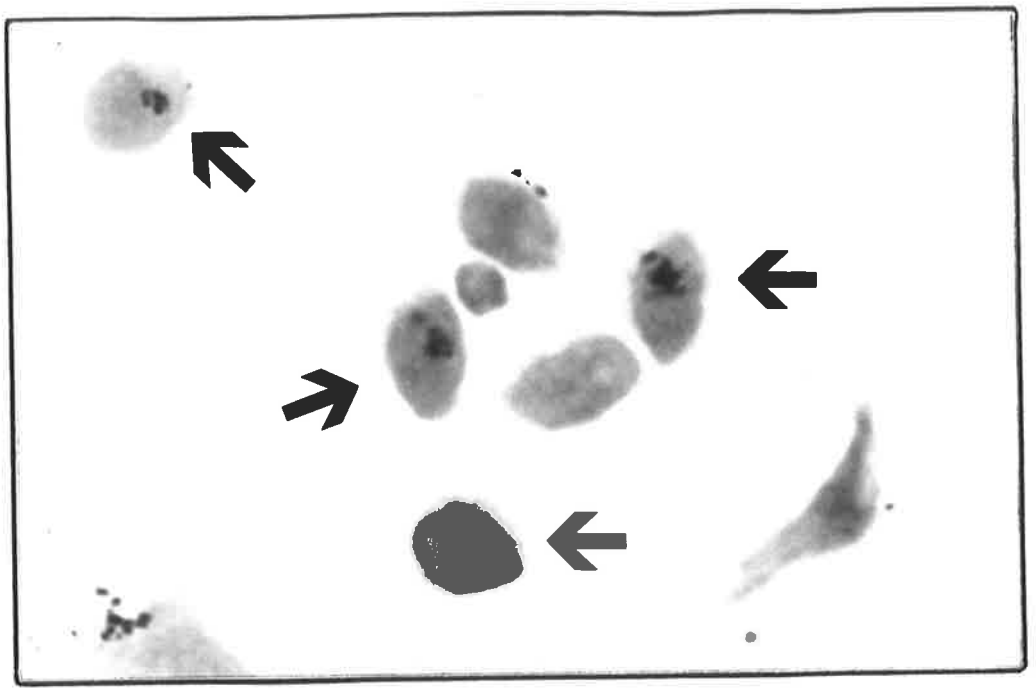


Figure 3.3: Frequency distribution of sperm with varying numbers of grains showing two separate peaks following *in situ* hybridization with ^{3}H-TRX probe



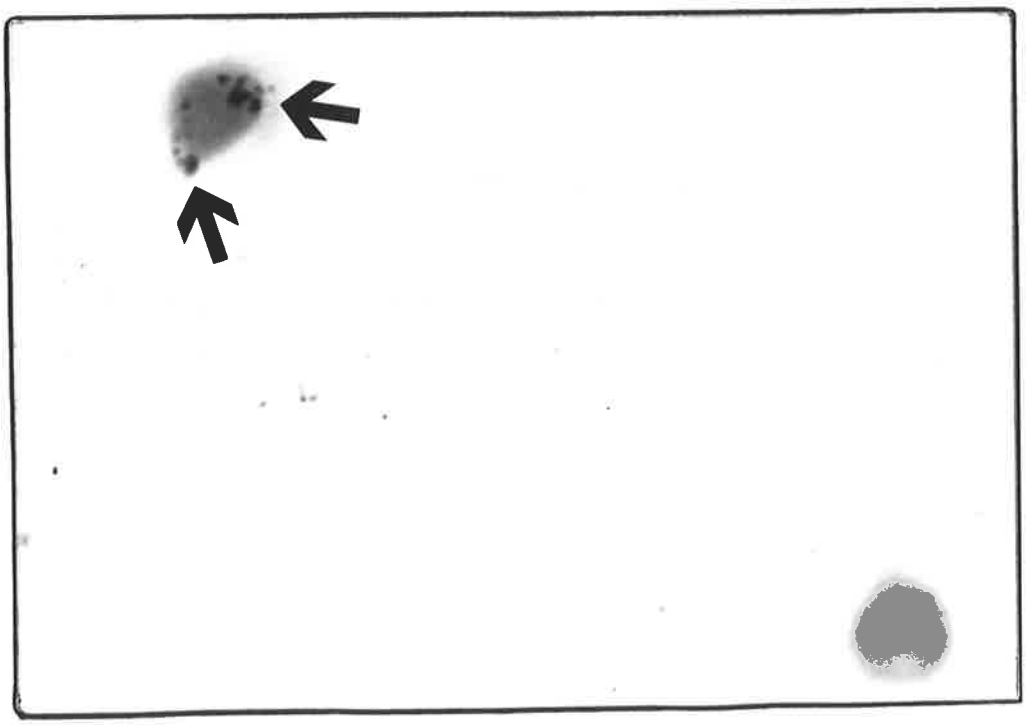


Figure 3.4 (top): Sperm nuclei labelled with ^{3}H-TRX had one "hot spot" (arrows).

Figure 3.5 (bottom): Sperm nucleus labelled with ^{3}H-TRX had two "hot spots" (arrows).

Table 3.1: Frequency of sperm labelled with the varying numbers of grains (using ^{3}H-TRX probe)

grains	0	1	2	3	4	5	6	7	8	9	10	11-15	16-20
sperm	455	40	8	0	14	31	28	25	27	33	282	30	11

Table 3.2: Comparison of sperm labelled with the ^{3}H-TRX probe, pretreated and untreated

No. slide	Probe	SDS/DTT	No. one hot spot	No. two hot spot	No. not labelled	Total scored
6#	^{3}H-TRX	-	6 (1.1%)	0	508 (98.9)	514
8#	^{3}H-TRX	30 min	479 (48.7%)	2 (0.2%)	503 (51.9)	984

Table 3.3: Variation in the frequency of sperm labelling with ^{3}H-TRX probe among 6 normal individuals (after pretreatment with SDS/DTT)

Donor	No. one hot spot	No. two hot spot	Total X-labelled	No. not labelled	Total scored
M.J.	527 (48.5%)	0	527 (48.5%)	560 (51.5%)	1,087
HH66	515 (48.3%)	1 (0.09%)	516 (48.4%)	550 (51.6%)	1,066
CB33	344 (34.3%)	2 (0.2%)	346 (34.5%)	656 (65.5%)	1,002
KV66	254 (25.3%)	1 (0.1%)	255 (25.4%)	749(74.6%)	1,004
NA22	117 (11.6%)	0	117 (11.6%)	890 (88.4%)	1,007
W.H.	84 (8.1%)	1 (0.1%)	85 (8.2%)	946 (91.8%)	1,031
Total	**1,841 (29.7%)**	**5 (0.08%)**	**1,846 (29.8%)**	**4351 (70.2%)**	**6,197**

3.4 Discussion

The initial use of a tritiated probe was considered to be advantageous because it utilised the established techniques of the laboratory at the time (Ford et al., 1988). In the study it was found that a high stringency post-hybridization wash, which included 3 changes of 50% formamide/0.5 x SSC, followed 5 changes of 0.5 X SSC, at 44°C for 3 min each, instead of the more usual 50% formamide/2 x SSC, 3 changes and 5 changes of 2 x SSC at the same temperature, gave a clear distinction between the labelled and non-labelled sperm, and produced a relatively clear background. It was assumed that sperm labelled with ^{3}H-TRX probe sperm were X-bearing sperm.

In the first experiment, with no pretreatment almost all (98.9%) of the sperm remained unlabelled with the probe. This exposed the necessity for pretreatment of the ejaculated sperm which had not been recognised previously, as the experience of the laboratory was limited to testicular sperm (Webb and Fabb, 1985). The results confirmed the failure to label human ejaculated spermatozoa following an isotopic *in situ* hybridization similar to the report of Joseph et al. (1984). The most likely explanation for this finding was that the chromatin of ejaculated mammalian sperm is packaged in a condensed and inactive state, the somatic histones being replaced by sperm-specific protamines during late spermiogenesis and the formation of intermolecular disulphide bounds between adjacent protamines during epididymal maturation serving to maintain the tightly packaged state of the chromatin (Ward and Coffey, 1991).

A pretreatment procedure to decondense the sperm nucleus was therefore essential to allow access of the probe. It was decided to try a combination of sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) to decondense the sperm nucleus (Calvin and Bedford, 1971). Using this treatment a variable response from sample to sample was obtained (from 8.2% to 48.5% for TRX probe, Table 3.3). Inter-sample

variability of decondensation of human sperm nuclei by SDS and DTT, assessed morphologically has been reported by Bedford et al. (1973). Inter-sample variation in the frequency of the labelling of sperm following hypotonic potassium chloride and fixation treatment, resulted in a variation of apparently biotinylated Y chromosome bearing sperm from 35%-66% in five normal fertile men (Burns et al., 1985). Coonen et al. (1991) investigated a larger number of semen samples (32 samples) using 25 mM DTT/0.1% trypsin pretreatment of sperm preparation for 5-20 min and a chromosome 1-specific DNA probe with a FISH method. Positive hybridization reactions varied from 40% to over 90% for the different samples. These results were explained by Coonen et al. (1991) as due to sperm nuclei in the human ejaculate being at different stages of maturation and stability, which reflected a variable accessibility of the cells to the DNA probes (Coonen et al., 1991).

Considering the wide range of percentage of sperm labelled with the TRX probe (Table 3.3) in the present study it was concluded that the SDS/DTT pretreatment method for sperm was not satisfactory. West et al.(1989) had obtained efficient labelling with a Y-specific probe, using EDTA/DTT pretreatment sperm and isotopic *in situ* hybridization. His results ranged from 37.5%% to 54.5%% with a mean 46.7% labelling with Y probe from eight different preparations of spermatozoa. It was presumed that this pretreatment regime would allow improvement. As a consequence, the pretreatment method of West et al. (1989) was adopted and was immediately successful when experimentation was switched to fluorescent *in situ* hybridization.

CHAPTER FOUR

LOCALIZATION OF CHROMOSOMES (X AND 17) IN HUMAN SPERM BY SINGLE LABEL FISH

4.1 Introduction

In situ hybridization techniques have been used widely to visualize specific DNA or RNA sequences in preparations of chromosomes, single cells or tissue sections. Initially, the nucleic acid probes were labelled isotopically and detected post-hybridization by autoradiography. However, non-isotopic techniques have become increasingly popular in recent years. Mainly because they are rapid; the total FISH procedure requires only 2 days whereas autoradiographic methods require between 1 week and 3 months (Joseph et al., 1984). Only recently have single label fluorescence *in situ* hybridization (FISH) experiments using DNA probes specific for chromosomes 1 and Y been successfully performed on human semen ejaculates; Wyrobek et al. (1990) found 50.1% Y-bearing human sperm in three donors examined; and Guttenbach and Schmid (1990; 1991) found 49.3% Y-bearing sperm and 0.27% disomic Y from eight donors, with an incidence of 0.41% disomic for chromosome 1 (7 donors). FISH permits both the detection of the expected frequencies of normal chromosomes and aneuploidy in human sperm nuclei simply by counting the number of fluorescent spots (Eastmond and Pinkel, 1989).

No information was available relating to chromosomes other than the chromosome 1 and Y in human spermatozoa using the FISH method. Thus the aim of the study was to determine the feasibility of detecting the normal frequency and the incidence of aneuploidy in human ejaculated sperm using probes specific for the 17 and X chromosomes by FISH.

4.2 Methods

4.2.1 Subjects

Single semen samples were obtained from thirteen healthy donors who attended the Andrology Laboratory at The Queen Elizabeth Hospital. Eleven of the thirteen donors were of proven fertility. The samples were produced by masturbation, allowed to liquefy at room temperature for at least 20 minutes and then analysed according to standard procedures (World Health Organization, 1987). Sperm parameters from thirteen normal male showed detail in Table 4.1.

Table 4.1: Sperm parameters from 13 normal male

DONOR	SEMEN VOLUME (ml)	COUNT (x 10^6/ml)	MOTILITY (%)	NORMAL MORPHOLOGY (%)
1	2.1	23	56	52
2	2.0	132	57	66
3	3.0	45	52	41
4	1.5	242	54	59
5	3.0	117	53	39
6	1.7	124	58	59
7	4.0	150	58	50
8	1.5	59	67	56
9	5.4	97	58	50
10	2.0	108	59	52
11	1.8	185	58	54
12	6.0	66	50	39
13	4.0	317	58	48
Total	**38.0**	**1,665**	**738**	**687**
Mean	**2.9**	**128**	**57**	**53**

4.2.2 Labelling DNA probes with Biotin-7-dATP by nick translation

The probes were labelled by nick translation with biotin-7-dATP (Bethesda Research Laboratories) according to the supplier's instructions (*Non-radioactive Nucleic Acid Detection system, Cat. No. 8279SA, BluGENE™, BRL).

4.2.2.1 A trace of [1', 2', 2, 8-^{3}H] dATP (1 µCi) was added to an Eppendorf tube and dried down in a vacuum desiccator for 10 min.

4.2.2.2 The following were added to the dry tube: TRX, TR17 or λDNA (Amersham) control probe 1 µg in H_2O or $T_{10}E_1$, H_2O to 37.5 µl with probe volume, 5 µl of Solution A1 (0.2 mM dNTPs in 500 mM Tris-HCl), 2.5 µl of Biotin-7-dATP (0.4 mM, Cat. No. 9509SA, RBL), 5 µl of Solution C (0.4 units/µl Polymerase I, 40 pg/µl DNA Pol I/DNAse I) and mixed thoroughly.

4.2.2.3 The nick mix was incubated at 15°C for 90 min.

4.2.2.4 5 µl of solution D (300 mM Na_2 EDTA pH 8.0) and 1.25 µl of 5% SDS were added to stop the reaction.

4.2.2.5 The nick mix was layered onto a Sephadex G 50 column (gel was washed 3 times with 1 x SSC/0.1% SDS before mix added). The fractions were diluted with 1 x SSC / 0.1% SDS. The collection of fractions in each tube as follows:

tube 1-19 . .150 µl

tube 20 . . . 600 µl

4.2.2.6 2 µl of each fraction in 0.3 ml H_2O and 3 ml of scintillant was counted for 1 min.

4.2.2.7 The tubes containing the labelled probe were determined, pooled and divided into convenient size. The labelled probe was ethanol-precipitated with 1/10 vol. 3 M Na acetic (pH 5.2) 2 vol. cooled 95% ethanol, and stored at -20°C.

4.2.2.8 The following formula was used to calculate the fraction incorporated with Biotin.

$$\text{Fraction} = \frac{\text{Total counts in probe}}{\text{Total counts in probe plus unincorporated bases}}$$

4.2.3 Immunocytochemical detection of biotin labelled probes

The sperm pretreatment and *in situ* hybridization procedures see Chapter Two (2.3 and 2.5) was used. Then a modification of the method of Arnoldus (1990) was followed. After post-hybridization washing the slides were preincubated with 5% non-fat dry milk (NFDM) in 4 x SSC for 20 minutes at room temperature. A sandwich labelling method consisting of 30-minute incubation steps at 37°C was then followed.

4.2.3.1 The first step consisted of 0.8 ng of fluorescein (FITC)-labelled avidin DCS (cell sorter grade) (2 mg/ml; Vector Laboratories) in 100 µl of 5% NFDM/4 x SSC for each slide, followed by 3 washes with 4 x SSC containing 0.05% Tween 20.

4.2.3.2 The second step consisted of 2.5 ng of biotinylated anti-avidin (0.5 mg/ml; Vector Laboratories). The reagents were diluted in 50 µl of 0.5% blocking reagent (Boehringer-Mannheim) in 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5. Then the slides were washed 3 times in 0.1 M Tris-HCl, 0.15 M NaCl (pH 7.5) containing 0.05% of Tween 20.

4.2.3.3 The final step was FITC-conjugated avidin DCS. Dilution and washes were the same as step 4.2.3.2.

The slides were then dehydrated through an ethanol series and mounted with a 9:1 mix of glycerol and PBS which also contained 1 μg/ml propidium iodide counterstain and 20 μg/ml, 4-diazo bicyclo [2,2,2] octane (DABCO, Sigma) anti-fade reagent, sealed and stored in the dark at 4°C.

4.2.4 Fluorescence Microscopy and Analysis

The slides were examined at a magnification of 1,250 X with a Leitz microscope equipped for epifluorescence. Under conditions of blue excitation at 450-490 nm and emission at 520 nm, propidium iodide fluoresced red while the hybridization signals (FITC) appeared as yellow-green spots. To establish the percentage of labelled cells, at least 1,000 sperm were scored from each preparation (Eastmond and Pinkel, 1989).

Spermatozoa with either a distinct yellow-green spot in the nucleus or no spot were classified as positive or negative haploid cells respectively. The identification of disomic and diploid spermatozoa was based on head size as proposed by Joseph et al. (1984); sperm which had two distinct positive hybridization signals within a nucleus of normal size were considered disomic, whereas those which had two spots in a large nucleus (2-4 times the area) were scored as diploid.

4.2.5 Statistics

Regression analysis method was used to study the relationship between percentages of labelling by TR17 and TRX. The regression equation and correlation coefficient and its significance level were analysed.

4.3 Results

The positive controls, which consisted of slides of mitotic chromosomes, exhibited a signal in the centromeric region of both chromosomes 17 in male and female blood preparations when probed with TR17 (Figure 4.1). When mitotic chromosome preparations were probed with TRX, a signal was located in the centromere of the single X chromosome in male preparations and in both X chromosomes in female preparations (Figure 4.2). Negative controls utilized biotinylated lambda DNA hybridized to either mitotic chromosomes or decondensed sperm nuclei and showed no specific labelling (Figure 4.3).

Hybridization signals to TR17 (Figure 4.4-4.6) were detected in > 90% of the sperm nuclei in each semen sample and > 95% of the sperm were positive in 9 of the 13 samples. The TR17 labelling frequencies for each sample are shown in Table 4.2. Overall, 96.1% were positive (range = 91.1 - 99.7). The different TR17 labelling patterns are shown in Figures 4.4-4.6 and include 95.4% haploid (89.8-99.3) (Figure 4), 0.33% disomic (0.16 -0.54) (Figure 4.5) and 0.37% diploid (0.09 - 0.89) (Figure 5.6).

The TRX labelling frequencies for each semen sample are presented in Table 4.3. In all, 48.22% were positive for the X chromosome (range = 43.97 - 50.99). The different TRX labelling patterns are shown in Figures 4.7-4.9 and include 47.73% haploid (range=43.38-50.39) (Figure 4.7), 0.29% disomic (0.18-0.40) (Figure 4.8), 0.20% diploid (0 - 0.55) (Figure 4.9) and a frequency of 51.78% presumptive Y-bearing sperm.

A comparison of Tables 4.2 and 4.3 shows 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 (Figure 4.10).

Table 4.2: Percentage of Sperm-Labelled by the biotin-labelled TR17 Probe (13 Normal Donors)

	NUMBER(%) TR17 POSITIVE			TOTAL	NUMBER(%)	TOTAL
DONOR	HAPLOID	DISOMIC	DIPLOID	(%)	NON-FLUOR.	SCORED
1	1010 (99.31)	3 (0.29)	1 (0.10)	1014 (99.71)	3 (0.29)	1017
2	1010 (98.25)	5 (0.49)	4 (0.39)	1019 (99.12)	9 (0.88)	1028
3	1013 (98.06)	3 (0.29)	2 (0.19)	1018 (98.55)	15 (1.45)	1033
4	1006 (97.67)	2 (0.19)	5 (0.49)	1013 (98.35)	17 (1.65)	1030
5	1016 (97.79)	3 (0.29)	2 (0.20)	1021 (98.27)	18 (1.73)	1039
6	1202 (97.80)	2 (0.16)	3 (0.24)	1207 (98.21)	22 (1.79)	1229
7	1017 (96.12)	3 (0.28)	7 (0.66)	1027 (97.07)	31 (2.93)	1058
8	1123 (96.07)	3 (0.26)	4 (0.34)	1130 (96.66)	39 (3.34)	1169
9	1027 (95.36)	3 (0.28)	1 (0.1)	1031 (95.73)	46 (4.27)	1077
10	1010 (92.83)	5 (0.46)	3 (0.28)	1018 (93.57)	70 (6.43)	1088
11	1006 (91.21)	6 (0.54)	4 (0.36)	1016 (92.11)	87 (7.89)	1103
12	1001 (90.51)	5 (0.45)	6 (0.54)	1012 (91.50)	94 (8.50)	1106
13	1004 (89.80)	4 (0.36)	10 (0.89)	1018 (91.06)	100 (8.94)	1118
TOTAL	**13,445**	**47**	**52**	**13,544**	**551**	**14,095**
(%)	**(95.39)**	**(0.33)**	**(0.37)**	**(96.09)**	**(3.91)**	

Table 4.3: Percentage of Sperm Labelled by the biotin-labelled TRX Probe (13 Normal Donors)

	NUMBER(%) TRX POSITIVE				NUMBER(%) TOTAL		
DONOR	HAPLOID	DISOMIC	DIPLOID	TOTAL(%)	CORRECTED TOTAL(%)	NON-FLUORO	SCORED
1	501(48.74)	3(0.29)	1(0.10)	505(49.12)	49.26	523(50.88)	1028
2	516(50.29)	4(0.39)	0	520(50.68)	51.13	506(49.32)	1026
3	48848.70)	4(0.40)	20.20)	494(49.30)	50.03	508(50.70)	1002
4	509(49.37)	3(0.29)	1(0.10)	513(49.76)	50.59	518(50.24)	1031
5	514(49.00)	3(0.29)	2(0.19)	519(49.48)	50.35	530(50.52)	1049
6	511(50.39)	2(0.20)	4(0.39)	517(50.99)	51.92	497(49.01)	1014
7	474(47.16)	4(0.40)	4(0.40)	482(47.96)	49.41	523(52.04)	1005
8	503(48.79)	4(0.39)	0	507(49.18)	50.88	524(50.82)	1031
9	488(46.12)	3(0.28)	0	491(46.41)	48.48	567(53.59)	1058
10	464(45.41)	3(0.29)	1(0.10)	468(45.88)	49.03	552(54.11)	1020
11	477(45.95)	2(0.19)	2(0.19)	481(46.34)	50.31	557(53.66)	1038
12	510(47.13)	2(0.18)	6(0.55)	518(47.87)	52.32	564(52.13)	1082
13	439(43.38)	2(0.20)	40.40)	445(43.97)	48.29	567(56.03)	1012
TOTAL	**6,394**	**39**	**27**	**6,460**	**50.15**	**6,936**	**13,396**
(%)	**(47.73)**	**(0.29)**	**(0.20)**	**(48.22)**		**(51.78)**	

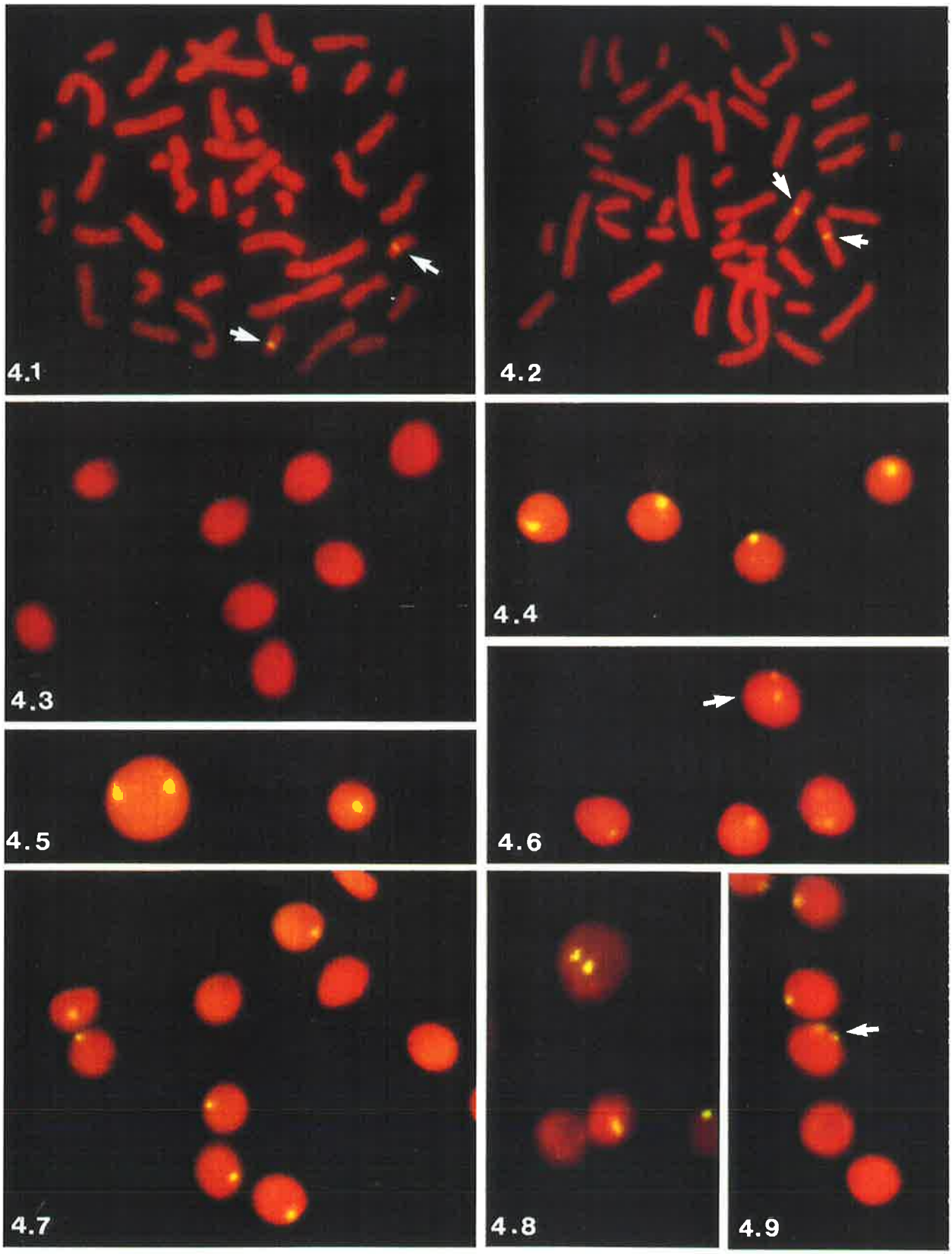
Figure 4.1: Two hybridization signals (arrows) localized to chromosome 17 after hybridization of TR17 to mitotic lymphocyte preparations.

Figure 4.2: Hybridization of TRX to mitotic chromosome preparations from lymphocytes of a human female, a hybridization signal (arrow) is at the centromeres of both X chromosomes.

Figure 4.3: Control hybridization: there is no specific labelling in sperm which have been incubated with biotinylated lambda DNA prior to FISH.

Figures 4.4-4.6: Signals of TR17 hybridized to decondensed human sperm nuclei. Figure 4: single signals in haploid sperm. Figure 5: a diploid cell on the left. Figure 6: a sperm which is disomic for chromosome 17 (arrow).

Figures 4.7-4.9: Signals of TRX hybridized to decondensed human sperm nuclei. Figure 7: haploid sperm, 6 out of 10 are labelled. Figure 8 shows a sperm which is diploid at the top (arrow). Figure 9: a sperm which is disomic for the X (arrow).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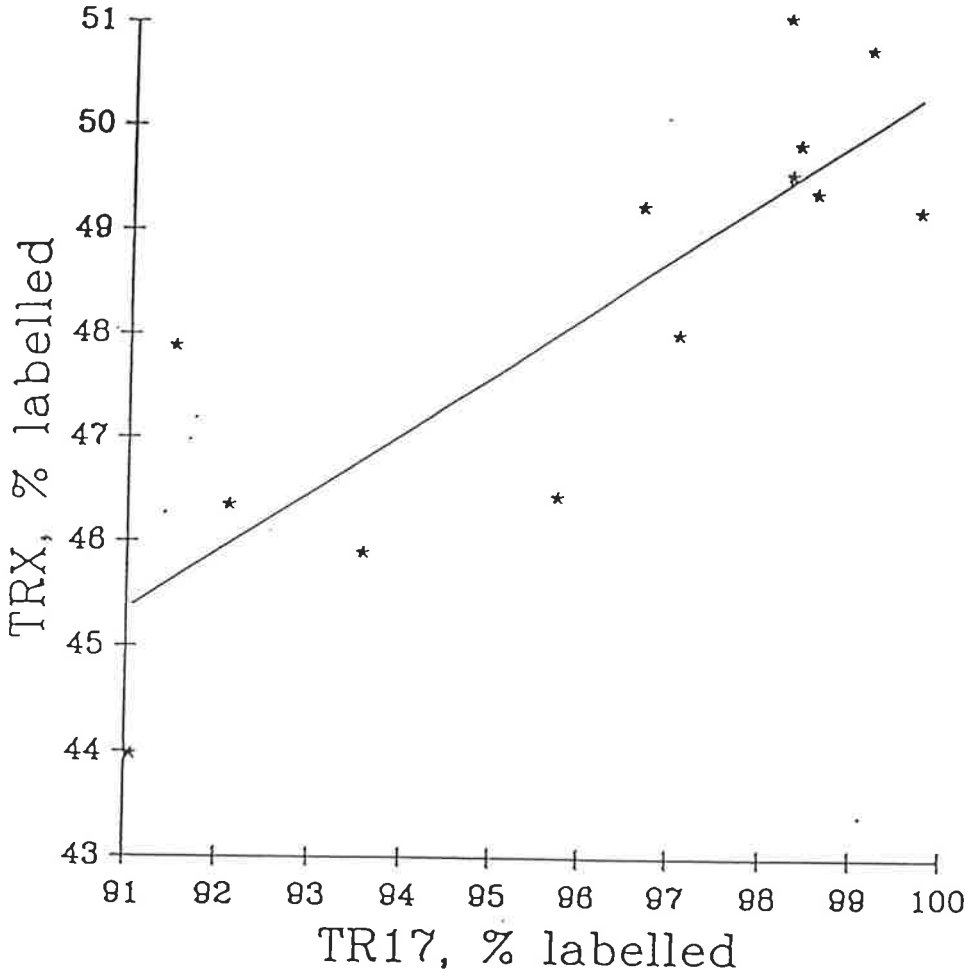


Figure 4.10: Plot of the percentage of sperms labelled by the TRX probe against the percentage labelled by the TR17 probe in all 13 individuals investigated. The regression line TRX% = 0.557 x TR17% - 5.36 is shown.

4.4 Discussion

In this study, pretreatment of sperm with EDTA followed by DTT (West et al., 1989) proved to be an immediately successful technique for decondensing sperm nuclei. Other methods, which were not tried, have included trypsin and DTT (Joseph et al., 1984; Coonen et al., 1991), a combination of sodium dodecyl sulphate (SDS) and DTT (Calvin and Bedford, 1971; Bedford et al, 1973), and a prolonged denaturation of the sperm slide (Guttenbach and Schmid, 1990,1991). The results of SDS/DTT pretreatment and isotopic *in situ* hybridization were reviewed in chapter 3 of this thesis. Guttenbach and Schmid (1990, 1991) found that denaturation of sperm preparations with a DNA probe mixture similar to the one used for the present work at 72°C for 10 min could cause all sperm nuclei to swell, so pretreatment was found to be unnecessary in their work. However this finding could not be repeated in the present study. Wyrobek et al (1990) treated human sperm nuclei with mixed alkymethylammonium bromide and DTT followed by decondensation with lithium di-iodosalicylate. This method produced good results when combined with FISH using a Y-specific probe, but the method is time-consuming and complex.

In this and other studies, the frequency of cells not labelled with the probe was the most variable factor and is probably affected by the efficiency of nuclear decondensation. In the present study only 3.9% of the sperm were not labelled with TR17 (Table 4.2). The 96.1% of sperm labelled with TR17 is the closest to 100% ever obtained for an autosome. Nonetheless the discrepancy may be much greater than that attributable simply to aneuploidy of chromosome 17, and the FISH technique using a single probe appears to be too insensitive to detect nullisomy due to normal levels of aneuploidy of any chromosome (Eastmond and Pinkel, 1989).

Overall, 48.2% of the sperm were labelled with TRX, again close to the expected 50%. The close correlation of the percentage scores for both these probes (Fig. 10) is also an indication that technical factors such as decondensation affected each probe.

If a simple correction of the TRX data by the formula fTRX/fTR17 X 100 is applied, (where f=a form of base-line straightening) then the average of the 13 corrected TRX scores (Table 4.3) is 50.15%; very close to the expected result. This demonstrated the importance of including an autosomal control such as TR17 to establish whether shifts in the ratio of X and Y sperm were real or merely an artifact of incomplete nuclear decondensation. The results correlate well with the following X:Y sperm ratios obtained using Y-specific probes and FISH method: 50.3 to 49.4 (Guttenbach and Schmid, 1990); 48.6 to 51.4 (Pieters et al., 1990); 49.9 to 50.1 (Wyrobek et al. 1990).

Several studies have assessed the frequency of autosomal disomy in human sperm. In the present study, an overall disomy frequency of 0.33% was found for chromosome 17 which is slightly lower than the reported disomy frequency of 0.41% (Guttenbach and Schmid, 1991) and 0.8% (Pieters et al., 1990) for chromosome 1 in human sperm nuclei. In addition, a disomy frequency of 0.29% for the X chromosome was detected which is close to a Y chromosome disomy frequency of 0.27% reported by Guttenbach and Schmid (1990).

In this study, diploid and disomic sperm were distinguished by the size of the sperm head, as proposed by Joseph et al. (1984). Sperm nuclei of normal size with two hybridization signals were considered disomic while large nuclei with two signals were scored as diploid. A diploid frequency of 0.37% was found using TR17 and 0.20% using TRX. The 0.20% diploid frequency with the X chromosome-specific probe should be corrected to 0.40% since the X chromosome is present in only half of the sperm. The corrected frequency of diploidy for the two chromosomes are thus very similar and as expected from other work.

To date the target sequences used for the chromosomal analysis of sperm have all been repetitious. In addition to the present report, and those utilizing the Y-chromosome, four groups have investigated chromosome 1 using ISH detection of a

repetitive target (Joseph et al., 1984; Guttenbach and Schmid, 1991; Pieters et al., 1990; Coonen et al, 1991).

Fluorescence *in situ* hybridization has many potential applications in infertility diagnosis and human *in vitro* fertilization research. Future simultaneous use of specific X, Y and autosomal chromosome probes should enable rapid and accurate assessment of X:Y ratios and disomy and diploidy. It should also be possible to evaluate sperm selection procedures such as albumin columns and Percoll gradients that have been reported to alter the ratio of X and Y sperm (Ericsson et al., 1973; Iizuka et al., 1987). In addition the development and application of multiple labelling FISH methods will improve the accuracy of studies of the frequency of aneuploidy in human spermatozoa. In particular the methodology will allow questions relating to chromosomal complement of pathological semen samples to be addressed.

CHAPTER FIVE

IDENTIFICATION OF X- AND Y-BEARING HUMAN SPERM BY DOUBLE FLUORESCENCE *IN SITU* HYBRIDIZATION

5.1 Introduction

Since the 1970's, quinacrine staining has been used to distinguish X- and Y-bearing sperm. Sperm with two fluorescent spots were considered to be disomic for the Y chromosome due to non-disjunction at the second division in meiosis and were found with a frequency of 1-5% (Sumner et al., 1971; Klasen and Schmid, 1981). However, this apparently high non-disjunction rate was not confirmed by studies of human sperm chromosomes after sperm penetration of hamster eggs (Martin et al., 1983) which observed no 24,YY complements in 1000 sperm. It may be that YY sperm failed to penetrate the hamster oocyte.

DNA probes for specific sequences on the Y chromosome subsequently became available. Joseph et al. (1984) used isotopic *in situ* hybridization to estimate the frequency of aneuploidy in human testicular sperm. In their first *in situ* experiments, DNA probes were applied to chromosomes 1 and Y and 48.3% of sperm were found to be Y-bearing of which 0.18% were disomic. In 1990, fluorescence *in situ* hybridization (FISH) was used to hybridize the Y chromosomes in ejaculated sperm with specific probes. Guttenbach and Schmid (1990) reported 49.4% sperm successfully labelled with the specific Y chromosome probe and 0.27% disomy. Wyrobeck et al. (1990) reported 50.1% Y-bearing sperm but did not report on disomy. In chapter 4 of this thesis the experiments with FISH and the X chromosome specific probe, TRX, found 48.22% sperm to be labelled and 0.29% were disomic (Han et al., 1992).

To date, all the reported ISH studies to date of sex chromosomes in sperm have considered the unlabelled cells to be X- or Y- bearing for Y and X probes respectively. When only one probe is used doubt always will exist as to whether the negative labelled cells are either nullisomic, contain the unlabelled sex chromosome or have failed to hybridize.

The following experiments describe a double FISH technique with both X and Y probes which has allowed the simultaneous detection of X- and Y-bearing sperm. This technique has greatly improved the reliability of the analysis, and allowed a more accurate estimation of sex chromosome aneuploidy in ejaculated human sperm.

5.2 Methods

5.2.1 Subjects

Ejaculates from 12 normal healthy donors, aged 20-45 years old, were obtained from the Andrology laboratory at The Queen Elizabeth Hospital. The donors had normal semen according to standard analytical procedures for ejaculate volume, sperm concentration, progressive motility and morphology (World Health Organization, 1987) and ten of the twelve donors were of proven fertility (see table 5.1). The semen was pretreated with EDTA/DTT, the details have been described in Chapter Two (Section 2.3).

Table 5.1: Sperm parameters from 12 normal male

DONOR	SEMEN VOLUME (ML)	COUNT (x 10^6/ml)	MOTILITY (%)	NORMAL MORPHOLOGY (%)
1	1.8	185	58	54
2	3.0	45	52	41
3	3.0	117	53	39
4	1.5	59	67	56
5	2.0	132	57	66
6	5.4	97	58	72
7	1.7	124	58	59
8	4.0	150	58	50
9	2.1	232	56	52
10	6.0	66	50	39
11	2.0	108	59	52
12	4.0	317	58	48
Total	**26.5**	**1,423**	**684**	**628**
Mean	**2.2**	**119**	**57**	**52**

5.2.2 Exposure of lymphocytes to sperm pretreatment method

Lymphocytes were separated from whole blood by ficol-paque and were pretreated in the same way as the sperm (Chapter Two, Section 2.3).

5.2.3 Labelling of DNA probes with Digoxigenin-dUTP by random priming method

The HRY probe was labelled by random priming with Digoxigenin dUTP (DIG DNA labelling and Detection Kit, Cat. No. 1093 657, Boehringer Mannheim Biochemical, according to the supplier's instruction).

5.2.3.1 The HRY probe was denatured by heating in a boiling water bath (100°C) for 10 min and then chilled quickly on ice for 10 min.

5.2.3.2 A trace of [1', 2', 5-^{3}H] dCTP (1 µCi) was added to an Eppendorf tube and dried down in a vacuum desiccator for 10 min.

5.2.3.3 The following were added to the dry tube:

Denatured HRY probe 1 µg in H_2O or $T_{10}E_1$ H_2O to 15 µl with probe volume 2 µl of Hexanucleotide mixture (vial 5, 10 x concentrated) 2 µl of dNTP labelling mixture (vial 6, 10 x concentrated, containing dATP, 1 mmol/L; dCTP, 1 mmol/L; dGTP, 1 mmol/L; dTTP 0.65 mmol/L; DIG-dUTP, 0.35 mmol/L; pH 7.5) 1 µl of Klenow enzyme (vial 7, 2 units/µl)

5.2.3.4 The reaction mixture was carefully mixed and then incubated at 37°C for 20 hrs.

5.2.3.5 2 µl of EDTA solution (0.2 mol/L, pH 8.0) was added to stop the reaction.

5.2.3.6 The mix was layered onto a Sephadex G 50 column (gel washed 3 times with 1 x SSC/0.1% SDS before mix added). The fractions were diluted with 1 x

SSC/0.1% SDS. The fractions were collected as follows:

tube 1-19	150 µl
tube 20	600 µl

5.2.3.7 2 µl of each fraction in 0.3 ml H_2O and 3 ml of water-miscible in a scintillation was counted for 1 min.

5.2.3.8 The tubes containing the labelled probe were determined, pooled and divided into convenient size. The labelled probe was ethanol-precipitated with 1/10 vol. 3 M Na Acetate (pH 5.2) and 2 vol. cooled 95% ethanol, and stored at -20°C.

5.2.3.9 The following formula was used to calculate the fraction incorporated with Digoxigenin.

$$\text{Fraction} = \frac{\text{Total counts in probe}}{\text{Total counts in probe plus unincorporated bases}}$$

5.2.4 Simultaneous immunocytochemical detection of Biotin and Digoxigenin labelled probes

For the sperm pretreatment and *in situ* hybridization procedures see Chapter Two (2.3 and 2.5). For simultaneous use of two probes a modification of the method of Arnoldus (1990) was followed. The slides were pre-incubated with 5% non-fat dry milk (NFDM) in 4 x SSC for 20 minutes at room temperature. This was followed by four consecutive immunocytochemical steps of 30 minutes each at 37°C:

5.2.4.1 0.8 ng of fluorescein (FITC)-labelled avidin DCS (cell sorter grade, 2 mg/ml, Vector Laboratories, Cat. No. A-2011) in 100 µl of 5% non fat dry milk in 4 x SSC for each slide, followed by 3 washes with 4 x SSC containing 0.05% Tween 20.

5.2.4.2 2.5 ng of biotinylated goat anti-avidin (0.5 mg/ml, Vector Laboratories, Cat. No. BA-0300) plus 0.1 µg mouse anti-digoxigenin (0.1 mg antibody/ml; Boehringer Mannheim Biochemical, Cat. No. 1333 062. This was diluted with 50 µl of 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Boehringer blocking reagent, 5% goat serum for each slide and then washed 3 times for 5 minutes each with 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween 20.

5.2.4.3 0.8 ng of FITC-Avidin DCS (as for 5.2.4.1) plus 1:40 of TRITC (tetramethylrhodamine isothiocyanate) conjugated rabbit anti-mouse immunoglobulins (0.4 mg antibody/ml, DAKO, Code No. R 270, Lot. No. 091) diluted in 100 µl as for (5.2.4.2), but with 5% of rabbit serum and washed as described in (5.2.4.2) above.

5.2.4.4 1:40 of TRITC-conjugated swine anti-rabbit immunoglobulins (0.2 mg antibody/ml, DAKO, Code No. R 156, Lot. No. 018). The dilution was as in (5.2.4.2) but with 5% swine serum. Washes were the same as (5.2.4.2).

Finally the slides were dehydrated through 35%, 70%, 95% and 100% ethanol, dried and mounted with 25 µl of a 9:1 glycerol:PBS mixture containing 0.5 µg/ml DAPI as a chromosomal counter-stain and 2% 1,4-diazabicyclo [2,2,2] octane (DABCO, Sigma) as an antifade reagent.

5.2.5 Fluorescence microscopy and photography

The slides were examined at 1250x with a Leitz microscope equipped with epifluorescence and three different filter blocks (A,UV; I-2/3,blue; N-2,green). The three fluorochromes under the different excitation wave lengths showed three emission colour (see Table 5.2).

To establish the percentage of labelled sperm, at least 1,000 sperm were scored from each preparation. Sperm cells with a distinct green spot in the nucleus were classified as haploid X-bearing sperm and those with a distinct red spot as haploid Y-bearing sperm. The identification of disomic and diploid sperm were based on the head size as proposed by Joseph et al.(1984). Sperm which had two distinct hybridization spots within a nucleus of normal size were considered disomic, whereas those which had two spots in a large nucleus (approximately 2-4 times the normal size) were scored as diploid.

Table 5.2: Fluorescence microscopy

	Fluorochrome	Filter block	Excitation	Emission colour
Nuclei	DAPI	A	UV	Blue
TRX	FITC	2/3	Blue	Green
HRY	TRITC	N-2	Green	Red

Photomicrographs were taken with Fujichrome DX 400 colour slide film using triple exposure (Nederlof et al., 1989). Exposure times were usually 10-30 seconds for TRITC, 2 minutes for FITC and 10-30 seconds for DAPI. Generally, TRITC and FITC were photographed first to diminish the effect of the fading which can occur with the two dyes; DAPI was photographed last because its fading was least critical.

Figure 5.1: Chromosome spread from lymphocytes in males exhibiting clear double labelling signals; the green FITC spot over the centromere of the X chromosome (top arrow) and the red TRITC spot over the long arm of the Y chromosome (bottom arrow) can be seen in the same metaphase.

Figure 5.2: Biotin-TRX and Digoxigenin-HRY labelling of decondensed human sperm nuclei. Three of the sperm with each show a green FITC spot are X-bearing sperm (top arrow), and three of the sperm which each show a red TRITC spot are Y-bearing sperm (lower arrow).

Figure 5.3: A disomic X sperm (arrow). Two distinctive green FITC spots are seen in one normal size sperm nucleus.

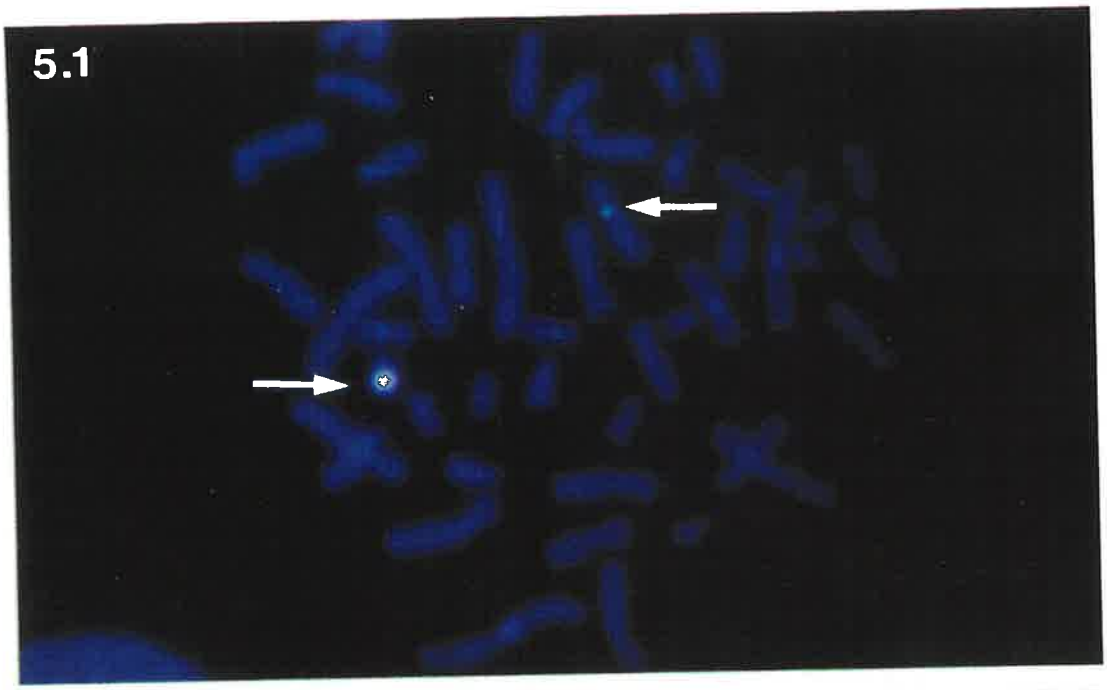
Figure 5.4: A diploid X sperm (arrow) has two distinctive green FITC spots in a nucleus which is larger than normal.

Figure 5.5: A disomic Y sperm (arrow). Two distinctive red TRITC spots are seen in a normal size nucleus.

Figure 5.6: A diploid Y sperm (arrow) has two distinctive red TRITC spots in a nucleus which is larger than normal.

Figure 5.7: Diploid cell (arrow) containing one X and one Y chromosome. (The mirrored appearance of the adjacent cell suggests that these are nuclei which have recently divided).

Figure 5.8: Tetraploid cell. Two pairs of green and red spots in one large cell which is four times the size of a normal sperm head.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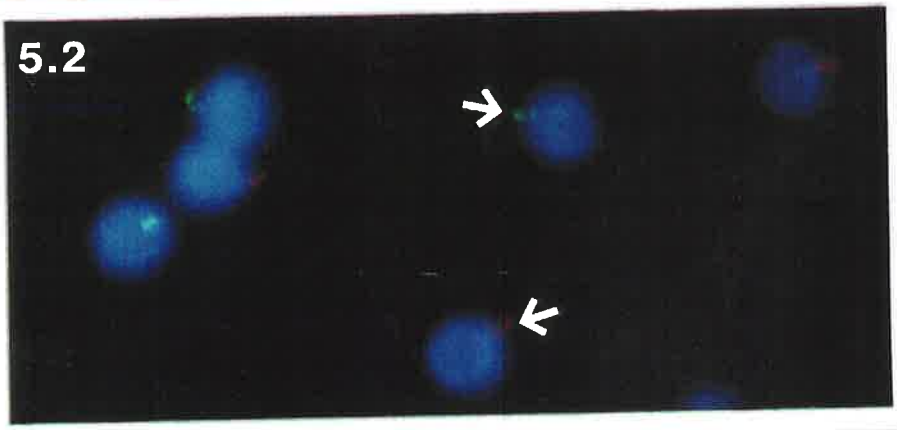
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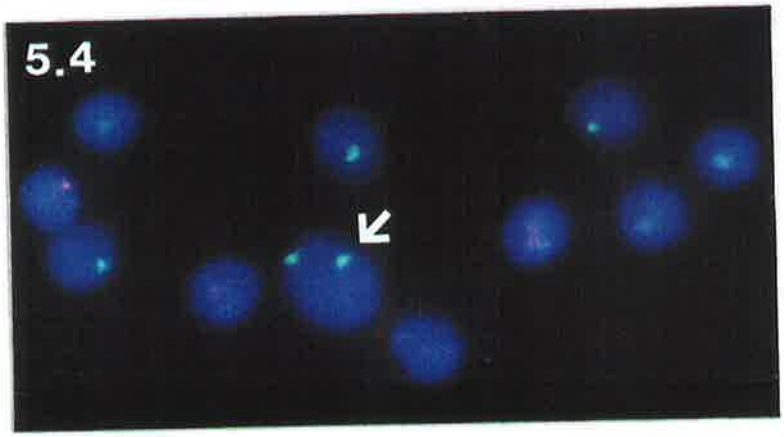
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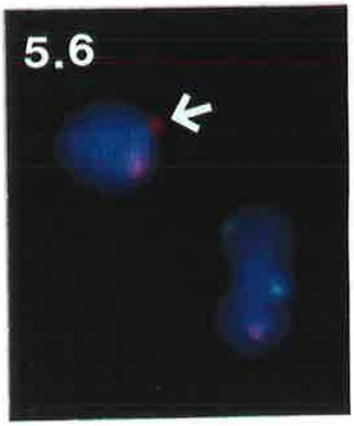
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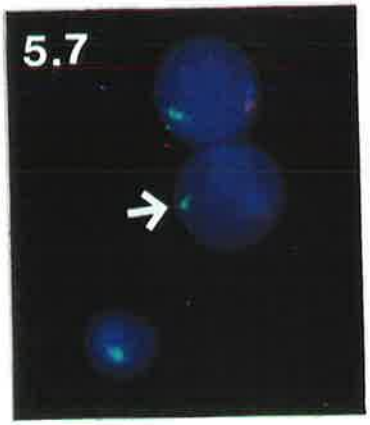
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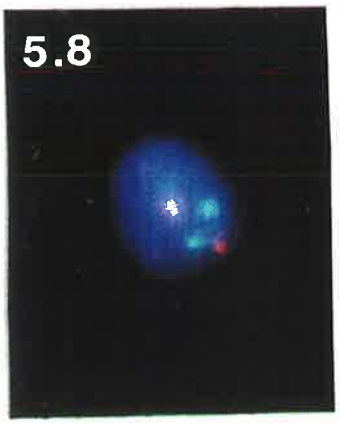
5.8

5.3 Statistical analysis

A chi-squared (χ^2) test for heterogeneity (Table 5.3) was applied on paired total scores of X- and Y-bearing sperm for the 12 donors.

5.4 Results

Chromosome spreads and interphase cells from lymphocytes in males exhibited clear double labelling signals; the green FITC spot over the centromere of the X chromosome and the red TRITC spot over the long arm of the Y chromosome could be readily seen in the same cell.

Ninety six per cent of sperm were fluorescently labelled among 12,636 scored (Table 5.3). Frequencies of 47.4% haploid X- and 46.8% haploid Y- sperm (Figure 5.1) were found (Table 5.1). A frequency of 0.28% haploid sperm disomic for the X-chromosome (Figure 5.2) and 0.18% diploid sperm were found (Figure 5.3); giving a total of 0.46% XX-sperm (Table 5.1). There were 0.21% of haploid sperm disomic for the Y (Figure 5.4) and 0.17% of diploid sperm (Figure 5.5), giving a total of 0.38% YY-sperm.

There was little variation between the 12 donors (Table 5.1). For the X-chromosome, the range of X bearing sperm was 46.3-49.3% (mean 47.9). For the Y chromosome, the range of Y-bearing sperm was 44.5-48.6% (mean 47.2). Overall 47.9% of sperm were labelled with the X probe and 47.2% with the Y probe. On chi-squared (χ^2) testing, none of the individual ratios of X- and Y- bearing sperm were significantly different from a 1:1 ratio, nor was the overall ratio, nor was there significant heterogeneity between the 12 individuals.

In addition to the X- or Y-bearing cells, 0.83% of cells contained both an X and a Y chromosome (Figure 5.6). These consisted of 0.21% of haploid XY-bearing sperm (with sperm head of normal size) and 0.62% with a diploid-sized nuclei. These latter cells may be large immature sperm, epithelial or leucocytes. In a separate control experiment, exposure of lymphocytes to the sperm pretreatment method did not markedly change their morphology so diploid XY sperm could not be distinguished from immature germ cells or contaminating non-sperm cells such as leukocytes.

Finally, a frequency of 0.05% XXYY-bearing cells was found (Figure 5.7). Five of the six XXYY-bearing cells were four times as large as a normal sperm head and one cell was eight times the size of a normal sperm head. These were considered to be tetraploid cells.

Table 5.3: Sperm Labelled with TRX and HRY Probes using Double FISH

DONOR	NUMBER OF TRX LABELLED X(N)	XX(N)	XX(2N)	TOTAL	NUMBER OF HRY LABELLED Y(N)	YY(N)	YY(2N)	TOTAL	NUMBER OF TRX & HRY LABELLED XY(N)	XY(2N)	XXYY(4N)	TOTAL	TOTAL NUMBER LABELLED	TOTAL NUMBER UNLABELLED	TOTAL NUMBER SCORED
1	502	2	0	504	500	1	1	502	2	3	1	6	1012	20	1032
2	502	2	1	505	501	1	2	504	2	18	1	21	1030	29	1059
3	493	4	3	500	476	2	2	480	3	10	0	13	993	32	1025
4	505	4	0	509	506	4	3	513	3	5	0	8	1030	33	1063
5	503	4	4	511	501	3	3	507	0	9	0	9	1027	36	1063
6	472	3	3	478	486	2	4	492	5	5	0	10	980	36	1016
7	504	3	2	509	500	2	1	503	1	3	1	5	1017	38	1055
8	500	4	0	504	504	2	2	508	2	2	0	4	1016	45	1061
9	505	4	4	513	473	3	0	476	0	5	0	5	994	47	1041
10	499	2	4	505	459	3	1	463	5	9	1	15	983	57	1040
11	502	2	0	504	500	2	1	503	4	3	0	7	1014	64	1078
12	508	1	2	511	511	1	2	514	0	6	2	8	1033	70	1103
TOTAL	5995	35	23	6053	5917	26	22	5965	27	78	6	111	12129	507	12636
Percent	47.4	.28	.18	47.9	46.8	.21	.17	47.2	.21	.62	.05	.88	96.	4	

5.5 Discussion

In earlier experiments using single-FISH a frequency of 96.1% of sperm labelling with a probe (TR17) to autosome 17 was reported (Han et al., 1992). This percentage is very close to the frequency obtained using the double-FISH technique for both the X and Y chromosome (96%). Thus the method used to decondense sperm nuclei prior to hybridization ensured efficient hybridization with both autosomal and sex chromosome probes and was reliable from experiment to experiment.

The proportion of X- and Y-bearing spermatozoa in human ejaculates has attracted considerable interest. Deviations from the theoretical 1:1 expectation, (with excess of X-sperm) were observed by Y-body detection using the quinacrine staining method (Pawlowitzki and Pearson, 1972) and early studies using the human sperm/hamster-egg system (Martin et al., 1983). However more recent larger studies of semen from normal men using the hamster egg penetration system, have observed that the ratio of X- and Y-bearing sperm did not differ significantly from the expected one to one ratio (Brandriff et al., 1985; Kamiguchi and Mikamo, 1986; Martin et al., 1987). The average proportions of X- and Y-bearing human sperm in the present study were 47.9% and 47.2%. This was consistent with recent chromosome studies and does not differ from the expected 1:1 ratio.

A frequency of 0.28% sperm with disomy X was found. This is similar to our previous study (Han et al., 1992) in which a disomic frequency of 0.29% with a single TRX probe was found. A frequency of 0.21% sperm with disomic Y was also found which is similar to the 0.27% reported by Guttenbach and Schmid (1990) using a Y specific probe and FISH method.

A frequency of 0.21% sperm had one X and one Y chromosome in cells judged to be haploid by their normal size. It seems likely these cells resulted from meiosis I errors during spermatogenesis. However the frequencies of XX-, YY–, and XY-

bearing haploid sperm which were detected exceed those found in studies following the hamster egg penetration technique. Martin et al. (1983) found 0.1% (1 in 1,000) XX-sperm in humans, and Brandriff and co-workers found 0.11% (1 in 909) of YY-sperm (Brandriff et al., 1984) and 0.08% (1 in 1,324) of XY- sperm in their studies (Brandriff et al., 1985). The non-disjunction frequencies also exceed those found in newborns where 0.06% of female births are 47,XXX, 0.17% of male births are 47,XXY and 0.1% of male births are 47,XYY (Rothwell, 1983). However, an excess of YY cells was also found by Guttenbach and Schmid (1990) using the FISH method. The aneuploidy excess may be due to technical artefacts in FISH or due to a reduced fertilizing capacity of aneuploid sperm in the hamster egg penetration system. A possible alternative technical reason for the apparently increased rate of aneuploidy might be that individual X or Y chromosomes sometimes show a two-dot signal which may be attributable to a splitting of the alpha repeat sequences of a single X or the heterochromatin of a single Y chromosome. Some cells did have double signals with each signal comparatively smaller than those in other sperm nuclei. In such cells, whenever the spots were touching they were counted as one (monosomic) but where they were completely separate, they were counted as two (disomic). If misclassification did occur then disomy would have been overestimated.

In newborn males, the incidence of 47,XXY individuals (the products of fertilization with XY sperm) is 0.17%, thus the incidence in all newborns is about 0.09%. However, since only about 50% of 47,XXY individuals are the product of paternal error (Hassold et al., 1991). less than 0.05% of newborns have received both an X and Y chromosome from their father. Since there is no proof of preferential loss of 47,XXY individuals *in utero* (Bond and Chandley, 1983) the newborn frequency is close to the frequency in all "recognized" pregnancies (i.e. those post six weeks gestation).

Hassold et al. (1991) have shown that when 47,XXY is paternally derived the XY gamete results from failure of pairing between the X and Y chromosomes in meiosis I. Failure of XY pairing was observed in an average of 15% of spermatocytes undergoing diakinesis-metaphase I in testicular biopsies (Skakkabaek et al., 1973) and in an average of 10% of these spermatocytes (range 0-30%) present in ejaculates from three oligospermic men (Peters, G.B., personal communication). It seems likely, therefore, that 24,XY gametes are generated in meiosis at a reasonably high frequency. The frequency with which these gametes are observed is then gradually reduced during the stages of maturation and fertilization.

About 0.62% of nuclei with one X and one Y chromosome were considerably larger than normal sperm nuclei. Since it had been established that lymphocyte nuclei remained intact during the pretreatment procedure, it was possible that some of these large XY-bearing cells were contaminating leukocytes or immature germ cells (spermatogonia and primary spermatocytes). The techniques which isolate motile sperm (e.g. swim-up technique) would be expected to largely eliminate these non-sperm cells from analysis (see later Chapter 6).

In conclusion, the double FISH method is a more powerful technique for identifying X- and Y-bearing sperm than either single FISH or quinacrine staining. Double FISH is not limited to sex chromosomes and will be a valuable tool in future studies of aneuploidy in human sperm.

CHAPTER SIX

DETECTION OF X- AND Y-BEARING HUMAN SPERMATOZOA FOLLOWING MOTILE SPERM ISOLATION BY SWIM-UP

6.1 Introduction

The swim-up technique (Drevius, 1972) is routinely used to obtain populations of highly motile human spermatozoa in many clinical intrauterine insemination (IUI), *in-vitro* fertilization (IVF) and gamete intrafallopian transfer (GIFT) programmes (Kirby et al. 1991; Avery et al., 1992). Previous studies have addressed the motility, morphology, fertilization rate and nuclear maturity of human sperm prepared by swim-up and other methods (Russell and Rogers, 1987; Englert et al., 1992; Ng et al., 1992; Le Lannou and Blanchard, 1988). However, little is known about the sex status and incidence of chromosomal abnormalities in populations of motile human sperm prepared by this procedure, although Check et al. (1989; 1993) recently reported that babies derived from IUI using sperm prepared by a modified swim-up procedure have a skewed male to female ratio.

In this study, a recently developed double label fluorescent *in situ* hybridization (FISH) technique (Han et al., 1993) has been used to detect simultaneously X- and Y-bearing sperm. This method allows the quick and efficient sexing of many thousand sperm allowing statistical methods to be applied to the assessment and avoids the uncertainties of sexing by quinacrine staining or single label FISH methods. It was used to compare the X:Y ratio in whole semen samples and in motile populations of sperm prepared from the same samples by the swim-up technique. The results suggest that these is no change in the X/Y ratio following motile sperm isolation.

6.2 Methods

6.2.1 Subjects

Single semen samples were obtained from ten normal healthy donors who attended the Andrology Laboratory at The Queen Elizabeth Hospital. The samples were produced by masturbation, allowed to liquefy at room temperature for 15-30 minutes and then analysed according to standard procedures (World Health Organization, 1987). Sperm morphology was assessed using WHO criteria on smears stained by the modified Papanicolaou technique. The semen values for the ten donors are summarized in Table 6.1.

Each sample was divided into two portions. One portion of semen (neat semen, as control) was prepared for double label FISH, while a motile fraction was isolated from the other aliquot of semen using the swim-up technique. The motile fraction was then prepared for FISH.

Table 6.1: Semen characteristics (WHO) of the samples examined

Donor	Volume (ml)	Concentration (x 10^6 sperm/ml)	Progressive Motility (%)	Morphology (% normal)
1	4.0	35	51	35
2	5.2	52	40	48
3	1.5	122	61	32
4	3.8	110	65	nd*
5	6.5	74	54	32
6	6.5	74	54	32
7	5.0	142	51	53
8	1.9	113	60	37
9	3.2	135	67	39
10	4.0	98	63	50
Mean	**3.8**	**99**	**57**	**42**

nd*= not determined

6.2.2 Swim-up technique

Motile sperm were isolated by swim-up from a washed pellet, as outlined by Kirby et al.,(1991). One ml of liquefied semen was diluted with 2 ml of HEPES-buffered human tubal fluid medium (Quinn et al., 1985) containing 7.5% human serum, and centrifuged at 300 x g for 10 min. The pellet was resuspended in 2 ml of medium

and the centrifugation step was repeated. The final pellet was carefully overlaid with 0.5 ml of medium, taking care not to disturb the loose pellet and the tube was then incubated upright at 37°C for 30 to 60 min. Up to 0.25 ml of the motile upper layer fraction was carefully removed and analyzed as described above. The progressive motility of this fraction was routinely 80-95%.

6.2.3 Statistical Analysis

A Chi-squared (χ^2) test was used to compare the proportions of haploid X, Y, XY cells and diploid XY cells in the control and swim-up groups. The χ^2 test for heterogeneity was used to compare the overall proportions of X- and Y-bearing sperm for the ten samples. A Fisher's exact test was used to compare the proportions of diploid XX and tetraploid XXYY cells in the control and swim-up groups.

6.3 Results

Mitotic chromosome spreads and interphase cells from lymphocyte preparations in males exhibited clear double labelling signals. In chromosome spreads there was a distinct green (FITC) spot over the centromere of the X chromosome and a red (TRITC) spot over the long arm of the Y chromosome.

A total of 10,046 sperm (control) and 10,059 (swim-up) were scored. In the neat semen, 95% of the sperm exhibited a positive hybridization signal and this varied from 90.1% to 98.5%. Similarly, 95.9% of sperm in the swim-up fractions were labelled, with individual samples giving hybridization efficiencies of 91.6% to 99% (Table 6.2).

The overall ratio of X- to Y- bearing sperm in the neat samples was 47.3% to 46.9% neat which was not significantly different from a 1 to 1 ratio. The ratios for individual samples were also not significantly different from 1 to 1 ratio. The ratio of X- to Y-bearing sperm in the swim-up fractions was 48.4% to 47.1% which was also not significantly different from the ratio in the neat semen or from a 1 to 1 ratio (Table 6.3).

Frequencies of 0.26% for disomic X, 0.27% disomic Y, and 0.16% haploid XY were found in the swim-up fraction (Table 6.3). There were no significant differences in the frequencies of disomic X, disomic Y or haploid XY sperm between the neat semen and swim-up fraction. However, the frequency of 0.12% diploidy for X or Y sperm in the motile fraction was significantly reduced compared to the frequency of 0.24% diploid X or 0.25% diploid Y in the neat semen (Table 6.4, $P<0.05$); Diploid XY cells were reduced from 0.67% in the neat semen to 0.18% in the swim-up fraction (Table 6.4, $P<0.00001$); and no tetraploid cells were found in any of the swim-up samples (Table 6.4, $P=0.03$).

Table 6.2: X and Y labelling results from ten semen samples

	Original semen				Swim-up			
Donor	% X	% Y	total(%) labelled	Total scored	% X	% Y	Total % labelled	Total scored
1	48.8	49.1	98.5	1,001	49.2	49.6%	99.0	1,030
2	47.6	49.0	97.1	1,006	48.9	49.6	98.6	1,000
3	49.5	47.1	97.6	1,004	49.2	47.8	96.5	1,005
4	48.2	47.1	96.2	1,004	49.1	46.9	96.5	1,004
5	47.1	47.6	95.6	1,001	48.4	47.7	96.5	1,000
6	46.7	46.0	94.4	1,000	49.6	46.2	96.3	1,000
7	44.0	46.6	91.4	1,005	47.5	47.7	95.5	1,010
8	47.5	46.9	95.6	1,010	46.8	46.3	93.5	1,004
9	48.6	44.4	93.6	1,015	47.9	45.3	93.4	1,006
10	44.7	44.9	90.1	1,000	47.2	44.1	91.6	1,000

Table 6.3: Overall results of X and Y labelling of sperm

	Original semen	Swim-up
No. of sperm	10,046	10,059
% labelled	95%	95.9%
% X-bearing	47.3%	48.4%
% Y-bearing	46.9%	47.1%
X to Y ratio	47.3 to 46.9*	48.4% to 47.1%*

* Not significantly different from a ratio of 1:1.

Table 6.4: Frequency of haploid, disomic, diploid and tetraploid spermatozoa before and alter motile sperm isolation

	Original semen	Swim-up	P value
Haploid X	46.8%	48.0%	ns
Haploid Y	46.4%	46.7%	ns
Disomic X	0.25%	0.26%	ns
Disomic Y	0.23%	0.27%	ns
Haploid XY	0.15%	0.16%	ns
Diploid X	0.24%	0.12%	< 0.05
Diploid Y	0.25%	0.12%	< 0.05
Diploid XY	0.67%	0.18%	<0.00001
Tetraploid XXYY	0.05%	0.0%	< 0.05

ns = not significant

6.4 Discussion

The ratio of X- to Y-bearing sperm in normal human semen is 1:1 (Brandriff et al., 1985; Kamiguchi and Mikamo, 1986; Martin et al., 1987; Han et al., 1993). Since reports exist which suggest that X- and Y-bearing sperm can be separated on the basis of the differential motility of the two populations of sperm (Ericsson et al, 1973), it was of interest to assess whether or not the routine swim-up technique altered the ratio of X- to Y-bearing sperm.

In this study, a double label FISH technique (Han et al., 1993) was utilized to simultaneously localize X- and Y-bearing human sperm. This technique has several advantages including the fact that many thousand spermatozoa can be examined without difficulty thus the entire population of sperm is studied rather than only a subgroup such as that which is capable of fertilizing zona-free hamster eggs. Additional advantages include the specificity of the probes used for the X and Y chromosomes rather than the use of a general staining such as quinacrine; and the simultaneous double labelling technique partly controls for the difference between nullisomy and non-labelling due to technical problems. In addition the high labelling efficiency (95%) allows confidence that the X:Y ratio is not unduly biased by inefficient labelling. To exclude non-labelling however, a third probe specific for an autosome must be used simultaneously. Although our technique can easily be extended to three chromosomes, our current equipment cannot accommodate this analysis.

The results of this study clearly show that the swim-up procedure isolates motile populations which retain a 1 to 1 ratio of X- to Y-bearing sperm. This result agrees with Brandriff et al. (1986b) and Benet et al. (1992) who determined the ratio of X- to Y-bearing sperm by examining chromosomes after penetration of hamster eggs by human sperm prepared by the swim-up method.

Check and co-workers (Check et al., 1989; Check and Katsoff, 1993) have reported a high percentage of male births following insemination with sperm isolated by a modified swim-up procedure. These workers found that the proportion of sperm which possessed a Y body after quinacrine staining was greatly increased by the modified swim-up procedure, but they did not verify that the ratio of X- to Y-bearing sperm was different using DNA probes. Thus the present results contrast with their findings. This may be due to the lack of specificity of quinacrine staining, or to technical differences in the swim-up procedure since the method I used (swim-up from a washed pellet) is only one of several ways of performing a swim-up.

Nevertheless, clinical results from our IVF-ET and Gift programs also indicated that there is no change in the ratio of X- to Y-bearing sperm after swim-up. Between 1982 and 1987, a total of 350 livebirths from 262 IVF-ET/GIFT pregnancies from Reproductive Medicine Unit at The Queen Elizabeth Hospital were derived from sperm isolated by swim-up (from a washed pellet). The data consists of 186 singletons, 64 twins, 11 triplets and 1 quadruplet pregnancy, and the proportion of male to female births was 92 to 94 (singletons) and 78 to 86 (multiple pregnancies). Overall, 170 male and 180 female infants were born, giving a male to female ratio of 48.6% to 51.4% which is not significantly different from a 1 to 1 ratio.

The frequency of aneuploidy (disomy, haploid XY) was similar in the control (neat semen) and swim-up samples which suggests that the level of aneuploidy in the isolated motile sperm was comparable to the total sperm population. The frequencies of disomic X (0.25-0.26%, disomic Y (0.23-0.27%) and haploid XY (0.15-0.16%) in the control and swim-up samples were comparable to my earlier FISH studies on human semen (chapter 4 and 5) and to other studies which used single label FISH and Y chromosome-specific DNA probes (Guttenbach and Schmid, 1990). These results also agree with those of Benet et al. (1992) and Brandiff et al. (1986a) who found that there were similar levels of chromosomal abnormalities in the motile sperm population and the total sperm population.

A significant reduction in the proportion of diploid and tetraploid cells in the swim-up fractions was noted; the most pronounced decrease occurred in the diploid XY group. The exact identity of these small population of cells is uncertain, they may comprise a mixed population of non-motile sperm, immature germ cells and leukocytes which mostly remain in the pellet after swim-up. This would explain the current finding that the frequency of these cells decreased in the swim-up fractions.

In conclusion, the experiments of double FISH with chromosome specific X and Y probes clearly demonstrate that the swim-up procedure does not alter the 1:1 ratio

of X- to Y-bearing human sperm. Furthermore, the study observed that isolation of a population of motile sperm by the swim-up procedure did not reduce the level of aneuploidy, but it significantly reduced the levels of diploid and tetraploid cells. Since the isolation of motile spermatozoa is an important procedure for routine IUI, IVF, and GIFT, the results of this study are important reassurance that the sex ratio is not altered.

CHAPTER SEVEN

CHROMOSOMES OF HUMAN EJACULATED SPERM IN A 47,XYY MALE

7.1 Introduction

The frequency of the 47,XYY karyotype occurs in the male neonatal population is approximately 0.1% (Hecht and Hecht, 1987). The extra Y chromosome is believed to arise through non-disjunction at the second paternal meiotic division. Considerable interest in this condition occurred during the mid-1960s because of reports that XYY males were more prone to criminal behaviour and of lower intelligence than chromosomally normal males (Jacobs et al., 1965; Casey et al., 1966).

Meiotic studies in testicular tissue in XYY males showed that some subjects had XY/XYY mosaicism in the gonadal tissue (Tettenborn et al., 1970; Hulten and Pearson 1970; Luciani et al., 1973) while in others, all the primary spermatocytes had an apparently normal 46,XY constitution (Thompson et al., 1967; Melnyk et al., 1969; Evans et al., 1970). Evans et al (1970) hypothesized that the loss of the extra Y chromosome from a primitive germ-cell or spermatogonium of a 47,XYY male is either a frequent but random event or that the 46,XY germ cell has a strong proliferative advantage over the 47,XYY germ cell. Burgoyne (1979), however, suggested that since the spermatogonial cells are usually 47,XYY, there might be a directed loss of the extra Y chromosome at a late stage of spermatogenesis.

Only one small study of the chromosomal constitution of ejaculated sperm from an XYY male has been published thus far. Using the hamster zona-free oocyte system, Benet and Martin (1988) karyotyped 75 cells from the ejaculated semen of an XYY man and found a normal incidence of X- and Y-bearing sperm. In this study we

used DNA probes specific to chromosomes 17, X and Y and the fluorescent *in situ* hybridization (FISH) to examine two ejaculates from a XYY man. The results confirm the 1:1 ratio of X:Y spermatozoa.

7.2 Materials and methods

A 47,XYY karyotype was detected in 1988 following a consultation by him and his partner for infertility. The man, now 42, who has a long history of criminal offences, is currently single, and although he has not achieved a pregnancy with a series of previous partners, there is no definitive evidence that he is infertile. Mitotic chromosomes were prepared from peripheral blood lymphocytes using routine cytogenetic procedures and 100 metaphases were analysed.

Two semen samples were studied. They were produced by masturbation, allowed to liquefy at room temperature, then analyzed using standard procedures (World Health Organization, 1992). A motile sperm fraction was also prepared from the second semen sample so possible differences in the X:Y ratio, aneuploidy and polyploidy between the motile and total sperm populations could be assessed. Motile sperm were isolated using the swim-up procedure (Kirby et al., 1991) (see 6.2.2).

The sperm were pretreated with EDTA/DTT as outlined by Han et al (1992)(see 2.3), fixed with methanol:acetic acid (3:1) and air dried on slides. Each preparation was studied in the following ways: (1) Chromosome 17 was detected by single FISH using a biotin-labelled TR17 probe; (2) X and Y chromosomes were detected by double labelled FISH using a biotin-labelled TRX probe and a digoxigenin-labelled HRY probe. The DNA probes and the single and double FISH procedures have been fully described by Ford et al (1993a) (see 2.5, 4.2.2-4.2.4 and 5.2.3-5.2.5).

7.2.1 Microscopical analysis

To establish the percentage of labelled cells, at least 1,000 sperm were scored from each preparation.

Cells with one or more fluorescent spots in the nucleus were described as labelled and those without spots were described as unlabelled (this group presumably includes some hypoploid cells). An eye-piece graticule was used to measure the size of each cell nucleus. The normal nuclear diameter (or length) for pretreatment haploid sperm is approximately 6-8 μm. The majority of these cells had one nuclear spot. Cells with two or more spots were classified as diploid or disomic according to the sizes of the cell nuclei (Joseph et al., 1984). Cells which were considered disomic had a nucleus of normal size (diameter or length 6-8 μm) with two distinct spots; while those containing two spots in a large nucleus (diameter or length 9 μm-20 μm) were considered to be diploid. The few cells with three distinct spots and a large size nucleus (9 μm-20 μm) were presumed to be hyperdiploids. Cells with four distinct spots and a very large diameter were scored as tetraploids.

7.2.2 Statistics

The results from the 47,XYY male were compared both with the results from neat semen (12 normal donors for chromosomes X and Y and 13 donors for chromosome 17 reported previously (Han et al., 1992; 1993)) and with the results from the motile fraction in ten normal donors (Chapter 6). The results were compared using the Chi-squared (χ^2) analysis.

7.3 Results

Lymphocytes from conventional cultures of whole blood were again karyotyped in the previously identified 47,XYY subject. One hundred cells were scored as 47,XYY. No mosaicism was detected. This result is identical to the previous analysis five years earlier.

The results from two semen samples and their swim-up fractions are shown in Table 7.1. Both samples contained a volume of 1 ml semen, approximately half the normal WHO parameter of 2 ml or more (World Health Organization, 1992). All other parameters were normal; the sperm concentrations were 150 and 350 x 10^6 sperm/ml. The progressive motility measurements were 55% and 44%, and 36% and 24% sperm were morphologically normal range. The second estimate of 24% was slightly below the usually accepted value of >30%.

In a total of 2,006 cells in the neat semen samples 96% of the sperm were fluorescently labelled with either the TRX or HRY probes. In the swim-up fractions 98% of total of 1,003 sperm were labelled (Table 7.2). The overall ratio of X- to Y-bearing sperm was 47% to 45.3% in the neat samples, and 48.4% to 49.3% in the swim-up fraction. Neither ratio was significantly different from 1:1.

The frequencies of sperm, haploid and disomic for chromosomes X, Y and 17 in the two semen samples are shown in Table 7.3. The frequencies of haploid cell types were 46.4% haploid X, 44.4% haploid Y, and 94% haploid 17. The frequencies of disomy in otherwise haploid cells 0.3% disomic X, 0.4% disomic Y, 0.25% XY, 0.05% XYY and 0.49% disomic 17. None of these frequencies of haploidy and disomy are significantly different from those found in 12 and 13 normal healthy donors (Han et al., 1992; 1993). The results of the different types of diploid, hyperdiploid and tetraploid cells in the XYY man are shown in Table 7.4. Labelling with the 3 different probes identified 9 to 20 μm-sized sperm at the following frequencies:

0.3% XX, 0.35% YY, 2.7% XY and 2.6% diploid 17. These are all assumed to be diploid. One cell trisomic for chromosome 17 was identified and one cell of diploid size was XYY. The frequencies of tetraploidy were: 0.05% XYYY, 0.15% XXYY and 0.2% for chromosome 17. The frequencies of diploid, hyperdiploid and tetraploid cells were increased and in most cases were significantly different from the control samples (Table 7.4).

The frequencies of haploid, disomic, diploid sperm from the swim-up fractions of the 47,XYY male and controls are shown in Table 7.5. The frequencies of haploidy detected were: 47.8% X, 48.5% Y and 97.5% haploid 17. The frequencies of disomy were: 0.4% X, 0.5% Y, 0.1% XY, and 0.5% disomic 17. The frequencies of diploidy detected were: 0.2% XX, 0.3% YY, 0.6% XY, and 0.7% diploid 17. No hyperploid and tetraploid cells were found in the motile fraction from the 47,XYY male. The frequencies of haploid and disomic cells in the swim-up fraction were not different from the ten controls (Table 7.5). The frequency of diploid XX and YY did not differ between the samples but the frequency of XY diploid sperm was increased in the 47,XYY male.

Table 7.1: Seminal characteristics of 47,XYY subject

Sample	Volume (ml)	Sperm concentration (x 10^6 sperm/ml)	Motility (%)	Normal morphology (%)
1 Semen	1.0	150	55	36
2 Semen	1.0	350	44	24
2 Swim-up	0.5	30	85	31

Table 7.2: Overall results of X and Y labelling sperm in a 47,XYY man

	Original semen	Swim-up
No. of sperm	**2,006**	**1,003**
% labelled	95.6%	98.3%
% X-bearing	47%	48.4%
% Y-bearing	45.3%	49.3%

* Not significantly different from a ratio of 1:1.

Table 7.3: Frequencies of monosomic and disomic (but otherwise haploid) spermatozoa from neat semen in 47,XYY man and control***

	47,XYY		Control		P value
	%	No. of sperm	%	No. of sperm	
X	46.4	2,006	47.4	12,636	ns
Y	44.6	2,006	46.8	12,636	ns
Monosomy 17	94	2,035	95.4	14,095	ns
XX	0.3	2,006	0.28	12,636	ns
YY	0.4	2,006	0.21	12,636	ns
XY	0.25	2,006	0.21	12,636	ns
XYY	0.05	2,006	0	12,636	ns
Disomic 17	0.49	2,035	0.33	14,095	ns

* control= 12 normal semen donors for X and Y, 13 normals donors for chromosome 17 (Han et al., 1992; 1993)
ns = Not significant.
** as judged by sperm nuclear size

Table 7.4: Frequencies of diploid and tetraploid cells from original semen in 47,XYY man and control*

	47,XYY		Control		P value
	%	No. of cells	%	No. of cells	
Diploid on size					
XX	0.3	2,006	0.18	12,636	ns
YY	0.35	2,006	0.17	12,636	ns
XY	2.7	2,006	0.62	12,636	<0.00001
Diploid 17	2.6	2,035	0.37	14,095	<0.00001
XYY	0.05	2,006	0	12,636	ns
Trisomy 17	0.1	2,035	0	14,035	ns
Tetraploid on size					
XYYY	0.05	2,006	0	12,636	ns
XXYY	0.15	2,006	0.05	12,636	ns
Tetrasomy 17	0.2	2,035	0	14,095	<0.00001

* control= 12 normal donors for X and Y; 13 normal donors for chromosome 17 (Han et al., 1992; 1993).

Table 7.5: Frequencies of haploid and diploid spermatozoa in swim-up fraction from 47,XYY man and control*

	47,XYY		Control		P value
	%	No. of sperm	%	No. of sperm	
Haploid on size					
X	47.8	1,003	48	10,059	ns
Y	48.5	1,003	46.7	10,059	ns
Monosomy 17	97.5	1,019	nd		
XX	0.4	1,003	0.26	10,059	ns
YY	0.5	1,003	0.27	10,059	ns
XY	0.1	1,003	0.16	10,059	ns
Disomy 17	0.5	1,019	nd		
Diploid on size					
XX	0.2	1,003	0.12	10,059	ns
YY	0.3	1,003	0.12	10,059	ns
XY	0.6	1,003	0.18	10,059	P<0.05
Diploid 17	0.7	1,019	nd		

* control = ten normal semen following swim-up procedure in the experiment of Chapter 6.

nd = not done.

7.4 Discussion

Meiotic studies from human testes have suggested that the extra Y chromosome in XYY men is eliminated during spermatogenesis (Melnyke et al., 1969; Evans et al., 1970). Our results from ejaculated sperm are consistent with this. Equal proportions of X- to Y-bearing sperm were observed (47%:45.3% in neat semen samples and 48.4%:49.3% in the motile fraction) in this 47,XYY male. The frequencies were not significantly different from a 1:1 ratio and are similar to those obtained from 75 sperm after hamster egg penetration (Benet and Martin, 1988).

The frequencies of haploidy and disomy for chromosomes 17, X and Y from both whole semen and the swim-up fraction were all within the normal ranges observed in the whole samples from 12 normal donors and motile fractions of the ten normal donors.

However, we observed significantly higher frequencies of diploid (as XY and diploid 17), and tetraploid (as tetrasomy 17) cells in the XYY subject's whole semen samples than in those normal healthy donors. This finding is most likely due to a higher relative frequency of immature (premeiotic) germ cells and somatic cells in this man's semen which could not be distinguished from sperm because of the effect of pretreatment. Alternatively, if those diploid cells are indeed mature sperm, then the present data suggests that failure of meiosis I, rather than meiosis II failure would produce XX or YY classes, neither of which is especially common in the XYY male.

Frequencies of 0.1% haploid XY and 0.6 % diploid XY-bearing sperm were observed in the swim-up fraction from this 47,XYY man, but no disomic and diploid cells were detected by Benet and Martin (1988). The small sample of cells analysable by the hamster technique precludes the detection of minor cell populations present at

only one to a few cells per thousand. It is also possible that such cells are unable to fertilise hamster eggs (Ford et al. 1993b).

The use of double labelled FISH and sampling large number of cells has allowed us to confirm that the extra Y chromosome is eliminated during or prior to spermatogenesis in 47,XYY men. By implication, perhaps those men who achieve normal fertility have displayed a much higher efficiency of elimination than those who are oligospermic and azoospermic. Whether there is some difference between the Y chromosomes that are efficiently eliminated and those that are not could be the subject of further studies.

CHAPTER EIGHT

DETECTION OF 17, X AND Y CHROMOSOMES IN HUMAN SPERM FROM TERATOZOOSPERMIC SEMEN

8.1 Introduction

A particular feature of human semen, in contrast to other species (Wildt et al, 1983, 1986) is the high proportion of morphologically abnormal sperm (Bedford et al., 1973; Le Lannou et al., 1986). Sperm morphology is an important correlate of fertility in human (Rogers et al., 1983; Kruger et al., 1986) and the World Health Organization (1992) recently proposed that a cut-off value of 30% normal forms should be used to distinguish normal and abnormal semen.

To date the mechanism(s) responsible for the high percentage of morphologically abnormal sperm in human semen are unknown, and only limited information relates to any possible association between abnormal morphology and aneuploidy. Martin and Rademaker (1988) examined the karyotypes of human sperm which had fertilized hamster eggs from samples with a range of morphologies (6-52%), and did not find any association between morphology and chromosomal abnormalities. Since the great majority of samples had >30% normal morphology the issue could be considered still unresolved. Given that many programmes of assisted reproduction are now treating couples with severe male infertility with microassisted fertilization techniques such as Zona Cutting and intracytoplasmic microinjection (Yates and De Kretser, 1987; Depypere et al., 1988; Payne et al., 1991; Cohen et al., 1992), the chromosomal complement of sperm becomes an important issue.

In addition some physical and chemical agents can induce a high frequency of morphologically abnormal sperm (MacLeod, 1974; Lancranjan et al., 1975) and

Wyrobek and Bruce (1978) have suggested that sperm morphology might prove a useful indicator of man at risk for an increased frequency of sperm chromosomal abnormalities.

These experiments have examined sperm from severely teratozoospermic samples using chromosome-specific probes (17, X and Y) and single or double FISH to evaluate if there is an increased level of autosomal and sex chromosome aneuploidy in morphologically abnormal sperm, and to determine whether the normal 1:1 ratio of X- to Y-bearing sperm is disturbed.

8.2 Materials and methods

A semen sample was obtained from men who were undergoing infertility treatment at the Reproductive Medicine Unit at The Queen Elizabeth Hospital. The men were selected on the basis of several previous semen analyses which indicated a consistent severe teratozoospermia (<10% normal morphology) defect. Samples were routinely analysed according to WHO standards (1992). The semen profile for the samples are shown in Table 8.1. While sperm density and motility indices were variable, the % sperm with normal morphology were all <10%.

After analysis, the semen samples were pretreated with EDTA/DTT (see 2.3) and chromosome 17 was localized using biotin-labelled TR17 probe and a single FISH procedure (Han et al., 1992) (see 2.5, 4.2.2-4.2.4). The X and Y chromosomes were localized using biotin-labelled TRX and digoxigenin-labelled HRY probes and the double FISH procedure (Han et al., 1993) (see 5.2.2-5.2.5).

A chi-squared (χ^2) test was used to compare results from the poor morphology samples with the results from normal donor samples which have been previously published (Han et al., 1992; 1993) (see Chapters 4 and 5).

8.3 Results

A total of 3,062 sperm were scored from 3 of the 4 samples after double FISH using the TRX and HRY probes. A total of 4,100 sperm from 4 samples were assessed with the TR17 probe. In both series, the hybridization efficiency was 96%. The overall ratio of X- to Y-bearing sperm was 47.7% to 46.2% in the teratozoospermic semen which was not significantly differently different from a 1:1 ratio (Table 8.2).

The results for chromosomes X, Y, and 17 in the teratozoospermic semen are shown in Tables 8.3 and 8.4. The frequencies of haploidy were 46.4% haploid X, 45% haploid Y and 92.9% haploid 17. The frequencies of disomy were 0.46% disomic X, 0.36% disomic Y, 0.26% haploid XY, and 0.46% disomic for chromosome 17 (Table 8.3). None of these frequencies for haploidy or disomy were significantly different from the frequencies reported for normal healthy donors (Han et al., 1992; 1993).

The frequencies of diploid (two complements) and tetraploid cells (four complements) in poor morphology semen are shown in Table 8.4. The diploid frequencies were 0.9% diploid X, 0.8% diploid Y, 2.2% diploid XY, and 2.8% diploid for chromosome 17. Frequencies of 0.07% XXY-bearing, 0.1% XYY-bearing, and 0.2% for three chromosome 17 bearing cells with large nuclei were also found. Finally, 0.2% of the cells were XXYY-bearing, and 0.34% were tetraploid for chromosome 17. The frequencies of diploid and tetraploid groups from these samples were significantly increased compared to previously published (Han et al., 1992; 1993) normal donor semen (see Table 8.4) (Chapters 4 and 5).

Table 8.1: Seminal values for the four teratozoospermic semen samples

Patient	Volume (ml)	Sperm concentration (x 10^6 sperm/ml)	Motility (%)	Abnormal head morphology (%)
1	1.8	7	16	97
2	2.0	37	36	92
3	6.7	16	46	96
4	0.7	3.7	14	99
Mean	**2.8**	**15.9**	**28**	**96**

Table 8.2: Overall results of X and Y labelling sperm in teratozoospermic semen and normal semen

	Teratozoospermic semen	Normal semen**
No. of sperm	3,062	12,636
% labelled	96.7%	96%
% X-bearing	47.7%	47.9%
% Y-bearing	46.2%	47.2%
X to Y ratio	47.7% to 46.2%*	47.9% to 47.2%*

* Not significantly different from a ratio of 1:1

** Normal semen=12 normal donors (Han et al., 1993).

Table 8.3: Frequencies of haploid and disomic spermatozoa from teratozoospermic semen and normal semen*

	Teratozoospermic Semen			Normal Semen			P value
	Number	Total Scored	(%)	Number	Total Scored	(%)	
Haploid X	1,420	3,062	46.4	5,995	12,636	47.4	ns[1]
Haploid Y	1,378	3,062	45.0	5,917	12,636	46.8%	ns
Haploid 17	3,800	4,100	92.9	13,445	14,096	95.4	ns
Disomic X	14	3,062	0.46	35	12,636	0.28	ns
Disomic Y	11	3,062	0.36	26	12,636	0.21	ns
Haploid XY	8	3,062	0.26	27	12,636	0.21	ns
Disomic 17	19	4,100	0.46	47	14,095	0.33	ns

* Normal semen=12-13 normal donors (Han et al., 1992;1993).

ns[1]=not significant.

Table 8.4: Frequencies of diploid and tetraploid cells from teratozoospermic semen and normal semen*

	Teratozoospermic Semen			Normal Semen			P value
	Number	Total Scored	(%)	Number	Total Scored	(%)	
Diploid X	28	3,062	0.9	23	12,636	0.18	P<0.00001
Diploid Y	25	3,062	0.8	22	12,636	0.17	P<0.00001
Diploid XY	67	3,062	2.2	78	12,636	0.62	P<0.00001
Diploid 17	114	4,100	2.8	52	14,095	0.37	P<0.00001
XXY	2	3,062	0.07	0	12,636	0	P<0.05
XYY	3	3,062	0.1	0	12,636	0	P<0.01
triple 17	8	4,100	0.2	0	14,095	0	P<0.00001
Tetraploid XXYY	6	3,062	0.2	6	12,636	0.05	P<0.05
Tetraploid 17	14	4,100	0.34	0	14,095	0	P<0.00001

*Normal semen= 12-13 normal donors (Han et al., 1993).

8.4 Discussion

Hugenholtz and Bruce (1979) have suggested that it is possible that chromosomal abnormalities could be associated with an increase in the frequency of abnormal sperm morphology. The present studies used TR17 probe to assess the incidence of aneuploidy for autosomes, and TRX/HRY probes to assess aneuploidy of sex chromosomes and to determine whether or not the X:Y ratio was altered in men with semen showing poor morphology. The results indicate that the frequencies of aneuploidy for chromosomes X, Y and 17 were within the range previously established for donors with normal semen (Han et al., 1992; 1993). The results also agrees with the limited findings of Martin and Rademaker (1988), that no relationship exists between sperm chromosome abnormalities and sperm morphology.

The results of this study have important implications for clinical infertility treatment programmes. In recent years, a number of assisted fertilization techniques have been developed to enable fertilization to be achieved with severely teratozoospermic semen samples (Cohen 1992). There has been concern about the chromosomal normality of sperm with abnormal morphology and the potential impact of this on embryo viability and pregnancy outcome. For this purpose the results of the present study are encouraging, and provide preliminary reassurance that sperm showing abnormal morphology may not necessarily lead to problems because of aneuploidy. This is reinforced by recent clinical studies on the outcome of sperm microinjection. Van Steirteghem et al.(1993a) have reported that no abnormalities have been detected in the 57 fetal karyotypes from 48 clinical pregnancies derived from intracytoplasmic sperm injection (ICSI). They also obtained twenty 46,XY and nineteen 46,XX in the 39 normal cytogenetic results of prenatal diagnoses after ICSI (Van Steirteghem et al, 1993b). These outcomes of micro-injection are an additional indication that fertilization with morphologically

abnormal sperm will probably not give rise to an increased number of abnormal embryos.

Higher frequencies of polyploidy (including diploid) cells for chromosomes 17, X and Y were observed in the semen samples showing poor morphology than from normal donors. These increased frequencies were also found in a 47,XYY man in the previous investigation (Chapter 7).

Polyploid spermatocytes were frequently seen in air-dried preparations from testes of mice and human (Ford and Evans, 1971; Hulten et al., 1970). Skakkebaek et al. (1973) investigated the meiotic chromosomes of infertile men and controls with a normal karyotype and found that the frequency of polyploid cells in infertile men was higher than in a control group (Table 8.5). It seems that this increase in higher ploidy cells is associated with abnormality and might indicate some disorder at the stage of spermatogenesis, possibly meiotic arrest, although the mechanism is still not clear. Also in the present study, it was not possible to distinguish between somatic cells and spermatogonial in early meiotic cells.

The conclusions of this limited studies are (1) Morphologically abnormal human sperm do not have an increased incidence of aneuploidy (disomy). (2) An increased incidence of polyploidy in teratozoospermic semen samples was seen but the exact identity of the cells is still not clear.

Table 8.5: Contribution of polyploid cells from ejaculated sperm and meiotic chromosomes from testis

c/s probe	Sample	Total No. of cells scored	Polyploidy Number	Polyploidy (%)
	SEMEN			
17	Normal	14,095	52	(0.37)
	47,XYY	2,035	57	(2.8)
	teratozoospermic	4,100	136	(3.3)
X & Y	Normal	12,636	129	(1.0)
	47,XYY	2,006	72	(3.6)
	teratozoospermic	3,062	131	(4.2)
	TESTIS*			
	Normal	1,774	46	(2.6)
	Infertility	7,185	319	(4.4)

* Skakkebaek et al., 1973

CHAPTER NINE

DISCUSSION AND CONCLUSIONS

9.1 Hybridization/detection efficiency

In the present study, it was demonstrated that a frequency of about 96% (range 91-99%) of sperm labelled with either single FISH or double FISH can be regularly obtained. Hybridization efficiencies were comparable with the results obtained in lymphocytes and cultured cells since 85%-95% nuclei of the displayed at least one hybridization signal (Klinger et al., 1992). These results are an improvement on those obtained with tritiated ISH (following SDS/DTT pretreatment) and other work reporting FISH in human sperm (Burns et al., 1985; Guttenbach and Schmid, 1990; Pieters et al., 1990; Coonen et al., 1991). The high efficiency allowed an accurate detection of aneuploidy in human ejaculated sperm. During the course of the present study, Wyrobek developed an air-dried smear procedure for swelling sperm nuclei, and obtained 99% labelling efficiency (personal communication). Thus an ideal pretreatment procedure for swelling sperm nuclei is essential to obtain high hybridization results.

9.2 Advantage of sperm in hybridization efficiency

Because sperm contains only one set of chromosomes (haploid), it is of advantage for FISH in that higher efficiencies can be achieved than for other somatic cells (diploid cells) and trisomic cells. For example, if the aggregate probability of detecting a target chromosome is 0.9, then the probability of detecting two chromosomes in a nucleus is 0.9 x 0.9, or 0.81, and the probability of detecting three chromosomes is 0.9 x 0.9 x 0.9, or 0.729. Thus decreased efficiency must accompany an increased number of target chromosome sites. Since the majority of sperm contains only a single sex chromosome; even the double FISH with X and Y probes still target one chromosome in most cells. Thus using the double FISH and two sex

chromosome probes an efficiency of 96% can be achieved and is comparable to the single FISH and an autosome probe (eg. TR17).

9.3 Incidence of aneuploidy in normal human ejaculated sperm by FISH

A frequency of 0.33% disomic chromosome 17 was found using single FISH in this study (Chapter 4), which is comparable with the result (0.41%) for chromosome 1 reported by Guttenbach and Schmid (1991) and is slightly lower than 0.67%; and 0.8% for chromosome 1 reported by Coonen et al. (1991) and Pieters et al. (1990). All three papers were using the single FISH method and a repetitive specific chromosome 1 probe. Using the multiple FISH method, Wyrobek (1993) recently found much lower frequencies of disomic autosome chromosomes (0.14% for chromosome 1 and 0.07% for chromosome 8) in his study of human ejaculated sperm.

The frequencies of 0.28% disomic X and of 0.21% disomic Y, and of 0.21% haploid XY were found in the double FISH experiment for this study. The frequency of disomic Y is similar to that reported by Guttenbach and Schmid (1990) using a Y specific probe and the single FISH method. However Wyrobek et al. (1993) found both much lower frequencies (0.055% disomic X, 0.06% disomic Y and 0.09% haploid XY sperm) and lower frequencies of diploidy and polyploidy in his multiple FISH study.

A few different laboratories have now achieved results with FISH in sperm. They have often used different procedures and have reported various results. This variability, at least in part, may reflect the quality of the reagents and facilities used or the degree to which experimental parameters were optimized. However it is also likely that some of the differences reflect patient selection. Whilst the variability

between studies requires explanation, the present results are consistent. Thus FISH and the use of specific DNA probes has allowed us to establish the performance criteria for the assay of chromosome aneuploidy in this laboratory.

9.4 The advantages and limitations of FISH in sperm chromosome detection

Specific DNA probes and FISH techniques have several advantages in the detection of individual chromosomes in human sperm. Firstly, there is no radioactivity in the procedure, making it quick and simple. Secondly, the entire population of sperm is studied allowing for the examination of many thousands of spermatozoa without difficulty, instead of only examining a subgroup capable of fertilizing zona-free hamster eggs.

Like any other scientific method, FISH has its limitations. When alpha satellites sequence DNA probes are used FISH does not detect structural chromosomal abnormalities in sperm. For example, a translocation in the long arm of a chromosome can not be detected by a alpha satellite probe as it only localizes the centromeric region of chromosome.

Chromosome paints may overcome this problem. During the time of this thesis, I hybridized a chromosome 8 library probe to metaphase spreads from lymphocytes and sperm nuclei, pretreated with EDTA/DTT. The paint successfully identified chromosome 8 in lymphocyte metaphases but generated a very large diffuse signal in sperm, so large that the edge of the signal was difficult to delineate. It is therefore unlikely that chromosome paints will be helpful for identification of chromosome abnormalities in sperm particularly if multiple FISH is used.

9.5 Conclusion

In conclusion, the use of chromosome specific probes and FISH provides a valuable tool for the investigation of aneuploidy in human ejaculated sperm. The double FISH technique with X and Y chromosome probes is a reliable method for assessing the ratio of X- and Y-bearing sperm, and to evaluate the sex selection method.

The present study shows that the ratio of X- and Y-bearing sperm in human ejaculates is one to one. It is not altered by the swim-up procedure. The swim-up procedure did not change the aneuploid level, but reduced the frequencies of diploid and tetraploid cells. In the study on a 47,XYY male FISH detected neither the extra Y chromosome in ejaculated sperm nor increased disomy Y sperm. There was also no relationship between abnormal morphology and increased aneuploidy in human sperm.

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