



Detection of chromosomes and chromosomal abnormalities in human sperm

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OVERVIEW

The technique of Intracytoplasmic Sperm Injection (ICSI), whereby a single sperm is selected and injected directly into the cytoplasm of an oocyte, has revolutionised the treatment of severe male infertility. ICSI bypasses the natural barriers to fertilisation by dysfunctional sperm, and this has raised concerns about the karyotypic normality of the sperm used for ICSI. It is therefore important to determine the chromosomal content of such sperm to ascertain the potential risk to embryos of transmission of chromosomal abnormalities.

The overall aim of this work was to study chromosomal abnormalities and the localisation of chromosomes in human sperm, especially from men with TSD, using fluorescence *in situ* hybridization (FISH). At the time this project commenced in 1994, the study of sperm chromosomes using FISH was relatively new, and there was very little published information about the incidence of numerical chromosomal abnormalities (aneuploidy) in sperm from men with triple semen defects (TSD), who typically require ICSI. Therefore, this project entailed: (i) development of reliable FISH protocols, (ii) determination of baseline frequencies of aneuploidy, (iii) analysis of chromosomal abnormalities in men with severe TSD, and (iv) assessment of the localisation of individual chromosomes within the sperm head.

(i) Development of FISH protocols. Multi-probe FISH protocols were developed using combinations of probes for 11 different chromosomes. Two reliable protocols were developed successfully, a triple-probe FISH protocol for

chromosomes 3, X and Y, and a double-probe FISH protocol for chromosomes 7 and 16. This work was undertaken at a time when commercial probes were not available or were very new and often unreliable, thus several other protocols failed due to inconsistencies in signal intensity, hybridization failure and cross-hybridization difficulties.

(ii) Baseline frequencies of aneuploidy. The incidence of aneuploidy in sperm from 10 normospermic men (NS) was estimated for chromosomes 3, 7, 16, X and Y, using the two FISH protocols developed in (i). To establish a baseline frequency of aneuploidy in normal human sperm it was important to assess a variety of chromosomes in sperm from a range of normospermic men, standardise scoring criteria, and analyse inter-donor differences and inter-chromosomal differences. Low incidences of disomy (0.05-0.20% per chromosome) and diploidy (0.27-0.35%) were obtained for this normospermic population.

(iii) Chromosomal abnormalities in men with severe TSD. In collaboration with the Lawrence Livermore National Laboratory (LLNL), Livermore, California, semen samples from 10 men with TSD and 10 NS men (controls) were prepared to investigate chromosomal abnormalities for chromosomes 1, 18, 21, X and Y. Two FISH procedures developed by the LLNL laboratory were used. The specific aim of this study was to estimate disomy for chromosomes 1, 18, and 21, sex-chromosome disomy, terminal telomeric duplications and deletions for chromosome 1p36.3 region and diploidy in sperm from both groups of men. The objective was to ascertain if there were higher frequencies of chromosomal abnormalities in sperm from men with

TSD than in the NS group. This study demonstrated that the incidence of specific abnormalities for these chromosomes was not significantly elevated in sperm from men with TSD. The incidences of chromosomal abnormalities (means for AM18 and XY21 assays, respectively) in sperm from both groups of men were very low (0.21% and 0.23% in TSD vs 0.20% and 0.15% in NS). Marked increases over the mean values were found for some individuals in the TSD group.

(iv) Localisation of chromosomes in sperm. The hypothesis tested was that the arrangement of individual chromosomes is more random and less regularly organised in morphologically abnormal sperm (TSD group) than in morphologically normal sperm (NS group). The centromere and telomere of chromosome 1 was used as a marker of the position of chromosome 1, and the centromeres of chromosomes 18, 21, and the sex chromosomes, were used as markers of these chromosomes within the sperm head. The positions of the chromosomes were localised to the anterior, middle, or posterior regions of the sperm head. The distribution of each of the five chromosomes appeared to be random throughout the sperm head in both groups of men, although the telomeric region of chromosome 1, and the sex chromosomes were very rarely present in the posterior region of the sperm head.

Major differences were not detected in the incidence of chromosomal abnormalities or in the localisation of chromosomes in sperm from TSD and NS men. This has important implications for couples undergoing ICSI in reproductive medicine clinics, as some other studies have reported 10-fold increases in chromosomal abnormalities in sperm from infertile men. The present study shows that in this group

of ICSI candidates, there was no greater risk of transmission of chromosomal abnormalities via their sperm.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

The author consents to this thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Sarah Elizabeth Downie

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Published articles

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Glossary/Abbreviations

Å	Angstrom
α	Alpha, anti-
Aneuploidy	Numerical chromosomal abnormalities
AMCA	Aminomethyl coumarin acetic acid
ANOVA	Analysis of variance
ART	Assisted Reproduction Techniques
Asthenozoospermia	Reduced progressive sperm motility
Azoospermia	Absence of sperm in the ejaculate
bp	Base pairs
β	Beta
BSA	Bovine serum albumin
chr.	Chromosome
cm	Centimetre(s)
°C	Degrees Celsius
CTAB	Cetyl trimethyl ammonium bromide
DAPI	4,6-diamidino-2-phenyl indole
Disomy	An extra chromosome present ($n + 1$)
Diploidy	Twice the normal haploid chromosome complement ($2n$)
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FISH	Fluorescence <i>in situ</i> hybridization, a technique whereby DNA probes are hybridized to chromosomes and

	fluorescently labelled
FITC	Fluorescein isothiocyanate
g	Gram(s)
Haploid	Set of chromosomes whereby $n = 23$
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HTF	Human Tubal Fluid (culture medium)
hr	Hour(s)
ICSI	Intracytoplasmic Sperm Injection, whereby a single sperm is selected and injected directly into the cytoplasm of an oocyte
IVF	<i>In vitro</i> fertilisation
k	Kilo
kb	Kilobase pairs
l	Litre(s)
LIS	Lithium diidosalicylic acid
m	Metre(s)/ mole(s)/milli (10^{-3})
MESA	Microsurgical epididymal sperm aspiration
μ	Micro (10^{-6})
KCl	Potassium chloride
min	Minute(s)
M	Moles per litre
MW	Molecular Weight
n	Nano (10^{-9})
NS	Normozoospermic men: >20 million/ml sperm concentration, >20% normal sperm morphology, >50% progressive motility
NS group	Control group of normozoospermic men
Nullisomy	A chromosome is missing ($n - 1$)

OAT	Oligoasthenoteratozoospermia (TSD), reduced sperm concentration, reduced progressive motility and reduced normal sperm morphology
Oligozoospermia	Reduced sperm concentration
%	Percent
p	Pico (10^{-12})
PBS	Phosphate buffered saline
PESA	Percutaneous epididymal sperm aspiration
PZD	Partial zona dissection
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SUZI	Subzonal insemination
Teratozoospermia	Reduced normal sperm morphology
TESA	Testicular sperm aspiration
TESE	Testicular sperm extraction
TR	Texas Red
TRITC	Tetramethyl rhodamine isothiocyanate
TRIS	Tris(hydroxymethyl)-aminomethane
TSD	Triple semen defect: <13 million/ml sperm concentration, <10% normal sperm morphology, <50% progressive motility
TSD group	Sub-fertile men with TSD
UV	Ultraviolet
WCP	Whole chromosome paint



CHAPTER 1

Literature review

The following discussion is a review of the literature on significant aspects related to this thesis: the spermatozoon, chromosomal abnormalities, male infertility, ICSI, and FISH.

1.1 The Spermatozoon

Spermatozoa are produced in the testes and are the final product of spermatogenesis. Several types of differentiated male germ cells can be found throughout the seminiferous epithelium, including spermatogonia, spermatocytes, spermatids and spermatozoa (Overstreet and Blazak, 1983). For the completion of spermatogenesis, the spermatid must undergo a maturation phase before it is released from the seminiferous epithelium as an independent cell, the spermatozoon.

Within the sperm head of all mammals there is a highly condensed chromatin in which the DNA is associated with small, basic proteins, of molecular weight approximately 8000 Daltons, called protamines. The anterior part of the sperm head is covered by a membrane-bound structure, the acrosome, which is filled with enzymes that aid in the passage of the spermatozoon through the extracellular coats around the egg (Setchell, 1982). This whole cell structure is enclosed by a plasma membrane, and only a small amount of cytoplasm is found within the cell. Attached to the head is the sperm tail, which consists of a midpiece, principal piece and endpiece. The midpiece contains mitochondria that produce energy for flagellar movement and the whole tail

contains the flagellar apparatus that generates motility.

Spermatozoa of different species vary in size. Spermatozoa of humans, rabbits and some mammals, such as ungulates, are approximately 50 μ m long whereas rodent spermatozoa are approximately 150-250 μ m long (Setchell, 1982). The shape of the sperm head has been found to be characteristic of each species (Fawcett, 1970). The human sperm head is ovoid in outline and wedge shaped in longitudinal section. Its dimensions are approximately 5 μ m long, 2.5 μ m wide and 1.5 μ m thick.

1.1.1 Spermatogenesis

Spermatogenesis involves three stages: mitotic division of progenitor stem cells (spermatogonia), meiotic divisions to form spermatids and the differentiation of spermatids into spermatozoa. Spermatogonia are diploid cells situated in the basal compartment of the seminiferous tubule. In the rat and mouse, four type A spermatogonia (type 1-4), intermediate spermatogonia, and type B spermatogonia have been described (Monesi, 1962; Clermont and Trott, 1969). Clermont (1966) described three types of spermatogonia in the human testis, type A-dark (Ad), type A-pale (Ap) and type B.

The actual process of stem cell renewal and multiplication of spermatogonia has yet to be clarified. In humans a model has been suggested whereby the type Ad spermatogonia are the stem cell spermatogonia involved in renewal (Clermont, 1970). Thus, type Ad spermatogonia undergo continual mitotic divisions for renewal, with some differentiating into Ap spermatogonia and then into B spermatogonia. The type

B spermatogonia mitotically divide to yield preleptotene primary spermatocytes which are tetraploid (Overstreet and Blazak, 1983). This characterises the beginning of meiosis.

Leptotene spermatocytes characterise the next step of meiosis with the visual organisation of chromatin fibres into thin filaments. During this stage the spermatocytes are transferred across the 'Sertoli junctions' into the adluminal compartment of the seminiferous epithelium. As meiosis progresses chromatin condensation occurs, until at pachytene, each chromosome divides longitudinally into two chromatids (Overstreet and Blazak, 1983; Guraya, 1987). The chromosomes continue to condense during the final stages of prophase, and at maximal condensation prophase is completed. The chromosomes align on the spindle fibres at metaphase and segregate from one another during anaphase of the first division, resulting in two secondary spermatocytes. This marks the end of the first meiotic division.

Each of the secondary spermatocytes undergoes a second meiotic division, in which the chromosomes divide at anaphase II to produce haploid spermatids containing either the X or the Y chromosome (Setchell, 1982; Overstreet and Blazak, 1983; Guraya, 1987). Spermatids are small round cells with a characteristic nucleus. As spermatids move adluminally in the seminiferous epithelium, they undergo a number of morphogenetic changes that are necessary for their differentiation into spermatozoa. This process is known as spermiogenesis, and it involves condensation of the nuclear chromatin to form the sperm head, formation of the acrosome around

the nucleus, arrangement of mitochondria into the sperm midpiece, development of a tail for motility and loss of most of the cytoplasm (Overstreet and Blazak, 1983).

1.1.2 Formation of the sperm nucleus

1.1.2.1 Unique packaging of DNA in the sperm head

Replacement of lysine-rich histones by arginine-rich protamines occurs in the nucleus of mammalian spermatids as they undergo spermiogenesis. Protamines are much smaller than histones and are extremely basic. In most mammalian species, the amino acid composition of protamines is 47-61% arginine, 8-16% cysteine, and 6-8% serine with relatively little lysine (Bellvé *et al.*, 1975; Calvin, 1976). Although, histones are replaced by protamines during spermiogenesis, a small portion of the DNA in human sperm is still packaged with histones (Tanphaichitr *et al.*, 1978). It was found that the chromatin of human sperm contains approximately 15% histones, which suggests that histones are replaced by protamines in a specific manner during spermiogenesis, and that the two types of chromatin in sperm nuclei are functionally distinct.

Biochemical studies have shown that more than one type of protamine molecule may be found in sperm nuclei. Rabbit, rat and guinea pig sperm contain only one protamine molecule (protamine 1), whereas human and mouse sperm contain two protamine molecules (protamines 1 and 2) (Koehler *et al.*, 1983). Protamine 1 is rich in arginine and cysteine, and contains serine and tyrosine. When present, protamine 2 has a high histidine content in addition to arginine and shows only 50% homology

with protamine 1 (McKay *et al.*, 1986; Arkhis *et al.*, 1991). It is possible that these two molecules arose from a common ancestral molecule encoded by a gene that underwent duplication, deletion or mutation, but at this stage the two protamine types, and therefore the gene, appear to be only distantly related.

Protamines are synthesised during the final stages of spermiogenesis in mammalian species and they are incorporated into the sperm nucleus during chromatin condensation (Kopécny and Pavlok, 1975). Disulphide cross-links form between cysteine residues of adjacent protamine molecules during the final transit period through the epididymis (Bedford and Calvin, 1974; Bedford *et al.*, 1973). These processes result in condensation of the sperm chromatin and serve to maintain this structure as transcriptionally inert during epididymal maturation and transport throughout the female tract (Perreault, 1992).

Due to the interaction of protamines, rather than histones, with sperm DNA, the characteristic DNA packaging model of somatic cell nuclei does not explain sperm DNA packaging (Figure 1). Structures called 'nucleosomes' are formed in somatic cells when DNA, approximately 200bp, wraps around an octamer of histone molecules, consisting of two copies each of the histones H2a, H2b, H3, and H4 (Figure 1B, McGhee and Felsenfeld, 1980; Ramakrishnan, 1994; Ward, 1994). Evidence from electron microscopy studies suggests that nucleosomes are further coiled together to form a solenoid structure, resulting in a supercoiled molecule (Figure 1C, 1D, Finch and Klug, 1976; Ward, 1994). It has been shown that there is insufficient nuclear volume to package sperm DNA in this manner. Pogany *et al.*

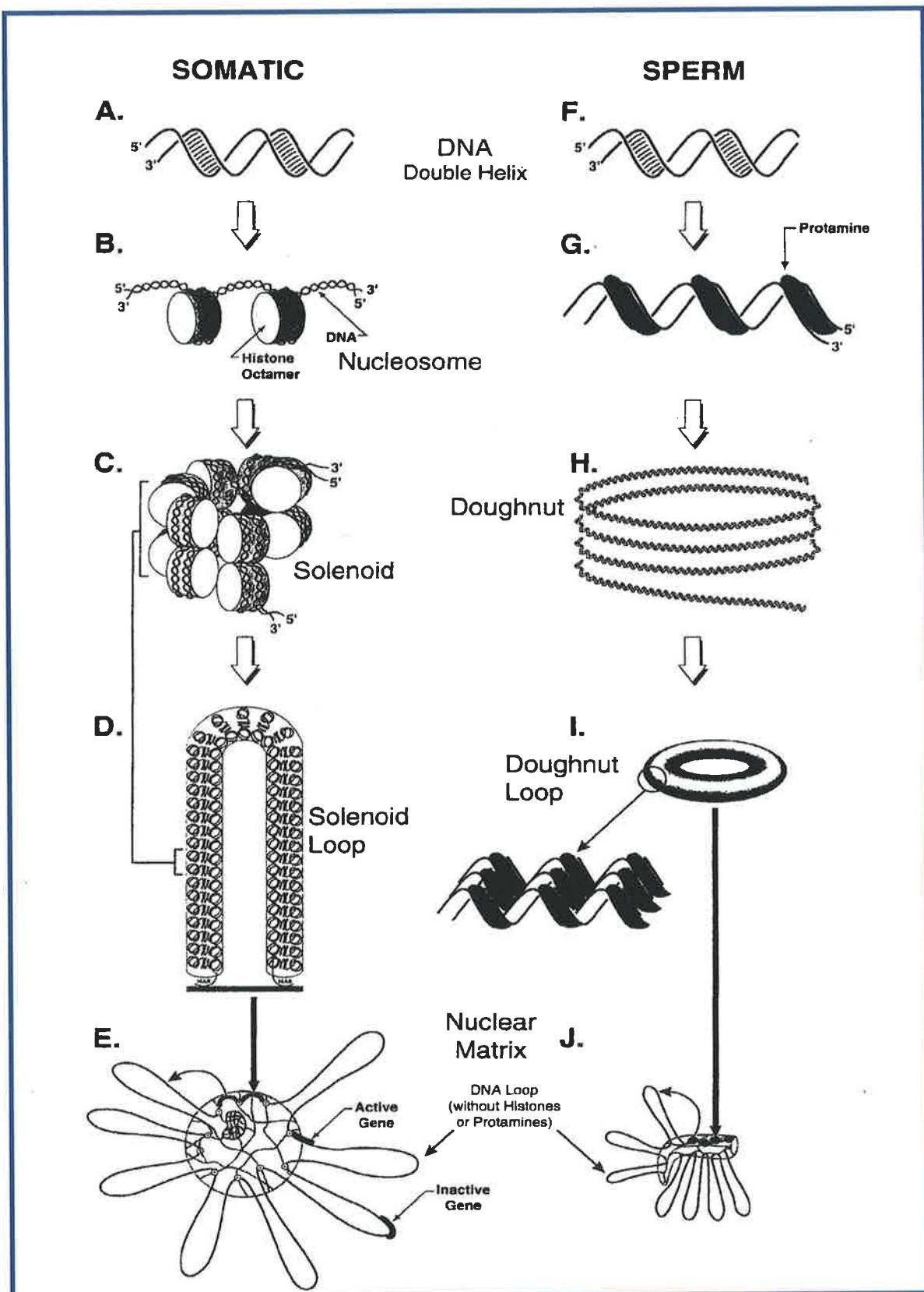


Figure 1: Comparison of DNA packaging models for somatic and sperm cells (reprinted Ward, 1994).

(1981) found that the mouse sperm nucleus contains 3.3pg of DNA, which would require approximately four times the volume of the sperm nucleus to be packaged as a solenoid. They suggested that the DNA molecules lie next to each other with protamines cross-linked within the grooves of DNA, to minimise the volume required to fit the DNA into the sperm head.

Balhorn (1982) proposed a model for the structure of chromatin in mammalian sperm. Balhorn's model postulates that sperm DNA is packaged into linear, side-by-side arrays, interacting with protamines, that neutralise the phosphodiester backbone of the DNA molecule, thus reducing the normal electrostatic repulsion that occurs between adjacent DNA molecules (Figure 1G-I, Ward, 1994). Protamine molecules bind to the minor groove of DNA. X-ray diffraction studies have demonstrated that the major groove is too wide to achieve specific binding of the protamine molecule with DNA in the necessary conformation to maintain the neutral charge (Suwalsky and Traub, 1972). However, the correct binding is achieved when protamine is bound to the minor groove (Suwalsky and Traub, 1972; Pogany *et al.*, 1981). Structural studies of sperm nuclei support Balhorn's model that sperm DNA is packaged in a linear side-by-side manner. Studies on rat (Koehler *et al.*, 1983), rabbit (Koehler, 1970) and cricket sperm (Suzuki and Wakabayashi, 1988) demonstrated that "lamellae" were present in the nucleus and found the comparative volumes of nucleus to DNA content supportive of the Balhorn model. Balhorn later verified his own protamine-binding model and showed the involvement of disulfides (Balhorn *et al.*, 1991).

Atomic force and electron microscopy studies (Hud *et al.*, 1993) have shown that sperm DNA is packaged in a toroidal structure, 900Å outside diameter with a 150Å diameter hole, which contains up to 60kb of DNA (Figure 1I, Ward, 1994). This supports Balhorn's model of linear side-by-side arrays of protamine-DNA molecules, with the linear arrays wrapped around to form a toroid. Taking into consideration the size of the sperm genome and the calculated DNA content of these toroids, it has been estimated that approximately 50,000 closely packed toroids are contained within each sperm nucleus (Hud *et al.*, 1993).

1.1.2.2 DNA organisation in the sperm nucleus

The arrangement of DNA in a cell is dependent on how it is packaged. In somatic cell nuclei, each solenoid structure is attached to a structure called the nuclear matrix, resulting in DNA loop domains, each 60 to 100kb in length (Figure 1E, Ward *et al.*, 1989; Ward, 1994). These DNA loop domains are thought to be important in replication and RNA transcription (Vogelstein *et al.*, 1980). The nuclear matrix is thought to organise the DNA three-dimensionally throughout the nucleus, however, this organisation may vary among cell types.

Sperm DNA, although packaged differently, also appears to be arranged into loop domains by a nuclear matrix (Figure 1J, Figure 2B, Ward and Coffey, 1989; Ward, 1994). Ward *et al.* (1989) demonstrated using hamster spermatozoa the presence of a loop domain structure, with slight differences to that of somatic cells. That is, sperm DNA loop domains were only about half the size of somatic cell loop domains, and they were not supercoiled, as a result of differences induced by

NUCLEAR STRUCTURES IN THE HAMSTER SPERMATOZOON

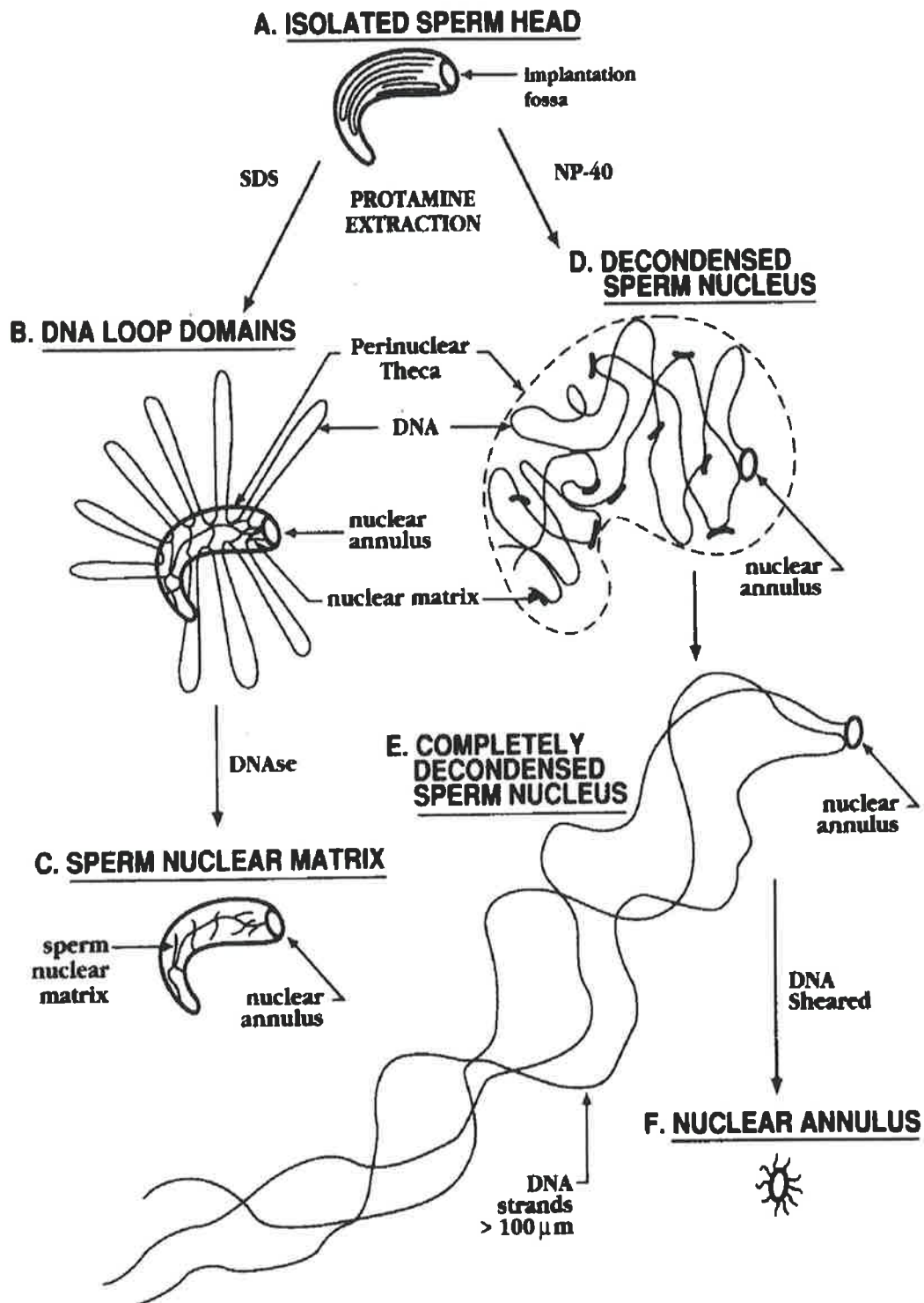


Figure 2: DNA organisation in the hamster sperm nucleus (reprinted from Ward and Coffey, 1989).

protamine binding. It has been predicted that, in sperm, a single DNA loop domain is packaged into a toroid structure, suggesting that protamine binding condenses and protects the DNA loop domains (Ward, 1993). DNA loop domains in somatic cells are often associated with transcription, however, the function of DNA loop domains in sperm, which are 'transcriptionally inert', has not yet been determined. Studies are ongoing to determine if DNA loop domains in sperm are structures remaining after transcription and replication during spermatogenesis or are involved in regulating transcription and replication during embryogenesis (Ward, 1997).

At a higher level of DNA organisation in the hamster sperm nucleus, another structure has been found, termed the nuclear annulus (Ward and Coffey, 1989). The nuclear annulus is located at the implantation fossa, the point at which the tail is joined to the sperm head, inside the nucleus, adjacent to the inner nuclear envelope and is shaped like a bent ring about 2µm in length (Figure 2D, Ward and Coffey, 1989). This structure remains attached to the DNA after the sperm nucleus has been completely condensed, suggesting that every chromosome has at least one attachment site to the nuclear annulus (Figure 2E, Ward and Coffey, 1989). Studies have shown that unique DNA sequences are bound to the nuclear annulus (Ward *et al.*, 1996) but that telomeres, centromeres and ribosomal DNA are not bound (De Lara *et al.*, 1993; Barone *et al.*, 1994; Nadel *et al.*, 1995). Together these studies suggest that the nuclear annulus plays a role in organisation of the DNA.

Studies by Barone *et al.* (1994) on human spermatozoa found that DNA was also organised into loop domains attached at their bases to a nuclear matrix. The

average DNA loop domain size of a human spermatozoon was approximately 27kb (consistent with that found for hamster spermatozoa), but only 50% as large as the size reported for mammalian somatic cells (Vogelstein *et al.*, 1980; Barone *et al.*, 1994). A nuclear annulus-like structure was also indicated as all the human sperm DNA remained anchored to the base of the tail when completely decondensed. However, attempts to isolate this structure failed due to structural instability when separated from the tail.

1.1.2.3 Species-specific sperm head shape

At the conclusion of spermiogenesis, the sperm head takes on the characteristic shape of the respective species. Primitive types of spermatozoa (marine and freshwater invertebrates) have a rounded or conical sperm nucleus, whereas amphibian sperm mostly have long, cylindrical, or tapering heads. Mammalian sperm are characterised by flattened, ovoid heads, although rodents often have hooked sperm heads (Fawcett, 1970; Fawcett *et al.*, 1971).

The mechanisms that regulate sperm head shape and the pattern of nuclear condensation during spermiogenesis are poorly understood. An organelle composed of clustered microtubules called the manchette is involved with nuclear condensation and it has been suggested that these microtubules may be involved in the species-specific formation of the sperm head. However, Fawcett *et al.* (1971), through studies on a number of species, showed that this process is not directly involved with shaping of the sperm head as the manchette microtubules later detach themselves and move away from the nucleus. It is proposed that the microtubules act as a support to

nuclear condensation rather than as a guide to formation of the sperm head.

The pattern of DNA packaging during condensation may be responsible for controlling the shape of the sperm head (Calvin, 1976). The species-specific determination of the sperm nucleus shape has been linked to the biochemical change from histones to protamines. However, the sequence similarity found in human and mouse protamine 2 does not appear to relate to the shape of their nuclei as the mouse sperm nucleus is falciform in shape while that of the human is discoid. Similarity in sperm nuclear shape is found between mouse and rat sperm even though protamine 2 is not present in the rat. Thus it appears that protamines alone do not determine nuclear shape as previously suggested by Fawcett *et al.* (1971). It has also been suggested that the differing histidine components in various eutherian protamines may relate to the species-specific shape of the sperm head (Calvin, 1976). That is, protamines derived from flattened, spatulate sperm nuclei have a low content of histidine (bull, boar, ram, stallion and rabbit), whereas mouse and human nuclei who have a more ovoid sperm head contain at least one protamine with high histidine content.

1.1.2.4 Chromosome organisation in the sperm nucleus

Several studies in insects (Taylor, 1964) and amphibians (MacGregor and Walker, 1973) have suggested that chromosomes may be packed in a precise sequential order within sperm nuclei. Taylor (1964) used autoradiography to label chromosomes in the sperm nucleus of the grasshopper (Orthoptera), and concluded that the chromosomes were organised in a tandem end-to-end arrangement within the

mature sperm nucleus. MacGregor and Walker (1973) have shown that a specific chromosome organisation also existed in the nucleus of mature sperm from the Plethodontid salamanders. *In situ* hybridization was used to show that the centromeres of all chromosomes in Plethodontid sperm are clustered together in the basal portion of the sperm nucleus. Based on this result, they suggested that the chromosomes are arranged in a U formation with their centromeres at the rear of the nucleus and their arms stretching forwards along the length of the nucleus.

Some studies have examined the dispersion of centromeric DNA within the sperm head to hypothesise on chromosome organisation. Powell *et al.* (1990) reported a non-random organisation of chromosomes in the bovine sperm nucleus as they localised centromeric sequences in the equatorial region of the sperm nucleus. Studies in human sperm have suggested centromeric DNA is distributed throughout the nucleus (Barone *et al.*, 1994) or localised in specific regions within the sperm head (Zalensky *et al.*, 1993). Whereas, hybridization of telomeric DNA appeared to localise the telomeres to the periphery of the nucleus (Zalensky *et al.*, 1993; 1995). Conclusions have yet to be reached on whether chromosome organisation exists within the sperm nucleus, but models of DNA packaging have been suggested based on the location of centromeres in the central region and telomeres closer to the periphery of the nucleus (Zalensky *et al.*, 1993; 1995; Ward and Zalensky, 1996; Ward, 1997).

1.2 Numerical and Structural chromosomal abnormalities

1.2.1 Chromosome errors

Chromosomal abnormalities are categorised as those that affect the number of chromosomes (aneuploidy) and those that affect the structure of chromosomes (structural). Human sperm are haploid cells ($n = 23$) which contain 22 autosomes and one sex chromosome, either the X or Y. Disomy (hyperhaploidy) is the condition in which a spermatozoon has an extra chromosome ($n+1$) while nullisomy (hypohaploidy) indicates that it is missing a chromosome ($n-1$). Disomy and nullisomy are examples of aneuploidy, the condition in which a cell does not have an exact multiple of the haploid number (Bond and Chandley, 1983). Ploidy relates to the number of sets of chromosomes in a cell, thus a diploid sperm will have 44 autosomes and two sex chromosomes (XX, YY or XY).

Structural chromosomal abnormalities might involve one but more usually two or more rearranged breaks in the DNA, and are characterised as chromosome translocations, inversions, insertions, duplications and deletions. It has been suggested that most de novo structural rearrangements arise during spermatogenesis (Olson and Magenis, 1988). A likely mechanism is that, as sperm mature, they lose their DNA repair mechanisms, so breaks persist until after fertilisation when repair mechanisms in the egg come into action (Generoso *et al.*, 1979). DNA strands might be inappropriately repaired to generate rearrangements or, if unrepaired, DNA distal to the break might be lost in subsequent cell divisions, resulting in genetic defects in the embryo.

The effects on an embryo as a result of fertilisation by a sperm carrying structural abnormalities is dependent on the severity and type of chromosomal abnormality carried. In the case of balanced rearrangements that have no gain or loss of vital genetic material, no disruption to critical genes, a normal embryo results. However, miscarriage and chromosomally abnormal conceptuses result from balanced Robertsonian translocations, due to essentially complete aneuploidy and only those effectively trisomic for chromosome 13 or 21 can survive full-term (McKinlay-Gardner and Sutherland, 1996). Unbalanced rearrangements, where the content of genetic material is altered, will have an effect on the embryo and this is dependent on the type of abnormality involved. The majority of unbalanced reciprocal translocations that result from mal-segregation of the autosomes end in miscarriage but some may result in the birth of an abnormal child, eg: Down's Syndrome. The effects that other rearrangements, such as inversions, insertions, duplications and deletions have on an embryo is dependent on the genetic content of the chromosomal material being exchanged, with loss of chromosomal material exerting a greater effect on growth of the embryo than an excess of chromosomal material.

Aneuploidy, where a chromosome is gained or lost in the embryo, can be responsible for infertility, pregnancy loss, infant death, congenital malformations, mental retardation and behavioural abnormalities (Epstein, 1986). The effects result from changes in gene dosage and genetic imbalance (Bond and Chandley, 1983). In human embryos, aneuploidy results either from irregular meiotic division during gametogenesis, from mistakes during gametogenesis, or from mistakes during or

following the process of fertilisation (Carr, 1965). Triploidy seems to be more commonly associated with disturbances during or just after fertilisation, whereas mono- and trisomy are mainly caused by abnormal meiotic segregation during oogenesis and spermatogenesis (Edwards *et al.*, 1967). Non-disjunction is a mechanism that affects the chromosome number, whereby either an autosome or a sex chromosome does not separate from its sister chromatid during the first or second meiotic division. Another mechanism that occurs more rarely is one in which a chromosome is lost during cell division due to 'lagging' at anaphase (Dean, 1983).

Clinically recognised pregnancies can be divided into three categories (Table 1). Firstly, spontaneous abortions are mostly pregnancies from about 5 weeks to 14 weeks but losses occur up to 24-28 weeks gestational age; secondly, stillbirths are pregnancies >28 weeks gestational age that do not result in livebirth; and finally, livebirths (Hassold and Jacobs, 1984).

Studies on early spontaneous abortions have shown that around 50% have chromosomal abnormalities, with 9% missing a sex chromosome, 26% trisomic and 2% with structural chromosome abnormalities (Jacobs, 1992). Thus, aneuploidy is responsible for the majority of spontaneous abortions (Hassold *et al.*, 1996). Aneuploidy has been observed for almost every chromosome, except chromosome 1, in human spontaneous abortions (Hassold *et al.*, 1980; Hassold and Jacobs, 1984). Trisomy 16 (7.5%) is the most frequent human trisomy and trisomies 21 and 22 are also frequent (2.3% and 2.7% respectively), with less frequent occurrences of trisomy 5, 11, 12, 17 and 19 (Hassold and Jacobs, 1984).

Table 1: The frequency of trisomy (Jacobs, 1992)

Chromosome	%Spont.aborts.	% Stillbirths	% Livebirths
1	-	-	-
2	1.1	-	-
3	0.3	-	-
4	0.8	-	-
5	0.1	-	-
6	0.3	-	-
7	0.9	-	-
8	0.8	-	-
9	0.7	0.1	-
10	0.5	-	-
11	0.1	-	-
12	0.2	-	-
13	1.1	0.3	0.005
14	1.0	-	-
15	1.7	-	-
16	7.5	-	-
17	0.1	-	-
18	1.1	1.2	0.01
19	<0.1	-	-
20	0.6	-	-
21	2.3	1.1	0.12
22	2.7	0.1	-
XX	0.2	0.4	0.05
YY	0.1	0.3	0.05
XY	-	-	0.05
Total	26.1	4.0	0.3

The overall rate of trisomy in stillbirths is approximately 4.0%, with trisomies 13, 18, 21, X and Y the most common (Jacobs, 1992; Hassold *et al*, 1996). Chromosomally abnormal pregnancies have little chance of progressing through to term, which accounts for the fact that only seven aneuploid chromosomal abnormalities have been recorded in stillbirths. Previous studies on the probable incidence of aneuploidy at conception have shown much higher frequencies than in liveborns (Bond and Chandley; 1983; Hassold and Jacobs, 1984; Jacobs, 1992;

Hassold *et al*, 1996;).

Studies on newborns show that the incidence of structural abnormalities is the most common abnormality (0.6%). The overall trisomy rate is approximately 0.3% and chromosomes 13, 18, 21, X and Y account for 95% of all numerical chromosomal abnormalities (Jacobs, 1992; Hassold *et al*, 1996). The incidences in newborns are 1 in 800 for trisomy 21, 1 in 1100 for sex chromosome trisomy, 1 in 8000 for trisomy 18 and 1 in 20,000 for trisomy 13 (deGrouchy and Turleau, 1984). Therefore a question arises as to whether the chromosomes prominent in liveborn trisomies are those particularly susceptible to nondisjunction or whether aneuploidy for other chromosomes is incompatible with survival to term.

1.2.1.1 Parental origin of chromosomal abnormalities

A number of nondisjunction products have been previously studied for their paternal inheritance patterns. Totally paternal in origin are all cases of 47,XYY. Determination of the origin of all types of sex chromosome aneuploidy was possible with the advent of X-linked Restriction Fragment Length Polymorphisms (RFLP). The highest frequency of paternally inherited sex-chromosome aneuploidy is found for 45,X cases, where 80% are found to be paternal in origin (Hassold *et al.*, 1988). Other studies have shown that 50% of 47,XXY cases (Harvey *et al.*, 1991) and 5% of 47,XXX cases (May *et al.*, 1990) are also paternal in origin, presumably due to 22,XY and 22,XX sperm respectively. The paternal origin of the extra chromosome in trisomies 13, 16, 18 and 21 has also been studied, initially using chromosomal heteromorphism and more recently using DNA markers. A recent study stated that

3/25 cases (12%) of trisomy 13, no cases of trisomy 16, 8% of trisomy 18 and 9% of trisomy 21 are paternal in origin (Hassold, 1998). In summary, data from such studies indicates that paternal errors are more likely to be involved in the generation of sex chromosome aneuploidies, whereas maternal errors are responsible for most of the autosomal aneuploidies.

With respect to the origin of structural abnormalities, Olson and Magenis (1988) have shown in human newborn infants that more than 80% of *de novo* chromosomal structural abnormalities are paternal in origin. They suggest that this predominance of paternally derived structural abnormalities may be due to increased chromosome breakage and rearrangement induced by environmental action on the process and location (testes) of spermatogenesis during adult life. Another mechanism mentioned previously was that, as sperm mature, they lose their DNA repair mechanisms, so breaks persist until after fertilisation when repair mechanisms in the egg come into action (Generoso *et al.*, 1979).

1.3 Male Infertility and ICSI

1.3.1 Clinical causes of infertility

Infertility is generally defined as the inability, of couples within the reproductive age, to conceive after 12 months of unprotected sexual intercourse. Infertility is thought to affect approximately 15% of people, with up to 50% being due to male factor infertility (Bhasin *et al.*, 1994). There are many clinical causes for male infertility which can be broadly grouped into untreatable sterility (12.5%), potentially

treatable conditions (12.5%) and untreatable subfertility (75%) (Baker, 1994).

Untreatable sterility can be defined as conditions causing severe primary seminiferous tubule failure with persistent azoospermia, but some cases may now be treated with assisted reproduction if sperm are present (Baker, 1994).

Potentially treatable conditions include some types of male genital tract obstruction which can be remedied by surgery, with up to 88% success rate (Silber, 1989), or with artificial reproductive techniques where sperm can be collected from the epididymis or testis (Silber *et al.*, 1994; 1995). A common treatable condition is sperm autoimmunity which may be treated by the administration of Prednisolone, whereas gonadotropin deficiency is an uncommon condition which will respond to gonadotropin hormone therapy. Other conditions involve coital disorders such as impotence, failure to ejaculate and retrograde ejaculation, and are not readily treatable conditions but pregnancy can be achieved by assisted reproduction (Baker, 1994).

Treatment via assisted reproductive techniques is possible for subfertility due to oligozoospermia (low sperm concentration), asthenozoospermia (low sperm motility), teratozoospermia (abnormal sperm morphology) and in cases of unexplained infertility (Baker, 1994).

1.3.2 Assisted Reproduction Techniques (ART)

ART has classically referred to *in vitro* fertilisation (IVF) which involves inseminating oocytes *in vitro* with sperm and subsequently transferring one or more embryos to the uterus. Men with severe sperm defects were unable to be treated with

IVF and their only alternatives were donor insemination or adoption. Assisted reproductive techniques such as zona drilling, partial zona dissection (PZD) and subzonal insemination (SUZI) increased fertilisation rates for couples with severe male infertility (Payne, 1995). Pregnancies have been achieved with these techniques, rarely after zona drilling (Jean *et al.*, 1992), generally low after PZD (Tucker *et al.*, 1991) but good pregnancy rates (9-31% per transfer) were achieved using SUZI (reviewed in Payne, 1995). However, there were still many instances in which couples did not achieve fertilisation using these techniques.

With the advent of a new technique called ICSI, most cases of severe male infertility are now treatable. ICSI requires only a few sperm in the ejaculate so that single sperm can be collected and injected directly into the cytoplasm of each oocyte (Van Steirteghem *et al.*, 1993; Payne and Matthews, 1995). Palermo *et al.* (1992) were the first to report successful pregnancies using this technique. Good fertilisation rates (~65%) have been reported for ICSI, comparable to those obtained by routine IVF, and pregnancy rates of 30-40% have been achieved (reviewed in Payne, 1995). Men who have obstructive or non-obstructive azoospermia can also be treated with ICSI in combination with microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), testicular sperm extraction (TESE) or testicular sperm aspiration (TESA) (Silber *et al.*, 1994; 1995).

1.3.3 Sperm morphology and fertilisation

Fertilising ability is related to sperm morphology and significant morphological differences are seen in sperm from fertile and sub-fertile men (Kruger *et al.*, 1988; Liu

et al., 1988; Grow *et al.*, 1994). Significantly lower fertilisation and pregnancy rates were reported after IVF in men with <9% normal sperm morphology than in men with normal semen parameters (Ombelet *et al.*, 1994). Studies in the Reproductive Medicine laboratory at The Queen Elizabeth Hospital have also shown that the percentage normal morphology has the strongest positive correlation with the fertilisation rate after IVF (Duncan *et al.*, 1993).

One of the benefits of ICSI over other forms of ART is that fertilisation can occur with sperm of very poor morphology. No correlation between chromosomal abnormalities and morphologically abnormal sperm has been found in most studies on human sperm (Martin and Rademaker, 1988; Rosenbusch *et al.*, 1992). However, Lee *et al.* (1996) studied chromosomal abnormalities in human spermatozoa after injection into mouse oocytes and found that the incidence of structural chromosomal abnormalities was four times higher in sperm with amorphous, round and elongated heads (26.1%) than in sperm with normal morphology (6.9%). No differences were found in the incidence of numerical abnormalities. Subsequently, this group studied whether mouse oocytes could develop normally after they were injected with mouse spermatozoa which had abnormal head shapes (Burrue *et al.*, 1996). Development of some of the embryos into normal fertile mice suggested that a proportion of these abnormal spermatozoa carry all the genome and organelles necessary for normal embryonic development and growth to fertile maturity.

1.3.4 Chromosomal abnormalities and male infertility

There is an increased incidence of constitutional chromosomal abnormalities in

subfertile and infertile men and it appears that spermatogenesis is affected as a result of these abnormalities.

The incidence of reciprocal translocations is 7-10 fold higher in infertile men (De Braekeleer and Dao, 1991; reviewed in Van Assche *et al.*, 1996) and 16 times more prevalent in infertile men who require ICSI (Mau *et al.*, 1997). During normal fertilisation, a reciprocal translocation carrier will have a 0-50% risk of miscarriage and/or an abnormal child. The risk in ICSI patients has not yet been extrapolated. Estimates of 40-60% unbalanced sperm have been reported from sperm karyotyping studies on balanced translocation carriers (McKinlay-Gardner and Sutherland, 1996). The difference in rates of reciprocal translocation carriers in newborns compared to sperm might be explained by affected sperm being less likely to fertilise and by increased rates of spontaneous abortion of carriers. These rates may change with ICSI.

The incidence of Robertsonian translocations has also been reported to be 13 times higher in infertile men than in newborns, with the 13q14q translocation occurring 26 times more frequently (Mau *et al.*, 1997). Previous studies have found 10-fold increases in oligozoospermic men compared to newborns, whereas in azoospermic men no differences were found (De Braekeleer and Dao, 1991). It has been suggested from meiotic studies of sterile carriers of 13q14q (Luciani *et al.*, 1984; Johannisson *et al.*, 1993) and 14q21q (Rosenmann *et al.*, 1985) that the spermatogenic impairment is related to an increased frequency of association of the XY bivalent and the Robertsonian trivalent during the pachytene stage.

A predominance of sex chromosome abnormalities has been reported in many studies on infertile men. Chandley (1979) studied an unselected group of 2372 infertile couples, and found that 2.1% of males had a karyotypic abnormality and over half of these were 47,XXY, 47,XYY or mosaic 46,XY/47,XXY. Most of the 47,XXY men were azoospermic and untreatable, however occasionally such men have sperm present in the testes and can be treated with ICSI. A review of the literature by De Braekeleer and Dao (1991) reported a 4.6% incidence of 47,XXY karyotype among infertile men which was 44 times higher than that previously reported in newborns; the majority of these men were azoospermic with very few oligozoospermic. Similar results were also reported by Van Assche *et al.*, 1996. A more recent study by Yoshida *et al.* (1997) found 3.8% sex chromosomal abnormalities in 1007 males presenting with infertility, with nearly three quarters accounted for by Klinefelter's syndrome (47,XXY). They also found that the incidence of chromosomal abnormalities rose in parallel with the severity of the infertility condition; 2.2% for men with normal sperm concentration, 5.1% for oligozoospermic men, 14.6% for azoospermic men and 20.3% for men with non-obstructive azoospermia.

1.3.5 Chromosomal abnormalities transmitted by ICSI

With the advent of ICSI, natural barriers against fertilisation by abnormal sperm were removed and there is recognition of the potential risk of transmission of genetic abnormalities from sperm to embryos and offspring (Engel *et al.* 1996). Preliminary clinical results have suggested that there is an inheritance of structural abnormalities

from the father and an increased incidence of sex chromosomal abnormalities in some ICSI children. In 1995, In't Veld *et al.* reported an extremely high incidence (33%) of sex chromosomal abnormalities (47,XXY(2), 45,X(2), 45,X/46,X.dic(Y)(q11)/46,X.del(Y)(q11)) in 15 prenatal karyotypes from ICSI patients who underwent prenatal screening due to increased maternal age. The parents were all found to be karyotypically normal. At the same time, Liebaers *et al.* (1995) reported a much lower incidence of sex chromosomal aneuploidy (1%) in a larger number of prenatal karyotypes after ICSI (n = 585), although this was still higher than that found in the newborn population (0.19%) (Jacobs, 1992).

Since then, studies have been published on the outcome of ICSI pregnancies. Concern has been voiced that the process of ICSI might be responsible for the anomalous sex chromosome results (Liebaers *et al.*, 1995). Persson *et al.* (1996) suggested that the high rate of sex chromosomal abnormalities may be the result of a proportion of azoospermic men being treated with ICSI having Klinefelter's (47,XXY) syndrome or 46,XY/47,XXY mosaicism. A pregnancy has been reported after ICSI with sperm from a man with a 47,XXY karyotype, however the pregnancy stopped developing in the ninth week but the foetus had a normal 46,XX karyotype, therefore fertilisation with a haploid 23,X sperm had occurred (Hinney *et al.*, 1997). Since then, there have been reports of normal livebirths following ICSI using sperm from men with Klinefelter's syndrome (Bourne *et al.*, 1997; Palermo *et al.*, 1998; Ron-El *et al.*, 1999).

The chromosomal content of sperm from men with 47,XXY karyotype has been

examined using FISH and these studies reported much higher frequencies of aneuploidy than in a control group. The most significant differences were in the incidence of disomy XY sperm, from 6-fold higher (2.1%, Chevret *et al.*, 1996) to 78-fold higher (14.6%, Foresta *et al.*, 1998). Guttenbach *et al.* (1997b) found increased frequencies of XY sperm (1.36%), XX sperm (1.22%), and diploid sperm (0.23%), but not YY sperm (0.09%) in sperm from a man with a 47,XXY karyotype. It is believed that meiosis of 47,XXY germ cells is possible (Cozzi *et al.*, 1994), therefore one could conceivably inject 24,XX or 24,XY sperm in such cases, leading to a much higher transmission of sex chromosomal abnormalities after ICSI in this subgroup.

An inheritance of paternal structural abnormalities has also been identified in a number of studies. Bonduelle *et al.* (1998) published the latest report on 1082 prenatal tests conducted up to August, 1997, and 10 cases (0.92%) of paternal transmission of structural abnormalities were reported. They also reported 18 cases (1.7%) of *de novo* chromosomal abnormalities, with 9 cases of autosomal abnormalities (5 x trisomy 21, 4 x *de novo* structural aberrations) and the other 9 cases due to sex chromosomal abnormalities (1 x 45,X; 1 x 46,XX/47,XXX; 2 x 47,XXX; 4 x 47,XXY; 1 x 47,XYY).

The increase in the incidence of trisomy 21 (0.46% in ICSI fetuses vs 0.19% in newborns; Jacobs, 1992) is also a cause for concern. Although the transmission of an extra chromosome 21 is predominantly linked with increased maternal age, a recent report described the first case of a paternally-derived trisomy 21 conceptus conceived by ICSI (Bartels *et al.*, 1998). However, a previous study had reported on two ICSI

foetuses with trisomy 21 and 18, and transmission was maternal in origin (Van Opstal *et al.*, 1997).

Recently, male infertility caused by non-obstructive azoospermia has been successfully treated by ICSI using spermatids (Fishel *et al.*, 1995; Tesarik *et al.*, 1995). To date, no studies have been reported on the incidence of chromosomal abnormalities in human spermatids associated with normal or abnormal spermatogenesis. One difficulty is in accurately identifying round spermatids prior to injection into oocytes (Tesarik and Mendoza, 1996). Angelopoulos *et al.* (1997) have described a method that selects spermatids based on cell size, morphological characteristics of the nucleus and cytoplasm, and on the nucleus/cytoplasm ratio. These workers used FISH to identify the chromosomal content of these selected cells. Of the cells selected, 84% were haploid, 8% were aneuploid and 6% were diploid, and in one individual sample, a much higher incidence of disomy XY cells was observed. It is not clear from the study what the exact aetiology of the clinical conditions examined but may have included known causes of non-obstructive azoospermia including Klinefelter syndrome (XXY), 46XY/47XXY mosaicism (Persson *et al.*, 1996) or aneuploidy confined to the germ cell line (Hendry *et al.*, 1976) as well as unknown causes. If spermatids are to be used for ICSI then the incidence of chromosomal abnormalities in spermatids and subsequent embryos needs to be ascertained.

1.4 Aneuploidy and structural abnormalities in sperm

There exists the potential for an increased rate of transmission of chromosomal

abnormalities using ICSI, hence it is important to accurately determine the incidences of chromosomal abnormalities in human sperm.

Historically, the first technique used to study chromosomes in human sperm was differential staining of specific regions of the chromosomes (Table 2). Pearson and Bobrow (1970) used fluorescent quinacrine to stain the distal two thirds of the long arm of the Y chromosome (Y body) and estimated that 1.4% of sperm were aneuploid for the sex chromosomes. Subsequently, the incidence of two Y bodies in human sperm was reported to be 1.3% (Sumner *et al.*, 1971) and 5% (Klasen and Schmid, 1981). Autosomes have also been studied using a Giemsa stain for the secondary constriction of chromosome 9 (Bobrow *et al.*, 1972; Pawlowitski and Pearson, 1972) and a Leishman's stain for chromosome 1 (Geraedts and Pearson, 1973). An average aneuploidy rate of approximately 2% per chromosome was reported, giving a total aneuploidy rate of 38% if all chromosomes were considered together (Pawlowitski and Pearson, 1972). These estimates were excessive and unreliable, presumably due to non-specific staining of chromosomes.

TABLE 2: Summary of cytological staining studies in sperm

Author(s)	Year	Chrom.	Method	%
Pearson and Bobrow	1970	Y	Fluorescent 'quinacrine'	1.4
Sumner <i>et al.</i>	1971	Y	"	1.3
Klasen and Schmid	1981	Y	"	4 - 5
Bobrow <i>et al.</i>	1972	9	'Giemsa 11' staining	2
Pawlowitski and Pearson	1972	9	"	1.3
Geraedts and Pearson	1973	1	Leishman's solution	1 - 2

A remarkable technique to visualise human sperm chromosomes in the ooplasm

of zona-free hamster oocytes was introduced by Yanagimachi *et al.* (1976). Hamster ova are collected and the cumulus cells and zona pellucida removed before insemination with human spermatozoa. After incubation for 4-5hr, fixation of the fertilised eggs enables the visualisation of sperm metaphases within the egg cytoplasm. Rudak *et al.* (1978) subsequently analysed 60 sperm complements and 3 were aneuploid, giving a frequency of 5%. Since then, over 20,000 sperm chromosome complements have been analysed by this technique from men with normal karyotypes, and almost 6,000 sperm chromosome complements have been analysed from men with constitutional chromosomal abnormalities (Guttenbach *et al.*, 1997c; Table 3).

It was frequently found that the number of nullisomic sperm was twice that of disomic sperm. This discrepancy was generally attributed to loss of chromosomes during fixation, so a conservative estimate of aneuploidy was derived by doubling the disomy rate, and this yielded total aneuploidy frequencies of 0.0 to 5.1% (Martin, 1986; Pellestor *et al.*, 1987; Martin and Hulten, 1993). A more realistic estimate of 1.4% aneuploidy can be found in the two largest studies (Brandriff *et al.*, 1985; Martin, 1990). Men who have a constitutional chromosomal abnormality were previously thought to have an increased risk of having aneuploid offspring due to the interference of the rearranged or extra chromosomes with the normal pairing or disjunction of homologous chromosomes (Martin, 1989). However, the studies outlined in Table 3 have not shown any significant increase in the incidence of aneuploidy in sperm from these men (Guttenbach *et al.*, 1997c).

The incidence of structural abnormalities determined using the hamster

technique was much higher than aneuploidy and ranged from 1.2 to 13% (Kamiguchi and Mikamo, 1986; Pellestor *et al.*, 1987). Incidences of 7.7% and 9.4% were found in the larger studies (Brandriff *et al.*, 1985; Martin, 1990). The majority of the structural abnormalities found in sperm were chromosome breaks, followed by fragments and less frequently chromatid exchanges, chromatid breaks, deletions, dicentrics, translocations and duplications (Brandriff *et al.*, 1985; Rosenbusch and Sterzik, 1994; Estop *et al.*, 1995). Rosenbusch and Sterzsik (1994) compared sperm karyotypes in three groups of men; normal men with no reproductive dysfunction, partners of women with habitual abortion and men with impaired sperm quality. No differences were found in aneuploidy but they found a significant difference in chromosome breaks (2.4 vs 5.8%) and acentric fragments (2.4 vs 8.1%) for normal men and partners of women with habitual abortion, respectively.

TABLE 3: Cytogenetic studies on human sperm after penetration of hamster eggs

* = values expressed as %.

AUTHOR	YEAR	N	Hypo.*	Hyper.*	Aneup.*	2xhype.*	Struct.*
Rudak et al.	1978	60	-	-	5	-	-
Martin et al.	1983	1000	2.7	2.4	5.2	4.8	3.3
Brandriff et al.	1985	2468	0.9	0.7	1.7	1.4	7.7
Martin ^a	1986	94	5.3	0	5.3	0	5.3
Kamiguchi and Mikamo	1986	1091	0.45	0.45	0.9	0.9	13.0
Jenderny and Rhorborn	1987	129	0.8	0.8	1.6	1.6	6.2
Martin et al.	1987	1582	3.4	1.3	4.7	2.4	6.2
Pellestor et al. ^b	1987	78	12.8	2.5	15.4	5.1	1.2
Martin (inc 1983/1987)	1990	5629	3.5	0.6	4.2	1.4	9.4
Martin and Rademaker ^c	1990	6821	3.3	0.7	3.9	1.5	-
Estop et al.	1991	555	6.3	2.0	8.3	4.0	3.6
Martin et al. ^d	1991	3259	5.3	1.1	6.5	2.3	9.7
Pellestor	1991	1561	-	-	6.1	-	3.5
Benet et al.	1992	505	9.1	2.0	11.1	4.0	6.9
Martin and Hulten ^e	1993	275	2.2	0.4	2.9	0.8	12
Martin and Hulten ^f	1993	268	3.0	0	3.0	0	7.8
Martin and Hulten ^g	1993	152	4.0	0	4.0	0	8.6
Rosenbusch and Sterzik ^h	1994	413	1.0	1.0	1.9	2.0	7.0
Rosenbusch and Sterzik ⁱ	1994	308	1.9	1.6	3.6	3.2	14.6
Rosenbusch and Sterzik ^j	1994	146	1.4	0.7	2.1	1.4	10.3
Estop et al. ^k	1995	2389	-	-	-	-	9.3
Templado et al. ^l	1996	3446	8.6	1.7	10.2	3.3	-

N=number of karyotypes analysed, Hypo.=nullisomic sperm, Hyper.=disomic sperm, Aneup.=sum of nullisomic and disomic sperm, 2xHype.=double the disomy rate (conservative estimate of aneuploidy), Struct.=sperm with structural abnormalities.

^aman heterozygous for a paracentric inversion of chromosome 7 (q11q22), ^bman heterozygous for a t(13;14) Robertsonian translocation, ^c83 normal donors and 15 men with constitutional chromosome abnormalities, ^dmen with constitutional chromosome abnormalities, ^eman heterozygous for a reciprocal t(7;20)(q33.2;p13) translocation, ^fman heterozygous for a reciprocal t(3;11)(q25.3;q25), ^gman heterozygous for a reciprocal t(15;22)(q26.1;q11.2), ^hnormal men, no reproductive dysfunction, ⁱpartners of women with habitual abortion, ^jmen with impaired sperm quality, ^k11 normal men, six reciprocal translocation carriers and one pericentric inversion carriers, ^l14 normal men, eight reciprocal translocation carriers and two pericentric inversion carriers.

Sperm karyotyping using the hamster technique yielded valuable data because the entire chromosome complement of each spermatozoon was examined and structural and numerical abnormalities were detected. However, sperm karyotyping is labour-intensive and time-consuming (Jacobs, 1992; Martin, 1993), and the results are potentially biased in that only those human sperm which can fertilize hamster oocytes are karyotyped - this may eliminate sperm with genetic mutations or morphological disadvantages that preclude them from fusing with oocytes. Nevertheless, it provided useful baseline data with which to compare results obtained using its successor, FISH (Martin *et al.*, 1993; Robbins *et al.*, 1993; Martin *et al.*, 1996; Spriggs *et al.*, 1996; Van Hummelen *et al.*, 1996). FISH has now largely replaced all other methods for assessing sperm aneuploidy.

1.5 Fluorescence In-Situ Hybridization (FISH)

1.5.1 Technique

Chromosomal *in situ* hybridization (ISH) involves hybridization of a chromosome-specific DNA probe to complementary sequences on a target chromosome followed by detection of the bound probe. The ISH technique was originally developed in 1969 by Pardue and Gall and radioactively-labelled probes and detection by autoradiography was the only available technology. Radioactive *in situ* hybridization (RISH) was mostly used for research purposes and was rarely applied clinically due to problems associated with safety measures, limited shelf life of the labelled probes, and the time and labour required for autoradiography. RISH remains the most suitable technique for very short DNA probes, of 150-1000 bp (Webb,

1999), but for larger probes, RISH was largely replaced in the 1980s by non-isotopic methods, in particular FISH, a technique in which the probes are detected using fluorochromes (red, green or blue) and indirect or direct detection procedures (Trask, 1991).

Indirect FISH utilises a DNA probe which contains a hapten such as digoxigenin (DIG) or biotin. After hybridization of the probe to the target DNA, the hapten is detected using a fluorochrome-conjugated binding protein such as avidin, for biotinylated probes, or a fluorochrome-conjugated antibody, for DIG probes (Figure 3a and 3b).

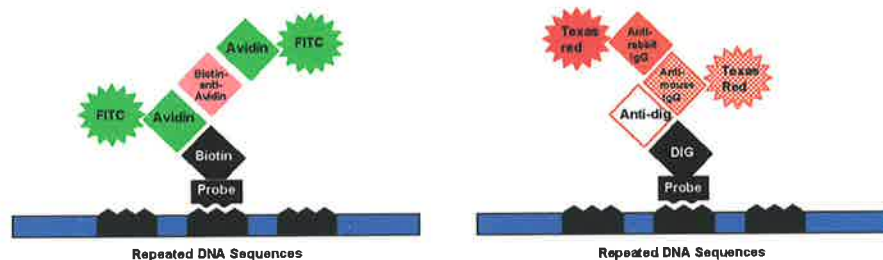


Figure 3: Indirect FISH using (a, left) biotinylated probes and detection reagents or (b, right) DIG-labelled probes and detection reagents.

The main advantages of indirect FISH are high sensitivity, the ability to intensify the signal using sandwich techniques in which consecutive amplifications of the signal are achieved using antibodies, and the availability of many combinations of detection reagents. Disadvantages are cost, extended staining times and higher background labelling (reduced signal to noise ratio).

In the direct FISH procedure, the fluorochrome is incorporated directly into the probe so that the DNA-probe complex can be visualised by fluorescence microscopy

without additional detection steps (Figure 4).

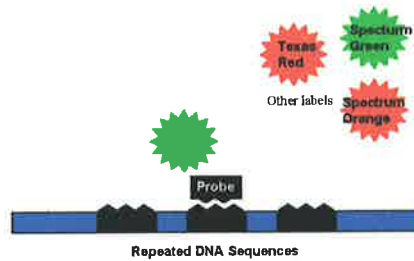


Figure 4: Direct FISH

Probes labelled with fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC), aminomethyl coumarin acetic acid (AMCA), Texas red (TR) and cyanine dyes (Cy3 and Cy5) have been used (Trask, 1991; Yurov *et al.*, 1996). Vysis (Framingham, MA, USA) supplies probes which are directly labelled with variants of these fluorochromes called Spectrum Orange[®], Spectrum Green[®] and Spectrum Aqua[®]. Direct FISH eliminates the time-consuming post-hybridization detection steps and reduces non-specific labelling. The only disadvantage is the decreased sensitivity of detection (Reid *et al.*, 1992a).

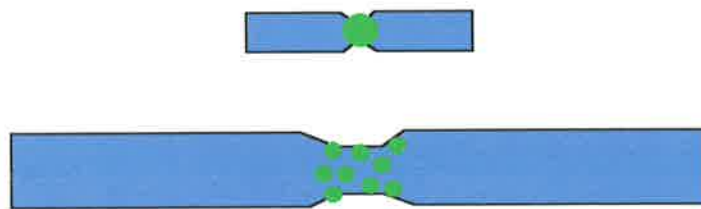
Earlier studies used single-probe FISH, whereby one chromosome per cell was detected, but it is preferable to simultaneously localise several chromosomes in each cell (multi-probe FISH) to increase the power of detection. Double-probe FISH is the simultaneous hybridization of two probes, while triple-probe FISH indicates hybridization of three probes.

There are several approaches that can be used for multi-probe FISH. (i) Up to three chromosomes can be detected simultaneously by direct and/or indirect FISH using three different probes and the three basic fluorochrome emission colours: green,

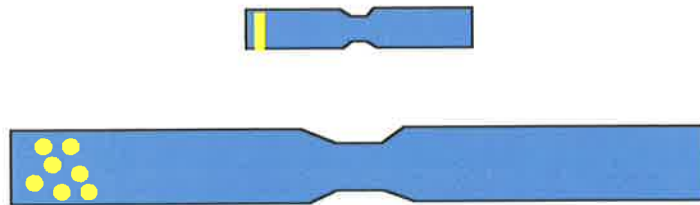
red and blue. The only drawback is that a nuclear counterstain is also required, and since this is usually either DAPI which fluoresces blue or propidium iodide which fluoresces red, it restricts detection to only two chromosomes. (ii) Each probe is labelled with up to 3 different haptens (or fluorochromes), so that the probe will produce up to 3 different signals *in situ*, either as separate coloured signals in the same location if single bandpass filters are used or as a signal of composite colour if a double or triple bandpass filter is employed (Nederlof *et al.*, 1990). Up to seven different probes have been visualised simultaneously on human metaphase chromosomes using a combination of three single-labelled probes, three double-labelled probes and one triple-labelled probe (Reid *et al.*, 1992b). (iii) Ratio labelling. Aliquots of a probe are directly labelled with different fluorochromes and the aliquots are then mixed in varying ratios prior to hybridization so as to produce a different coloured composite signal. This method can produce up to 12 different colours from the three primary colours (Dauwerse *et al.*, 1992) and recently a different colour was produced for every human chromosome (Lichter, 1997).

Three types of DNA probes are available for FISH (Figure 5). (i) Centromeric probes recognise repetitive DNA sequences in the centromeric region and have been developed for most human chromosomes (Willard and Waye, 1987). These alphoid or satellite repeat sequences produce a small signal in the vicinity of the centromere (Figure 5a) and are routinely used to detect aneuploidy. They are not very useful for detecting structural abnormalities which, with the exception of Robertsonian translocations, occur on the p or q arms of chromosomes. (ii) Sequence-specific

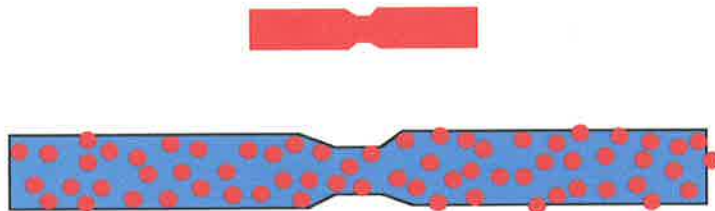
probes can be used to detect unique sequences, containing one or more genes, on chromosomes (Pinkel *et al.*, 1988; Reid *et al.*, 1992a). These sequences are unique to a particular chromosome, so the probes can be used to detect aneuploidy. Moreover, chromosome-specific centromeric probes are not available for some chromosomes (13, 14, 21, 22) and therefore sequence-specific probes must be used to detect these chromosomes. Chromosome-specific telomeric probes (Figure 5b) have recently been used to estimate structural abnormalities in human sperm chromosomes (Van Hummelen *et al.*, 1996). (iii) Whole chromosome painting (WCP) probes are chromosome-specific DNA libraries which label whole chromosomes (Figure 5c) in combination with Cot-I DNA to suppress repetitive sequences common to all chromosomes. They can be used to detect structural rearrangements in metaphase chromosomes (Pinkel *et al.*, 1988; Dauwerse *et al.*, 1992; Kearns and Pearson, 1994) and to study the organisation of chromosomes and chromatin in interphase nuclei (Pinkel *et al.*, 1988; Brandriff and Gordon, 1992). Dauwerse *et al.* (1992) applied this technique to bone marrow spreads and showed that half of the chromosomes could be painted in 12 different colours using WCP probes carrying three distinct labels mixed in multiple ratios. This implies that, in theory, two separate hybridizations would allow the full complement of metaphase chromosomes to be analysed. This technique is widely used for the detection of complex chromosome rearrangements, cryptic translocations, translocations involving small chromosome segments and the identification of marker chromosomes.



(a) Centromeric DNA probe



(b) Sequence-specific DNA probe



(c) Whole chromosome paint DNA probe

Figure 5: Different types of DNA probes.

1.5.2 Sperm nuclear decondensation

Mammalian sperm are haploid interphase cells which have a unique packaging and arrangement of DNA that differs significantly from somatic cells (Ward and Coffey, 1991; Barone *et al.*, 1994). The linear, side-by-side arrays of DNA, cross-linked by disulphide bonds between adjacent protamines, create a condensed, genomically inert nucleus (Bedford and Calvin, 1974; Balhorn, 1982) which is mostly inaccessible to DNA probes.

Due to the condensed nature of the nucleus the earliest ISH studies on human sperm were problematical and mostly unsuccessful. Joseph *et al.* (1984) used probes that were specific for the Y chromosome and chromosome 1, but achieved no hybridization in ejaculated sperm and variable results in testicular sperm. They used untreated sperm and ejaculated sperm which had been pretreated with 1% trypsin for 30-60 sec followed by 0.01% dithiothreitol (DTT) for 60-90 sec. Seunanez *et al.* (1976) reported that hybridization occurred in immature sperm, but the hybridization efficiency decreased as sperm matured.

It is now well recognised that to achieve efficient hybridization, the sperm nucleus must be made accessible to probes by reducing disulphide bonds between protamine molecules. This has been achieved using a variety of protocols (Table 4).

The earliest successful reports on non-isotopic ISH with ejaculated sperm were Pieters *et al.* (1990) and Coonen *et al.* (1991) who found that pretreatment of sperm with 25mM DTT and 0.1% trypsin for 5-20 min promoted nuclear decondensation.

Table 4: Pretreatment (nuclear decondensation) of human sperm for ISH and FISH

Method and Studies	Method *	Comments
DTT		
Rousseaux and Chevret (1995)	10 mM DTT in 0.05M Tris, pH 8, 10-50 min	Adequate nuclear swelling, intact tail morphology.
Martini <i>et al.</i> (1995)	25 mM DTT in 1M Tris, pH 9.5, 5 min	
TRYPSIN + DTT		
Coonen <i>et al.</i> (1991)	0.1% trypsin + 25 mM DTT, 5-20 min	Many tails lost. Optimal time (5-15 min) determined for each sample.
Goldman <i>et al.</i> (1993)	0.1% trypsin + 25 mM DTT, 12 min, RT	
Bischoff <i>et al.</i> (1994)	0.1% trypsin + 25 mM DTT, 2 min, 37°C	
LIS + DTT		
Wyrobek <i>et al.</i> (1990)	(1) Sperm nuclei isolated with MATAB and DTT; (2) 10 mM LIS + 1 mM DTT, 3 h	Concentrations as low as 1 mM LIS induced swelling of isolated sperm nuclei. Swelling was uniform and the oval shape was maintained.
Robbins <i>et al.</i> (1993)	(1) 10 mM DTT, 30 min on ice; (2) 4 mM LIS, 90 min, RT	No fixation. Sperm air dried onto slides before decondensation.
Williams <i>et al.</i> (1993)	(1) 10 mM DTT in 0.1M Tris, pH 8, 30 min, RT; (2) 10 mM LIS + 1 mM DTT, 1-3 h	Modification of Wyrobek <i>et al.</i> (1990)
Miharu <i>et al.</i> (1994)	(1) 5 mM DTT, 10 min; (2) 10 mM LIS + 0.5 mM DTT, 70 min	Modification of Wyrobek <i>et al.</i> (1990). Sperm nuclei were swollen to 1.5 times the original nuclear diameter.
EDTA + DTT		
Han <i>et al.</i> (1992)	(1) 6 mM EDTA; (2) 2 mM DTT, 45 min	Preferable to trypsin + DTT and SDS + DTT.
Pang <i>et al.</i> (1994)	6 mM EDTA + 2 mM DTT, 45 min, 37°C	
Wang <i>et al.</i> (1994)	(1) 6 mM EDTA; (2) 2-4 mM DTT, 45 min	Modification of Han <i>et al.</i> (1992).
CTAB + DTT		
Holmes and Martin (1993)	(1) 10 mM DTT in 0.05M Tris, pH 8; (2) sonication, 4°C; (3) 1% CTAB, 30 min at 4°C.	Modification of Balhorn <i>et al.</i> (1977). Swelling to 1.5 times nuclear area. Tail and nuclear membrane were removed by CTAB and DTT.
NO PRETREATMENT		
Guttenbach and Schmid (1990)	Denatured with probe in formamide, 10 min, 72°C	Denaturation prior to hybridization caused all sperm nuclei to swell.
Guttenbach <i>et al.</i> (1994a)	3M NaOH, 3-10 min, RT	3M NaOH allowed decondensation to be controlled.

* Numbers in parentheses indicate the order of sequential treatments; RT = room temperature

However, this only resulted in approximately half of the sperm nuclei being accessible to probes. Most researchers have subsequently used trypsin, lithium diiodosalicylic acid (LIS), ethylene diaminetetracetic acid (EDTA), or cetyl trimethylammonium bromide (CTAB) in combination with a disulphide reducing agent, DTT, to release the disulphide bonds between adjacent protamine molecules and thereby induce swelling of the sperm head (Table 4).

Wyrobek *et al.* (1990) sought a reproducible pretreatment procedure for human sperm and found that 10mM LIS and 1mM DTT induced uniform swelling of the nucleus from 1.5 to 2.5 times its normal area and maintained the characteristic oval shape of the human sperm nucleus. This pretreatment procedure, or a modification of it, has subsequently been used in many studies and has proven to be a reliable pretreatment procedure (Robbins *et al.*, 1993; Williams *et al.*, 1993; Wyrobek *et al.*, 1993a; Miharu *et al.*, 1994; Spriggs and Martin, 1994; Wyrobek *et al.*, 1994; Spriggs *et al.*, 1995; Martin *et al.*, 1996; Van Hummelen *et al.*, 1996; Martin *et al.*, 1997a; 1997b; Robbins *et al.*, 1997; Griffin *et al.*, 1996; Van Hummelen *et al.*, 1997).

Two other approaches have also been used. Chevret *et al.* (1994) and Martini *et al.* (1995) used DTT alone to disrupt the disulphide bonds without excessive swelling of the sperm head, thus retaining sperm morphology while ensuring efficient hybridization. In contrast, Guttenbach and co-workers did not pretreat human sperm in any way prior to hybridization, relying instead on an extended denaturation in formamide, or denaturation in 3M NaOH to swell the sperm heads (Guttenbach and Schmid, 1990; 1991; Guttenbach *et al.* 1994a; 1994b). However, the hybridization

efficiencies were considerably lower ($> 80\%$) than those obtained using DTT (95-99%) (Table 4).

Irrespective of which pretreatment method is used, there are several requirements. First, pretreatment should enable hybridization of probes to a very high proportion of the sperm, in practice most researchers use a lower limit of between 95 and 98%, to minimise scoring biases. Second, excessive loss of sperm from the slides should not result. Third, pretreatment should maintain the oval shape of the sperm head and not result in excessive distortion of the nucleus which might lead to disruption of DNA integrity and hence signal splitting. Finally, the sperm tail should remain attached to the sperm head so that sperm can be readily distinguished from non-sperm cells such as leucocytes and immature germ cells which are also present in semen.

1.5.3 Single-probe versus multi-probe FISH.

Single-probe FISH has been used to estimate aneuploidy in sperm, however, it is now recognised that this method has several technical limitations which reduce its efficacy. First, it is impossible to differentiate accurately between disomy and diploidy when only a single probe is used. Some researchers have attempted to distinguish these conditions on the basis of nuclear size by assuming that diploid sperm have a large nucleus and two hybridization signals, whereas disomic sperm have a normal size nucleus and two signals (Coonen *et al.*, 1991; Han *et al.*, 1992, 1993a, 1993b; Guttenbach *et al.*, 1994a, 1994b; Wang *et al.*, 1994). This association has never been proven, and it would therefore seem prudent to avoid using nuclear size to estimate

the ploidy status, especially in view of the fact that Williams *et al.* (1993) found that the rate of diploidy for many donors was higher than the disomy rate. Second, in the absence of a hybridization signal it is impossible to make an interpretation when only a single probe has been used; either nullisomy or failure of hybridization is indicated. This is an even greater problem when a single sex chromosome probe is used because only half of the sperm should exhibit a hybridization signal (Fig. 6).

Multi-probe FISH overcomes these limitations, and enables reliable distinction between diploid and disomic sperm, and between nullisomy and failed hybridization (Figs. 7-9). More accurate estimates of autosomal disomy can be obtained using double-probe FISH because each spermatozoon should generate two signals, one for each of the autosomes. Thus, sperm with two signals for each chromosome are diploid, whereas sperm with a single signal for one probe and two signals for the other probe are disomic for the latter autosome. Similarly, sperm with only one signal are nullisomic for the other autosome, whereas those with no signals are likely to have failed to hybridize (Fig. 7).

Estimation of sex chromosome aneuploidy engenders further difficulties. Double-probe FISH with X and Y probes has been used to estimate sex chromosome aneuploidy (Goldman *et al.*, 1993; Han *et al.*, 1993a, 1993b; Chevret *et al.*, 1994; Wang *et al.*, 1994; Wyrobek *et al.*, 1994), however, this approach cannot differentiate between disomic (22 XX, 22 YY, 22 XY) and diploid (44 XX, 44 YY, 44 XY) sperm (Fig. 8). Furthermore, one cannot determine whether unlabelled sperm are nullisomic for the sex chromosomes or failed to hybridize, and disomy and diploidy estimates are

indirectly reduced if nullisomic sperm are incorrectly classified as unlabelled.

Triple-probe FISH using probes for the sex chromosomes and one autosome can be used to accurately determine sex chromosome aneuploidy in sperm and differentiate between unlabelled sperm which are nullisomic for the sex chromosomes and sperm which are unlabelled because the probes did not hybridize (Fig. 9). With the inclusion of the third autosomal probe, each spermatozoon should exhibit one autosomal signal and one sex chromosome signal (X or Y). Sex chromosome disomy is characterised by one autosomal signal and two sex chromosome signals, whereas a diploid spermatozoon has two autosomal signals and two sex chromosome signals. Sex chromosome nullisomy is indicated by the presence of only a single autosomal signal in the spermatozoon, whereas sperm that are unlabelled due to hybridization failure exhibit no signals at all. It is also possible to distinguish between sex chromosome disomy and diploidy of meiosis I and II origin; non-disjunction at meiosis II results in only one disomic spermatozoon (XX or YY) whereas non-disjunction at meiosis I results in two disomic XY sperm. Rademaker *et al.* (1997) recently demonstrated that comparable diploidy frequencies are obtained using double-probe FISH and triple-probe FISH on the same sperm samples.

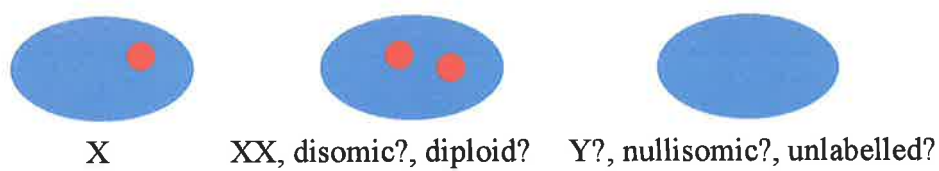


Figure 6: Single-probe FISH using a X chromosome-specific probe. Only 50% of the spermatozoa exhibit a signal, and the gender-determining capacity of the unlabelled spermatozoon is uncertain. Furthermore, it is impossible to distinguish disomy from diploidy.

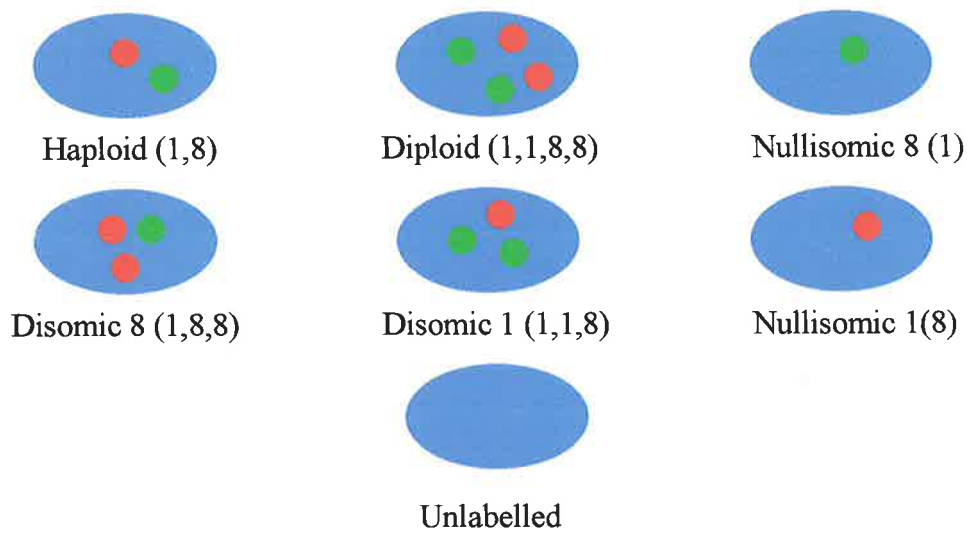


Figure 7: Double-probe FISH using autosomal probes (chromosomes 1 and 8). All spermatozoa should exhibit at least one signal unless hybridization failure has occurred (unlabelled). Disomy, nullisomy and diploidy can be distinguished by the number and colours of signals (perceived signals shown in parentheses).

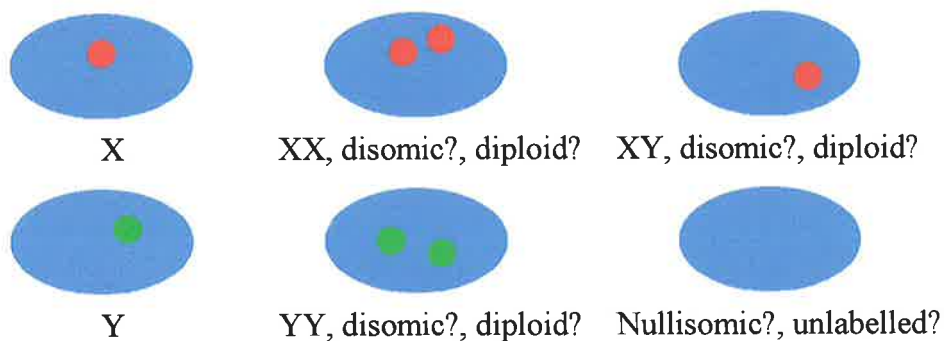


Figure 8: Double-probe FISH using X- and Y-specific probes. While XX, YY and XY spermatozoa can be identified, it is impossible to distinguish disomic from diploid spermatozoa, and the sex chromosome status of unlabelled spermatozoa is unclear.

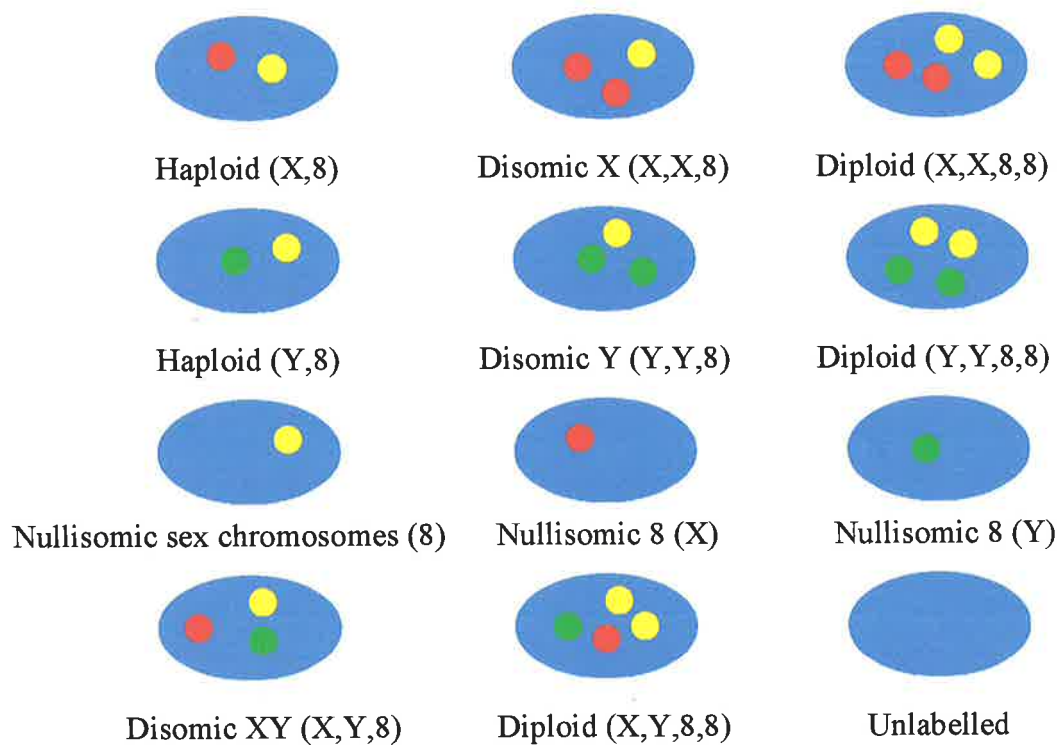


Figure 9: Triple-probe FISH using sex chromosome probes and an autosomal probe (chromosome 8) enables accurate determination of sex chromosome aneuploidy in human spermatozoa. Haploid spermatozoa can be distinguished from disomic, nullisomic and diploid spermatozoa, and the status of spermatozoa can be determined (perceived signals shown in parentheses).

1.5.4 Estimation of aneuploidy in sperm using FISH

When this study commenced in 1994, there had been 5 reports using ISH and 13 reports using FISH on the estimation of aneuploidy in human sperm. The results of these studies are summarised in Tables 5, 6 and 7. For ease of comparison, single- and multi-probe studies have been grouped together.

Table 5: Frequency of two signals (disomy or diploidy[♦]) using single-probe ISH or FISH in human sperm from normospermic men

Study	Hyb. eff. (%) [*]	Number of samples used	Number sperm counted /donor	1	12	13/21	15	16	17	X	Y
Joseph <i>et al.</i> (1984)	80/48	1 [#]	3733	0.35							0.18
West <i>et al.</i> (1989)	47	8	3,900								0.03
Guttenbach and Schmid (1990)	49	8	8,061								0.27
Pieters <i>et al.</i> (1990)	40-60	?	3,000	0.8							
Coonen <i>et al.</i> (1991)	40-90	32	1,000	0.67							
Guttenbach and Schmid (1991)	-	7	1500	0.41							
Jackson-Cook and Haller (1991)	-	8	25,666 ^a			0.5		0.6	0.1	0.6	0.2
Han <i>et al.</i> (1992)	96/48	13	1,000						0.33	0.29	
Holmes and Martin (1993)	99/49	1	10,000	0.06	0.04					0.03	
Martin <i>et al.</i> (1993)	98-99	1	10,000				0.14	0.17			0.11
Robbins <i>et al.</i> (1993)	98/50	3	10,000	0.14							0.06

[♦] mean values

^{*} Hyb. eff. = hybridization efficiency, ranges or separate values for each of the chromosomes are given

[#] Testicular sperm in air-dried meiotic preparations

^a Total number of sperm counted for 8 donors

Table 6: Studies on disomy[♦] using double-probe FISH in human sperm from normospermic men

Study	Hyb. eff. (%) [*]	Number of samples used	Number sperm counted/donor	16	18	X	Y	XY
Goldman <i>et al.</i> (1993)	99.8	3	10,000			0.08	0.1	0.23
Han <i>et al.</i> (1993a)	96	12	1,000			0.28	0.21	0.21
Han <i>et al.</i> (1993b)	95	10	1,000			0.25	0.23	0.15
Schattman <i>et al.</i> (1993)	99.1	10	1,000			0.04	0.09	0.17
Williams <i>et al.</i> (1993)	96-97	9	5,000	0.13	0.08	0.08	0.11	

[♦] mean values

^{*} Hyb. eff. = hybridization efficiency, ranges or separate values for each of the chromosomes are given

Table 7: Studies of disomy[♦] using triple-probe FISH in human sperm from normospermic men

Study	Hyb. eff. (%) [*]	Number of samples used	Number sperm counted /donor	1	8	18	X	Y	XY
Schattman <i>et al.</i> (1993)	98	10	10,000			0.0	0.1	0.2	0.39
Williams <i>et al.</i> (1993)	95	9	5,000				0.04	0.055	0.09
Wyrobek <i>et al.</i> (1993a)	-	?	10,000	0.14	0.07		0.055	0.061	0.089
Wyrobek <i>et al.</i> (1993b)	-	14	10,000		0.07		0.038	0.042	0.091

[♦] mean values

^{*} Hyb. eff. = hybridization efficiency, ranges or separate values for each of the chromosomes are given

While isotopic ISH was used in the earliest studies (Joseph *et al.*, 1984; West *et al.*, 1989), it is now common practise to use FISH. Guttenbach and Schmid (1990, 1991) also used a non-isotopic ISH method which involved hybridization of biotinylated DNA probes followed by detection of the probes with streptavidin-peroxidase and diaminobenzidine. Signals were viewed under brightfield microscopy and disomy was identified by two brown signals, easily distinguishable from the Giemsa-stained chromatin. The advantages of non-isotopic methods are that sperm morphology is well preserved, slides are scored using brightfield microscopy, and the preparations are permanent. However, the disomy frequencies (0.27 to 0.41%) were much higher than those obtained using single-probe FISH (Holmes and Martin, 1993; Robbins *et al.*, 1993).

Initial aneuploidy estimates obtained using FISH were compared with that of 'the gold standard' sperm karyotyping. Robbins *et al.* (1993) used FISH to analyse samples from donors whose sperm had previously been karyotyped using the hamster oocyte technique. Disomy frequencies obtained using FISH for chromosome 1 (0.14%) and chromosome Y (0.057%) were not significantly different from those obtained for the same donors by karyotyping, which demonstrated the efficacy of FISH for scoring aneuploidy. In contrast, Holmes and Martin (1993) examined 10,000 sperm from only a single donor using FISH and reported a chromosome 1 disomy frequency of only 0.06%, which illustrated the importance of studying sperm from more than one donor, and suggests that some of the variation in Table 5 is due to inter-donor variation.

The application of multi-probe FISH enabled more accurate estimates of sperm aneuploidy to be obtained. Before this study commenced, reports using multi-probe FISH estimated rates of disomy up to 0.4% per chromosome, although the majority were 0.02 to 0.2% (Tables 6 and 7). Williams *et al.* (1993) used double-probe FISH (18, Y or 18, X) to evaluate sex chromosome disomy from meiosis II, and chromosome 16 and 18 probes to study autosomal disomy. To study sex chromosome disomy from meiosis I, they used triple-probe FISH for chromosomes X, Y and 8. To account for different rates of non-disjunction at meiosis I and meiosis II, they corrected the disomy estimates to 0.055% for the Y chromosome and 0.04% for the X chromosome. In this study, they reported that it seems likely that, in an unselected population of males, at least 0.2 to 1.0% of all sperm are diploid. Therefore it seems probable that, in earlier single-colour FISH studies, the majority of sperm with two hybridization signals were diploid, not disomic, justifying the use of multi-probe FISH to study sperm aneuploidy.

In general, single-probe FISH and ISH has yielded higher (and less reliable) estimates of aneuploidy than multi-probe FISH, and there has been considerable variability in the estimates for specific chromosomes. Many of the single-probe studies were undertaken during the formative years of this technology when probes, pretreatment procedures, hybridization protocols, sample sizes and scoring criteria were less well developed and less standardised than they are now. When using single-probe FISH, one cannot differentiate between disomy and diploidy, so disomy estimates obtained using single-probe FISH undoubtedly included diploid sperm.

1.6 AIMS OF THIS PROJECT

When this project commenced in 1994, FISH was a relatively new tool for studying aneuploidy in human sperm. Many of the hybridization and scoring procedures had not been standardised, and this was reflected by inconsistent aneuploidy estimates in published reports. Furthermore, very few studies had been performed on sperm from 'infertile' men, and the few that had been, suggested that there was an increase in sperm aneuploidy levels. ICSI had recently been introduced and was quickly becoming a front line treatment for male infertility, so there was clearly an important need in Reproductive Medicine to investigate the incidence of chromosomal abnormalities in sperm from men undergoing ICSI.

The principal hypothesis examined in this study was that men with triple semen defects (TSD) would demonstrate an increased frequency of chromosomal abnormalities in their sperm compared with a control group of fertile, normospermic donors (NS). Based on the results, some conclusions could be drawn with respect to the potential risk of transmission of such abnormalities to the embryo.

The specific aims of this project were:

1. To develop reliable double- and triple-probe FISH protocols for a range of chromosomes.
2. To determine the baseline frequencies of numerical chromosomal abnormalities (autosomal and sex chromosome aneuploidy, and diploidy) in sperm from normospermic donor samples, and to examine differences in disomy between donors and between chromosomes.

3. To investigate the frequency of numerical and structural chromosomal abnormalities in sperm from men with TSD, and thereby ascertain whether or not there was an increased risk of transmission of abnormalities.

Further to these aims, the specific localisation of individual chromosomes was examined in morphologically abnormal and normal sperm to identify if chromosome(s) were arranged more randomly in morphologically abnormal sperm and therefore postulate whether or not chromosome packaging is involved in the formation of sperm head shape.

CHAPTER 2

Development of FISH protocols for human sperm

2.1 Introduction

The application of FISH to human sperm began in the early 1990s and has continued to flourish as a prominent technique for the detection of aneuploidy.

Human chromosomes were originally categorised into groups, called Denver groups, based on chromosome size and the position of the centromere. The largest chromosomes (1-3) are categorised into Denver group A, and based on decreasing chromosome size, groups B (4-5), C (6-12, X), D (13-15), E (16-18), F (19-20), and G (21-22, Y) are subsequently categorised. Numbering each pair of human chromosomes largely superseded Denver groups when G-banding came into use (Paris Conference, 1971).

With the availability of indirectly-labelled and directly-labelled DNA probes for most chromosomes, it is now possible to develop multi-probe FISH techniques to study chromosomes from all of the Denver groups. At the commencement of this project in early 1994, multi-probe FISH had only been used to study aneuploidy in human sperm for chromosomes 1, 8, 16, 18 and the sex chromosomes (Goldman *et al.*, 1993; Han *et al.*, 1993a, 1993b; Schattman *et al.*, 1993; Williams *et al.*, 1993; Wyrobek *et al.*, 1993a, 1993b).

The aim of this project was to develop multi-probe FISH protocols for human

sperm to use for the detection of aneuploidy for a range of chromosomes, especially those not previously studied. A number of different FISH protocols and probes were evaluated. The application and/or development of other techniques, such as semen analysis criteria, preparation of semen samples on glass slides, pretreatment methods to decondense the sperm DNA, and preparation of lymphocyte smears as positive controls are also explained. In summary, this chapter describes the methodological procedures developed and trialled to determine the most suitable protocols for the subsequent study of aneuploidy in normospermic donors (chapter 3).

2.2 Standard techniques

2.2.1 Semen samples and analysis

Semen samples were obtained from 45 normal, healthy donors who regularly attended the Andrology Laboratory at The Queen Elizabeth Hospital.

Semen analysis techniques were relatively straightforward to learn, but training and expertise were required for accurate scoring of motility and morphology. Results obtained by the author for semen analyses were initially compared to parallel results obtained by trained staff of the Andrology laboratory. Comparisons of neat semen samples (n=18) and washed semen samples (n=5) were performed and differences obtained between the author's and trained members of the Andrology laboratory results ranged from -6% to +19%. Reliability and reproducibility are essential in semen analyses and for this reason it was decided that all analyses should be performed by experienced members of the Andrology laboratory and not by the

author. The advantages in this were that the laboratory regularly undergoes quality control procedures, is externally quality analysed and inter-technician variables are minimal, ensuring that accurate and reliable semen analyses were recorded for the samples used in the present and future studies.

All samples were produced by masturbation, allowed to liquefy at room temperature (RT) and then analysed by staff in the Andrology laboratory using standard procedures (World Health Organization, 1992). All of the donors routinely produced semen samples with >20% normal morphology which is in the normal range for the Reproductive Medicine laboratory at The Queen Elizabeth Hospital (Duncan *et al.*, 1993), and >20 million/ml sperm concentration, >50% progressive motility and >2.0 ml volume (World Health Organization, 1992).

The semen analysis results (mean $\pm$ standard deviation) are shown in Figure 10. The results were semen volume: 3.8 ± 1.3 ml, sperm concentration: 94 ± 53.9 million/ml, sperm motility: 57 ± 7.2 % progressive, and sperm morphology: 31 ± 8.6 % normal forms.

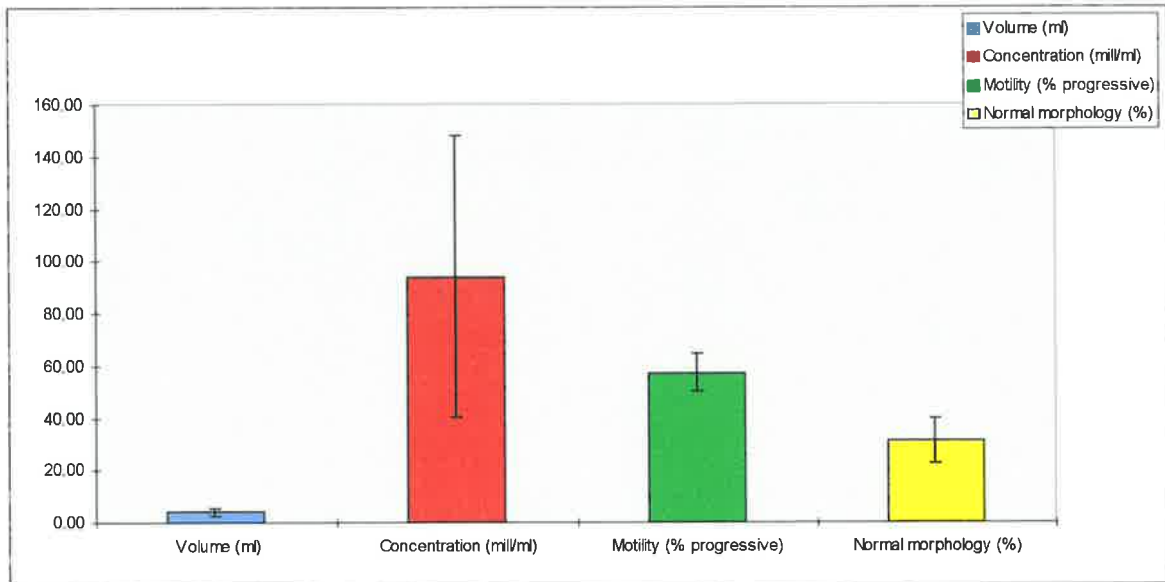


Figure 10: Semen analysis results (mean $\pm$ standard deviation) for 45 normospermic donor samples.

2.2.2 Preparation of semen samples

Prior to sample preparation, glass microscope slides were cleaned overnight in 5% Decon solution. Slides were rinsed under tap water until all Decon had washed off (approx. 2 hr), rinsed twice in MilliQ-H₂O (Millipore Corporation, Bedford, MA, USA), followed by 100% alcohol, and air-dried.

Sperm were washed three times for 10 min each at 300g in HEPES-HTF medium (Quinn *et al.*, 1985; prepared by staff in the IVF laboratory) with human serum added. Once the semen sample had been washed, the supernatant was removed and the pellet resuspended in 1-4ml of HEPES-HTF. Drops (20-50 μ l) of sperm suspension were smeared onto clean glass microscope slides and air-dried. Slides were stored with desiccant at -20°C.

2.2.3 Pretreatment (decondensing) of sperm

When applying FISH protocols to human sperm, it is necessary to chemically pretreat (decondense) the sperm to break the disulphide bonds linking the DNA. This causes the sperm head to swell, and thereby allows the probes to enter and hybridise to the sperm DNA. Studies have shown that the ideal degree of swelling is 1.5-2 times normal nuclear size (Wyrobek *et al.*, 1990). Without pretreatment, it would be very difficult to reliably visualise FISH signals in sperm.

2.2.3.1 Materials and Methods

The following decondensing methods were tested on sperm to determine the most reproducible method that would reliably decondense sperm nuclei to 1.5 to 2 times normal size:

1. A modification of the method of Williams *et al.* (1993), in which slides were taken from -20°C, allowed to equilibrate to RT and incubated for 30 min in 10mM dithiothreitol (DTT; Sigma Chemical Co., St Louis, MO, USA) in 0.1M Trizma base (Sigma), pH 8.0, and then for 1-3 hr in 1mM DTT and 5 or 10mM lithium 3,5-diiodosalicylic acid (LIS; Sigma) in 0.1M Trizma base, pH 8.0. After pretreatment, slides were washed in 0.1M Trizma base, pH 8.0, rinsed in MilliQ-H₂O and air-dried.
2. The method of Martini *et al.* (1995) in which slides were incubated for 5 min in 25mM DTT in 1M Tris-HCl, pH 9.5. After pretreatment, slides were washed 5 min in 2 x SSC, and 5 min in phosphate buffered saline (PBS) and then dehydrated in a 70-85-95-100% ethanol series and air-dried.



(a)



(b)



(c)

Figure 11: Normospermic samples after pretreatment using method of Williams et al. (1993), (a) before treatment, (b) after pretreatment, (c) after pretreatment.

3. The method of Robbins *et al.* (1993) in which slides were incubated for 30 min on ice in 10mM DTT in 0.1 M Trizma base, pH 8.0, and then 90 min at RT in 4mM LIS, 1mM DTT in 0.1M Trizma base, pH 8.0. Slides were washed three times in 300mM NaCl, 30mM sodium citrate, pH 7.0 (2 x SSC) for 5 min each, dehydrated in an ethanol series and air-dried.

2.2.3.2 Results

Method 1 (Williams *et al.*, 1993) was used in initial studies and resulted in the sperm heads swelling to 1.5-2 times their normal size (Figure 11). However, on occasion, inconsistent results were obtained in the degree of swelling, many sperm were underswollen and it was difficult to observe hybridization signals. It was necessary then to visualise sperm under the microscope whilst decondensing to ensure significant swelling had occurred and therefore the incubation times in LIS varied over a 1-3 hr period.

Method 2 (Martini *et al.*, 1995) produced very overswollen sperm heads which yielded split FISH signals when hybridized with centromeric probes. Martini *et al.* (1995) suggested “the dilution of DTT in Tris-HCl buffer gave a compact morphology but the pH of the buffer strongly influenced DTT’s reducing efficiency, range pH 6-9.5”. So decondensing was tested using this method with 1M Tris-HCl buffer at varying pH, range 6.0-9.5.

Table 8: Samples pretreated at various pH values; Martini et al. (1995).

Normospermic Donor	pH 6.0	pH 7.0	pH 8.0	pH 9.5
1	Under swollen ¹	Diffuse head ⁴	Over swollen	Blown apart ⁵
2	Swollen 1.5-2 times normal size ²	Diffuse head	Diffuse head	Diffuse head
3	Under swollen	Over swollen ³	Over swollen	Over swollen
4	Under swollen	Diffuse head	Diffuse head	Diffuse head
5	Under swollen	Diffuse head	Diffuse head	Diffuse head
6	Under swollen	Diffuse head	Diffuse head	Over swollen

¹ = sperm under swollen have normal nuclear size, fluoresce blue (DAPI counterstain) under UV filter and no hybridization signals seen, ² = swollen 1.5-2 times normal size have increased nuclear size, fluoresce pale blue under UV filter with hybridization signals seen clearly, ³ = sperm over swollen have 2.5+ times increased nuclear size, fluoresce pale blue under UV filter and split hybridization signals seen, ⁴ = sperm with diffuse head have 2.5+ times increased nuclear size, minimal blue fluorescence under UV filter and split or no hybridization signals seen, ⁵ = sperm blown apart have no nuclear shape and often just remnants of DNA attached to sperm tail, no blue fluorescence under UV filter and no hybridization signals seen.

The results, shown in Table 8, indicated that sperm were under swollen when pH 6.0 was used and when pH 7.0 or greater were used, the sperm head had a diffuse appearance and DNA under DAPI staining could be seen around the sperm membrane. The authors reported that this pretreatment method, using 1M Tris-HCl buffer at approximately pH 6.5, would result in reliably swollen sperm heads. However, in this study, this method proved to be unreliable and frequently resulted in the majority of sperm nuclei being over swollen.

Method 3 (Robbins *et al.*, 1993) was also evaluated and yielded similar results to method 1. It was used mainly in the process of developing the autosomal and sex chromosomal FISH protocols and gave consistent results. However, method 1 was the chosen method for future studies (chapter 3) because it was easier to replicate and generally produced more consistent swelling throughout the sample.

2.2.4 Mitotic chromosome spreads

Mitotic chromosome spreads from human male and female lymphocytes were used as positive controls for each hybridization procedure.

To prepare mitotic chromosome spreads, 10ml of blood was taken from the subject (male or female laboratory staff) and aliquots of 0.4ml were placed into culture bottles containing 5ml of PHA-stimulation medium and cultured for 72hr at 37°C in a 5% CO₂ incubator. PHA-stimulation medium consisted of RPMI 1640 cell culture medium to which was added 0.5g/L NaHCO₃, 20mM HEPES, 17% foetal bovine serum, 2mM glutamine, 100mg/L gentamycin and 15µl/ml phytohaemagglutinin (PHA) medium. After 72hr, 200µg/ml of (2)-5-bromodeoxyuridine was added to each culture bottle and under the same conditions incubation continued for a further 16 hr. Cultures were then light sensitive and had to be protected from strong light. Each culture suspension was then washed twice in PBS, pH 7.4, and once in fresh HEPES-HTF medium by centrifuging at 175g for 5 min each time. The final cell pellet was then resuspended in fresh HEPES-HTF medium containing 10⁻⁵ M thymidine and was cultured under the same conditions for 6hr. Colchicine (0.5µg/ml) was added to each culture bottle, incubated for 15 min, and centrifuged at 175g for 5 min to pellet cells. Pre-warmed (37°C) potassium chloride solution (KCl, 0.075M) was added to each pellet and incubated for 10 min, then Carnoy's fixative (3:1 methanol:acetic acid) was added to each tube and mixed thoroughly. This mixture was centrifuged at 175g for 5 min, the supernatant was removed and the pellet was resuspended in fixative and centrifuged again. This

procedure was repeated until the pellet was white in colour. The final pellet was resuspended in fixative to give a milky white suspension and then 1-2 drops were added to clean glass slides and air-dried. Slides were checked to ensure a consistent spread of metaphases and were then stored with desiccant at -20°C.

2.2.5 Signal detection

After FISH, slides were examined at a magnification of 1 250 X using a Leica Laborlux microscope equipped with epifluorescence and a triple band-pass filter block (Chroma Technology Corp., Brattleboro, VT, USA). Excitation and emission values (respectively) were 495nm and 530nm for FITC, 570nm and 625nm for Texas Red and 365nm and 480nm for the DAPI counterstain.

2.3 Development of multi-probe FISH protocols

A number of DNA probes for the sex chromosomes (X, Y) and the autosomes (2, 3, 4, 7, 15, 16, 17, 18, 20) were used (Table 9) in the development of FISH protocols and different combinations of probes were tested (Table 10) until reliable and reproducible methods (Table 11) were found. The aim was to develop double- and triple-probe FISH protocols for chromosomes from all the Denver groups to investigate the hypothesis that inter-chromosomal and inter-donor differences exist in sperm aneuploidy rates in normospermic donors (Chapter 3).

Table 9: DNA probes and detection reagents

DNA probe	Type	Label	Company*
X chromosome/ Y chromosome	α -satellite/ Satellite III	Spectrum-Orange®/ Spectrum-Green®	Vysis
Chromosome 3	α -satellite	Biotinylated	Oncor
Chromosome 7	α -satellite	FITC	Oncor
Chromosome 16	α -satellite	Biotinylated	Oncor
X chromosome	α -satellite	Digoxigenin (DIG)	Oncor
Y chromosome	Satellite III	Biotinylated	TQEHa
X chromosome	α -satellite	Texas Red	Oncor
Y chromosome	Satellite III	FITC	Boehringer Mannheim
Chromosome 16	α -satellite	DIG	Oncor
X chromosome	α -satellite	Biotinylated	Oncor
Chromosome 17	α -satellite	DIG – at TQEH.	TQEHb
Chromosome 20	α -satellite	DIG	Oncor
Chromosome 2	α -satellite	FITC	Boehringer Mannheim
Chromosome 15	α -satellite	DIG	Boehringer Mannheim
Chromosome 15	α -satellite	Spectrum-Orange®	Vysis
Chromosome 18	α -satellite	Spectrum-Aqua®	Vysis
Chromosome 4	α -satellite	Spectrum-Orange®	Vysis
Detection	Company*		Catalogue number
Spectrum CEP® hybridization buffer	Vysis		32-804828
Hybrisol IV buffer	Oncor		
BBR=blocking reagent	Boehringer Mannheim		1096-176
Texas Red avidin	Vector Laboratories		A-2006
FITC avidin	Vector Laboratories		A-3101
Biotinylated anti-avidin	Vector Laboratories		BA-0300
Anti-digoxigenin	Boehringer Mannheim		1333-062
Texas red conjugated rabbit anti-mouse IgG	Jackson ImmunoResearch		315-075-003
Texas red goat anti-rabbit IgG	Vector Laboratories		TI-1000
Anti-digoxigenin rhodamine or FITC	Boehringer Mannheim		1207-750 (rhodamine) 1207-741 (FITC)
Anti-digoxigenin rhodamine or FITC	Boehringer Mannheim		1207-750 (rhodamine) 1207-741 (FITC)
DIG DNA labelling kit	Boehringer Mannheim		1175-033
DIG Nucleic Acid Detection Kit	Boehringer Mannheim		1175-041
Texas Red® anti-sheep IgG	Vector Laboratories		TI-6000
Fluorescein anti-sheep IgG	Vector Laboratories		FI-6000

*Company addresses are Vysis: Downers Grove, IL, USA; Oncor: Gaithersburg, MD, USA; TQEHa: Han *et al.* (1993a); Boehringer Mannheim: Castle Hill, NSW, Australia; TQEHb: Han *et al.* (1992); Vector Laboratories: Burlingame, CA, USA; Jackson ImmunoResearch Laboratories Inc: West Grove, PA, USA.

Table 10: Development of FISH protocols

Protocol	Method	Probe 1	Probe 2	Probe 3	Hybridization mixture used for probes	Denat. temp. and Hybrid. temp.	Post-hyb. wash 1.	Post-hyb. wash 2.	Blocking step	Detection step 1.	Detection step 2.	Detection step 3.	Detection step 4.	Detection washing and mount 1.
1	Classic*	20ng of X-dig	20ng of Y-biotin	-	5µl ssDNA 5µl 10xSSCP 25µl DF + 20% DS Add 50µl/slide	75°C for 10 mins. 37°C for 16-18 hrs.	3 times in 50% DF /2xSSC at 45°C for 5 mins. each	3 times in 0.1xSSC at 60°C for 5 mins. each.	Blocking in 5% NF /4xSSC, 20 mins, RT. Detection wash (a).	100µl of FITC avidin (final 4µg/ml) in 5%NF /4xSSC, 30 mins, 37°C. Detection wash (b).	100µl of 5µg/ml biotinylated anti-avidin, 0.4µg/ml anti-dig in TN2, 37°C, 30mins. Detection wash (b).	FITC avidin as in step 1 + 11.6µg/ml texas red goat anti-rabbit IgG in TN2. Detection wash (b).	100µl of 15µg/ml texas red goat anti-rabbit IgG in TN2. Detection wash (b).	a) 3 times in 0.05% Tween 20/4xSSC at RT for 5 mins. b) 3 times in 0.05% Tween 20/TN1 at RT for 5 mins.
2	Classic	15ng X-dig or X-biotin	-	-	2µl ssDNA 2µl 20xSSC 4µl mqH ₂ O 10µl DF + 20% DS Add 20µl/slide	As in protocol 1	3 times in 2xSSC at 37°C for 5 mins. Each	-	(a) As in protocol 1 or (b) 1% BBR in PBS at 37°C for 1 hr.	(a) As in protocol 1 or (b) 200µl of 67µg/ml anti-dig rhodamine + 67µg/ml anti-dig FITC in PBS2.	(a) As in protocol 1	(a) As in protocol 1	(a) As in protocol 1	(a) As in protocol 1 or (b) 3 times in 0.1% Triton X-100 in PBS at RT for 10 mins. each.
3	Classic	2µl Y-FITC per slide	-	-	As in protocol 1 but 5µl ssDNA + 5µl 20xSSC, 25µl/slide	As in protocol 1	As in protocol 2	-	-	-	-	-	-	Mount 1 used
4	Codenat.**	1.5µl X-dig	2µl Y-FITC	-	Add 20µl/slide of 5µl stock (400µl ssDNA/400µl 20xSSC/0.4g 20%DS/200µl mqH ₂ O) + 10µl DF + 1.5µl mqH ₂ O	Preheated oven at 72°C for 10 mins. 37°C overnight.	As in protocol 2	-	As in protocol 2b	As in protocol 2b but only 4 - 67 µg/ml anti-dig rhodamine used.	-	-	-	As in protocol 2b
5	Codenat.	1.5µl X-dig	2µl or 4µl Y-biotin	-	As in protocol 4	As in protocol 4	As in protocol 4	-	As in protocol 2b	As in prot. 4 using anti-dig FITC + 80µg/ml texas red avidin.	-	-	-	As in protocol 2b
6	Codenat.	1.5µl X-dig	2µl Y-FITC	-	As in protocol 4	78°C, 5 or 10mins. 37°C overnight.	Protocol 4 or 3 x 0.1xSSC, 60°C, 5mins.	-	As in protocol 2b	Prot. 4, 4µg/ml anti-dig rhodamine.	-	-	-	As in protocol 2b

7	Codenat.	1.5µl X-biotin	-	-	As in protocol 4	72°C-78°C, 5 - 10 mins. 37°C overnight.	As in protocol 6b.		As in protocol 2b	Protocol 2b, 10 - 80 µg/ml texas red avidin used.	-	-	-	As in protocol 2b
8	Codenat.	1.5µl new X-dig	-	-	Add probe to 30 µl Hybrisol IV (Oncor)/slide	As in protocol 4	0.25 x SSC, 72°C for 5min.	PBS at RT for 1-2 mins.	As in protocol 2b	As in protocol 6	-	-	-	As in protocol 2b
9	Codenat.	2µl X-Spectrum Orange®/Y-Spectrum Green®	As in probe 1	-	Add probe to 7µl Spectrum CEP® hyb buffer (Vysis), 1µl mqH ₂ O/slide	As in protocol 4	As in protocol 2	-	-	-	-	-	-	Mount 1 used.
10	Classic Using triple-probe FISH with different probes.	20ng of X-biotin or X-texas red	20ng of Y-FITC	20ng of chr. 16-dig	As in protocol 1 but no ssDNA added. Add 15µl/slide	75°C for 2-5 mins. 37°C for 16-18 hrs.	Once in 50% DF /2xSSC at 50°C for 5 mins.	2 times in 0.1xSSC at 60°C for 5 mins. each.	As in protocol 2b	(1) As in protocol 4 at 40µg/ml for chr. 16/chr. Y (2) As in protocol 4 but using 40µg/ml anti-dig FITC for chr. 16/chr. X texas red. (3) As in 2b but at 40µg/ml for chr. 16/chr. X-texas red/chr. Y-FITC.	-	-	-	As in protocol 2b

* = classic FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides in 2 x SSC, pH 7.0, for 5 min. Slides were denatured at 70°C in 70% deionized formamide/2 x SSC, pH 7.0 for 10 min and cooled immediately in chilled 70% alcohol for 2 min. Slides were dehydrated in 80-95-100% ethanol and air dried.

** = codenaturing FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides three times in mqH₂O, dehydrating slides in 70-80-95-100% ethanol, air-drying, applying hybridization mixture with probes to slides, seal coverslips with rubber cement and denature simultaneously at a consistent temperature.

Abbreviations: chr. = chromosome, dig = digoxigenin, ssDNA = salmon sperm DNA (10 mg/ml stock), 10 x SSCP = 1.2 M NaCl, 0.15 M sodium citrate, 0.2 M sodium phosphate, pH 6.0, DF = deionized formamide, DS = dextran sulphate, Denat. temp. = denaturation temperature/time and all tubes immediately chilled on ice, mins. = minutes, Hybrid. temp. = hybridization temp and time, hrs. = hours, post-hyb. wash = post-hybridization wash conditions, 2 x SSC = 30 mM NaCl, 3.0 mM sodium citrate, pH 7.0, 20 x SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7.0, 0.1 x SSC = 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, , 0.25 x SSC = 37.5 mM NaCl, 3.75 mM sodium citrate, pH 7.0, 0.5xSSC = 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0, 4 x SSC = 60 mM NaCl, 6.0 mM sodium citrate, pH 7.0, NF = nonfat skim milk powder, BBR = Boehringer blocking reagent, PBS = phosphate buffered saline, PBS2 = 1.5 M NaCl, 0.2 M phosphate, pH 7.4 containing 1% Boehringer blocking reagent and 0.5% bovine serum albumin, RT = room temperature, TN = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, TN2 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% goat serum, 0.5% Boehringer blocking reagent, TN3 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% rabbit serum, 0.5% Boehringer blocking reagent, mount 1 = after final wash slides are dehydrated through a series of ethanol solutions (80 - 100%), air-dried and then mounted with a glycerol-based solution containing 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain and 20 mg/ml 1,4-diazabicyclo{2,2,2}octane (DABCO) as an antifade, coverslips added and sealed with DPX and stored at 4°C in dark petri dish.

Protocol	Method	Probe 1	Probe 2	Probe 3	Hybridization mixture used for probes	Denat. temp. and Hybrid. temp.	Post-hyb. wash 1.	Post-hyb. wash 2.	Blocking step	Detection step 1.	Detection step 2.	Detection step 3.	Detection step 4.	Detection washing and mount 1.
11	Classic	20ng of X-texas red	-	20ng of chr. 16-dig	Add probe to 30 µl Hybrisol IV (Oncor) mixture/slide	As in protocol 10	65%DF/2xSS X at 43°C for 10 mins.	0.5xSSC at 72°C for 10 mins.	As in protocol 2b	As in protocol 10 (2).	-	-	-	As in protocol 2b
12	Classic	20ng X-biotin or X-dig or 20ng(1.5µl) chr. 16-dig	-	-	As in protocol 1	As in protocol 1	As in protocol 1	As in protocol 1	(a) As in protocol 1 (b) As in protocol 2b	(a) As in protocol 1 (b) Protocol 2b at 50 or 67µg/ml	(a) As in protocol 1	(a) As in protocol 1	(a) As in protocol 1	(a) As in protocol 1
13	Classic	0.25µl XY-Spectrum Orange®/Spectrum Green®	As probe 1	0.38 µl of chr. 16-dig	Add probe to 8.4µl Spectrum CEP® hyb buffer (Vysis), 1µl mqH ₂ O/slide	As in protocol 1	As in protocol 1 but for 10 mins each.	2xSSC, 45°C for 10 mins, and 0.1xSSC, RT for 10 mins.	As in protocol 2b	As in protocol 2b at 24µg/ml	-	-	-	3 x 0.1%NP-40 (Sigma)/2xSSC, RT, 5 mins each.
14	Codenat.	1µl chr.16-dig or 1µl X-dig or 1µl Y-FITC	-	-	Probe added to 10 µl Hybrisol IV (Oncor) mixture, and total volume added to slide	90°C for 12 mins. 37°C overnight.	Once in 1.0 x SSC at 72°C for 5 mins.	-	As in protocol 2b	As in protocol 4.	-	-	-	As in protocol 2b
15	Codenat.	1-10µl of chr. 17-dig	Y-FITC	-	As in protocol 4	As in protocol 4	Protocol 1 (wash 2).	-	As in protocol 2b	As in protocol 4.	-	-	-	As in protocol 2b
16	Codenat.	0.05-0.1µl of chr. 15-dig/µl hybr. mixture	-	-	As in protocol 4	As in protocol 4	As in protocol 1 (wash 2) but for 10 mins. each.	-	(a) As in protocol 1. (b) As in protocol 2b. (c) As in protocol 2b.	(a) Protocol 1, step 2. (b) Protocol 4, 4-8µg/ml. (c) Prot 2b + 40µg/ml anti-sheep Texas Red or FITC	(a) As in protocol 1, step 3.	-	-	(a) As in protocol 1. (b) As in protocol 2b. (c) As in protocol 2b.
17	Codenat.	0.1µl chr. 15-dig/µl hyb mixture	0.1µl chr. 2-FITC/µl hyb mixture	-	As in protocol 4.	As in protocol 4.	As in protocol 1 (wash 2).	-	As in protocol 2b.	As in protocol 6.	-	-	-	As in protocol 2b.

18	Codenat.	0.25µl chr. 15- Spectrum Orange®	0.5µl of chr. 16- biotin	-	As in protocol 13.	As in protocol 4.	(a) Prot. 1, wash 2, (b) 3 x in 1xSSC, 50°C, 10mins (c) 0.4xSSC, 50°C, 5mins.	-	(a) As in protocol 2b but only for 30 mins. (b) As in (a). (c) As in (a).	(a) Protocol 2b, 40 µg/ml FITC avidin (b) Protocol 1 -FITC avidin (c) As in (b).	-	-	-	(a) Pprotocol 2b, 3 mins. (b) Prot 2b but 0.1% Tween 20, 3 mins. (c) As in (b).
19	Codenat.	1.5µl chr. 3-biotin or chr.20-dig	-	-	As in protocol 8.	As in protocol 4	As in protocol 8.	As in protocol 8.	As in protocol 2b but only for 30 mins.	Chr.3: Prot 7, 20-80µg/ml Texas red avidin. Chr 20:Prot 6	-	-	-	As in protocol 18b
20	Codenat.	1.5µl chr. 3-biotin or chr.20-dig	-	-	As in protocol 8.	Preheated oven at 75°C for 10 mins. 37°C overnight.	As in protocol 8.	As in protocol 8.	As in protocol 2b but only for 30 mins.	Chr. 3: (a) 200µl of 80µg/ml Texas red avidin/PBS2, 30mins, 37°C. (b) as (a) + 40µg/ml FITC avidin. (c) as (b) at 10µg/ml. Chr. 20: Prot 1, Texas red anti-dig, or prot 2b, 4µg/ml, or prot 16c. 5- 100µg/ml.	Chr. 3: (a) 200µl of 5µg/ml biotinylated anti-avidin in PBS2 for 1.5hrs at 37°C. (b) 200µl of 10µg/ml biotinylated anti-avidin in PBS2 for 30 mins. at 37°C.	Chr. 3: (a) 200µl of 80µg/ml Texas red avidin in PBS2 for 30 mins at 37°C. (a) 200µl of 80µg/ml Texas red avidin and 40µg/ml FITC avidin in PBS2 for 30 mins. at 37°C.	-	As in protocol 18b

* = classic FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides in 2 x SSC, pH 7.0, for 5 min. Slides were denatured at 70°C in 70% deionized formamide/2 x SSC, pH 7.0 for 10 min and cooled immediately in chilled 70% alcohol for 2 min. Slides were dehydrated in 80-95-100% ethanol and air dried.

** = codenaturing FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides three times in mqH₂O, dehydrating slides in 70-80-95-100% ethanol, air-drying, applying hybridization mixture with probes to slides, seal coverslips with rubber cement and denature simultaneously at a consistent temperature.

Abbreviations: chr. = chromosome, dig = digoxigenin, ssDNA = salmon sperm DNA (10 mg/ml stock), 10 x SSCP = 1.2 M NaCl, 0.15 M sodium citrate, 0.2 M sodium phosphate, pH 6.0, DF = deionized formamide, DS = dextran sulphate, Denat. temp. = denaturation temperature/time and all tubes immediately chilled on ice, mins. = minutes, Hybrid. temp. = hybridization temp and time, hrs. = hours, post-hyb. wash = post-hybridization wash conditions, 2 x SSC = 30 mM NaCl, 3.0 mM sodium citrate, pH 7.0, 20 x SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7.0, 0.1 x SSC = 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, , 0.25 x SSC = 37.5 mM NaCl, 3.75 mM sodium citrate, pH 7.0, 0.5xSSC = 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0, 4 x SSC = 60 mM NaCl, 6.0 mM sodium citrate, pH 7.0, NF = nonfat skim milk powder, BBR = Boehringer blocking reagent, PBS = phosphate buffered saline, PBS2 = 1.5 M NaCl, 0.2 M phosphate, pH 7.4 containing 1% Boehringer blocking reagent and 0.5% bovine serum albumin, RT = room temperature, TN = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, TN2 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% goat serum, 0.5% Boehringer blocking reagent, TN3 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% rabbit serum, 0.5% Boehringer blocking reagent, mount 1 = after final wash slides are dehydrated through a series of ethanol solutions (80 - 100%), air-dried and then mounted with a glycerol-based solution containing 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain and 20 mg/ml 1,4-diazabicyclo{2,2,2}octane (DABCO) as an antifade, coverslips added and sealed with DPX and stored at 4°C in dark petri dish.

Protocol	Method	Probe 1	Probe 2	Probe 3	Hybridization mixture used for probes	Denat. temp. and Hybrid. temp.	Post-hyb. wash 1.	Post-hyb. wash 2.	Blocking step	Detection step 1.	Detection step 2.	Detection step 3.	Detection step 4.	Detection washing and mount 1.
21	Codenat.	0.25µl of chr. 7-FITC	0.25µl chr. 18-Spectrum Aqua® and later 1µl/hyb mixture.	-	Probe added to 8µl Hybrisol IV (Oncor) mixture, and 1.5µl mqH ₂ O and total volume added to slide	As in protocol 20.	As in protocol 1 (wash 2) but for 10 mins. each.	-	-	-	-	-	-	-
22	Codenat.	0.5µl of chr. 7-FITC (d) 1.0µl of chr. 7-FITC	0.5µl chr. 4-Spectrum Orange® (d) 1.0µl of chr. 4-Spectrum Orange®	-	As in protocol 21. (e) Probe added to 7µl Hybrisol IV (Oncor) mixture, and 1µl mqH ₂ O and total volume added to slide	(a) As in protocol 20. (b) Preheated oven at 75°C for 10 mins. Hyb. 42°C for 5 hrs. (c) As in (b) (d) As in (a)	(a) As in protocol 20. (b) Once in 0.1xSSC at 60°C for 5 mins. (c) Once in 2xSSC at 48°C for 5 mins. (d) Once in 0.4xSSC at 68°C for 5 mins.	-	-	-	-	-	-	-

* = classic FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides in 2 x SSC, pH 7.0, for 5 min. Slides were denatured at 70°C in 70% deionized formamide/2 x SSC, pH 7.0 for 10 min and cooled immediately in chilled 70% alcohol for 2 min. Slides were dehydrated in 80-95-100% ethanol and air dried.

** = codenaturing FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides three times in mqH₂O, dehydrating slides in 70-80-95-100% ethanol, air-drying, applying hybridization mixture with probes to slides, seal coverslips with rubber cement and denature simultaneously at a consistent temperature.

Abbreviations: chr. = chromosome, dig = digoxigenin, ssDNA = salmon sperm DNA (10 mg/ml stock), 10 x SSCP = 1.2 M NaCl, 0.15 M sodium citrate, 0.2 M sodium phosphate, pH 6.0, DF = deionized formamide, DS = dextran sulphate, Denat. temp. = denaturation temperature/time and all tubes immediately chilled on ice, mins. = minutes, Hybrid. temp. = hybridization temp and time, hrs. = hours, post-hyb. wash = post-hybridization wash conditions, 2 x SSC = 30 mM NaCl, 3.0 mM sodium citrate, pH 7.0, 20 x SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7.0, 0.1 x SSC = 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, , 0.25 x SSC = 37.5 mM NaCl, 3.75 mM sodium citrate, pH 7.0, 0.5xSSC = 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0, 4 x SSC = 60 mM NaCl, 6.0 mM sodium citrate, pH 7.0, NF = nonfat skim milk powder, BBR = Boehringer blocking reagent, PBS = phosphate buffered saline, PBS2 = 1.5 M NaCl, 0.2 M phosphate, pH 7.4 containing 1% Boehringer blocking reagent and 0.5% bovine serum albumin, RT = room temperature, TN = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, TN2 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% goat serum, 0.5% Boehringer blocking reagent, TN3 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% rabbit serum, 0.5% Boehringer blocking reagent, mount 1 = after final wash slides are dehydrated through a series of ethanol solutions (80 - 100%), air-dried and then mounted with a glycerol-based solution containing 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain and 20 mg/ml 1,4-diazabicyclo{2.2.2}octane (DABCO) as an antifade, coverslips added and sealed with DPX and stored at 4°C in dark petri dish.

Table 11: Development of successful XY3 and 7/16 FISH protocols

Protocol	Method	Probe 1	Probe 2	Probe 3	Hybridization mixture used for probes	Denat. temp. and Hybrid. temp.	Post-hyb. wash 1.	Post-hyb. wash 2.	Blocking step	Detection step 1.	Detection step 2.	Detection step 3.	Detection step 4.	Detection washing and mount 1.
23	Codenat.	1.0µl of chr. 7-FITC (d) 0.5µl of chr. 7-FITC	0.5µl of chr. 16-biotin (c) 1.0µl of chr. 16-biotin (d) 0.5 µl of chr. 16-biotin	-	Probe added to 7.3µl Hybrisol IV (Oncor) mixture, and 1.2µl mqH ₂ O and total volume added to slide (d) Probe added to 8µl Hybrisol IV (Oncor) mixture, and 1µl mqH ₂ O and total volume added to slide	As in protocol 20.	(a) Once in 0.4xSSC at 65°C for 5 mins. (b) 3 times in 0.25xSSC at 60°C for 10 mins each. (c) 3 times in 0.1xSSC at 60°C for 5 mins each. (d) as in (c) but only once	-	As in protocol 2b but only for 30 mins at RT.	(a) As in protocol 20a at 40µg/ml (b) As in (a) but 25µg/ml (c) as in (a) (d) As in protocol 20a at 50µg/ml	(a) As in protocol 20a at 20µg/ml (b) As protocol 20a (c) as in (a) (d) As in protocol 20b	(a) As in protocol 20a at 40µg/ml (b) As in (a) but 25µg/ml (c) as in (a) (d) As in protocol 20a at 50µg/ml	-	As in protocol 18b.
24	Codenat.	0.5µl chr. 3-biotin.	0.25µl X-Spectrum Orange®/Y-Spectrum Green®	As in probe 2.	Probe added to 8.0µl Spectrum CEP® hybridization buffer (Vysis) and 1.25µl mqH ₂ O and total volume added to slide	As in protocol 20.	As in protocol 1 (wash 2) but for 10 mins. each.	-	As in protocol 23	200µl of 40µg/ml Texas red avidin + 40µg/ml FITC avidin for 1hr in PBS2 at 37°C.	As in protocol 20a but only for 1 hour	200µl of 40µg/ml Texas red avidin + 40µg/ml FITC avidin for 1hr in PBS2 at 37°C.	-	As in protocol 18b

* = classic FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides in 2 x SSC, pH 7.0, for 5 min. Slides were denatured at 70°C in 70% deionized formamide/2 x SSC, pH 7.0 for 10 min and cooled immediately in chilled 70% alcohol for 2 min. Slides were dehydrated in 80-95-100% ethanol and air dried.

** = codenaturing FISH methodology involved treating slides with 100 µg/ml of RNase A in 2 x SSC, pH 7.0, for 60 min at 37°C, washing slides three times in mqH₂O, dehydrating slides in 70-80-95-100% ethanol, air-drying, applying hybridization mixture with probes to slides, seal coverslips with rubber cement and denature simultaneously at a consistent temperature.

Abbreviations: chr. = chromosome, dig = digoxigenin, ssDNA = salmon sperm DNA (10 mg/ml stock), 10 x SSCP = 1.2 M NaCl, 0.15 M sodium citrate, 0.2 M sodium phosphate, pH 6.0, DF = deionized formamide, DS = dextran sulphate, Denat. temp. = denaturation temperature/time and all tubes immediately chilled on ice, mins. = minutes, Hybrid. temp. = hybridization temp and time, hrs. = hours, post-hyb. wash = post-hybridization wash conditions, 2 x SSC = 30 mM NaCl, 3.0 mM sodium citrate, pH 7.0, 20 x SSC = 300 mM NaCl, 30 mM sodium citrate, pH 7.0, 0.1 x SSC = 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, , 0.25 x SSC = 37.5 mM NaCl, 3.75 mM sodium citrate, pH 7.0, 0.5xSSC = 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0, 4 x SSC = 60 mM NaCl, 6.0 mM sodium citrate, pH 7.0, NF = nonfat skim milk powder, BBR = Boehringer blocking reagent, PBS = phosphate buffered saline, PBS2 = 1.5 M NaCl, 0.2 M phosphate, pH 7.4 containing 1% Boehringer blocking reagent and 0.5% bovine serum albumin, RT = room temperature, TN = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, TN2 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% goat serum, 0.5% Boehringer blocking reagent, TN3 = 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% rabbit serum, 0.5% Boehringer blocking reagent, mount 1 = after final wash slides are dehydrated through a series of ethanol solutions (80 - 100%), air-dried and then mounted with a glycerol-based solution containing 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain and 20 mg/ml 1,4-diazobicyclo{2,2,2}octane (DABCO) as an antifade, coverslips added and sealed with DPX and stored at 4°C in dark petri dish.

Probes for the sex chromosomes were initially tested as they contain larger and more frequent repetitive units, which increases their chances of binding to the chromosomes. Next, different centromeric probes for the autosomes were used individually, in combination with the sex chromosome probes or in combination with other autosomal probes (chromosomes 15 and 2, 15 and 16, 20 and 3, 7 and 18, 7 and 4, 7 and 16).

In the development of FISH protocols, classic FISH methodology was initially used, whereby sperm DNA was denatured in formamide solution and the denatured probe mixture was added to the slide and hybridized overnight. However, signal intensity was not always consistent using this method on sperm. To improve the hybridization efficiency and thereby the signal intensity, codenaturing hybridizations were subsequently used, whereby the probe mixture was added to the sperm slide and together they were denatured and hybridized overnight.

All FISH protocols were applied to both sperm and lymphocyte slides as it was often difficult to assess if the FISH method had worked on sperm slides alone because pretreatment of the sperm DNA may not have been successful. The inclusion of a lymphocyte control slide ensured that if the FISH method had worked, signals would definitely be seen in the lymphocyte slide and would be expected in the sperm slide if the pretreatment procedure had been successful.

2.3.1 Development of FISH protocols

Combining different probes (Table 9) and FISH methodologies was difficult because optimal hybridization conditions differed for each probe. This required testing

many protocols, many of which proved to be unsuccessful (Table 10).

Protocol 1 was already established and was applied to predecondensed sperm and lymphocyte slides to familiarise the author with FISH methodology.

A multi-probe FISH protocol was developed by combining an extra probe for chromosome 16 with protocol 1 (protocols 10 and 11). Irregular signals were seen on predecondensed sperm slides and lymphocyte slides, but signals were obtained for the X and 16 chromosome probes but not for the Y chromosome probe. A single-probe FISH (protocol 3) was developed to optimize the FITC signals for the Y chromosome probe, but it was difficult to reliably see these signals in sperm.

Commercially available probes for the X and Y chromosomes, labelled with Spectrum Orange® and Spectrum Green® respectively, were available combined in the one tube and were named direct-labelled dual XY probe. These probes produced stronger signals and were much easier to use than previous probes for the sex chromosomes. A triple-probe FISH (protocol 13) was developed using the sex chromosome probes and a chromosome 16 probe. Hybridization was successful for the sex chromosomes but not for the chromosome 16 probe. No signals were seen for the chromosome 16 probe in a single-probe FISH method (protocol 12) using either anti-digoxigenin (DIG) rhodamine (red) or anti-DIG FITC (green) antibodies. In summary, the DIG-labelled chromosome 16 probe was not working under these hybridization conditions.

Other probes for the X chromosome were also tried (protocol 2) as the original

Texas Red-labelled chromosome X probe (protocols 10 and 11) was no longer commercially available. Signals were seen for both the DIG-labelled chromosome X probe and the biotinylated chromosome X probe.

To increase the hybridization efficiency and signal intensity of the probes a new hybridization procedure was tried whereby the sperm DNA and probe DNA were simultaneously denatured (codenaturing) (protocol 9).

Single-probe codenaturing FISH using either a DIG-labelled chromosome 16 or X probe, or a FITC-labelled Y chromosome probe (protocol 14) was used on predecondensed sperm and lymphocyte slides. On the lymphocyte slides, no signals were seen for any of the probes and there was extensive background anti-DIG rhodamine labelling. On the sperm slides the same effect was seen and the DNA appeared to be damaged and displaced by the high denaturation temperature. The codenaturing FISH procedure was then evaluated at a lower temperature (72°C) using only probes for the sex chromosomes (protocol 4). Hybridization of the probes was successful but there was excess anti-DIG rhodamine labelling. The concentration of anti-DIG rhodamine was 67µg/ml, so different protocols using concentrations of 4µg/ml, 10µg/ml, and 20µg/ml were tested. Excess antibody labelling was still seen at these concentrations but to a lesser extent as concentrations decreased. The Y chromosome signal could be more readily seen when anti-DIG rhodamine detection was at concentrations of 4µg/ml. However, probably due to the decreased denaturation temperature, the X chromosome probe bound nonspecifically, as red centromeric signals could be seen on 4-5 other chromosomes on the lymphocyte

slides. The codenaturation temperature was increased slightly to 78°C for 5 and 10 min to decrease non-specific binding of the X chromosome probe whilst maintaining intensity of the Y chromosome FITC signal (protocol 6). Denaturing at 78°C for 10 min produced the best result with a strong fluorescent signal on the centromere of the X chromosome and nonspecific binding reduced to weaker red signals. A clear green signal was seen for the Y chromosome probe. This experiment was repeated with the stringency of the post-hybridization wash increased to 0.1xSSC at 60°C for 5 min. The result was successful and a single red signal was seen for the X chromosome probe without non-specific hybridization.

For flexibility in combining probes, it was necessary to also trial a biotinylated X chromosome probe (protocol 7). This probe produced a strong signal when 40µg/ml Texas Red avidin detection was used. However, if the original codenaturation temperature of 72°C for 10 min was used, the fluorescent signal was not as strong, so the Texas Red avidin concentration was increased to 80µg/ml. Other probes available were also trialled. A Y chromosome probe, produced at TQEH, was used (protocol 5), but did not produce adequate signals when used in combination with a DIG-labelled X chromosome probe. A chromosome 17 probe (TR17), produced at TQEH and labelled with DIG (Table 9), was used in combination with FITC-labelled Y chromosome probe (protocol 15). The chromosome 17 probe had been successfully used previously in a single-probe FISH (Han *et al.*, 1992) but no signals were seen consistently in either lymphocyte or sperm slides using double-probe FISH. Thus, both probes were considered unsuitable for further use.

New commercial probes were purchased for different Denver group chromosomes to develop a reliable triple-probe protocol for the sex chromosomes and an autosome and to develop double-probe protocols for various autosomes.

Initially, probes for chromosome 3 (biotinylated) and chromosome 20 (DIG-labelled) were used. To optimize the hybridization conditions, these probes were each used in a single-probe codenaturing FISH procedure (protocol 19) with the DIG-labelled X chromosome probe as a control (protocol 8). Under these hybridization conditions, the control probe (chromosome X) worked, the chromosome 20 probe only worked in lymphocyte cells, as did the chromosome 3 probe, but only when Texas Red avidin concentrations of 80µg/ml were applied. It was evident that all three probes were working as signals were seen in the control lymphocyte slides, although the autosomal probes (chromosome 3 and 20) had very small signals. Extra decondensation of sperm nuclei was required to produce signals in sperm. The above experiments were repeated on lymphocyte slides and newly decondensed sperm, with different post-hybridization detection regimes to increase signal intensity (protocol 20). Signals for the chromosome 20 probe improved with three-step anti-DIG Texas Red detection and when anti-DIG detection was used in conjunction with anti-sheep antibodies, but only at concentrations of 50µg/ml or 100µg/ml. The signals were very small and difficult to see consistently in sperm, so this probe was no longer used.

Signals improved for the chromosome 3 probe with Texas Red avidin labelling and a combination of Texas Red/FITC avidin labelling. Texas Red avidin and FITC avidin were combined to produce a yellow signal so that the probe could be used in a

triple-probe FISH procedure with other probes. Initial attempts produced a green signal because the three-step detection procedure (Texas red avidin/anti-avidin/FITC avidin) only fluoresced the final signal, FITC avidin (green). To produce a yellow signal it was necessary to apply both Texas Red avidin and FITC avidin simultaneously in a single- and three-step detection procedure (protocol 20). In summary, the biotinylated chromosome 3 probe worked successfully and was able to be used in combination with probes for the sex chromosomes in a triple-probe procedure (protocol 24).

So far, autosomal probes for chromosomes 3, 16, 17 and 20 had been used but only the chromosome 3 probe had worked successfully. Further probes were selected for Denver group A (chromosome 2, FITC-labelled) and D (chromosome 15, DIG-labelled) to use in FISH protocols. These probes were tried in a double-probe codenaturing protocol (protocol 17). Faint signals were seen for the chromosome 15 probe but it was very difficult to see signals for the chromosome 2 probe, and subsequently only the chromosome 15 probe was further developed. Single-probe FISH methods were tried (protocol 16) but signals were only seen using one-step anti-DIG rhodamine detection at concentrations of 4-8 μ g/ml. These signals were much brighter when amplified with anti-sheep texas red or FITC antibodies (40 μ g/ml).

The DIG-labelled chromosome 15 probe produced very small fluorescent signals and indirect anti-DIG rhodamine detection often produced background signals, so a new Spectrum Orange[®]-labelled chromosome 15 probe was evaluated. A double-

probe codenaturing protocol (protocol 18) was used in combination with a biotinylated chromosome 16 probe. Signals were very weak, so a less stringent post-hybridization wash was applied in combination with a three-step FITC avidin detection procedure (protocol 18b). Fluorescent signals were seen on the lymphocyte slides but the chromosome 15 signal was difficult to see in sperm. The post-hybridization wash stringency was decreased to 0.4xSSC at 50°C for 5 min, and the signals were much brighter in sperm (protocol 18c). The chromosome 15 probe appeared to be a very small red signal and was difficult to visualize in all experiments, whereas the chromosome 16 probe produced a strong fluorescent signal under these hybridization conditions. It was decided to trial other autosomal probes to use in combination with the biotinylated chromosome 16 probe.

Direct-labelled probes appeared to be easier to work with and produced better signals than some indirect-labelled probes, so new direct-labelled probes were purchased. A Spectrum Aqua[®]-labelled chromosome 18 probe, a Spectrum Orange[®]-labelled chromosome 4 probe and a FITC-labelled chromosome 7 probe were used in different double-probe protocols. Protocol 21 was initially tried for chromosomes 7 and 18, and under these hybridization conditions, a fluorescent signal was seen for chromosome 7 in both lymphocyte and sperm slides. The chromosome 18 Spectrum Aqua[®]-labelled probe was trialled as an alternative fluorescent signal (blue) to yellow. However, when the DAPI (blue) counterstain was used it was difficult to see the contrasting blue signal for the probe so an alternative counterstain that fluoresced orange, propidium iodide, was used. The chromosome 18 probe failed to produce

reliable signals, even when the recommended probe concentration was used in the hybridization mixture, so this probe was abandoned.

The FITC-labelled chromosome 7 probe was then tried in combination with the Spectrum Orange[®]-labelled chromosome 4 probe (protocol 22), and both probes produced reliable fluorescent signals. To improve time efficiency, a same-day hybridization protocol was tried (protocol 22b), whereby hybridization at 37°C was only for 5hr instead of 24hr and the stringency of the post-hybridization wash was decreased to 0.1xSSC at 60°C for 5 min. Fluorescent signals were seen for both probes but at a reduced intensity to normal. This experiment was repeated using a less stringent post-hybridization wash of 2xSSC at 48°C for 5 min (protocol 22c), which improved the signal intensity for both probes, but signal intensity was inconsistent in repeated experiments. Previously, successful hybridizations had been achieved using 1 in 4 dilutions of recommended direct-labelled probe concentrations. To improve signal intensity, probe concentrations for these new probes were increased to those recommended by the manufacturer, and a low stringency post-hybridization wash was also used, 0.4xSSC at 68°C for 5 min (protocol 22d), which resulted in brighter signals. However, non-specific binding of the chromosome 4 probe occurred which was difficult to remedy as increasing the stringency of the post-hybridization wash decreased the fluorescent signal for the chromosome 7 probe, so this combination was no longer used.

Previously, the biotinylated chromosome 16 probe had produced reliable bright green signals in combination with the chromosome 15 probe. A new double-probe

protocol was tried with the chromosome 16 probe in combination with the FITC-labelled chromosome 7 probe (protocol 23). Texas Red avidin labelling was applied to the chromosome 16 probe. Signals resulted under these hybridization conditions, but different combinations of post-hybridization washes and avidin labelling were trialled to maximise signal intensity. A successful protocol was developed for chromosome 7 and 16 (protocol 23d).

2.3.2 Double- and triple-probe FISH protocols

Using codenaturing hybridization, two successful FISH protocols were developed (Table 11); a triple-probe protocol for a biotinylated chromosome 3 probe and dual-labelled chromosome X and Y probes, and a double-probe protocol for a biotinylated chromosome 16 probe and a direct FITC-labelled chromosome 7 probe.

The probes used are shown in Table 9. Hybridization using protocol 24 produced fluorescent signals for chromosome 3 (yellow), chromosome X (red) and chromosome Y (green) and this was the chosen protocol for the triple-probe FISH (XY3) used in the study on sperm from 10 normospermic donors (Chapter 3). Hybridization using protocol 23 produced fluorescent signals for chromosomes 7 (green) and 16 (red) and this was the chosen protocol for the double-probe FISH (7/16) used in Chapter 3.

Protocol 24. Triple-probe FISH using probes for chromosome X, Y and 3.

- Sperm DNA was treated with 100µg/ml of RNase A in 2xSSC, pH 7.0, for 60 min at 37°C.
- RNase was rinsed three times in Milli Q-H₂O.
- Sperm DNA was dehydrated through an ethanol series (70 to 100%).
- Probe mixture (X, Y and 3 probes) was prepared in Vysis CEP hybridization buffer (0.25µl of a dual-labelled chromosome XY probe mixture, 0.5µl of chromosome 3 probe, 8µl hyb buffer and 1.25µl MilliQ-H₂O).
- 10µl of the probe mix was applied to each sperm slide, coverslip sealed in place using rubber cement, and codenaturation proceeded in an oven at 72-75°C for 10 min.
- Hybridization proceeded at 37°C for 16-18 hr.
- Post-hybridization washing was three times in 15mM NaCl, 1.5mM sodium citrate, pH 7.0 (0.1xSSC) at 60°C for 10 min each.
- A three-step detection procedure was employed for the biotinylated chromosome 3 probe.
- Non-specific binding was reduced by incubation for 30 min in PBS, pH 7.4 containing 1% blocking reagent (Boehringer Mannheim).
- The first and third labelling steps involved incubation in a mixture of 40µg/ml Texas Red-avidin and 40µg/ml FITC avidin (Vector Laboratories) in PBS containing 0.5% bovine serum albumin and 1% blocking reagent for 1 hr at 37°C.
- In the second labelling step, slides were incubated in 5µg/ml biotinylated anti-avidin (Vector Laboratories) for 1 hr at 37°C.
- Washing was three times, 5 min each wash, in 0.1% Tween 20 in PBS between each labelling step.
- After the final wash, the slides were dehydrated through a series of ethanol solutions (80 to 100%) and air dried.
- They were mounted with a glycerol-based solution containing 0.1µg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma) as a nuclear counterstain and 20mg/ml 1,4-diazobicyclo[2,2,2]octane (DABCO; Sigma) as an antifade.

Protocol 23. Double-probe FISH using probes for chromosomes 7 and 16.

The double-probe FISH protocol for chromosomes 7 and 16 was as described in protocol 24 with three modifications:

- (i) In 10µl, there was 0.5µl of chromosome 7 probe, 0.5µl of chromosome 16 probe, 8µl Oncor Hybrisol IV hybridization buffer and 1.0µl MilliQ-H₂O.
- (ii) The post-hybridization wash was 0.1 x SSC at 60°C for 5 min.

(iii) The post-hybridization detection procedure for the chromosome 16 probe involved incubations for 30 min at 37°C in Texas Red-avidin for steps 1 and 3 and biotinylated anti-avidin for step 2.

2.4 Summary

Many different single-, double-, and triple-probe FISH protocols were developed and tested using different indirect and direct-labelled DNA probes. Single-probe FISH was used to confirm that probes produced reliable signals under the specified hybridization conditions. This was relatively straightforward as most of the probes were purchased commercially and had recommended hybridization conditions. The development of multi-probe FISH protocols was not so straightforward as this involved the combination of different probes under certain hybridization conditions that were not always appropriate to each of the probes chosen. In the development of multi-probe FISH protocols, combining different probes brought with it problems, such as probe hybridization failure, reduced signal intensity, cross-hybridization, and background antibody labelling. A number of different FISH procedures were trialled with the most reliable results produced by codenaturing FISH methodology.

Successful multi-probe FISH protocols were developed for five chromosomes; the sex chromosomes (X, Y) and Denver groups A (3), C (7) and E (16).

CHAPTER 3

Estimation of disomy and diploidy for chromosomes 3, 7, 16, X and Y in spermatozoa from 10 normospermic men using FISH

3.1 Introduction

The introduction of FISH has made it possible to study aneuploidy in large numbers of human sperm (Downie *et al.*, 1997a). It can be used to detect aneuploidy in human sperm using two or three probes simultaneously to control for scoring errors and biases and it is a reliable method for estimating diploidy (Williams *et al.*, 1993).

To establish reliable baseline aneuploidy frequencies in human sperm, it is important to assess a variety of chromosomes in sperm from a number of normospermic men to account for inter-chromosomal and inter-donor variations. At the time this study commenced, there were a few published studies on aneuploidy for chromosomes 1, 8, 16, 18 and the sex chromosomes (Wyrobek *et al.*, 1993a; 1993b; Williams *et al.*, 1993), but most of the autosomes had not yet been adequately studied in human sperm.

In the present study, the incidence of aneuploidy in sperm from 10 normospermic men was scored using two protocols developed in chapter 2, a triple-probe FISH protocol for chromosomes 3, X and Y, and a double-probe FISH protocol for chromosomes 7 and 16. The specific aims were: (i) to estimate the incidence of disomy and diploidy for these 5 chromosomes in sperm from a reference population of normospermic men, (ii) to determine whether the disomy frequencies

differed for the 3 autosomes studied (chromosomes 3, 7, 16), and (iii) to compare disomy frequencies for the autosomes and the sex chromosomes.

The results of this chapter were published by Downie *et al.* (1997b).

3.2 Materials and methods

3.2.1 Semen samples

Semen samples were obtained, as in section 2.2.1, from 10 of the 45 healthy donors. Eight of the donors were of proven fertility. Their mean $\pm$ SD age was 34.7 ± 7.0 years. A total of sixteen samples were prepared (section 2.2.2), with one sample used from each of 5 donors, two samples used from each of 4 donors, and 3 samples used from the other donor. Intra-individual variation is an important consideration when using more than one sample from an individual, but it has been shown in a longitudinal analysis that aneuploidy estimates remain stable over time (4-55 months) (Robbins *et al.*, 1995).

Results of the semen analysis (mean $\pm$ SD) for the sixteen samples prepared and used in this study were:

Semen volume	3.7 ± 0.9 ml
Sperm concentration	$95 \pm 50 \times 10^6/\text{ml}$
Progressive motility	$54 \pm 5\%$
Normal morphology	$33 \pm 10\%$

3.2.2 Pretreatment of spermatozoa

A modification of Williams *et al.* (1993) was used (section 2.2.3.1).

3.2.3 Mitotic chromosome spreads

Mitotic chromosome spreads from human male and female lymphocytes were used as positive controls for each hybridization procedure (section 2.2.4).

3.2.4 Fluorescence *in situ* hybridization (FISH)

3.2.4.1 Triple-probe FISH for chromosomes X, Y and 3

A triple-probe FISH protocol was applied (section 2.3.1, protocol 24). Signals were examined at a magnification of 1 250 X using a Leica Laborlux microscope equipped with epifluorescence and a triple band-pass filter block (Chroma Technology Corp., Brattleboro, VT, USA). Fluorescent signals were produced for chromosome 3 (yellow), the X chromosome (red) and the Y chromosome (green) (Figure 12).

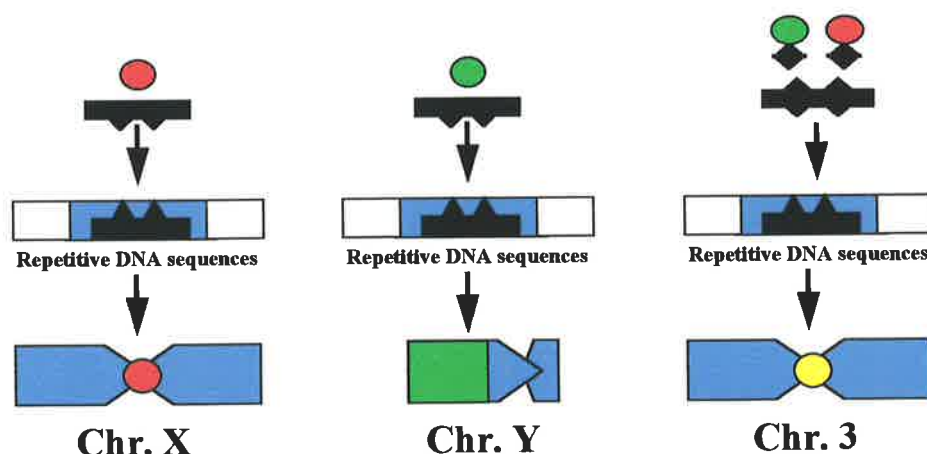


Figure 12: Triple-probe FISH for chromosome 3 and the sex chromosomes.

3.2.4.2 Double-probe FISH for chromosomes 7 and 16

A double-probe FISH protocol was applied (section 2.3.1, protocol 23), and

signals were examined in the manner described above. Fluorescent signals were produced for chromosome 7 (green) and chromosome 16 (red) (Figure 13).

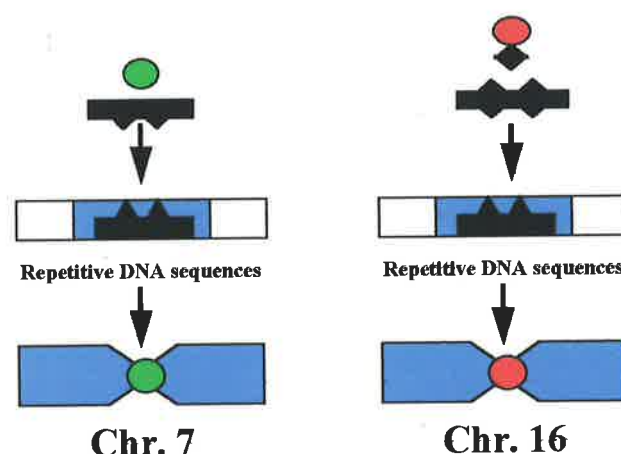


Figure 13: Double-probe FISH for chromosome 7 and 16.

3.2.5 Scoring criteria

Slides were only scored if the hybridization efficiency was $\geq 98\%$, and approximately 10 000 sperm were scored from each slide. The following scoring criteria were used:

- (i) Only nuclei with an attached tail were scored to eliminate non-sperm cells; the proportion of nuclei without tails was $< 0.06\%$.
- (ii) Overlapping or clumped sperm nuclei were not scored, nor were nuclei which were over decondensed or had indistinct boundaries.
- (iii) Two signals were scored as disomic if they were both of similar size and intensity and were separated by at least one signal domain, otherwise they were considered to be split signals and were scored as one.

3.2.6 Statistical analysis

Statistical analyses were performed using Excel 5.0 (Microsoft Corporation, Redmond, WA, USA). Differences in disomy and diploidy frequencies were analysed using a single factor ANOVA and paired t-tests. A P value < 0.05 was considered significant.

3.3 Results

3.3.1 Overall results

3.3.1.1 Triple-probe FISH for chromosomes X, Y and 3

A total of 101 273 sperm were scored for chromosomes 3, X and Y with an overall hybridization efficiency of 99.6%. Of the sperm scored:

97.83% were haploid 3,X  or 3,Y ,

0.35% were diploid 3,3,X,X  or 3,3,Y,Y 
or 3,3,X,Y , and

1.42% were aneuploid. Of the aneuploid sperm:

0.39% were disomic 3,3,X  or 3,3,Y 
or 3,X,X  or 3,Y,Y 
or 3,X,Y , and

1.03% were nullisomic 3,0  or 0,X 
or 0,Y .

3.3.1.2 Double-probe FISH for chromosomes 7 and 16

A total of 100 760 sperm were scored for chromosomes 7 and 16 with an overall hybridization efficiency of 99.8%. Of the sperm scored:

98.9% were haploid 7,16 ,

0.27% were diploid 7,7,16,16 ,

0.64 % were aneuploid. Of the aneuploid sperm:

0.11% were disomic 7,7,16  or 7,16,16 , and

0.53 % were nullisomic 7,0  or 0,16 .

3.3.2 Inter-chromosomal disomy differences

Disomy results for each donor are presented in Table 12. Using a single-factor ANOVA, it was found that there were significant inter-chromosomal differences in the frequency of disomy ($F = 6.40$, $P = 0.0014$). Paired t-tests confirmed that the frequency of disomy 3 was significantly higher than disomy 7 ($t = 2.844$, $P = 0.019$) and disomy 16 ($t = 2.765$, $P = 0.022$). Comparison of disomy for the sex chromosomes (0.19%) and the autosomes revealed that sex chromosome disomy was significantly higher than disomy 7 ($t = 0.186$, $P = 0.006$) and disomy 16 ($t = 3.500$, $P = 0.007$), but not disomy 3 ($t = 0.355$, $P = 0.73$).

Table 12. Disomy and diploidy estimates in sperm from 10 normospermic men

Donor	Disomy for chromosome:				Diploidy	
	3	7	16	X+Y	7,16	3,X,Y
1	0.39	0.02	0.05	0.11	0.06	0.13
2	0.52	0.05	0.07	0.42	0.25	0.42
3	0.31	0.07	0.03	0.23	0.13	0.35
4	0.22	0.03	0.05	0.26	0.27	0.40
5	0.08	0.05	0.00	0.05	0.33	0.25
6	0.15	0.04	0.08	0.30	0.25	0.42
7	0.17	0.09	0.14	0.11	0.43	0.63
8	0.04	0.06	0.07	0.09	0.31	0.39
9	0.06	0.04	0.04	0.17	0.37	0.37
10	0.07	0.06	0.06	0.13	0.29	0.15
Mean	0.20	0.05	0.06	0.19	0.27	0.35
SD	0.15	0.02	0.03	0.11	0.10	0.14

3.3.3 Inter-donor disomy differences

The frequencies of disomy for chromosomes 3, X and Y varied between individual donors, ranging from 0.04% to 0.52% for chromosome 3, and 0.05% to 0.42% for the sex chromosomes (Table 12). In contrast, the disomy frequencies for chromosomes 7 (0.02% to 0.09%) and 16 (0% to 0.14%) showed less variation. Two donors (#5, 8) showed low disomy frequencies for all five chromosomes, while donor #2 had markedly elevated frequencies of disomy 3 and disomy X+Y, but normal values for disomy 7 and disomy 16.

3.3.4 Diploidy estimates

The diploidy estimates (0.27%, 0.35%) obtained from the two FISH procedures were not significantly different (single-factor ANOVA, $F = 2.039$, $P = 0.17$). Marked inter-donor differences in diploidy were noted (see Table 12), with estimates ranging

from 0.13% to 0.63% with the triple-probe FISH and from 0.06% to 0.43% with the double-probe FISH procedure. Donors #1 and 7 showed very low and very high diploidy levels respectively.

3.4 Discussion

In this study, we have estimated baseline disomy and diploidy frequencies for chromosomes 3, 7, 16, X and Y in sperm from 10 normospermic men using double- and triple-probe FISH procedures. The incidence of sex chromosome disomy (0.19%) was significantly higher than disomy 7 (0.05%) and disomy 16 (0.06%), but not disomy 3 (0.20%). The incidence of disomy 3 was also significantly higher than disomy 7 and disomy 16.

3.4.1 Triple-probe vs double-probe FISH

The overall percentages of haploid and diploid sperm were similar for the two protocols, however, the incidences of aneuploid sperm differed. The triple-probe protocol resulted in 1.42% aneuploidy whereas only 0.64% of the sperm were scored as aneuploid using the double-probe procedure. Nullisomy estimates in sperm can be biased by localised hybridization failure of one or more probes, so it is routine practice to tabulate disomy and nullisomy separately (Downie *et al.*, 1997a). In this study, the incidence of nullisomy was 1.03% using three probes, over 2.5 times higher than disomy using the same protocol. For the double-probe FISH protocol, 0.53% of the sperm were scored as nullisomic, nearly 5 times higher than the incidence of disomy. Since an equal number of nullisomic and disomic sperm would be expected from

meiosis, it seems likely that nullisomy was overestimated. This may have been due to sperm which failed to hybridize with one probe due to inadequate pretreatment, uneven nuclear decondensation or localised failure of hybridization. Errors in the detection of biotinylated probes using avidin and anti-avidin reagents may also have contributed to this bias. In this study, 0.39% of sperm were disomic using the triple-probe method while 0.11% were disomic using a double-probe method. We would expect these values to differ due to differences in the rates of non-disjunction for individual chromosomes.

3.4.2 Comparison of aneuploidy estimates in sperm

Since the commencement of this project, other studies have been published on the incidence of aneuploidy in sperm from normospermic men using single-, double-, or triple-probe FISH (Tables 13, 14, and 15).

Chromosome 3 disomy in human sperm has only been reported in a few published studies but comparisons of the current results can only be made with those of Bischoff *et al.* (1994) and Lu *et al.* (1994) who used FISH and centromeric probes. Other studies used ISH (Guttenbach *et al.*, 1994a) or chromosome paint probes (Rives *et al.*, 1998), but it is difficult to compare the current results with these two studies as any differences may be due to the different probes and hybridization techniques. Bischoff *et al.* (1994) reported incidences of 0.41% and 0.27% in sperm from two donors, and Lu *et al.* (1994) reported an incidence of 0.16% in sperm from 33 donors. The incidence of disomy 3 found in the present study (0.20%) is comparable with these values, however, valid comparisons are difficult because much

Table 13: Frequency of two signals (disomy or diploidy) using single-probe ISH or FISH in human sperm from normal men (published after present study commenced)

Study	Hyb. eff (%)*	No. samples	No. sperm/donor	1	3	7	8	10	11	15	16	17	18	X	Y
Guttenbach <i>et al.</i> (1994a)	>80	7	1,500		0.31	0.31		0.32	0.34			0.31		0.34	
Guttenbach <i>et al.</i> (1994b)	>80	8	2,000										0.36		
Miharu <i>et al.</i> (1994)	>95	9	4,000	0.14							0.17			0.13	0.08
Martini <i>et al.</i> (1995)	98.7	7	1,600	0.69											
Morel <i>et al.</i> (1997)	78-89	97	500				0.18			0.06			0.22	0.03	0.07

* Ranges or separate values for each of the chromosomes studied are given

Table 14: Studies on disomy using double-probe FISH in human sperm from normal men (published after present study commenced)

Study	Hyb. eff. (%) [*]	No. samples	No. sperm/donor	1	2	3	4	6	7	8	9	10	12	15	16	17	18	20	21	X	Y	XY
Bischoff <i>et al.</i> (1994) ^{***}	99.9	2	1,000			0.41	0.30	0.15	0.09	0.04		0.28	0.29	0.23	0.54	0.19	0.39					
Bischoff <i>et al.</i> (1994) ^{***}	99.9	2	1,000			0.27	0.18	0.0	0.0	0.18		0.09	0.32	0.17	0.24	0.0	0.0					
Chevret <i>et al.</i> (1994)	99	1	10,638																	0.05	0.05	0.42
Lu <i>et al.</i> (1994)	-	33	500			0.16		0.17	0.16								0.15					
Martin <i>et al.</i> (1994)	92-95	1	?	0.8									0.4									
Wyrobek <i>et al.</i> (1994)	99.8	1	10,000																	0.04	0.04	0.06
Rousseaux and Chevret (1995) [*]	97-99	5	?		0.2			0.08											0.1			
Spriggs <i>et al.</i> (1995)	>98	5	10,000	0.10									0.16	0.11			0.11					
Blanco <i>et al.</i> (1996)	99	9	2,000					0.14											0.38			
Martin <i>et al.</i> (1996)	>98	10	10,000	0.11									0.16									
Spriggs <i>et al.</i> (1996)	>98	5	10,000		0.08		0.11				0.14				0.11			0.12	0.29			
Lahdetie <i>et al.</i> (1996)	98.8	24	10,000	0.12					0.64													
Morel <i>et al.</i> (1997a)	94-96	97	500											0.05			0.2			0.04	0.05	0.15
Rives <i>et al.</i> (1998) ^{**}	98.7	4	5,000	0.24	0.26	0.25	0.24	0.28	0.22	0.28	0.25	0.26	0.25	0.28	0.24	0.24	0.18	0.23	0.19	0.17	0.17	0.32

^{*} Disomy frequencies of 0.09% for chr. 11 and 0.17% for chr. 14 were also obtained

^{**} Disomy frequencies of 0.20% for chr. 5, 0.20% for chr. 11, 0.23 % for chr. 13, 0.25% for chr. 14, 0.24% for chr. 19 and 0.26% for chr. 22.

^{***} The two sets of data are for two different donors

Table 15: Studies of disomy using triple-probe FISH in human sperm from normal men (published after present study commenced)

Study	Hyb. eff (%)*	No. samples	No. sperm/ donor	1	8	12	13	18	21	X	Y	XY
Bischoff <i>et al.</i> (1994)	99.9	2	10,000							0.375	0.086	0.12
Lu <i>et al.</i> (1994)	-	33	500			0.12				0.25*	0.25*	0.25*
Martin (1994)	-	5	10,000	0.08		0.17				0.07	0.12	0.16
Chevret <i>et al.</i> (1995)	>99	4	11,584- 36,761	0.2						0.04	0.009	0.34
Martin <i>et al.</i> (1995b)	>98	10	10,000	0.11		0.16				0.07	0.18	0.16
Griffin <i>et al.</i> (1995)	-	24	7038- 25091					0.04		0.02	0.03	0.10
Robbins <i>et al.</i> (1995)	>98	14	10,000		0.065					0.031	0.031	0.095
Spriggs <i>et al.</i> (1995)	>98	5	10,000							0.07	0.21	0.15
Abruzzo <i>et al.</i> (1996)	-	28	13,000					0.033		0.018	0.027	0.094
Martin <i>et al.</i> (1996)	>98	10	10,000							0.07	0.18	0.16
Van Hummelen <i>et al.</i> (1996)	>99	3	10,000	0.017	0.019							
Griffin <i>et al.</i> (1996)	-	24	364,788 [#]					0.04	0.17			
Martinez-Pasarell <i>et al.</i> (1997)	99.7	1	20,570							0.03	0.01	0.09
McInnes <i>et al.</i> (1998b)	>98	18	10,000	0.09			0.13		0.37			

* The combined sex chromosome (X + Y) disomy frequency was 0.25%

[#] Total number of sperm scored for all donors

lower numbers of sperm were scored in those other studies, 1000 and 500 sperm per sample respectively (Bischoff *et al.*, 1994 and Lu *et al.*, 1994), which is insufficient for accurately estimating aneuploidy. Interestingly, four of the donors (#5, 8, 9 and 10) studied in the present study had low frequencies of disomy 3, comparable to disomy 7 and disomy 16, so inter-donor differences may have influenced the results in the present study. There is no evidence for a higher incidence of chromosome 3 aneuploidy in spontaneous abortions, liveborns or human sperm karyotypes (Martin *et al.*, 1991; Jacobs, 1992), so further studies on the incidence of chromosome 3 disomy in sperm from normospermic donors are required to clarify the present findings.

The frequency of disomy 7 in human sperm has been estimated in other studies (Guttenbach *et al.*, 1994a; Bischoff *et al.*, 1994; Lu *et al.*, 1994; Lahdetie *et al.*, 1996; Rives *et al.*, 1998), but as mentioned above it is difficult to make comparisons with those studies that used ISH and chromosome paint probes. Bischoff *et al.* (1994) reported frequencies of 0.09% and 0% in two donors, which is similar to this study, while Lu *et al.* (1994) reported a higher frequency of 0.17% and Lahdetie *et al.* (1996) a much higher frequency of 0.64%. However, the two earlier published studies only scored low numbers of sperm, making it difficult to compare with the baseline frequency reported in this study. On the other hand, Lahdetie *et al.* (1996) reported similar scoring criteria to the present study, which makes the 10-fold difference in the two reported frequencies surprising. They reported large inter-donor variation in their study, with 3 donors having much higher frequencies than the other donors, which could account for some of the differences.

Similarly, chromosome 16 disomy in human sperm has only been estimated in a few studies. Williams *et al.* (1993) reported an incidence of 0.13%, which is twice that found in this study. However, when we compare the range of disomy 16 estimates in the present study (0.0-0.14%) with their study (0.03-0.20%), we find that the ranges are very similar. Miharu *et al.* (1994) reported a disomy 16 frequency of 0.17%, similar to that reported in Spriggs *et al.* (1996) (0.11%), while Bischoff *et al.* (1994) reported much higher frequencies of 0.24% and 0.54% in two men, but only scored low numbers of sperm.

Sex chromosome aneuploidy in sperm has been reported many times in the literature (Williams *et al.*, 1993; Chevret *et al.*, 1995; Martin *et al.*, 1995; Griffin *et al.*, 1995; Robbins *et al.*, 1995; Spriggs *et al.*, 1995; Martin *et al.*, 1996). The results of these studies are outlined in Tables 13, 14, and 15. The frequencies in the present study (disomy XX, 0.03%; disomy YY, 0.03%; disomy XY, 0.13%) were similar to two other studies which scored 10 000 sperm per sample for at least 10 different donors and adhered to strict scoring criteria (Griffin *et al.*, 1995; Robbins *et al.*, 1995). However, other studies have estimated higher levels of disomy XX and disomy YY (Spriggs *et al.*, 1995; Martin *et al.*, 1995, 1996). In all the published triple-probe FISH studies to date, estimates for disomy XY have fallen within the range 0.09-0.34%. The higher incidence of disomy XY relative to disomy XX or YY is not entirely unexpected as there is evidence to suggest that the sex chromosomes are more susceptible to first meiotic segregation errors (Armstrong *et al.*, 1994).

More consistent values of sex chromosome aneuploidy have been reported with

the use of triple-probe FISH. The importance of using sex chromosome probes and an autosomal probe to estimate sex chromosome aneuploidy was demonstrated by Bischoff *et al.* (1994). The frequency of unlabelled sperm was 2.1 and 3.9% when double-probe FISH with X and Y probes was used, whereas it was only 0.19 and 0.76% when a chromosome 12 probe was included in a triple-probe procedure.

It is evident from trying to compare the results obtained in the present study with other published studies, that to accurately estimate the frequency of aneuploidy in sperm, it is important to consider two issues, (i) inter-chromosomal differences, and (ii) inter-donor variability.

3.4.3 Inter-chromosomal differences

There is preliminary evidence that some chromosomes such as X, Y, and 21 are predisposed towards higher rates of non-disjunction during spermatogenesis. Spriggs *et al.* (1995) reported that sex chromosome disomy (XX + YY + XY; 0.43%) was significantly higher than disomy for chromosomes 1, 12, 15 and 18 (0.12%). This confirmed the results of Williams *et al.* (1993) in which sex chromosome disomy (0.19%) was higher than disomy for chromosome 18 (0.08%). Spriggs *et al.* (1996) reported that the sex chromosomes and chromosome 21 had a significantly higher frequency of disomy than the other autosomes tested. Blanco *et al.* (1996) published a higher incidence of disomy 21 (0.38%) compared to disomy 6 (0.14%) and Griffin *et al.* (1996) found a higher incidence of disomy 21 (0.17%) than disomy 18 (0.04%). Griffin *et al.* (1996) suggested that the extra chromosome 21 preferentially segregated with the Y chromosome as >60% of all disomy 21 sperm were Y-bearing. When

whole chromosome paint probes were used to detect aneuploidy in sperm, an increased incidence of sex chromosome disomy (0.66%) compared with autosomal disomy (0.24%) was detected, although no differences between disomy rates for chromosomes 1-22 were reported. (Rives *et al.*, 1998).

Taken together, these results suggest that during male meiosis, the sex chromosomes and chromosome 21 may be more susceptible to non-disjunction than the other autosomes. Data from sperm karyotyping, spontaneous abortions and live births support this contention (Martin *et al.*, 1991; Jacobs, 1992; Templado *et al.*, 1996), and there is some evidence that the sex chromosomes are more susceptible to pairing and first meiotic segregation errors (Armstrong *et al.*, 1994).

Inter-chromosomal differences need to be considered when estimating the overall risk of aneuploidy in human spermatozoa. Assuming that a higher rate of non-disjunction exists for the sex chromosomes and chromosome 21 but that the other autosomes all have a similar non-disjunction rate, we can roughly estimate a total aneuploidy rate for spermatozoa. For instance, using mean autosomal, chromosome 21 and sex chromosome disomy rates of 0.11%, 0.29% and 0.27% respectively (Williams *et al.*, 1993; Spriggs *et al.*, 1995; 1996; Downie *et al.*, 1997b), the overall disomy frequency in spermatozoa from a normospermic man would be ~3%. If the incidence of aneuploidy is assumed to be twice the disomy rate (as disomy and nullisomy occur equally), then the total aneuploidy rate would be 6%. Given a mean diploidy rate of 0.30% (Williams *et al.*, 1993; Spriggs *et al.*, 1995; 1996; Downie *et al.*, 1997b), the overall incidence of numerical chromosomal abnormalities in

spermatozoa would be about 6.3%. This is a conservative estimate, however, as we have only assumed uniform disomy frequencies for the various autosomes, nevertheless it is similar to, or slightly higher than results obtained by karyotyping spermatozoa (Martin, 1993).

3.4.4 Inter-donor variability

Valid comparisons between studies are difficult because of differences in subject selection and FISH techniques, but several groups have examined inter-donor variation. Miharū *et al.* (1994) studied spermatozoa from 21 donors and found that chromosome 1 disomy varied from 0.07-0.20% for individual donors. Martin (1994) analysed sperm from 5 donors and found consistent disomy frequencies for chromosomes 12, Y and XY, however, there were significant inter-donor differences in the frequencies of diploidy and disomy for chromosomes 1 and X. Robbins *et al.* (1995) examined inter-donor variation amongst 14 donors, and reported significant individual variation for disomy YY and diploidy (XY88). Spriggs *et al.* (1995) studied sperm using double-probe FISH and probes to chromosomes 1, 12, 15, 18 and the sex chromosomes. They reported significant inter-donor variation for disomy 1, disomy 15, and sex chromosome disomy (YY and XY), but not disomy 12 and 18. Similar inter-donor variation was seen for disomy 1, XX, YY and diploidy in 10 normal men (Martin *et al.*, 1996) and for disomy 1, 13, 21, and duplications and deletions of telomeric 1p36.3 region in men of various ages (McInnes *et al.*, 1998b). However, another study investigating the incidence of duplications and deletions of telomeric 1p36.3 region, found no inter-donor variation for disomy of chromosomes 1 and 8, or

duplications of 1p36.3 (Van Hummelen *et al.*, 1996). They did report variation amongst individuals for deletions of 1p36.3, but an absence of a signal may also reflect hybridization failure and this result could be artifactual.

These studies confirm that inter-donor variability is an important consideration when making intra- and inter-study comparisons of disomy for specific chromosomes in sperm, but clearly there is a need for additional, well-designed studies to clarify the extent of this variation in normospermic men.

3.5 Summary

In the present study, the use of multi-probe FISH, efficient chromosome-specific probes and stringent scoring criteria, facilitated the estimation of aneuploidy. The results demonstrate that the incidence of aneuploidy for chromosomes 3, 7, 16, X and Y is low (<0.20% per chromosome) in sperm from normospermic men. Inter-chromosomal differences were recorded and the influence of inter-donor variability on aneuploidy estimates was also emphasised.

CHAPTER 4

Comparison of chromosomal abnormalities in sperm from subfertile and fertile men

4.1 Introduction

The introduction of ICSI has necessitated the study of sperm from sub-fertile men due to concerns that these men may have an increased incidence of aneuploidy in their sperm. Studies on children born through ICSI have shown an increase in sex chromosome numerical abnormalities and an inheritance of structural abnormalities from the father (In't Veld *et al.*, 1995; Liebaers *et al.*, 1995; Bonduelle *et al.*, 1996).

To date, there have been a number of FISH studies, reporting conflicting data, on the incidence of chromosomal abnormalities in sperm from infertile men (Pang *et al.*, 1994; 1998; Miharu *et al.*, 1994; Finkelstein *et al.*, 1995; 1998; Moosani *et al.*, 1995; Bernardini *et al.*, 1997; Guttenbach *et al.*, 1997a; Martin *et al.*, 1997b; Martin, 1998; McInnes *et al.*, 1998; Rives *et al.*, 1997; Veiga *et al.*, 1997; Bernardini *et al.*, 1998; Storeng *et al.*, 1998; Luetjens *et al.*, 1999). However few, if any, properly controlled FISH studies on numerical and structural abnormalities in sperm from ICSI candidates have been published.

In collaboration with Dr. Andrew Wyrobek's laboratory at Lawrence Livermore National Laboratory (LLNL) in California, a strictly controlled study of chromosomal abnormalities in sperm from subfertile men (ICSI candidates) was undertaken. A FISH procedure using probes for chromosomes X, Y and 8 had been used previously in their laboratory to study sex chromosome aneuploidy in sperm from mice (Lowe *et*

al., 1995) and normal healthy men (Robbins *et al.*, 1995). Van Hummelen *et al.* (1996) developed a new four-probe, triple-colour FISH technique in their laboratory to simultaneously estimate aneuploidy for chromosomes 1 and 8 and telomeric duplications and deletions of chromosome 1, region p36.3, in human sperm.

In this study, similar FISH methodology was used to investigate the incidence of sex chromosome aneuploidy, disomy 21, disomy 18, disomy 1 and duplications and deletions in the 1p36.3 region. The specific aims of this study were to investigate the frequency of numerical and structural chromosomal abnormalities in sperm from men with TSD. The hypothesis tested was that sperm from sub-fertile men with TSD exhibit an increased frequency of chromosomal abnormalities compared with sperm from a control group of NS men.

4.2 Materials and methods

4.2.1 Subjects

This study was approved by the Ethics of Research Committee at The Queen Elizabeth Hospital and informed written consent was obtained from each subject.

All subjects had to meet the following strict selection criteria: (i) specific semen values; (ii) age <35 y.o.; (iii) non-smoker; (iv) no chemotherapy, radiotherapy, fever and/or sulphur drugs.

Potential subjects were identified from semen analyses performed between January 1995 and March 1998. Sub-fertile men seeking infertility treatment at the

Reproductive Medicine Unit's at The Queen Elizabeth Hospital and Wakefield Clinic were recruited for the TSD group. Healthy, fertile normospermic donors who regularly attended the Andrology Laboratory at The Queen Elizabeth Hospital were recruited for the NS group. Potential subjects were contacted by letter (TSD group) or at the time of routine donations (NS group).

For each subject, at least two semen samples were analysed (section 2.2.1), at least one month apart, and with at least 2 days abstinence before each sample. The following specific semen criteria had to be met in both analyses:

TSD group: Sperm concentration: <13 million/ml

Motility: <50% progressive

Morphology: <10% normal.

NS group: Sperm concentration: >20 million/ml

Motility: >50% progressive

Morphology: >20% normal.

The TSD group in this study refers to men with defects in sperm concentration, progressive motility and morphology, which are typical of patients classified as oligoasthenozoospermic (OAT), that require ICSI. Previous clinical studies have shown a strong association between <10% normal sperm morphology, failed fertilisation after IVF (Duncan *et al.*, 1993) and a reduced pregnancy rate after IUI (Burr *et al.*, 1996) so routine clinical practice is to offer ICSI to these patients. Sperm concentration and progressive motility values are often less than WHO criteria (1992) in these men, and therefore patients are also offered ICSI if $<0.5 \times 10^6$ motile sperm

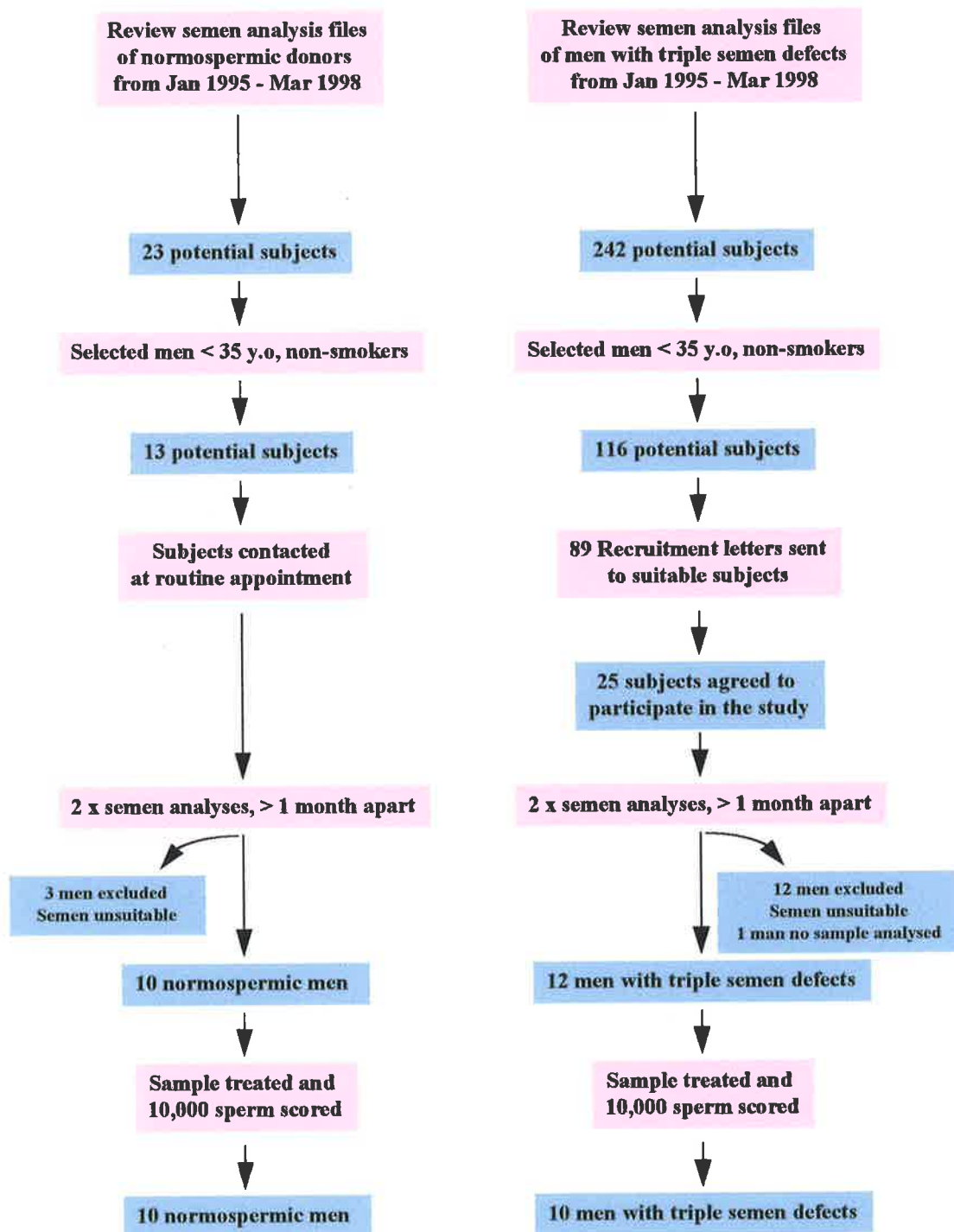


Figure 14. Flow diagram of recruitment process of normospermic men and men with triple semen defects.

are recovered from the ejaculate.

Age and non-smoking were included as selection criteria because previous studies have shown that there is an increased incidence of chromosomal abnormalities in sperm from older men and that smoking causes mutations in sperm that lead to birth defects and genetic disease in the offspring (Griffin *et al.*, 1995; Robbins *et al.*, 1995; Fraga *et al.*, 1996).

Each subject completed a questionnaire about their exposure to chemotherapy, radiotherapy, fever and/or sulphur drugs. Individuals were excluded if they had been exposed to any of these factors because of their detrimental effects on spermatogenesis.

After the recruitment process (Figure 14), 25 sub-fertile men had agreed to participate in the study. After two subsequent semen samples were analysed, at least one month apart, twelve men were excluded from the TSD group as at least one of their semen parameters was higher than the stringent TSD criteria. Semen samples were collected from 12 sub-fertile men (TSD group) and 10 normospermic donors (NS group). Samples from two of the TSD men were subsequently found to be unsuitable (see 4.3.1). The semen analysis results for the two groups are shown in Table 16.

Table 16. Semen analysis results

	Volume (ml)	Concentration (million/ml)	Morphology (% normal)	Motility (% progressive)
1	3.9	8.0	0	6
2	3.3	11.5	0	14
3	5.5	4.7	9	34
4	2.5	7	1	17
5	2.3	6.5	2	19
6	2.0	11	4	21
7	3.6	2.3	1.5	13
8	5.0	4.4	0.5	45
9	6.8	13	5	34
10	1.4	1.4	1	11
TSD group*	3.6 ± 1.7	7.0 ± 3.9	2.4 ± 2.9	21.4 ± 12.3
1	5.0	91	37	59
2	2.3	135	32	61
3	4.6	177	54	63
4	3.9	155	28	59
5	3.1	151	36	49
6	3.6	69	30	55
7	3.1	70	38	59
8	2.0	110	33	62
9	2.8	177	38	51
10	6.0	162	42.5	54
NS group*	3.4 ± 1.0	129.7 ± 41.9	36.4 ± 7.5	57.2 ± 4.7

* = mean ± standard deviation

4.2.2 Preparation of semen samples for FISH

Semen samples were prepared according to a new method from LLNL laboratory. Each sample was mixed well and stored in 250µl aliquots at -80°C. Samples were thawed at RT and a 7µl drop was placed on a clean glass slide, smeared and air-dried over 2 days. For samples with low sperm concentration (1.4 million/ml was the minimum sperm concentration used in this study), the whole sample was left at 4°C overnight to allow the sperm to settle. The next day, a drop was taken from the bottom of the tube and smears were made. If this was unsuccessful, PBS was

added, the sample centrifuged at 81g at 4°C for 15 min, the supernatant removed, and the sperm pellet resuspended in a small amount of PBS and smears made. Slides were stored at -20°C with nitrogen gas and desiccant.

4.2.3 Pretreatment (decondensing) of sperm samples

To partially decondense sperm nuclei, a pretreatment method was developed at LLNL laboratory that was a modification of Wyrobek *et al.* (1990). Slides were incubated for 30 min in 10mM DTT in 0.1M Tris-HCl, pH 7.8, on ice and then for 30 min (when using probes for chromosome 1 and 18) or 90 min (when using probes for the sex chromosomes and chromosome 21) in 4mM LIS in 0.1M Tris-HCl, pH 7.8, at RT. After pretreatment, slides were allowed to air-dry at RT.

4.2.4 FISH protocols

4.2.4.1 Chromosomes 1 and 18 (AM18 assay)

Four probes were used:

- (i) A biotinylated chromosome 1 α -satellite probe (D1Z5) from Oncor (Gaithersburg, MD, USA).
- (ii) Two chromosome 18 α -satellite probes, one labelled with Spectrum Orange® and the other with Spectrum Green® from Vysis (Downers Grove, IL, USA).
- (iii) A DIG-labelled chromosome 1 midi satellite probe specific for the telomeric region p36.3, kindly prepared, labelled and donated by Xiu Lowe (LLNL).

The chromosome 1 centromeric probe fluoresced green when indirectly-labelled with FITC avidin, the combination of two chromosome 18 centromeric probes fluoresced yellow as one was directly labelled with Spectrum Orange® and the other was directly labelled with Spectrum Green®, and the telomeric probe fluoresced red

when indirectly-labelled with rhodamine (Figure 15).

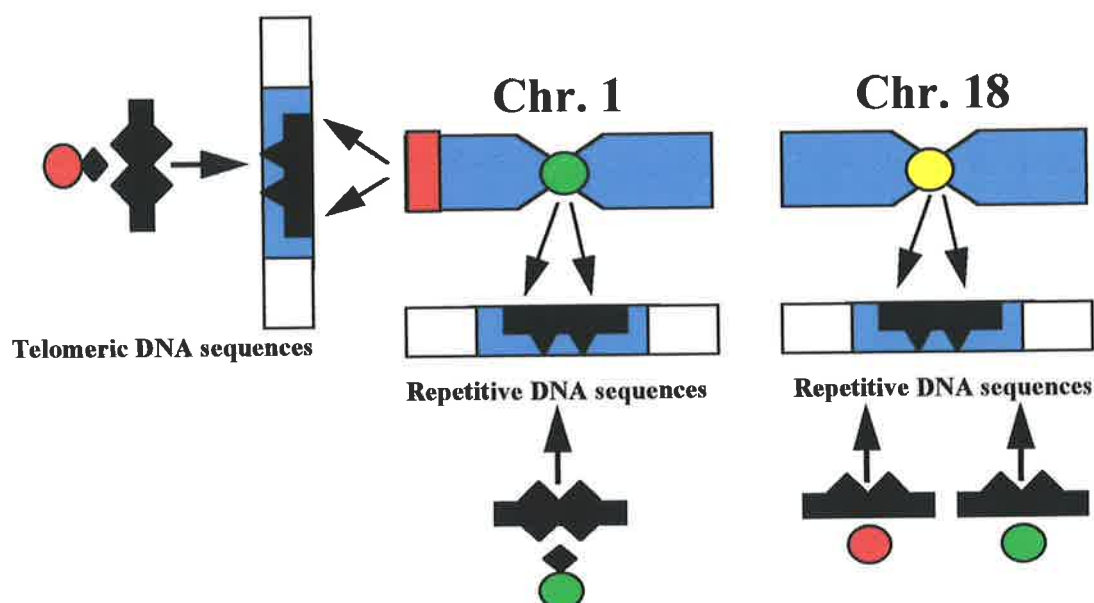


Figure 15. Three-colour FISH using four probes for chromosomes 1 and 18.

Classic FISH methodology was applied to probes for chromosomes 1 and 18:

- Slides immersed in 70% formamide, 2xSSC, pH 7.0 denaturation solution (35 ml formamide, 5ml 20 x SSC, pH 7.0 + MilliQ-H₂O to 50ml) at 76-78°C for 6 min.
- Ethanol dehydration in a series of 70%, 85% and 100% ethanol, each for 2 min.
- Sperm numbers checked and a hybridization area marked.
- DNA probe hybridization mixture was made per slide:
 - 1µl of chromosome 1 (D1Z5) probe
 - 1µl of chromosome 1 midi (p36.3) probe
 - 0.5µl of chromosome 18 Spectrum Orange® probe
 - 0.5µl of chromosome 18 Spectrum Green® probe
 - 7µl of premade master mixture, pH 7.0 (In 7ml, 5.5ml formamide, 0.5ml 20 x SSC, pH 7.0 + 1g dextran sulphate, stored at -20°C in 1 ml aliquots).
- Probe mixture denatured in a waterbath at 76-78°C for 6 min, then chilled immediately on ice.
- Add 10µl of probe mixture/slide at 37°C, seal coverslips with rubber cement.
- Hybridization proceeded at 37°C overnight.
- Post-hybridization washing:
 - (i) 3 times in 60% formamide, 2xSSC washing solution, pH 7.0 (In 150ml, 90ml formamide, 15ml 20xSSC, pH 7.0 + MilliQ-H₂O) at 45°C for 10 min each.

- (ii) Twice in PN buffer (0.1M Na₂HPO₄·12H₂O, 0.1M NaH₂PO₄, 0.1% NP-40, pH 8.0) at 45°C for 10 min each.
- Post-hybridization detection of the biotinylated and DIG-labelled chromosome 1 probes:
 - a) Blocking step: Add to each slide 40µl of PMN buffer (5% nonfat milk powder, 0.02% Na Azide in PN buffer), cover with plastic coverslip, and incubate for 20 min at RT.
 - b) First detection step: Add to each slide 40µl of FITC avidin diluted in PMN buffer to 5µg/ml for 20 min at RT.
 - c) Second detection step: Add to each slide 40µl of biotinylated anti-avidin diluted in PMN buffer to 5µg/ml and anti-DIG rhodamine antibody diluted in PMN buffer to 0.8µg/ml for 20 min at RT.
 - d) Third detection step: Add to each slide 40µl of FITC avidin diluted in PMN buffer to 5µg/ml for 20 min at RT.
- Washing between detection steps was 2 x in PN buffer for 3 min each.
- After the final wash, slides were wiped each side of the marked hybridization area and 7µl of antifade (Vectashield®, Vector Laboratories, Burlingame, CA, USA) was added which contained 0.05µg/ml DAPI as a nuclear counterstain. The hybridization area was covered with a 22 x 22mm coverslip and stored at 4°C.

4.2.4.2 Chromosomes X, Y and 21 (XY21 assay)

Four probes were used, all purchased from Vysis:

- (i) A Spectrum Orange®-labelled chromosome 21 locus-specific identifier (LSI) probe (loci D21S259, D21S341, D21S342).
- (ii) Two X chromosome α-satellite probes, one labelled with Spectrum Orange® and the other with Spectrum Green®.
- (iii) A Spectrum Green®-labelled Y chromosome α-satellite probe.

The chromosome 21 probe fluoresced red as it was directly labelled with Spectrum Orange®, the combination of two X chromosome centromeric probes fluoresced yellow as one was directly labelled with Spectrum Orange® and the other was directly labelled with Spectrum Green®, and the Y chromosome centromeric probe fluoresced green as it was directly labelled with Spectrum Green® (Figure 16).

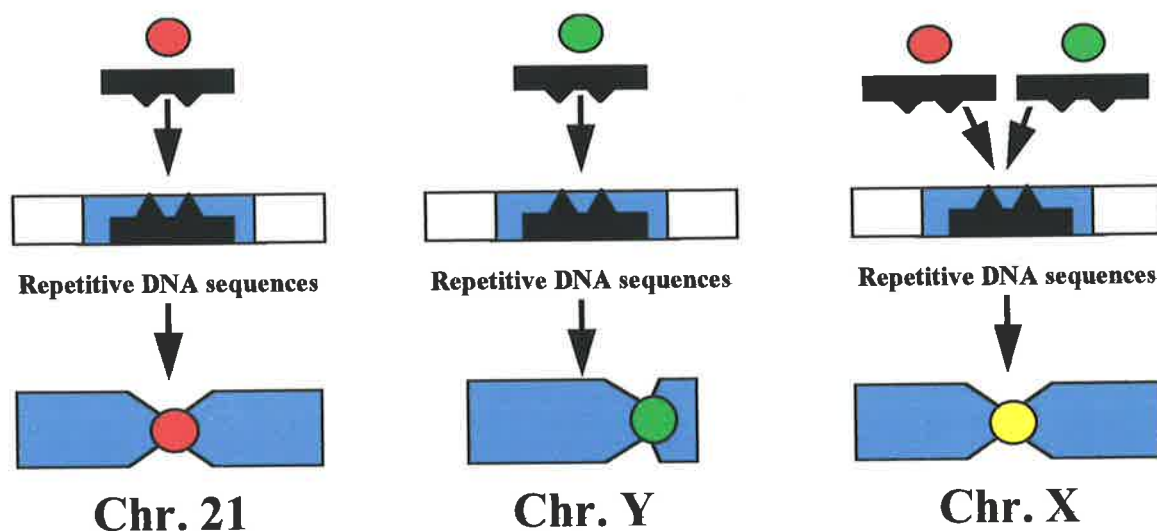


Figure 16. Three-colour FISH using four probes for chromosomes 21, X and Y.

The FISH protocol was similar to that used for the chromosome 1 and 18 probes with some modifications. The X, Y and 21 probes were concentrated using sodium acetate/ethanol to increase hybridization efficiency.

- DNA probe hybridization mixture was made per slide:
 - 1µl of chromosome 21 probe
 - 1µl of Y chromosome probe
 - 0.5µl of X chromosome Spectrum Orange® probe
 - 0.5µl of X chromosome Spectrum Green® probe
 - add 1/10 volume of probes (0.3µl) 3M sodium acetate and 2.5 total volume (8.25µl) absolute ethanol
 - Centrifuge at 15,000 rpm for 30 min
 - Remove supernatant and dry pink pellet in fume hood for 30 min to 2 hr
 - Add to pellet, 7µl/slide of LSI hybridization buffer (Vysis, Downers Grove, IL, USA) and 3µl/slide MilliQ-H₂O and store at 4°C overnight.
- Denaturation and hybridization was as previously described in 4.2.4.1 except it proceeded at 37°C for two nights to improve signal intensity.
- Post-hybridization washing:
 - (i) Once in 50% formamide, 2 x SSC washing solution, pH 7.0 (25 ml formamide, 5 ml 20 x SSC, pH 7.0 + MilliQ-H₂O) at 45°C for 10 min.
 - (ii) Once in 2 x SSC at 37°C for 10 min.
 - (iii) Once in 2 x SSC at RT for 10 min.
- Slides mounted as in 4.2.4.1.

4.2.5 Scoring of sperm slides

As in the previous studies (chapters 2 and 3), slides were examined at a magnification of 1 250 X using a Leitz Laborlux microscope equipped with epifluorescence and a triple band-pass filter block (Chroma Technology Corp., Brattleboro, VT, USA). This enabled simultaneous observation of the blue nuclear counterstain and the red (p36.3 region of chr 1, chr 21), green (centromeric region of chr 1, chr Y) and yellow (chr 18, chr X) hybridization signals. Slides were only scored if the hybridization efficiency was $\geq 98\%$, and approximately 10 000 sperm were scored from each slide.

Stricter scoring procedures were introduced for this study to minimise biases in scoring chromosomal abnormalities. All scoring was done without knowledge of the specimen (blinded) by the author. Groups of four slides (2 TSD; 2 control) were coded using a standardised numbering system by someone other than the scorer and 5000 sperm were then scored in one area of the slide (Figure 17). These slides were recoded and 5000 sperm were scored in a different area. The two sets of 5000 sperm scored from each slide were examined using Cochran's equal proportion test. If $P > 0.05$, the two sets of values were considered to be comparable, otherwise slides were recoded and scored again using the same procedure.

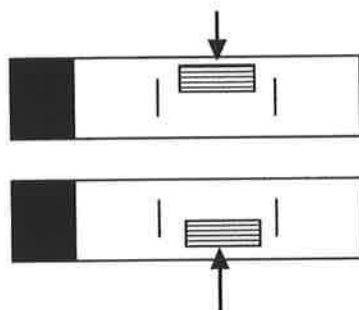


Figure 17. Areas of sperm slides scored in a blinded fashion.

4.2.6 Scoring criteria

Scoring results were recorded using a Macintosh computer and the Cytoscore® scoring program (LLNL, Livermore, CA). Only single, unclumped sperm which clearly possessed a tail were scored so as to avoid confusion about the origin of the signal and to exclude non-sperm cells. Sperm with over-swollen nuclei characterised by ≥ 2.5 times increase in nuclear size and split fluorescent signals were excluded because it was difficult to identify the number of fluorescent signals present within the sperm nucleus. Signals were only scored if they lay within the DAPI stained perimeter of the nucleus. A sperm cell was scored as disomic if the two signals were of equal size and intensity and were separated by at least half a signal domain; otherwise they were considered to be a split signal and were scored as only one signal.

The presence of a fluorescent signal was scored using letter abbreviations in the Cytoscore® program.

4.2.6.1 AM18 assay

A normal haploid sperm was classified as AM18, ie: the presence of 'A' (alpha) chromosome 1 centromeric green signal, 'M' (midi) chromosome 1p36.3 red signal

and '18' for the chromosome 18 centromeric yellow signal. A sperm with a duplicated midi region (p36.3) was classified as AMM18 whereas a sperm with a deleted midi region was classified as AO18. Disomic sperm were classified as AAMM18 for disomy 1 and AM1818 for disomy 18. Diploidy was classified as AAMM1818.

4.2.6.2 XY21 assay

A normal haploid sperm was classified as X21 or Y21, ie: the presence of 'X' chromosome X centromeric yellow signal and '21' chromosome 21 red signal, or the presence of 'Y' chromosome Y centromeric green signal and '21' chromosome 21 red signal. Disomic sperm were classified as XX21 for disomy X, YY21 for disomy Y and XY21 for disomy XY. Diploidy was classified as XX2121 if the X chromosome was present, YY2121 if the Y chromosome was present and XY2121 if both the sex chromosomes were present.

4.2.7 Statistical analysis

Statistical analyses were performed using Excel 5.0 (Microsoft Corporation, Redmond, WA, USA). Differences in disomy and diploidy frequencies were analysed using two sample *t*-tests. A *P* value < 0.05 was considered significant.

4.3 Results

4.3.1 Sample processing and pretreatment

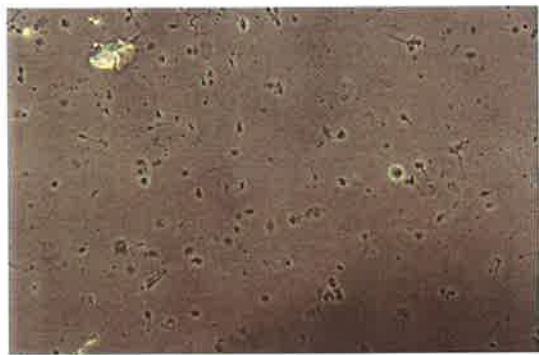
A database was compiled for each semen sample. Tables 17a, 17b and 17c are a record of this information and show which slides hybridized successfully and had



(a)



(c)



(b)



(d)

Figure 18: Pretreatment of TSD and NS samples, (a) TSD sample before pretreatment, (b) TSD sample after pretreatment, (c) NS sample before pretreatment, (d) NS sample after pretreatment.



(a)



(b)

Figure 19: (a) Low sperm numbers in TSD sample before pretreatment, (b) Low sperm numbers in TSD sample after pretreatment.

10,000 sperm scored and which slides did not hybridize and were discarded.

Preparation of TSD samples was difficult due to the low sperm concentrations (1.4-13 million/ml), and as a result the number of scoreable slides was low. It was important to ensure that sperm from the TSD samples (Figure 18a,b) were smeared onto glass slides at a similar density to the NS group (Figure 18c,d) so that no biases were introduced into the coded scoring of chromosomal abnormalities. Thus, slides with hardly any sperm on them, before or after treatment, were discarded and were not used for FISH (Figure 19a, b). In the TSD group, one subject (# 7) was recalled for a second sample due to very low numbers of sperm on slides prepared from the first sample. Slides from two subjects (# 11, 12) were found to be unsuitable and were discarded (Table 17c).

Sample preparation in the NS group was much easier due to the high sperm concentration (69-177 million/ml) of these samples. Unexpectedly, some variability was seen in pretreatment and FISH results on some slides but this was due to inconsistencies in the pretreatment procedures and hybridization of probes. Some slides that had been successfully hybridized and coded (subjects # 1, 3, 6) were repeated due to scoring difficulties.

Table 17a. Inventory of sample preparation, slides prepared, treatment and outcome for the TSD group.

Slide	Sample collected	Slides made	Number of slides	Slides used	Sperm number	Decon	AM18	XY21	Discarded slide	No FISH signals	Inconsist. signals	Scoreable slide
TSD												
1	24/1/97	21/4/97	2	2	Not many	2	0	0	2			
		3/5/97	3	3	Good	3	1	0	2	1		
		19/5/97	2	1	Low	1	0	1				1
		8/1/98	3	2	Low-avg	2	1	1	1		1	
		9/1/98	10	8	Good	8	1	3	5		3	
		22/5/98	10	7	Avg-ok	7	1	2	4		2	1
2	9/2/97	7/4/97	4	4	Good	4	4	0	3	1		
		2/5/97	4	2	Good	2	1	1				2
3	20/2/97	21/4/97	4	2	Low-gd	2	1	0	1			1
		11/9/97	5	0	Low							
		18/9/97	9	1	Low-avg	1	0	1				1
4	26/2/97	15/4/97	6	3	Low-avg	3	0	0	3			
		3/5/97	2	2	Avg	2	1	0	2			
		8/1/98	7	0	Low-avg							
		9/1/98	9	2	Avg-gd	2	1	1				2
5	28/2/97	4/7/97	6	3	Avg-ok	3	0	0	3			
		5/3/97	4	4	Good	4	1	0	3		1	
		15/5/97	6	6	Okay	6	0	0	6			
		27/5/97	9	9	Avg-gd	9	4	4	1	2	3	1-redone 2
		16/4/98	7	3	Low-Av	3	0	2	2	1		
		17/4/98	11	5	Avg-gd	5	0	0	5			
6	1/3/97	15/4/97	4	4	Okay	4	4	0	2	1		1-redone
		2/5/97	3	1	Avg	1	1	0				1
		11/9/97	8	6	Avg-gd	6	3	0	4		2	
		16/4/98	22	8	Avg-gd	8	0	6	3	2	2	1
7a	18/2/97	2/5/97	2	0	Very low							
		13/5/97	2	1	Very low	1	1				1	
		8/1/98	3	0	Very low							
		22/5/98	2	0	Very low							
7b	22/6/98	22/6/98	16	2	Okay	2	1	1				2
8	26/6/98	26/6/98	14	6	Avg-okay	6	2	2	2		2	2
9	3/8/98	3/8/98	19	12	Good	12	2	5	6	1	3	2
10	8/10/98	8/10/98	9	8	Okay	8	1	3	1	4	2	2

Abbreviations: Decon = decondensing pretreatment of sperm, AM18 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 1 and 18, XY21 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 21, X and Y, NM = normospermic men, TSD = men with triple semen defects.

Table 17b. Inventory of sample preparation, slides prepared, treatment and outcome for the NS group.

Slide	Sample collected	Slides made	Number of slides	Slides used	Sperm number	Decon	AM18	XY21	Discarded slide	No FISH signals	Inconsist. signals	Scoreable slide
NM												
1	29/8/96	7/4/97	4	4	Good	4	4	0		1	2	1 x 5000
		20/5/97	5	5	Good	5	3	2			5	
		2/2/98	4	2	Good	2	1	1		2		
		21/5/98	4	1	Gd-avg	1	1	0			1	
		27/7/98	5	3	Good	3	1	0	2		1	
		6/9/98	6	3	Good	3	1	1	1			1 x 5000 1 x 10,000
2	19/11/96	15/4/97	4	4	Good	4	3	1		2	1	1
		2/2/98	6	6	Okay	6	1	5	1	2	3	
		16/4/98	6	2	Good	2	0	2			1	1
3	22/11/96	7/4/97	4	4	Good	4	3	0	1	1		2 x 5000
		20/5/97	4	4	Good	4	0	4			3	1
		2/2/98	6	6	Good	6	4	2		2	4	
		16/4/98	7	1	Good	1		1			1	
4	7/2/97	21/4/97	4	4	Good	4	3	1			2	2
		2/2/98	6	2	Okay	2	2	0			2	
5	10/2/97	21/4/97	4	4	Good	4	2	1	1	1		2
6	27/2/97	15/4/97	4	4	Good	4	4	0	1	2		1 x 5000
		6/9/97	6	2	Low-gd	2	2	0	1		1	
		6/11/97	7	1	Low-gd	1	1	0				1 x 5000
		18/9/97	13	3	Good	3	0	3			2	1
7	4/3/97	21/4/97	4	4	Good	4	4	0		2	2	
		2/2/98	6	6	Ok-avg	6	3	0	4	1	1	
		21/5/98	4	4	Okay	4	2	1	1	1	2	
		27/7/98	5	4	Ok-avg	4	0	2	2		2	
		6/9/98	8	7	Good	7	1	3	3	1	1	2
8	4/3/97	13/5/97	4	4	Good	4	3	0	1	2	1	
		21/5/98	4	4	Okay	4	2	1	1	1	2	
		27/7/98	5	4	Good	4	2	1	1		2	
		6/9/98	8	7	Good	7	1	3	3	1	1	2
9	6/3/97	13/5/97	4	4	Good	4	3	0	1	2		1
		21/5/98	5	3	Okay	5	2	1	2	1	1	1
10	5/7/98	5/7/98	6	4	Good	4	1	2	1		1	2

Abbreviations: Decon = decondensing pretreatment of sperm, AM18 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 1 and 18, XY21 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 21, X and Y, NM = normospermic men, TSD = men with triple semen defects.

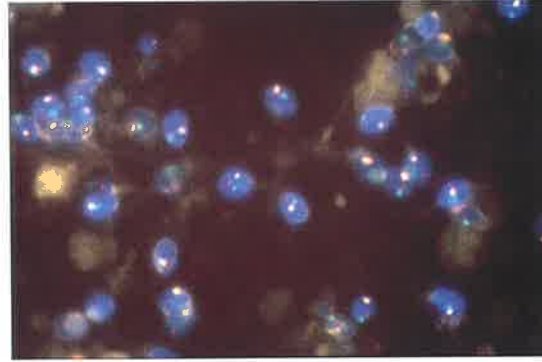
Table 17c. Inventory of sample preparation, slides prepared, treatment and outcome for discarded TSD samples.

Slide	Sample collected	Slides made	Number of slides	Slides used	Sperm number	Decon	AM18	XY21	Discarded slide	No FISH signals	Inconsist. signals	Scoreable slide
TSD	samples	not used	in study									
11	23/1/97	21/4/97	4	2	Not many	2	0	0	2			
		3/5/97	3	3	Avg	3	2	0	1		2	
		20/5/97	4	0	Low							
		27/5/97	5	1	Avg	1	1	0				1-redone
		11/9/97	2	1	Low	1	0	1			1	
12	25/2/97	2/5/97	2	1	Low	1	1	0	1			
		13/5/97	3	0	Low							
		19/5/97	3	0	Low							
		27/5/97	9	2	Low-avg	2	2	0			2	
		8/1/98	2	0	Not many							
		22/5/98	1	0	Very low							

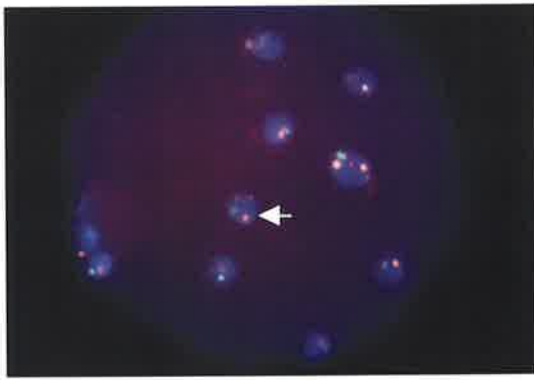
Abbreviations: Decon = decondensing pretreatment of sperm, AM18 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 1 and 18, XY21 = fluorescence *in situ* hybridisation using DNA probes for chromosomes 21, X and Y, TSD = men with triple semen defects.



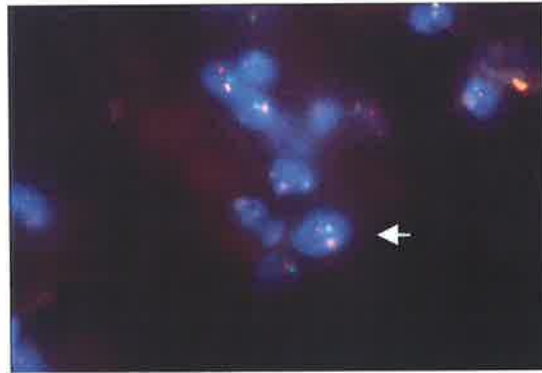
(a)



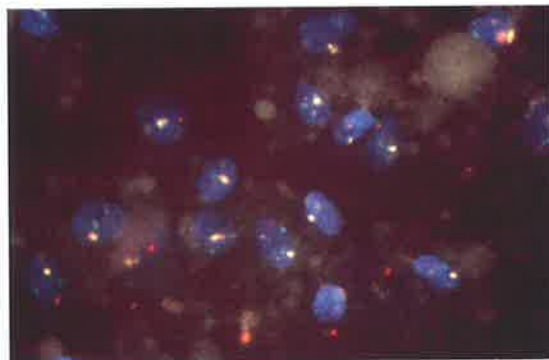
(c)



(b)



(d)



(e)

Figure 20: TSD and NS samples after FISH, (a) AM18 (haploid) sperm in NS sample, (b) Duplication mid 1p36.3 region, AMM18 sperm shown by arrow, in NS sample, (c) AM18 (haploid) sperm in TSD sample, (d) Duplication mid 1p36.3 region, AMM18 sperm shown by arrow, TSD sample, (e) Split FISH signals and sperm without tails in poor quality TSD sample.

4.3.2 Overall results

4.3.2.1 AM18 assay

A total of 200 603 sperm were scored for chromosomes 1 and 18 with an overall hybridization efficiency of 99.99% (Figure 20).

Results of the AM18 assay and the chromosomal abnormalities reported for each subject are presented (Table 18, Figure 21a, Figure 21b). In sperm from the TSD group the frequency of chromosomal abnormalities was 0.21%, which was comparable to that recorded in the NS group at 0.20%.

In the TSD and NS groups, respectively, the incidences of:

- ⇒ AM18 (haploid) sperm were 99.79% and 99.78%
- ⇒ AMM18 (1p36.3 duplication) sperm were 0.03% and 0.04%
- ⇒ A018 (1p36.3 deletion) sperm were 0.03% and 0.02%
- ⇒ AAMM18 (disomy 1) sperm were 0.01% and 0.01%
- ⇒ 0018 (nullisomy 1) sperm were 0.01% and 0.01%
- ⇒ AM1818 (disomy 18) sperm were 0.03% and 0.03%
- ⇒ AM0 (nullisomy 18) sperm were 0.03% and 0.04%
- ⇒ AAMM1818 (diploid) sperm were 0.06% and 0.05%

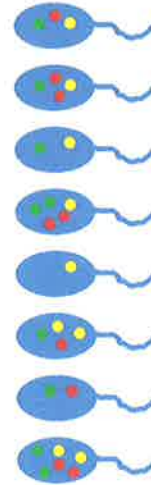


Table 18. Chromosomal abnormalities for chromosomes 1 and 18 in sperm from TSD and NS groups

Subject	Total sperm	Haploid	Total abn.	Dupl'n 1p36.3	Del'n 1p36.3	Disomy 1	Nullisomy 1	Disomy 18	Nullisomy 18	Diploidy
1	10016	99.89%	0.11%	0.01%	0.01%	0.04%	0.03%	0.00%	0.00%	0.01%
2	10050	99.80%	0.21%	0.07%	0.05%	0.00%	0.02%	0.01%	0.04%	0.02%
3	10010	99.61%	0.43%	0.04%	0.09%	0.00%	0.04%	0.04%	0.16%	0.06%
4	10051	99.68%	0.30%	0.09%	0.07%	0.03%	0.01%	0.02%	0.01%	0.06%
5	10047	99.70%	0.29%	0.02%	0.00%	0.01%	0.01%	0.04%	0.00%	0.21%
6	10017	99.84%	0.15%	0.02%	0.03%	0.00%	0.00%	0.03%	0.02%	0.05%
7	10019	99.86%	0.14%	0.03%	0.00%	0.02%	0.00%	0.04%	0.00%	0.05%
8	10026	99.83%	0.17%	0.00%	0.01%	0.00%	0.02%	0.03%	0.03%	0.08%
9	10023	99.83%	0.17%	0.04%	0.01%	0.02%	0.00%	0.05%	0.01%	0.04%
10	10019	99.87%	0.13%	0.03%	0.00%	0.01%	0.00%	0.03%	0.04%	0.02%
Totals, TSD	100278	99.79%	0.21%	0.03%	0.03%	0.01%	0.01%	0.03%	0.03%	0.06%
1	10017	99.87%	0.11%	0.04%	0.00%	0.00%	0.00%	0.02%	0.03%	0.02%
2	10030	99.66%	0.30%	0.08%	0.11%	0.01%	0.04%	0.01%	0.01%	0.04%
3	10043	99.86%	0.12%	0.02%	0.02%	0.00%	0.01%	0.02%	0.00%	0.04%
4	10025	99.90%	0.09%	0.00%	0.01%	0.00%	0.01%	0.01%	0.01%	0.05%
5	10045	99.70%	0.23%	0.10%	0.01%	0.01%	0.01%	0.03%	0.01%	0.06%
6	10062	99.52%	0.43%	0.07%	0.03%	0.03%	0.03%	0.04%	0.11%	0.12%
7	10016	99.90%	0.10%	0.01%	0.00%	0.03%	0.00%	0.00%	0.04%	0.02%
8	10021	99.86%	0.14%	0.02%	0.00%	0.00%	0.00%	0.05%	0.03%	0.03%
9	10036	99.73%	0.27%	0.05%	0.00%	0.02%	0.01%	0.06%	0.04%	0.09%
10	10030	99.83%	0.17%	0.00%	0.00%	0.00%	0.00%	0.03%	0.08%	0.04%
Totals, NS	100325	99.78%	0.20%	0.04%	0.02%	0.01%	0.01%	0.03%	0.04%	0.05%

Figure 21a. Chromosomal abnormalities in sperm from TSD group (chromosomes 1 and 18)

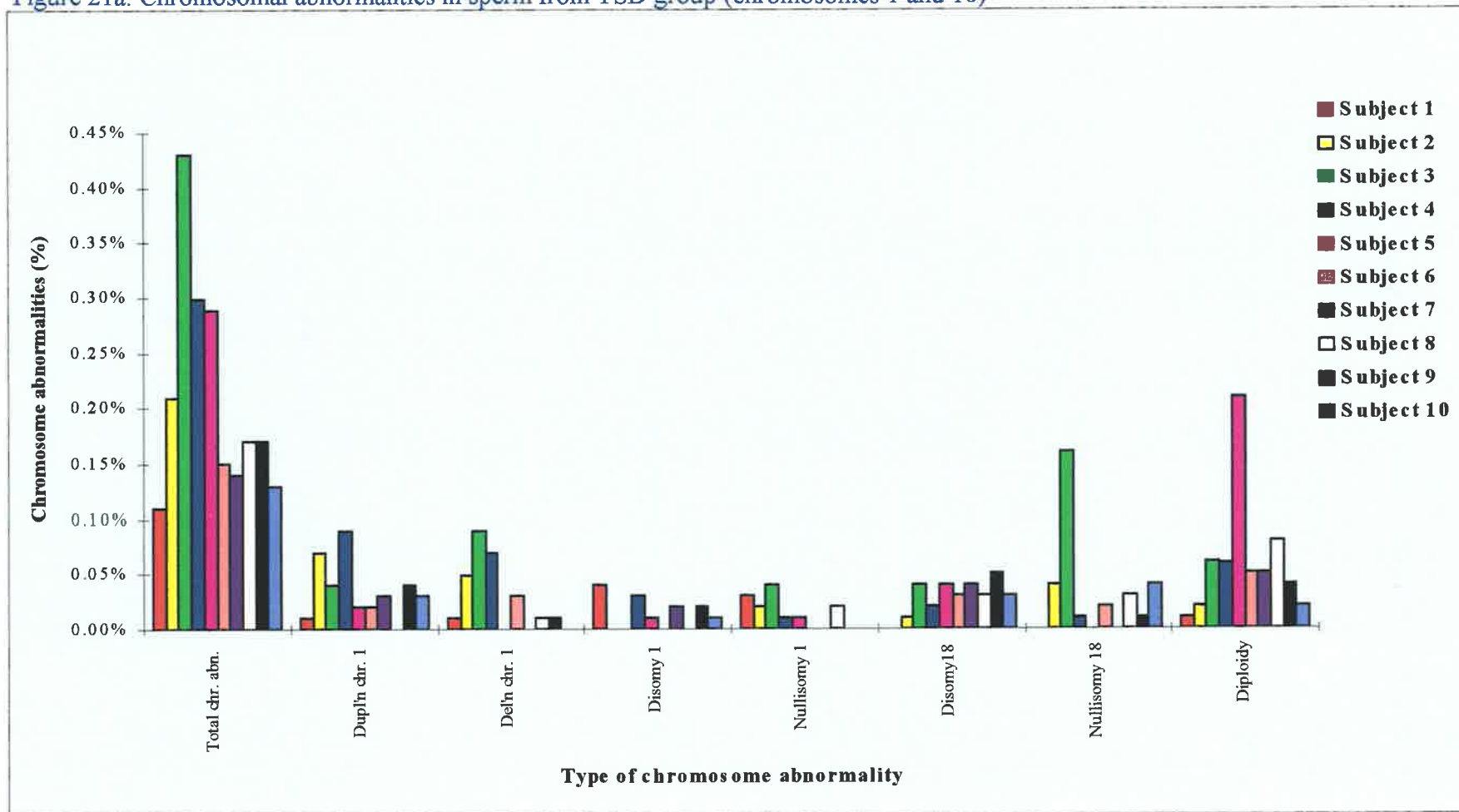
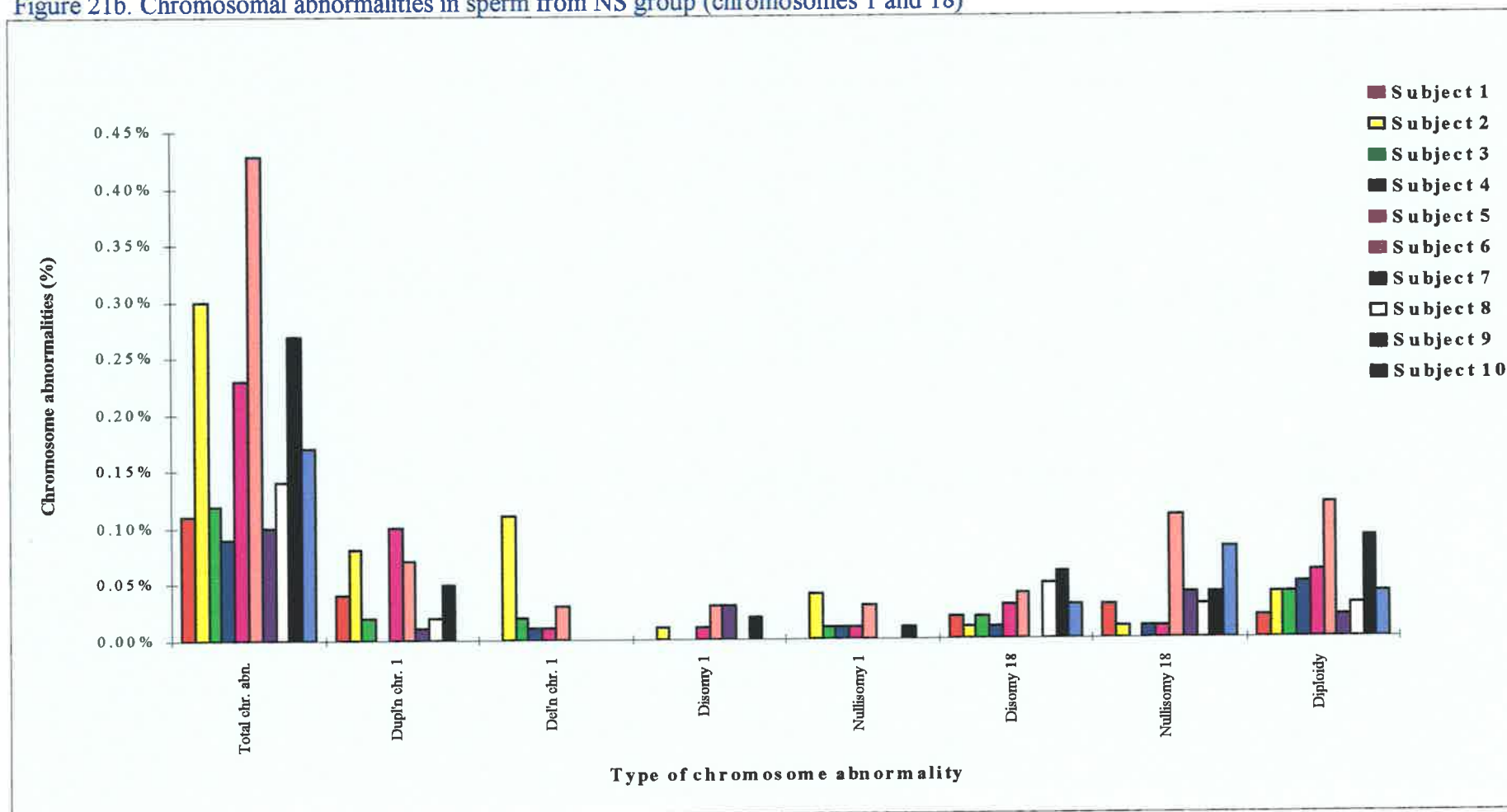
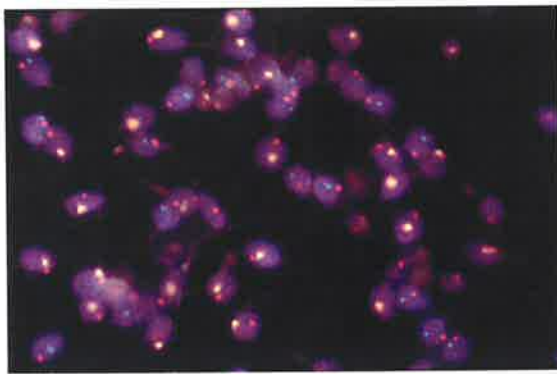


Figure 21b. Chromosomal abnormalities in sperm from NS group (chromosomes 1 and 18)

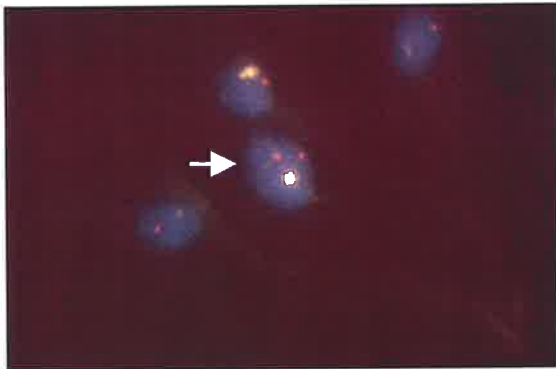




(a)



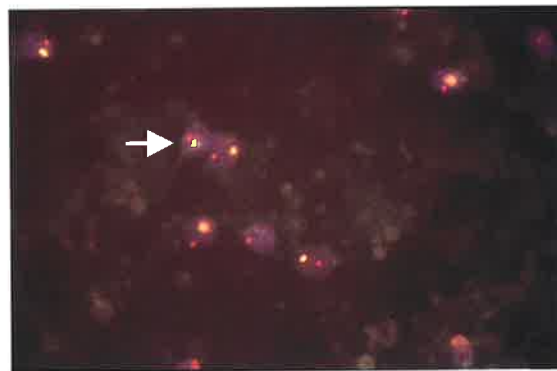
(c)



(b)



(d)



(e)

Figure 22: TSD and NS samples after FISH, (a) X21 and Y21 (haploid) sperm in NS sample, (b) XY2121 (diploid) sperm, shown by arrow, in NS sample, (c) X21 and Y21 (haploid) sperm in TSD sample, (d) XY2121(diploid) sperm, shown by arrow, in TSD sample, (e) XY21 (disomic) sperm, shown by arrow, in TSD sample.

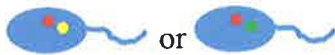
4.3.2.2 XY21 assay

A total of 200 651 sperm were scored for chromosomes X, Y and 21 with an overall hybridization efficiency of 99.99% (Figure 22).

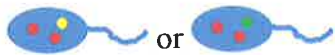
Results of the XY21 assay and the chromosomal abnormalities reported for each subject are presented (Table 19, Figure 23a, Figure 23b). In sperm from the TSD group, the frequency of chromosomal abnormalities was 0.23%, which was higher than that recorded in the NS group (0.15%), but not significantly different ($P = 0.18$).

In the TSD and NS groups, respectively, the incidences of:

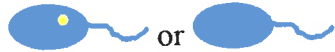
⇒ X21 or Y21 (haploid) sperm were 99.79% and 99.85%



⇒ X2121 or Y2121 (disomy 21) sperm were 0.06% and 0.05%



⇒ X0 or Y0 (nullisomy 21) sperm were 0.01% and 0.01%



⇒ XX21, YY21 or XY21 (sex chromosome disomy) sperm were 0.05% and 0.04%



⇒ 021 (sex chromosome nullisomy) sperm were 0.0% and 0.0%



⇒ XX2121, YY2121 or XY2121 (diploid) sperm were 0.09% and 0.05%



Table 19. Chromosomal abnormalities for chromosomes X, Y and 21 in sperm from TSD and NS groups

Subject	Total sperm	Y21	X21	Total Haploid	Total abn.	Disomy 21	Null 21	Sex chr disomy (sum of *)	XX21*	YY21*	XY21*	Sex chr null	Diploid (sum of #)	XX2121 #	YY2121 #	XY2121 #
1	10012	49.62%	50.31%	99.93%	0.08%	0.03%	0.01%	0.03%	0.01%	0.01%	0.01%	0.00%	0.01%	0.01%	0.00%	0.00%
2	10055	45.61%	54.23%	99.84%	0.21%	0.00%	0.03%	0.06%	0.01%	0.03%	0.02%	0.00%	0.04%	0.02%	0.01%	0.01%
3	10026	48.15%	51.72%	99.87%	0.13%	0.06%	0.00%	0.06%	0.02%	0.02%	0.02%	0.00%	0.01%	0.01%	0.00%	0.00%
4	10042	48.68%	50.93%	99.60%	0.42%	0.18%	0.00%	0.08%	0.00%	0.02%	0.06%	0.00%	0.15%	0.07%	0.02%	0.06%
5	10077	47.36%	52.11%	99.46%	0.55%	0.15%	0.03%	0.12%	0.06%	0.02%	0.04%	0.00%	0.25%	0.06%	0.07%	0.12%
6	10024	49.66%	50.22%	99.88%	0.12%	0.03%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.08%	0.03%	0.03%	0.02%
7	10037	48.08%	51.69%	99.77%	0.23%	0.08%	0.00%	0.02%	0.01%	0.00%	0.01%	0.00%	0.13%	0.04%	0.02%	0.07%
8	10036	49.06%	50.74%	99.80%	0.20%	0.02%	0.01%	0.07%	0.02%	0.01%	0.04%	0.00%	0.10%	0.01%	0.02%	0.07%
9	10022	49.19%	50.72%	99.91%	0.19%	0.06%	0.00%	0.07%	0.05%	0.01%	0.01%	0.00%	0.06%	0.01%	0.02%	0.03%
10	10022	50.06%	49.80%	99.86%	0.14%	0.03%	0.01%	0.02%	0.01%	0.00%	0.01%	0.00%	0.07%	0.03%	0.01%	0.03%
TSD	100353	48.55%	51.25%	99.79%	0.23%	0.06%	0.01%	0.05%	0.02%	0.01%	0.02%	0.00%	0.09%	0.03%	0.02%	0.04%
1	10019	49.68%	50.23%	99.91%	0.09%	0.02%	0.00%	0.03%	0.01%	0.01%	0.01%	0.00%	0.04%	0.02%	0.01%	0.01%
2	10029	49.62%	50.18%	99.80%	0.19%	0.08%	0.00%	0.03%	0.00%	0.00%	0.03%	0.00%	0.09%	0.01%	0.01%	0.06%
3	10023	49.08%	50.83%	99.91%	0.09%	0.01%	0.01%	0.05%	0.02%	0.00%	0.03%	0.00%	0.02%	0.00%	0.00%	0.02%
4	10039	49.82%	50.04%	99.86%	0.16%	0.04%	0.00%	0.07%	0.03%	0.01%	0.03%	0.00%	0.03%	0.02%	0.02%	0.01%
5	10070	48.59%	51.12%	99.71%	0.30%	0.06%	0.04%	0.06%	0.03%	0.01%	0.02%	0.04%	0.10%	0.01%	0.01%	0.05%
6	10040	48.91%	50.90%	99.81%	0.19%	0.07%	0.00%	0.07%	0.00%	0.02%	0.05%	0.00%	0.05%	0.00%	0.00%	0.05%
7	10022	49.61%	50.23%	99.84%	0.16%	0.09%	0.01%	0.02%	0.02%	0.00%	0.00%	0.00%	0.04%	0.01%	0.00%	0.03%
8	10022	48.73%	51.16%	99.89%	0.11%	0.05%	0.00%	0.03%	0.01%	0.01%	0.01%	0.00%	0.03%	0.01%	0.00%	0.02%
9	10014	49.34%	50.58%	99.92%	0.09%	0.03%	0.00%	0.02%	0.01%	0.01%	0.00%	0.00%	0.03%	0.01%	0.01%	0.02%
10	10020	50.13%	49.76%	99.89%	0.09%	0.01%	0.00%	0.03%	0.02%	0.01%	0.00%	0.00%	0.06%	0.01%	0.01%	0.02%
NS	100298	49.35%	50.50%	99.85%	0.15%	0.05%	0.01%	0.04%	0.01%	0.01%	0.02%	0.00%	0.05%	0.01%	0.01%	0.03%

Figure 23a. Chromosomal abnormalities in sperm from TSD group (chromosomes X, Y and 21)

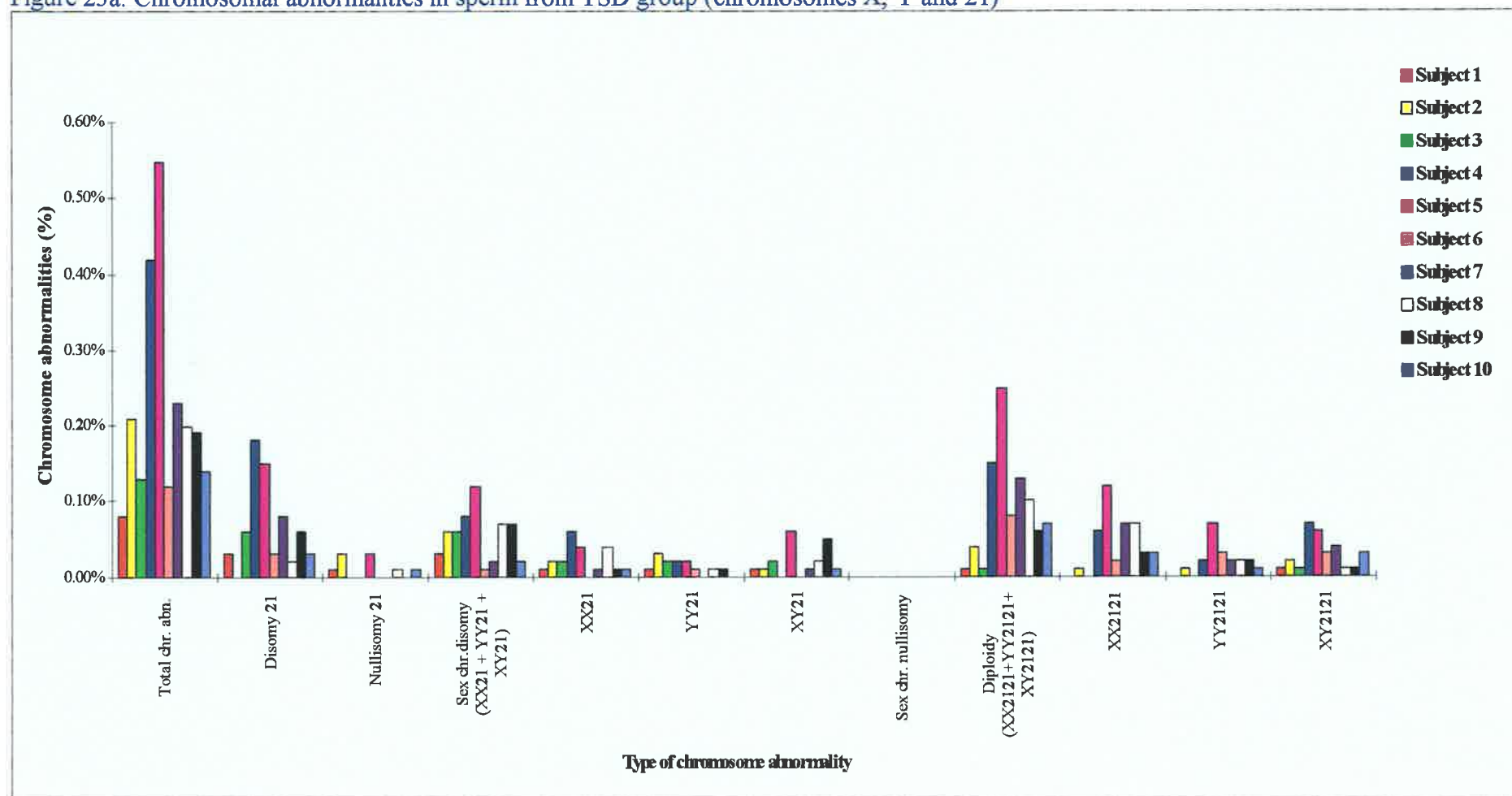
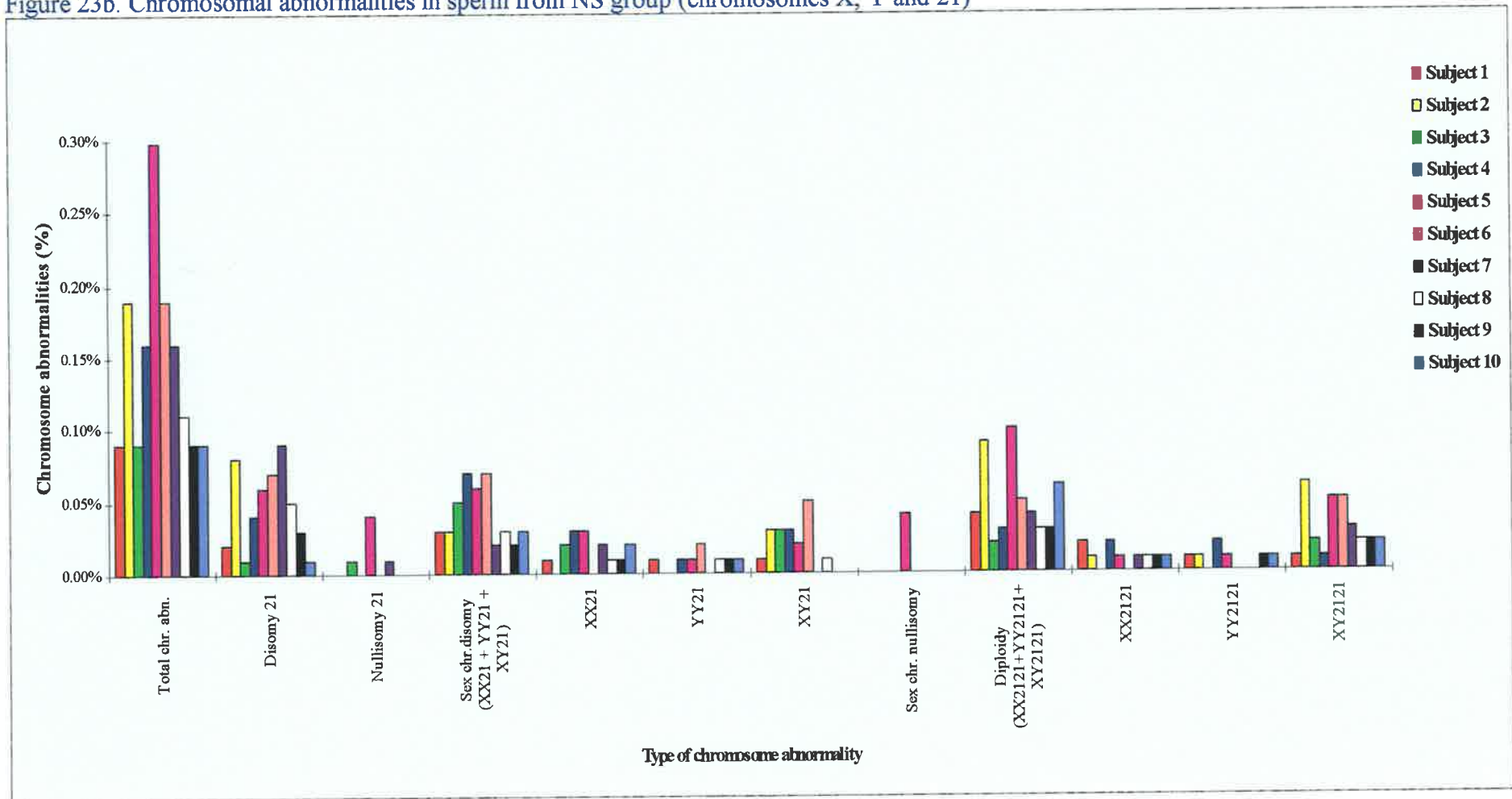


Figure 23b. Chromosomal abnormalities in sperm from NS group (chromosomes X, Y and 21)





4.3.3 Comparison of chromosomal abnormalities in sperm from TSD and NS groups

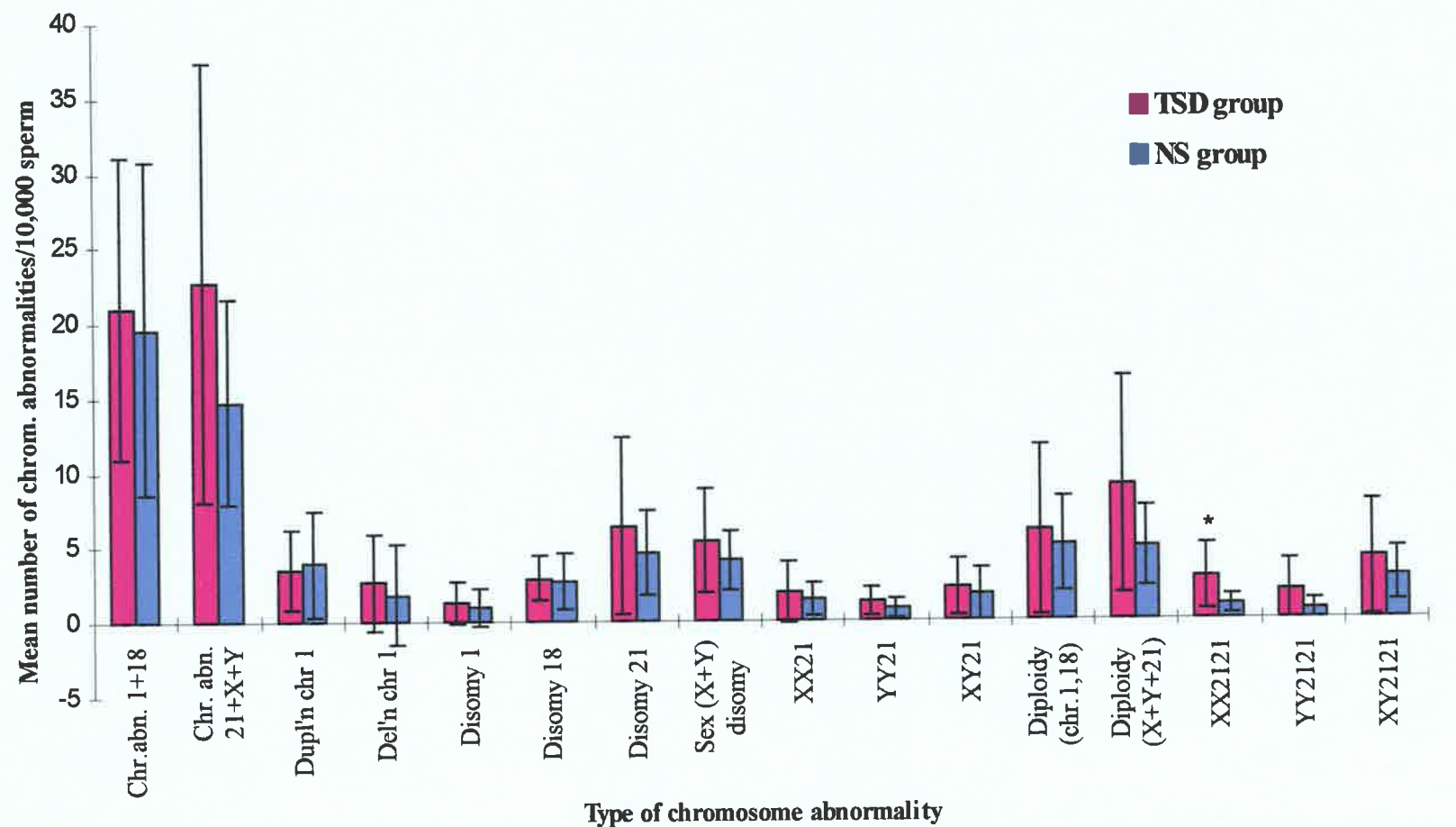
Estimates of chromosomal abnormalities are presented (Table 20, Figure 24).

Table 20. Chromosomal abnormalities (per 10,000 sperm, mean $\pm$ SD) for chromosomes 1p36.3, 1, 18, 21, X, and Y.

Chromosomal abnormality	TSD group	Control group	P-value
			* = sig. diff.
Total chr. 1, 18.	21 $\pm$ 10.1	19.6 $\pm$ 11.0	0.77
Total chr. 21, X, Y.	22.7 $\pm$ 14.7	14.7 $\pm$ 6.8	0.18
Duplication chr. 1p36.3	3.5 $\pm$ 2.7	3.9 $\pm$ 3.5	0.78
Deletion chr. 1p36.3	2.7 $\pm$ 3.2	1.8 $\pm$ 3.4	0.55
Disomy chr. 1	1.3 $\pm$ 1.4	1.0 $\pm$ 1.3	0.62
Disomy chr. 18	2.9 $\pm$ 1.5	2.7 $\pm$ 1.9	0.79
Disomy chr. 21	6.4 $\pm$ 5.8	4.6 $\pm$ 2.9	0.39
Sex chr. (X + Y) disomy	5.4 $\pm$ 3.4	4.1 $\pm$ 2.0	0.31
XX21	1.9 $\pm$ 2.0	1.5 $\pm$ 1.1	0.59
YY21	1.3 $\pm$ 1.0	0.8 $\pm$ 0.6	0.18
XY21	2.2 $\pm$ 1.9	1.8 $\pm$ 1.7	0.62
Diploidy (chr. 1, 18)	6.0 $\pm$ 6.0	5.1 $\pm$ 3.2	0.67
Diploidy (chr. 21, X, Y)	9.0 $\pm$ 7.3	4.9 $\pm$ 2.7	0.11
XX2121	2.9 $\pm$ 2.2	1.0 $\pm$ 0.7	0.02 *
YY2121	2.0 $\pm$ 2.0	0.7 $\pm$ 0.7	0.07
XY2121	4.1 $\pm$ 3.8	2.9 $\pm$ 1.8	0.38

For each chromosomal abnormality analysed, the data from both groups of men (2 x n=10) were calculated as a percentage and arcsin transformed to normalise the data. Two sample *t*-tests (two-tailed, assuming equal variance) were used to compare the incidence of chromosomal abnormalities in the two study groups (Table 20). There was an overall trend towards higher frequencies of all abnormalities in the TSD

Figure 24. Chromosomal abnormalities (mean $\pm$ standard deviations) in sperm from TSD and NS groups.



* = significant difference

group, however, the only significant difference was the frequency of diploid sperm (XX2121) which was significantly higher in the TSD group ($t=2.63$, $P=0.02$). To increase the stringency of detecting a significant difference, the Mann-Whitney U test was applied to XX2121, YY2121 and total abnormalities (X+Y+21), thereby assuming unequal variances. No change in significant differences between the two groups were found with a significant difference still evident for XX2121 ($P = 0.04$), but not for YY2121 ($P = 0.08$) or total abnormalities ($P = 0.12$).

4.3.4 Inter-individual differences

The frequencies of chromosomal abnormalities varied between individuals in both groups. For all chromosomal abnormalities, the 25th and 75th percentiles (data not shown) were calculated for both groups to assess the degree of variation.

For most subjects in both groups, the frequency of chromosomal abnormalities were within a normal distribution, but variations from the normal were observed for some subjects in the TSD group. Duplications of 1p36.3 were detected more frequently (0.07% and 0.09%) in sperm from two men (# 2 and 4 respectively) than the 75th percentile (0.04%), and deletions of 1p36.3 in sperm of two men (# 3 and 4) were found more frequently (0.09% and 0.07% respectively) than the 75th percentile (0.05%). Diploidy, detected in the AM18 assay, was seen more frequently in sperm of two men (# 5 and 8) than the 75th percentile (0.06%), with a high frequency (0.21%) in sperm from one subject (# 5). In two men (# 4 and 5) from the TSD group, a greater frequency of sperm with (i) disomy 21 (0.18% and 0.15% respectively) were seen than the 75th percentile (0.08%), (ii) sex chromosome disomy (0.08% and

0.12% respectively) were seen than the 75th percentile (0.07%) and (iii) diploidy (detected in the XY21 assay) at 0.15% and 0.25% respectively, were seen than the 75th percentile (0.12%). Another man (# 7) also had slightly more diploid sperm (0.13%).

These results show that chromosomal abnormalities in sperm can vary from one individual to another. This is important, especially for the TSD group, in predicting transmission of these abnormalities to the embryo. It is therefore crucial that estimates of chromosomal abnormalities in sperm are calculated from a representative sample size to take this variation into account.

4.4 Discussion

4.4.1 Technical considerations

This study was carefully designed to utilise a strict procedure for recruitment of subjects and stringent criteria for scoring aneuploidy in sperm. While multi-probe FISH has opened the way for extensive studies of aneuploidy in human sperm, there are technical considerations and certain limitations and pitfalls which must be addressed so as to achieve accurate estimates.

4.4.1.1 Paternal age effects

Several studies have used multi-probe FISH to examine whether paternal age influences the frequency of aneuploidy in human sperm. Martin *et al.* (1995) reported a significant age-related increase in disomy for chromosomes Y and 1, and Wyrobek

et al. (1994) also found a paternal age effect on the incidence of disomy Y, whereas Lahdetie *et al.* (1996) found no age-related increase in disomy for chromosomes 1 and 7 in a group of 24 men aged 20-46 y.o. Robbins *et al.* (1995) reported increased frequencies of disomy XX and YY but not disomy XY in older men (43-59 y.o.). Griffin *et al.* (1995) studied sperm from 24 men aged 18-60 and found that there was no relationship between age and disomy 18, but the incidence of sex chromosome disomy was elevated 2-fold in men 50 years and older. These results correlate well with a study which demonstrated an increased incidence of sex chromosome aneuploidy in sperm from aged mice (Lowe *et al.*, 1995). Further investigations are needed as other studies (Kinakin *et al.*, 1997; McInnes *et al.*, 1998b) have found no association between age and sperm aneuploidy in men aged 23-58 y.o.

4.4.1.2 Pretreatment procedures, probes and hybridization conditions

A wide variety of pretreatment procedures have been employed in FISH studies on human sperm. These methods differ significantly in their propensity to decondense sperm nuclei and thereby disrupt and alter the conformation of probe binding sites. As such, they may not yield comparable aneuploidy estimates. Furthermore, over-swelling of nuclei generates split signals which can lead to over-estimates of aneuploidy, so it is important that sperm pretreatment is standardised to minimise this bias. Swelling sperm nuclei to 1.5 to 2 times their original size minimises signal splitting without compromising hybridization efficiency (Holmes and Martin, 1993; Robbins *et al.*, 1993; Wyrobek *et al.*, 1994; Robbins *et al.*, 1995; Downie *et al.*, 1997b).

The choice of probes, hybridization conditions and post-hybridization washing procedures also affects the generation of signals, and at this stage, we do not fully understand the contribution of these variables to: (i) differences in aneuploidy estimates for the same chromosome in different studies, and (ii) differences in the frequency of aneuploidy for different chromosomes. The impact of these technical factors on the estimation of aneuploidy in spermatozoa requires careful evaluation.

4.4.1.3 Signals and scoring criteria

In FISH, an assumption is made that each spot indicates the presence of a chromosome, so the presence of two spots represents two chromosomes. However, under certain conditions this assumption may be invalid. First, two signals can arise from one chromosome due to signal splitting, and this can be a problem when centromeric probes are used because of the distribution of repetitive satellite DNA sequences in the centromeric region and the tendency of this region to fragment. Retention of chromatin integrity and the morphology of probe-binding domains is therefore an important issue.

A second problem relates to the arrangement of signals. Sperm nuclei are three dimensional structures and the centromeric regions of different chromosomes, and hence the FISH signals generated, are not always separate and clearly defined. Signals from different chromosomes, or from two copies of the same chromosome, can be very close together or completely overlapping and therefore cannot be distinguished. This will lead to incorrect estimates of aneuploidy and/or diploidy. The impact of this bias increases as more chromosomes are simultaneously studied in multi-probe FISH.

A third problem is that we assume that if a chromosome is present, the probe will always bind and we will always see a signal. However, localised hybridization failure of the probe to one or more chromosomes could lead to an incorrect assessment of the ploidy status of sperm. For example, if double-probe FISH with two autosomal probes (chromosomes 1, 8) were used and hybridization failure occurred with the chromosome 8 probe, a diploid sperm (1,1,8,8) would be misclassified as disomic (1,1,8), a haploid sperm (1,8) would be misclassified as nullisomic (1), and a disomic sperm (1,8,8) would be misclassified as haploid (1,8).

Aneuploidy for a given chromosome occurs at a very low frequency in human sperm, so large numbers of sperm must be evaluated to ensure that reliable aneuploidy estimates are obtained. Williams *et al.* (1993) stated that scoring 5000 sperm to determine the aneuploidy frequency for each chromosome was insufficient for comparisons of chromosome-specific disomy rates between donors, however this sample size was adequate for comparisons between chromosomes if the results from a group of donors were pooled. They recommended instead that a minimum of 10,000 sperm should be scored from each sample to provide an accurate estimate for each chromosome and enable inter-donor comparisons. In the present study, and in other recent studies, this recommendation was employed (Robbins *et al.*, 1993, 1995; Wyrobek *et al.*, 1994; Griffin *et al.*, 1995; Martin and Rademaker, 1995; Martin *et al.*, 1995, 1996; Moosani *et al.*, 1995; Spriggs *et al.*, 1995, 1996; Van Hummelen *et al.*, 1996; 1997; Downie *et al.*, 1997b). However, some researchers have only scored lower numbers of sperm with the attendant limitations (Schattman *et al.*, 1993;

Bischoff *et al.*, 1994; Lu *et al.*, 1994; Pang *et al.*, 1994; Morel *et al.*, 1997).

The statistical pitfalls associated with scoring small numbers of sperm are obvious. For example, if the true disomy rate were 0.2%, then this would equate to only 2 disomic sperm per 1000 scored but 20 per 10,000 scored. If the true disomy rate were 0.1%, then this would equate to only 1 disomic sperm per 1000 scored or 10 per 10,000 scored. However, if the true disomy rate were only 0.05%, then this would equate to < 1 disomic sperm per 1000 scored or only 5 per 10,000 scored. Clearly, there is great potential for error if only 1000 sperm are scored for each chromosome because the disomy rate will depend on how many disomic sperm are in the cluster of 1000 sperm scored. Scoring one or two more (or less) sperm would change the disomy rate significantly in this situation. This raises doubts about the validity of results obtained by scoring low numbers of sperm.

In some laboratories, efforts are being made to standardise scoring procedures so that inter- and intra-technician variation is minimised and meaningful intra- and inter-donor variations can therefore be compiled. Van Hummelen *et al.* (1996; 1997) scored a total of 10,000 cells per slide, and all slides were scored in two blinded steps. Slides were coded, 5000 sperm were scored, then the slides were re-coded and a second group of 5000 sperm in a different area of the slide were scored. This methodology was employed in the present study. Robbins *et al.* (1997) used a similar system in which two researchers each scored 5000 sperm per specimen; they were blinded to the identity and treatment status of the samples, as well as to individual scoring results. In the study of Chevret *et al.* (1997), slides were scored by two

independent observers who each counted about 3000 sperm per slide. No significant differences were detected between the results for the two observers.

Stringent scoring criteria are therefore needed to ensure accurate aneuploidy estimates and enable meaningful comparisons between chromosomes, donors and studies. It is now standard practise to employ stringent scoring criteria as outlined in section 4.2.5 and 4.2.6.

It is important that technological evolution continues in FISH methodology so that the limitations are resolved or better understood. Particular attention should be given to the establishment of optimal pretreatment and hybridization conditions, the influence of different probes, and meaningful scoring criteria should be established and verified. It is also important to determine the extent of inter-donor variability, and while some researchers have attempted to address this issue, it will only be achieved using standardised methodology and large numbers of men. Providing that adequate attention is given to these technical aspects and to experimental design, as has been attempted in the present study, FISH can provide useful estimates of aneuploidy in human spermatozoa under a variety of clinical conditions.

4.4.2 Incidence of chromosomal abnormalities in sperm

In the present study, FISH was used to detect chromosomal abnormalities in sperm from a clinically relevant group of men seeking infertility treatment. The incidences of numerical and structural chromosomal abnormalities for chromosomes 1, 18, 21 and the sex chromosomes (X,Y) were estimated in over 400,000 sperm from

TSD and NS men.

4.4.2.1 Structural abnormalities

This is the first study that we know of to investigate structural chromosomal abnormalities in men with TSD. A midi satellite probe for the telomeric region p36.3 of chromosome 1 was used to detect duplications and deletions of this region. Although chromosome 1 has not been identified in spontaneous abortions or liveborn chromosomal abnormalities, this region was useful because the probe was commercially available and the protocol had been standardised by Van Hummelen *et al.* (1996). The incidences of duplications (0.04%) and deletions (0.02%) of chromosome 1p36.3 in the present study (NS group), were in good agreement with that found previously by Van Hummelen *et al.* (1996), who found 0.03% duplications and 0.03% deletions of 1p36.3 in three normospermic donors.

In a recently published study on chromosomal abnormalities in sperm from normospermic men aged 23-58 years, McInnes *et al.* (1998b) reported high frequencies of duplications and deletions of 1p36.3 ($0.21 \pm 0.37\%$ and $0.22 \pm 0.14\%$ respectively), with quite high inter-donor variation. These values are much higher than those obtained in the present study and by Van Hummelen *et al.* (1996). While subject age may have a bearing on chromosomal abnormality rates, exclusion of men > 35 y.o from the study by McInnes *et al.* (1998b) does not alter the results (0.26% and 0.23% respectively). It was also reported that a significant difference in the frequency of telomeric duplications was seen between biotinylated and DIG-labelled probes, but not for the FITC direct-labelled probe. In their study, McInnes *et al.* (1998b)

incubated sperm in 10mM DTT for 5-30 min at RT followed by 1mM DTT/10mM LIS for 30 min to 2.5 hr. In the present study, this step was found to be critical when using the chromosome 1 centromeric and telomeric probes, as pretreatment in 4mM LIS for greater than 30 min would often result in split FISH signals. Hence, different pretreatment procedures may also have contributed to the differences in abnormality values.

4.4.2.2 Numerical abnormalities

In the present study, there was an overall trend towards a slight increase in all categories of chromosomal abnormalities in sperm from men with TSD. However, the incidences of specific numerical chromosomal abnormalities for chromosomes 1, 18, 21, X and Y were not statistically significantly elevated.

These findings are clinically important as some other researchers have reported highly elevated incidences of abnormal sperm in infertile men and this has led to increased concern about the risk of transmission of chromosomal abnormalities through the use of ICSI. Pang *et al.* (1994) reported a significant 10-fold increase in disomy 1 (9.9%) in sperm from men with oligoasthenoteratozoospermia (OAT) compared to control men (0.96%). In further studies using multi-probe FISH for chromosomes 1, 4, 6, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y, this group reported significantly more disomic sperm (0-5.7%/chromosome) in OAT men than in controls (0-0.3%/chromosome), and significantly more diploid sperm (0-9.6%) than in controls (0-1.2%) (Pang *et al.*, 1998). Other groups have reported significant increases in disomy XX, XY and diploidy (Veiga *et al.*, 1997), two sex chromosome signals

(XX+YY+XY) (Bernardini *et al.*, 1997) and aneuploidy for chromosomes 1, 13, 14, 18, X and Y (Rives *et al.*, 1997) in sperm from OAT men. In each case, the increases were much less than those reported by Pang *et al.* (1994; 1998). Storeng *et al.* (1998) found a significant difference in the total aneuploidy rate (1.59%) in 9 men with abnormal semen compared with controls (0.78%) but only counted a total of 1446 sperm in the abnormal group. All these groups scored very low numbers of sperm from infertile men and in many cases they used less stringent scoring criteria that can artefactually elevate estimates of aneuploidy.

Other published studies have counted at least 10,000 sperm per chromosome. Finkelstein *et al.* (1995) reported total aneuploidy rates of 0.73-1.01% in five infertile men compared with 0.06-0.11% in five fertile men. Moosani *et al.* (1995) reported significant increases in chromosome 1 disomy and disomy XY in 5 infertile men, although two-colour FISH was used for the sex chromosomes so disomy and diploidy could not be differentiated. Further studies by this group recorded significant increases in disomy 1, 13, 21, and XY in 10 infertile men (Martin *et al.*, 1997b; Martin, 1998; McInnes *et al.*, 1998a). Disomy values in the infertile group were 0.14% for disomy 1, 0.28% for disomy 13, 0.48% for disomy 21, and 0.42% for disomy XY. The aneuploidy values reported in these studies were up to 10-fold higher than in the present study, most likely as a result of less stringent scoring criteria and/or patient selection. In most of these studies the infertile patient group had many subjects with semen parameters approaching normal values (2.5-50 x 10⁶ million/ml sperm concentration, 11-69% motile forms, 14-54% normal sperm morphology),

however, this does not explain the increased aneuploidy rates (Moosani *et al.*, 1995; Bernardini *et al.*, 1997; McInnes *et al.*, 1998a).

The importance of scoring criteria is emphasised by two other studies, which like the present study, reported no significant increases in aneuploidy from sperm of infertile men. Miharu *et al.* (1994) used single-colour FISH on 12 infertile men and found 0.11-0.16% disomy for chromosomes 1, 16, X and Y, with 4000 sperm scored per subject. Of the subjects, 50% were classified as unexplained infertility and 50% were oligospermia, with one subject's infertility due to anti-sperm antibodies, but no mention was made of semen analysis values for each subject. Guttenbach *et al.* (1997a) scored 10,000 sperm per subject and reported disomy values of 0.10-0.14% for chromosomes 1, 7, 10, 17, X and Y in 45 infertile men whose infertility classification was based on a semenogram. The disomy and diploidy values estimated in both studies were 10-fold higher than those found in this study.

4.4.3 Inter-individual variability and total aneuploidy estimate

In the present study, inter-individual variability in the frequencies of chromosomal abnormalities was observed. In particular, it was noted that two men (# 4, 5) from the TSD group had much higher incidences of aneuploidy in their sperm and therefore would have a much higher risk of chromosomally abnormal sperm being used for ICSI than the remainder of the group. If a diploid sperm were selected, this would increase the chances of implantation failure and/or first trimester miscarriage, and if disomic sperm (chr. 21, X and Y) were used, this may result in liveborns with either Down Syndrome or sex chromosomal abnormalities, such as 47,XXX, 47,YYY

and 47,XXY (Klinefelter's syndrome). A study by Guttenbach *et al.* (1997a) also found two infertile men who had much higher incidences of diploidy (0.16% and 0.35%) than the other subjects (mean=0.10%).

In the TSD group, the overall risk of aneuploidy in their spermatozoa can be estimated, as was determined for normospermic men in chapter 3. From the present study, the mean autosomal, chromosome 21 and sex chromosome disomy rates in the TSD group were 0.02%, 0.06% and 0.05% respectively. If two assumptions are made, that differences exist in non-disjunction rates for the sex chromosomes and chromosome 21 and that each abnormality occurs in a different sperm, then the overall disomy frequency in spermatozoa would be 0.53%. If the incidence of aneuploidy is assumed to be twice the disomy rate, and given a mean diploidy rate of 0.08%, then the overall incidence of numerical chromosomal abnormalities in spermatozoa would be about 1%. Applying the same principles to the control group in the present study, the overall incidence of numerical chromosomal abnormalities (1.05%) compares favourably with that for men with TSD.

However, the total estimate in the control group is much lower than the rate previously estimated (chapter 3) for normospermic men (6.3%), which is comparable with aneuploidy estimates obtained by karyotyping spermatozoa. Total aneuploidy frequencies of 0.0 to 5.1% (Martin, 1986; Pellestor *et al.*, 1987; Martin and Hulten, 1993) have been estimated by karyotyping spermatozoa and in the two largest studies (Brandriff *et al.*, 1985; Martin, 1990) a total aneuploidy estimate of 1.4% was reported, which is consistent with that found in the present study. In the present study

careful attention was paid to stringent scoring criteria, sample preparation, FISH methodology and subject factors (age, smoking status, chemotherapy, radiotherapy, fever and/or sulphur drugs) that have been linked with increased aneuploidy estimates, all of which are likely to have contributed to the difference in aneuploidy rate between the two studies (chapter 3 and 4).

It is also important to consider inter-individual variation in these calculations. If the same principles are applied again to the TSD group but this time using only the disomy values recorded in this study for subjects #4 and #5, the overall incidence of numerical chromosomal abnormalities is double (2.1%) the initial rate we estimated. If the disomy frequencies reported by Pang *et al.* (1994; 1998) are used to estimate an overall frequency using the same principles, the potential risk of transmission of these abnormalities to the embryos is much greater. The overall disomy frequency in spermatozoa would be ~38% and the overall incidence of numerical chromosomal abnormalities in spermatozoa would be about 78%. The clinical data (discussed in 4.4.4) on ICSI outcomes are not reflective of such an extreme abnormality rate in sperm, so this estimate is most likely artefactual and possibly due to methodological differences in sperm preparation, hybridization and scoring criteria.

It is easy to overestimate the incidence of disomy by including cells without tails which have two signals and/or recording overlapping cells as disomic sperm. It is also possible to arbitrarily record a high incidence of aneuploidy in sperm (due to an increase in split signals) when the sample has been smeared inadequately. In this study, chromosomal abnormalities were recorded (data not shown) in two TSD

samples but no more than 1000 sperm per subject could be scored. The frequency of chromosomal abnormalities in sperm from these subjects was up to 0.8%. These samples were prepared again, and after hybridization, 10-fold lower frequencies of abnormalities, within the range reported for the rest of the group, were recorded.

4.4.4 Clinical outcomes of ICSI

The clinical outcomes (prenatal karyotypes, congenital malformations etc.) of pregnancies achieved using ICSI have been reported by some groups. Earlier studies raised concerns about an increased risk of sex chromosomal aneuploidy in children born after ICSI (In't Veld *et al.*, 1995; Liebaers *et al.*, 1995). A recent report from the Brussels group on ICSI outcomes up to August 1997, on a total of 1082 prenatal karyotypes, stated 1.66% de-novo chromosomal aberrations with 50% due to sex chromosome abnormalities (n=9) and the other half due to trisomies (n=5) or structural abnormalities (n=4) (Bonduelle *et al.*, 1998). They also found 10 cases (0.92%) of inherited structural aberrations. The second survey released by the ESHRE task force on ICSI (1998) involved 90 centres from 24 different countries and reported 98% of the prenatal karyotypes were normal 46,XX or 46,XY karyotypes, with the abnormal cases being 47,XXY, trisomy 21, 2x mosaicism and 4x paternally inherited structural abnormalities. Information was collected from a total of 93 postnatal karyotypes and only one abnormal karyotype, a trisomy 21 male, was reported.

4.5 Summary

The objective of this study was to ascertain if there were higher frequencies of chromosomal abnormalities in sperm from men with TSD, who are candidates for ICSI. The incidence of numerical abnormalities for chromosomes 1, 18, 21, X and Y and duplications and deletions of the telomeric region p36.3 on chromosome 1 were not significantly elevated in sperm from these men.

Data on ICSI outcomes indicates a slight increase in the sex chromosome aneuploidy rate, a slight increase in *de novo* structural abnormalities and trisomies, and a paternal inheritance of structural abnormalities. It is possible that there is an increased incidence of chromosomal abnormalities in sperm from the male partners of these couples, however, the clinical data do not support extreme sperm aneuploidy rates of up to 76% as reported by Pang *et al.* (1994; 1998) as the majority of ICSI pregnancies would have miscarried if this were true. In the present study, two TSD subjects were found to have double the chance (2% vs 1%) of chromosomally abnormal sperm being used for ICSI than the remainder of the group. It seems likely that a few individuals may contribute to the increased incidence of specific chromosomal abnormalities after ICSI, whereas the majority of men undergoing ICSI have no greater chance of transmitting chromosomal abnormalities to their offspring than fertile, normospermic men. It is most likely that the increase in autosomal trisomies after ICSI is a reflection of the increased maternal age of these couples (Van Opstal *et al.*, 1997; Bonduelle *et al.*, 1998; Meschede *et al.*, 1998).

More information is required on prenatal karyotypes and pregnancy outcomes

from ICSI before firm conclusions can be drawn. As information accumulates the literature is starting to suggest that males undergoing ICSI should be screened for chromosomal abnormalities in their blood (Tournaye *et al.*, 1997; Munne *et al.*, 1998). If a translocation carrier is identified, it would be wise to suggest preimplantation diagnosis to prevent the replacement of unbalanced embryos. However, this screening procedure would not detect any chromosomal abnormalities specifically localised to the sperm cells due to meiotic errors during spermatogenesis. It may be necessary to use FISH to detect chromosomal abnormalities (sex chromosome or autosomal) in sperm from selected individuals, and if an abnormal frequency is detected, it would be wise to use preimplantation diagnosis in these cases also. Obviously, as time progresses more reports on the implications and results of ICSI will evolve, and as generations age the effects on transmission of chromosomal abnormalities and infertility will unfold.

CHAPTER 5

Localisation of chromosomes in sperm

5.1 Introduction

Mammalian spermatozoa have highly compacted nuclei resulting from condensation of the nuclear chromatin during spermiogenesis. Sperm DNA is packaged into linear, side-by-side arrays which are maintained by disulphide cross-links that form between adjacent protamine molecules during epididymal maturation (Bedford *et al.*, 1973; Balhorn *et al.*, 1982; 1991).

In hamster and human spermatozoa, the DNA is arranged into loop domains by a nuclear matrix (Ward and Coffey, 1989; Ward *et al.*, 1989; Ward, 1994; Barone *et al.*, 1994). Furthermore, the loop domains appear to be anchored to another structure near the implantation fossa, called the nuclear annulus, and remain attached to this structure after decondensation of the sperm nucleus (Ward and Coffey, 1989). This suggests that each chromosome has at least one attachment site to the nuclear annulus, and studies have shown that unique, but as yet unidentified DNA sequences other than telomeres, centromeres or ribosomal DNA are bound to the nuclear annulus (De Lara *et al.*, 1993; Barone *et al.*, 1994; Nadel *et al.*, 1995; Ward *et al.*, 1996).

These studies suggest that DNA packaging in the sperm nucleus is non-random and a higher level of organisation exists. Studies have suggested that chromosomes may be packaged in a precise sequential order within the sperm nucleus in insects

(Taylor, 1964), amphibians (MacGregor and Walker, 1973), planarians (Joffe *et al.*, 1998), mammals (Powell *et al.*, 1990; Jennings and Powell, 1995) and humans (Zalensky *et al.*, 1993), although some studies have found it to be more random (Barone *et al.*, 1994; Ward *et al.*, 1996). It is evident that nuclear structures play a role in the organization of sperm DNA, but whether chromosome organization within the sperm nucleus is specific or random, and whether this has any effect on post-fertilisation events, is yet to be determined.

At the conclusion of spermiogenesis, the sperm head takes on the characteristic shape of the respective species. The mechanisms which regulate sperm head shape and the pattern of nuclear condensation during spermiogenesis are poorly understood. Previous studies have suggested that the species-specific shape of the sperm head could be due to involvement of the manchette (Fawcett *et al.*, 1971; Yoshida *et al.*, 1994), DNA packaging (Calvin, 1976), and the peri-nuclear theca (Bellve *et al.*, 1992). However, if and how these structural and biochemical processes influence the shape of the sperm nucleus is unclear.

Within a given human semen sample, there is marked morphological variation, with most morphologically abnormal sperm having head defects but some also having defects in the neck or tail regions. Significant morphological differences can also be seen in sperm of fertile and sub-fertile men and it is well known that sperm morphology correlates with fertilising ability (Kruger *et al.*, 1988; Liu *et al.*, 1988; Grow *et al.*, 1994). Fertilisation and pregnancy outcomes after IVF were assessed in men with <9% normal sperm morphology and men with normal semen parameters,

and significantly lower fertilisation rates (69.2 vs 79.4% respectively), pregnancy rates per cycle (12.0 vs 42%), pregnancy rates per transfer (13.9 vs 42.0%) and implantation rates per embryo transferred (6.1 vs 14.8%) were reported (Ombelet *et al.*, 1994). In men with <20% normal sperm morphology, fertilisation rates were still significantly lower (45 vs 72%) than in fertile, normospermic men respectively, but comparable rates were seen for the other outcomes (Terriou *et al.*, 1997). Studies in the Reproductive Medicine laboratory at The Queen Elizabeth Hospital have also shown that sperm morphology has the strongest correlation with the fertilisation rate after IVF (Duncan *et al.*, 1993).

In this study, the positions of individual chromosomes (centromeres) were used to study if a random or organised packaging existed and whether specific chromosomes have a defined location within the sperm nucleus. This was to identify whether abnormal chromosome packaging during spermiogenesis could influence the morphology of morphologically abnormal sperm. If this were the case, then a more random arrangement of chromosomes should be seen in morphologically abnormal sperm than in morphologically normal sperm. It is difficult to directly compare morphologically abnormal and normal sperm as marked morphological variations are seen in a given semen sample and sperm morphology is altered during FISH. Thus, chromosomes were localised in sperm from two groups of men with markedly different sperm morphology, sub-fertile men (<10 % normal morphology) and fertile men (>20% normal morphology). The specific aims of this study were to investigate the localisation of chromosomes in human sperm and examine whether: (i) a given

chromosome has a defined localisation in the sperm head, (ii) there are inter-chromosomal differences, (iii) there are differences in localisation between morphologically normal and abnormal sperm.

5.2 Materials and methods

5.2.1 Subjects and FISH procedures

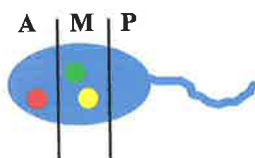
Marked differences in sperm morphology were seen between the two groups of men used in chapter 4, $2.4 \pm 2.9\%$ normal morphology in the TSD group and $36.4 \pm 7.5\%$ normal morphology in the NS group. These samples were therefore used to generate data representative of morphologically abnormal and normal sperm respectively.

The centromere and telomere of chromosome 1, and the centromeres of chromosomes 18, 21, X and Y were used to indicate the relative positions of chromosomes in the nucleus. Protocols for sample preparation, decondensing, hybridization of probes and examination of signals are detailed in chapter 4.

5.2.2 Scoring criteria

A minimum of 500 haploid (AM18 and X21 or Y21) sperm were scored from each slide and all scoring was carried out using the Cytoscore® program. For each sperm, the position of each chromosome was scored in one of three regions, anterior (A), middle (M), or posterior (P). These regions were arbitrarily defined as equidistant sub-divisions of the longitudinal axis of the sperm head to determine if one

or more of the chromosomes was more likely to be situated towards the acrosomal, central or tail region of the sperm nucleus.



5.2.3 Statistical analysis

Statistical analyses were performed using Excel 5.0 (Microsoft Corporation, Redmond, WA, USA). Differences in chromosome localisation were analysed using two-sample *t*-tests. A *P* value < 0.05 was considered significant.

5.3 Results

Chromosomes 1p36.3, 1, 18, 21, X and Y were localised in a total of 10,225 sperm from the NS group and 10,005 sperm from the TSD group. The chromosome distribution was 24-31% in the anterior region, 45-62% in the middle region and 9-27% in the posterior (tail) region (Table 21, Figure 25).

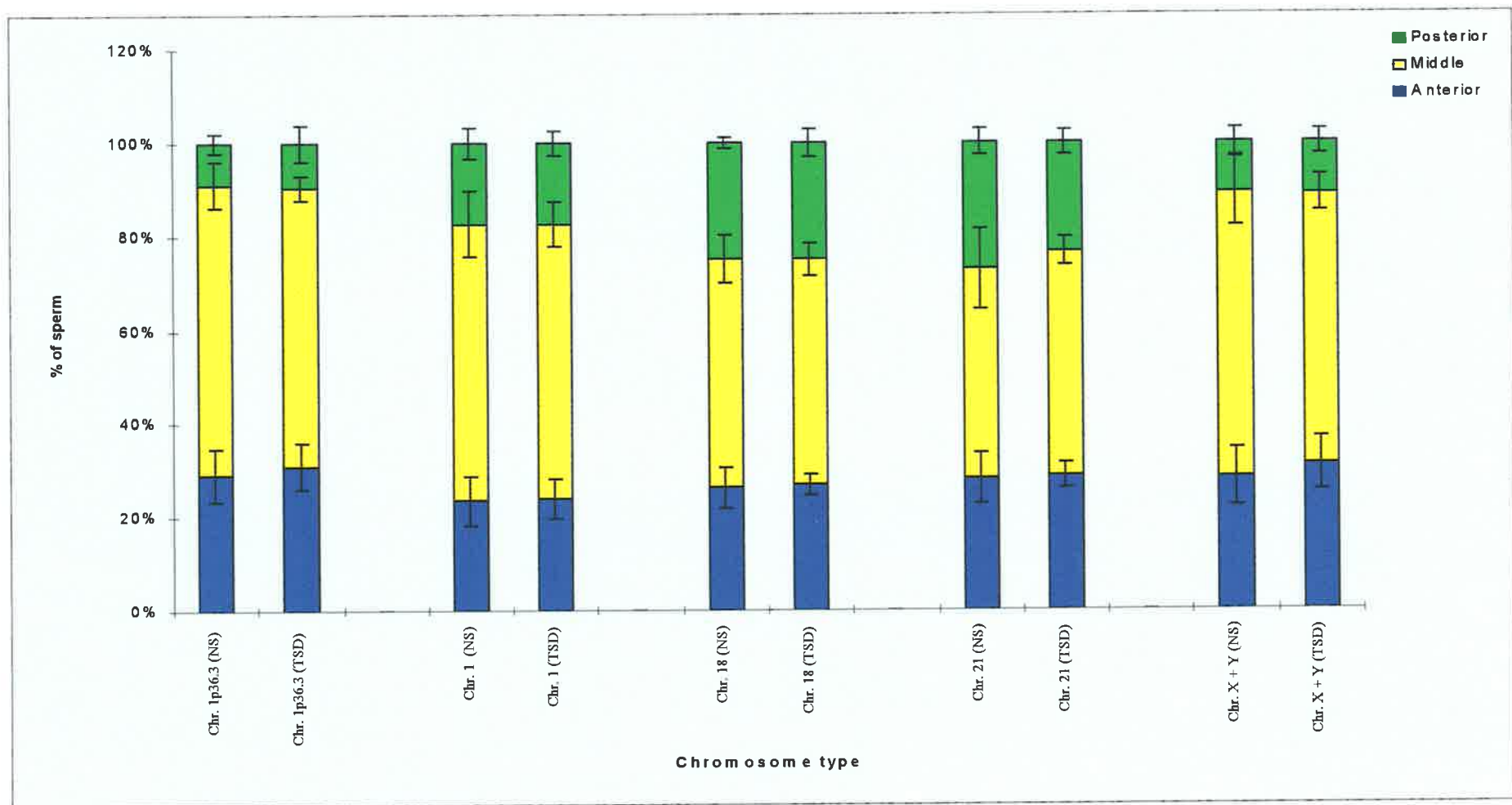
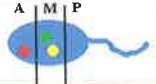


Figure 25. Localisation of chr. 1p36.3, 1, 18, 21, X and Y in morphologically normal sperm (NS group) and morphologically abnormal sperm (TSD group), mean $\pm$ SD.

Table 21. Localisation of chromosomes in sperm from NS and TSD groups, mean values expressed as a percentage of the total.

		Anterior (%)	Middle (%)	Posterior (%)	Total (%)
Chr. 1p36.3	NS	29.09	62.00	8.91	100
	TSD	30.93	59.43	9.64	100
Chr. 1	NS	23.51	59.23	17.26	100
	TSD	23.95	58.60	17.45	100
Chr. 18	NS	24.86	49.73	25.41	100
	TSD	26.84	48.24	24.92	100
Chr. 21	NS	28.12	44.85	27.03*	100
	TSD	28.69	47.87	23.44	100
Chr. X + Y	NS	28.30	60.94	10.76	100
	TSD	31.15	57.68	11.17	100

* = significant difference, $p=0.007$.

The 25th and 75th percentiles were calculated to detect any markedly different values for individuals in each group. In the TSD group the distribution of chromosomes was similar for each area, except for one man where the sex chromosomes were less frequently localised to the anterior region (19.2%) than the 25th percentile (29.0%). No inter-individual differences were detected in the NS group. The only difference between the TSD and NS groups was the frequency of chromosome 21 in the posterior region, which was greater in the NS group (27.03%) than the TSD group (23.44%), $t\text{-test}=2.1$, $P=0.007$.

The positions of the five chromosomes, in both groups of men, can be described as essentially random as there was a fairly consistent distribution throughout the three areas (24-31% in the anterior region, 45-62% in the middle region and 9-27% in the posterior region), and none of the chromosomes were excluded from any region. However, there were significant differences in the distribution of chromosomes in the

anterior, middle and posterior regions (Table 22).

Table 22. Differences detected in distribution of chromosomes in sperm in the anterior, middle and posterior regions for both groups (NS vs TSD).

ANTERIOR	Chr. 1p36.3		Chr. 1		Chr. 18		Chr. 21		Chr. X + Y	
	NS	TSD	NS	TSD	NS	TSD	NS	TSD	NS	TSD
Chr. 1p36.3	-	-	P<0.05	P<0.05	-	P<0.05	-	-	-	-
Chr. 1	P<0.05	P<0.05	-	-	-	-	P<0.05	P<0.05	P<0.05	P<0.05
Chr. 18	-	P<0.05	-	-	-	-	-	P<0.05	-	-
Chr. 21	-	-	P<0.05	P<0.05	-	P<0.05	-	-	-	-
Chr. X + Y	P<0.05	P<0.05	-	-	-	-	-	-	-	-

p<0.05 = significant difference, - = no difference

MIDDLE	Chr. 1p36.3		Chr. 1		Chr. 18		Chr. 21		Chr. X + Y	
	NS	TSD	NS	TSD	NS	TSD	NS	TSD	NS	TSD
Chr. 1p36.3	-	-	P<0.01	-	P<0.01	P<0.01	P<0.01	P<0.01	-	-
Chr. 1	P<0.01	-	-	-	P<0.01	P<0.01	P<0.01	P<0.01	-	-
Chr. 18	P<0.01	P<0.01	P<0.01	P<0.01	-	-	-	-	P<0.01	P<0.01
Chr. 21	P<0.01	P<0.01	P<0.01	P<0.01	-	-	-	-	P<0.01	P<0.01
Chr. X + Y	-	-	-	-	P<0.01	P<0.01	P<0.01	P<0.01	-	-

p<0.01 = significant difference, - = no difference

POSTERIOR	Chr. 1p36.3		Chr. 1		Chr. 18		Chr. 21		Chr. X + Y	
	NS	TSD	NS	TSD	NS	TSD	NS	TSD	NS	TSD
Chr. 1p36.3	-	-	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	-	-
Chr. 1	P<0.01	P<0.01	-	-	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
Chr. 18	P<0.01	P<0.01	P<0.01	P<0.01	-	-	-	-	P<0.01	P<0.01
Chr. 21	P<0.01	P<0.01	P<0.01	P<0.01	-	-	-	-	P<0.01	P<0.01
Chr. X + Y	-	-	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	-	-

p<0.01 = significant difference, - = no difference

The distributions of the five chromosomes were similar in the anterior region, although some differences were seen between chromosomes. The most notable difference was in the posterior region, where the telomeric region (1p36.3) of chromosome 1 and the sex chromosomes were rarely (<13%) present (Figure 25), and significant differences were detected between these chromosomes and the other

autosomes (chrs. 1, 18, 21). The differences in the posterior region were also reflected in the middle region.

To summarize, these five chromosomes are essentially randomly distributed within the human sperm nucleus. The majority of the chromosomes were localised to the middle region, with notable differences in the posterior region. The telomeric region (1p36.3) of chromosome 1 and the sex chromosomes were less likely to be localised to the posterior region than the autosomes (chromosomes 1, 18, and 21).

5.4 Discussion

Throughout this study two assumptions were made.

(i) Sperm chromosomes cannot be visualised without decondensation of the nucleus, either in the oocyte or by chemical degradation of the disulphide cross-links. It was therefore necessary to decondense sperm nuclei prior to hybridization, and the unavoidable assumption therefore had to be made that the relative positions of the chromosomes were not disrupted by this procedure.

(ii) At the time this study was performed, it was only possible to use centromeric or unique sequence DNA probes in sperm, as probes that hybridize to the whole chromosome were difficult to apply to the condensed sperm nucleus. Furthermore, because centromeric and telomeric probes were used, it was assumed that the positions of these sequences were representative of the chromosomal position. Since then, FISH using WCP probes has been successfully applied to human sperm (Rives *et al.*, 1998) and in the future it may be more useful to use this method

as less decondensation is involved and whole chromosomes can be visualised within the sperm head.

The decondensing procedure used in this study involved treating sperm with DTT and LIS to consistently swell the sperm head. This procedure has been used for several years and reliably results in >99% hybridization efficiency of all the DNA probes used (Robbins *et al.*, 1993; Lowe *et al.*, 1995; Van Hummelen *et al.*, 1996, Downie *et al.*, 1997b). Using phase contrast, swelling of the sperm head is 1.5-2 times that of an untreated nucleus, the tails are still attached, and the shape is rounder but no other changes to the morphology of the sperm head are seen. Other studies have also assumed that decondensation doesn't change the location of chromosomes. Powell *et al.* (1990) were confident that the DNA was evenly distributed over the sperm head after decondensing and that hybridization would not be biased if, "...DNA decondensation proceeded equally throughout the head to allow uniform access of probes and thus obviate artefacts." Zalensky *et al.* (1993) tested hybridization on untreated and heparin decondensed nuclei, and found a similar pattern for the centromeres in untreated cells that hybridised, and were confident that the nuclear isolation and heparin decondensation procedures had not produced this result. Ward *et al.* (1996) stated that an intact sperm nucleus after decondensation and FISH, and a consistent distribution of DNA, were indicators that the *in vivo* arrangement of the DNA sequences had not been altered.

In this study, few differences were seen in chromosomal location between morphologically abnormal and normal sperm, except for chromosome 21 which

occurred more frequently in the posterior region of sperm in the NS group (27.03%) than in the TSD group (23.44%), $P=0.007$. It is difficult to directly interpret these results as we have looked at two different groups of men and not at individual morphologically normal or abnormal sperm. It could have been possible to compare morphologically normal and abnormal sperm within an individual sample by using Percoll gradient separation (40% vs 80%) as it has been shown that fewer abnormal cells are found in the bottom layer than at the top (7% vs 82% respectively) (Makler *et al.*, 1998). However, this would have resulted in intra-sample comparisons only, and in the present study there was adequate differences in sperm morphology between the two sample groups ($2.4 \pm 2.9\%$ normal morphology in the TSD group vs $36.4 \pm 7.5\%$ normal morphology in the NS group) to detect variation in chromosomal location if it were present.

Thus, it can be inferred that the similarity in chromosomal localisation in both groups suggests that chromosome packaging does not significantly influence the morphology of sperm. A study by Lee *et al.* (1997) reported that factors such as DNA content of the sperm nucleus, differences in chromatin organization, and the extent of DNA compaction do not cause abnormal head morphologies in fertile males. Although they did not directly compare sperm morphologies between fertile and infertile males, their results indicate that similar DNA compaction is seen in sperm with abnormal morphologies and may also explain the similar distribution of chromosome localisation observed in the two groups of men in the present study.

Overall, in both groups, no specific location was identified for any of the five

chromosomes and there was essentially a random distribution of centromeres throughout the sperm nucleus. A notable difference was the less frequent occurrence of the telomeric region of chromosome 1 and the sex chromosomes in the posterior region.

Previous studies have found centromeric DNA localised throughout the sperm nucleus, as in the present study, and that nuclear structures organized the DNA (Zalensky *et al.*, 1993; Barone *et al.*, 1994). Telomeric DNA has been localised throughout the sperm nucleus, as in the present study, but mainly to the periphery of the nucleus (Zalensky *et al.*, 1993). Later reports by this group indicated that the peripheral localisation of telomeres is dependent on the treatment of sperm with nonionic detergents, suggesting an association with the nuclear membrane (Zalensky *et al.*, 1995; 1997). Ward *et al.* (1996) identified preferred locations for three genes within the hamster sperm nucleus and suggested that the relative arrangements of chromosomes were flexible. They suggest nuclear structures organise the localisations of centromeres and telomeres, but not the manner in which chromosomes are packaged. In contrast, Joffe *et al.* (1998) suggested that structures in the sperm nucleus selectively recognise specific DNA sequences on chromosomes producing their particular arrangement within the nucleus.

Models for chromosome packaging in mammalian sperm nuclei have been proposed (Ward and Zalensky, 1996). Three models have been suggested, characterized by the centromere clustering in the central region of the sperm nucleus, and distinguished by the positioning of telomeres. In one model based on human

sperm, the chromosomes are arranged in a hair-pin structure with homologous chromosome ends (telomeres) forming dimers around the periphery of the nucleus (Zalensky *et al.*, 1995). Another model based on rat and mouse sperm, suggests that the chromosomes are stretched across the nucleus with homologous telomeres opposite each other along the periphery of the nucleus. A third model based on rat and mouse sperm, suggests that the centromeres are situated more to one side, with chromosomes in a hair-pin structure and homologous telomeres forming dimers and clustering together opposite to the centromeres (Zalensky *et al.*, 1997). A further report by Ward (1997) suggested a similar model for DNA packaging in sperm and the organization of chromosomes by different sequences found along the length of the chromosome.

5.5 Summary

The results of the present study suggest that packaging of chromosomes in the sperm nucleus during spermatogenesis is essentially random and therefore not strictly regulated, as the centromeres for all five chromosomes and the telomeric region 1p36.3, were similarly distributed throughout the sperm head. It was interesting to note, though, that the telomeric region of chromosome 1 and the sex chromosomes were less frequently present in the posterior region of the sperm head, suggesting some degree of organized packaging. How this occurs is uncertain, but it may result from a protective mechanism packaging the sex-determining chromosome (X or Y) in the middle of the sperm nucleus, and as suggested in other studies, the telomeres located more at the periphery of the anterior and middle areas of the nucleus.

It is evident that sperm DNA is highly organized in the nucleus by various structures and it has been suggested that specific chromosome sequences are involved in this organization. Further work needs to be performed using FISH to determine the specific location of telomeres, different chromosomes (centromeres) and possibly whole chromosomes with the advent of suitable protocols for WCP probes, to determine the degree of chromosome organisation in the human sperm nucleus.

Concluding Statement

Male infertility is a common problem presenting to reproductive medicine units worldwide. There has been a significant improvement in the treatment of male infertility since the introduction of intracytoplasmic sperm injection (ICSI), which is now used routinely to treat many couples with severe male factor infertility and unexplained fertilisation failure, including men who have severe triple semen defects (TSD). It is now possible for these men to father their own children rather than consider donor insemination or adoption. However, the natural barriers to fertilisation by abnormal sperm have been removed by ICSI and this has raised concerns about whether there are increased genetic risks to the embryo and offspring. Published data to date, have shown a slight increase in sex chromosomal abnormalities and a paternal inheritance of structural abnormalities in some children conceived through ICSI. For this reason it is important to study the chromosomal content of sperm that are likely to be artificially selected for the ICSI procedure.

FISH is an important tool for the detection of chromosomal abnormalities in human sperm. Since its inception, the technique has developed so that it is now possible to screen large numbers of sperm with different DNA probes to reliably estimate the incidence of numerical (aneuploidy) and structural chromosomal abnormalities. There are now many published reports using FISH on sperm which have led to an appreciation of several important technical issues. Particular attention should be given to optimal pretreatment and hybridization conditions, the influence of different probes and the effect of technical variations on estimates of aneuploidy.

In this thesis, the principal hypothesis examined was that men with TSD have an increased frequency of chromosomal abnormalities in their sperm. This hypothesis was tested to elucidate whether men requiring ICSI were more likely to transmit chromosomal abnormalities to their offspring. This involved the development of many of the FISH procedures used. Once reliable results were being obtained, it was then possible, with confidence, to design and conduct a FISH study on sperm from sub-fertile men. In addition to these aneuploidy studies, the localisation of chromosomes was also compared in morphologically abnormal and normal sperm to examine whether there is a random or specific chromosome packaging and ascertain if this is influenced by sperm morphology.

Inadequate application of FISH to sperm makes it difficult to compare results from different laboratories. For this reason, considerable care was taken in this study to ensure that reliable and reproducible FISH protocols were developed. A large number of DNA probes were tried until different combinations produced reliable and compact fluorescent signals in sperm (Chapter 2). Successful protocols were developed for chromosomes 3, 7, 16, and the sex chromosomes (X, Y), and these were then applied to sperm from 10 normospermic men (Chapter 3) to obtain reliable baseline frequencies of chromosomal abnormalities in sperm, which was not available in the literature at that time. The frequency of aneuploidy was found to be quite low (<0.20% per chromosome). An important finding was that inter-chromosomal and inter-individual variability were significant considerations when estimating aneuploidy in sperm.

The most clinically relevant aspect of this thesis was the investigation of chromosomal abnormalities in sperm from men with severe TSD (Chapter 4). Extensive time was involved in recruiting men to this study to ensure that a specific subset of sub-fertile men, candidates for ICSI, were investigated. This study was conducted in collaboration with Lawrence Livermore National Laboratory (LLNL) as Dr. Andrew Wyrobek had many years expertise in the development and application of FISH to sperm. Two FISH techniques were used, the AM18 and XY21 assays, which enabled, for the first time, the study of structural abnormalities in sperm from men with TSD using a probe for the chromosomal region 1p36.3. Careful attention was given to selection of samples, preparation of samples, and scoring criteria. Intensive training in aneuploidy scoring was completed at LLNL prior to scoring the sample groups. To date, very few published FISH studies have used as strict a study design as was employed in this project.

Sperm from 10 men with severe TSD and 10 normospermic men were analysed for duplications and deletions of chromosome 1p36.3 and aneuploidy for chromosomes 1, 18, 21, and the sex chromosomes. No statistically significant increases in abnormalities for these chromosomes were found, which contrasts with many of the recent published studies which reported up to 10-fold increases in aneuploidy in sperm from infertile men. The results of the present study correlate well with the excellent clinical outcomes of ICSI pregnancies. Some evidence exists for a slight increase in the sex chromosomal aneuploidy rate in ICSI children, however, it appears that a few select individuals are responsible for this increase and that most

men undergoing ICSI are at no greater risk of transmitting chromosomal abnormalities to their offspring than normospermic men. Two subjects from the TSD group were found to have a higher incidence of chromosomal abnormalities than the rest of the group. It is possible that these men are at a greater risk of miscarriage or of conceiving a chromosomally abnormal child. It will be important to conduct further studies, using a strict study design similar to the present one, on subgroups of infertile men undergoing ICSI to confirm the risk of transmission of chromosomal abnormalities to their offspring and to provide an accurate basis for counselling couples prior to undertaking ICSI.

In the final chapter of this thesis, the arrangement of chromosomes within morphologically abnormal and normal sperm was investigated to ascertain whether chromosome packaging may have an influence on sperm head shape. No significant differences in the localisation of five chromosomes (1, 18, 21, X and Y) were found between the two groups of men, which suggests that chromosome packaging does not markedly influence sperm head morphology.

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