THE UNIVERSITY OF CALGARY

CHROMOSOMAL ANALYSIS OF SPERM FROM INFERTILE MEN USING SPERM KARYOTYPING AND MULTI-COLOUR FLUORESCENCE *IN SITU* HYBRIDIZATION

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

JUNE, 1995

THE UNIVERSITY OF CALGARY
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Chromosomal Analysis of Sperm From Infertile Men Using Sperm Karyotyping and Multi-colour Fluorescence *In Situ* Hybridization" submitted by Nafisa Moosani in partial fulfillment of the requirements for the degree of Master of Science.

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June 22, 1995

ABSTRACT

There is some concern that infertile men may have a higher frequency of chromosomal abnormalities in their sperm but there is no direct evidence linking abnormal semen parameters with chromosomal errors. Using the human sperm / hamster oocyte fusion technique and fluorescence *in situ* hybridization (FISH), sperm chromosomal studies were completed to determine if infertile men with oligo-, astheno- or teratozoospermia have an elevated risk of chromosomal abnormalities in their sperm.

Sperm chromosomal complements from five 46,XY infertile men were assayed using the human sperm / hamster oocyte fusion system. The sperm karyotypes from the infertile men were difficult to obtain because of suboptimal sperm parameters; however, a total of 518 karyotypes was analyzed from the five men. These karyotypes showed increased frequencies of numerical chromosomal abnormalities and total chromosomal abnormalities, when compared to those obtained from normal men with proven fertility. The frequency of numerical chromosomal abnormalities was high not only because of increased numbers of hypohaploid spreads, which may be a technical artefact, but also because of an increased frequency of hyperhaploid spreads. A conservative estimate of aneuploidy for the sperm karyotypes obtained from the five infertile men was significantly elevated at 3.1% ($p<0.005$) relative to that found in normal men with proven fertility (0.84%).

Using FISH analysis, 20,000 sperm nuclei were analyzed for each of 10 infertile men studied: 10,000 for chromosomes 1, 12, and an additional 10,000 for the sex chromosomes. Investigation of the autosomes was carried out using two-colour FISH and

three-colour FISH was utilized for the sex chromosomes with chromosome 1 as an internal hybridization control. The results obtained by FISH indicated a significant increase in the frequencies of disomy for chromosome 1 and XY disomy. These results support those obtained in the sperm karyotypes thereby suggesting that in the ejaculate of infertile men, there exists a higher frequency of chromosomally-abnormal sperm relative to that seen in normal men.

ACKNOWLEDGEMENTS

Much appreciation to my supervisor Dr. Renée Martin for her guidance, advice, and encouragement in facilitating my learning. She provided a model of excellence in research, professional conduct and personal growth. I also gratefully acknowledge all the members of my supervisory committee including, Dr. David Cox, Dr. Anthony Pattinson, and Dr. Birgitte Roland for sharing their valuable time, knowledge and expertise in furthering my project. Dr. Gil Schultz is gratefully acknowleged for his contribution as examiner.

Special thanks to the staff in the various departments without whom this work would not have been possible. This includes the staff at the Regional Fertility Clinic, and the staff in the Cytogenetics laboratories at both the Alberta Children's Hospital and the Foothills Hospital.

Many thanks also to Dr. Alfred Rademaker for statistical consultation and to Dr. Michael Carter for his sincere interest and recruitment of infertile patients.

There is not enough room to thank all the many people I have met in the past several years in the Genetics Department. Many thanks to Dr. Brian Lowry and all the department for providing a stimulating, encouraging, and friendly environment.

Thank you to all the members in Renée's lab. You all guided my learning and taught me about the various techniques while also providing me with an education on life and all that it entails. Beth, Evelyn, Leona: thank you for the stimulating environment in which, each day, I looked forward to seeing you all. Finally, thank you to Beth and Evelyn for reading this manuscript.

DEDICATION

This is to my parents, grandmother, and Karim. They have always given me the strength, motivation and encouragment to strive forward each day.

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ace	acentric fragment
BWW	Biggers-Whitten-Whittingham medium
°C	degree Celsius
cen	centromere
csb	chromosome break
csg	chromosome gap
ctb	chromatid break
cte	chromatid exchange
ctg	chromatid gap
Cy3™	Cyanine-3
DAPI	4, 6, diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dUTP	deoxyuridine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
del	deletion
dic	dicentric
EDTA	ethylenediamine tetraacetic acid
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
G	force of gravity
g	gram
ICSI	intracytoplasmic sperm injection
inv	inversion
ISCN	International System for Human Cytogenetic Nomenclature
IU	International Units
IUI	intrauterine insemination
IVF	in vitro fertilization
LIS	3, 5, diiodosalicyclic acid-lithium salt
M	molarity
mar	marker
MB	multiple breaks
MB+R	multiple breaks and rearrangements
mg	milligram
MI	meiosis I
MII	meiosis II
ml	millilitre
N	normality
ng	nanogram
p	short arm of a chromosome
PZD	partial zona dissection

q	long arm of a chromosome
Q-band	quinacrine dihydrochloride banding stain
SUZI	subzonal (sperm) insemination
TEST	TES-Tris
μg	microgram
μl	microlitre
μm	micrometre
X^2	chi square statistic

INTRODUCTION

"To God belongs
the kingdom of the heavens and the earth.
He creates whatsoever He wills,
bestows daughters on whosoever He will,
and gives sons to whom He choose.
On some He bestows both sons and daughters,
and some He leaves issueless.

-Qur'an 42:49-50

For centuries, societies have looked to a number of Holy Books for political, social, and theological messages which are incorporated into societies values and beliefs. Fertility is often perceived as a blessing from a higher being and thus infertile couples are faced with great emotional stress. Infertility, the inability to produce live offspring after attempting to do so for one year, affects 8% of all Canadian couples (Royal Commission on New Reproductive Technologies, 1993). In the past, the female partner was primarily investigated and only after her reproductive capabilities were determined to be normal was the male partner studied. It is now known that approximately one-half of the cases of infertility are due to male factors (Chandley *et al.*, 1975). The majority of these infertile men are considered untreatable, with infertility due to oligozoospermia ($\leq 2 \times 10^7$ sperm/ml), asthenozoospermia (<40% motile) or teratozoospermia (<40% normal morphology). In an attempt to overcome these problems by the juxtaposition of sperm and oocytes, some couples with male-factor infertility are offered *in vitro* fertilization (IVF). Unfortunately, IVF has a low rate of success for men with abnormal sperm characteristics (De Krester *et al.*, 1985). Microinjection of sperm under the zona pellucida (Ng *et al.*, 1991) or into the

cytoplasm (Van Steirteghem *et al.*, 1993) is now performed as an alternative to IVF.

A correlation between cytogenetic abnormalities and oligozoospermia exists (Chandley *et al.*, 1975), and consequently there are concerns about chromosomal abnormalities present in the sperm of infertile men. Few studies have investigated the frequency of chromosomal abnormalities in the sperm of infertile men. Thus, at the present time, it is unknown whether an infertile couple might carry a greater risk of having a chromosomally-abnormal child than that of a fertile couple.

A. Chromosomal Abnormalities Associated with Infertility

An individual with a constitutional chromosomal abnormality carries the abnormality in all the cells within the body. In a mosaic individual, the chromosomal anomaly is restricted to certain cell populations. Any loss or gain of chromosomal material in the cells of an individual has an effect; the severity of the effect depends on the nature of the chromosomal abnormality, the proportion of the cells expressing the abnormality, and the environmental influence present.

i. Constitutional Chromosomal Abnormalities

a. Liveborn Human Sex Chromosomal Aneuploidies

Sex chromosomal aneuploidy occurs at a higher frequency than any autosomal aneuploidy found in liveborns. Specifically, one in every 476 liveborn males and one in every 820 liveborn females has a sex chromosomal aneuploidy, whereas one in every 737 liveborns has an autosomal trisomy (Nielsen and Sillesen, 1975). The sex chromosomal aneuploidies which survive to term are 45,X and 47,XXX in females, and 47,XXY and 47,XYY in males. Though the effects of sex chromosomal

aneuploidy can be variable, infertility is a problem seen in a significant proportion of these individuals.

Females with a 45,X karyotype, associated with Turner Syndrome, are rare relative to the other sex chromosomal aneuploidies as a large proportion die in utero. Of those that survive, they are characterized with fibrous ovaries containing ovarian stroma devoid of follicles. Primary amenorrhoea and the lack of secondary sexual characteristics are also associated with the 45,X karyotype (Lindsten, 1963).

The phenotype of 47,XXX females is variable and physically unremarkable. Though it was first studied by Jacobs and associates (1959) in two amenorrhoeic women, most women with this condition menstruate normally and many have children (Stewart and Sanderson, 1960). However, examinations of 47,XXX women have shown that their ovaries contain fewer follicles, and are comparable to ovaries seen in women at or near menopause (Johnson *et al.*, 1961).

Males with the 47,XXY karyotype associated with Klinefelter syndrome are generally infertile, characterized with aspermatogenesis. The testes of these men are small, hyalinized and devoid of germ cells. However, fertile tubules have occasionally been found in the testes of men with Klinefelter syndrome (Skakkebaek *et al.*, 1969), and spermatozoa have been found in their ejaculate (Foss and Lewis, 1971). The frequency of Klinefelter syndrome amongst the men attending infertility clinics has been reported to be 10 times greater than the incidence of Klinefelter syndrome observed in the newborn male population (Chandley, 1979).

The phenotype associated with 47,XYY karyotype is not as well-characterized as that of men with Klinefelter syndrome. There is considerable variation in the histological appearance of the adult testis, with some individuals showing normal spermatogenic activity and others exhibiting varying degrees of impairment in sperm maturation (Skakkebaek *et al.*, 1973).

b. Liveborn Human Autosomal Aneuploidies

When comparing the aneuploidy frequency in spontaneously-aborted fetuses to the frequency seen among liveborns, it becomes apparent that embryonic mortality eliminates the majority of aneuploid fetuses. The relatively small proportion of autosomal trisomies that survive to term are mainly trisomies of chromosomes 13, 18 and 21. Few infants with trisomies of chromosomes 13 or 18 survive beyond six months of life; most individuals with trisomy 21 (Down syndrome) survive to adulthood.

Individuals with trisomy 21 show poorly-developed sexual characteristics and late sexual development (Smith and Berg, 1976). Female trisomy 21 individuals have abnormal ovaries containing fewer follicles than normal, and the number of follicles declines rapidly after the age of three years (Hojager *et al.*, 1978). Male trisomy 21 individuals show a range of phenotypic effects: some men have normal spermatogenesis and others are azoospermic. However, in trisomy 21 men with normally-descended testes, there is usually a degree of impaired spermatogenesis, and although sperm are present in the ejaculate, sperm counts are usually lower than that seen in normal fertile males (Stearns *et al.*, 1960).

c. Effects of Chromosomal Abnormalities on Sperm

In humans, structural rearrangements occur with a frequency of 1.7 per 1,000 liveborns (Jacobs, 1978). One effect of this type of chromosomal anomaly, particularly in male heterozygotes, is the disruption of spermatogenesis resulting in oligo- or azoospermia. Within the literature, there exist numerous reported cases where spermatogenesis is affected by inversions, reciprocal translocations between the X chromosome and an autosome or the Y chromosome and an autosome, reciprocal translocations between two autosomes, or Robertsonian translocations (Bourrouillou *et al.*, 1985; Andersson *et al.*, 1988; Micic *et al.*, 1992; Gabriel-Robez *et al.*, 1986; Chandley *et al.*, 1986).

Since the first study performed by Ferguson-Smith *et al.* (1957) that focused on men attending infertility clinics, many subsequent studies have focused on these men and have confirmed that chromosomal and genetic factors make a significant contribution to human male infertility. Retief and associates (1984) studied lymphocyte karyotypes from 106 azoospermic men and 390 oligozoospermic men. They determined that constitutional chromosomal abnormalities were found in 14.1% of azoospermic men and 5.1% in oligozoospermic men. Within the azoospermic group, all the abnormalities were associated with sex chromosomal aneuploidy. In the oligozoospermic men, 10 of the 20 chromosomal abnormalities found involved sex chromosomal aneuploidy, while the other 10 involved autosomal translocations, inversions or marker chromosomes. A similar trend was observed by Bourrouillou *et al.* (1985), who reported a major chromosomal abnormality in 10.3% of the 952

oligozoospermic men studied.

Most studies of infertile men have focused on problems associated with the number of sperm present in the ejaculate. Relatively few studies have determined the chromosomal content of sperm from men with qualitative disorders (asthenozoospermia and teratozoospermia). Having recognized the lack of knowledge available on qualitative disorders, Eiben *et al.* (1987) studied infertile, teratozoospermic males cytogenetically. Of the 109 patients studied, four men were found to carry chromosomal anomalies: three were mosaic for Klinefelter syndrome and one had a balanced translocation. Interestingly, 28 patients (25.7%) of the 109 men studied carried a heterochromatic variant, 9qh+. The authors suggested that this variant, which was found in a significantly higher proportion of these infertile men as compared to the control group, may be a factor which disrupts spermatogenesis. Matsuda and associates (1991) compared the sperm morphology of nine translocation carriers to that of infertile men with a normal karyotype. The two groups were matched for age, sperm concentration, sperm motility and the presence or absence of a varicocele. There was no significant difference in the percentage of abnormal sperm and it was concluded that structural chromosomal aberrations have no relation to sperm morphology. These results support prior findings by Martin and Rademaker (1988), who found no significant relationship between the proportion of morphologically-abnormal sperm and the frequency of chromosomally-abnormal sperm in normal men, and concluded that sperm morphology was not a valid indicator of the chromosomal content of the sperm.

ii. Mosaicism

It has been suggested that all infertile men should undergo routine cytogenetic investigation, since it is known that constitutional chromosomal abnormalities occur frequently in this group of men. Specifically, there is a ten-fold increase in the frequency of reciprocal translocations, an 18-fold increase in the incidence of Robertsonian translocations, and inversions are seen 37 times more often in the infertile male population than in newborn males (Bourrouillou *et al.*, 1985). However, cytogenetically-normal lymphocytes in an infertile man does not rule out the possibility of chromosomal mosaicism. Six large surveys have studied meiotic anomalies in infertile and sterile men with a somatically-normal karyotype. Investigations of meiotic anomalies detectable only through studies of meiosis have determined that the frequency of an anomaly limited to the germ cell line in males with fertility problems varies between 1.4% (Chandley *et al.*, 1976) and 17% (Hendry *et al.*, 1976), with a mean of 6.6% (Egozcue *et al.*, 1983), indicating that even infertile men with a normal lymphocyte karyotype are at risk for having a chromosomally-abnormal child as a consequence of cryptic mosaicism.

iii. Predisposition to Chromosomal Abnormalities

Apart from constitutional chromosomal abnormalities and mosaicism, there is a possibility that infertility is related to chromosomal instability. Studies using cytogenetic techniques on mitotic chromosomes from lymphocytes obtained from couples experiencing reproductive wastage reveal that these individuals show a significantly higher frequency of hypermodal chromosomal spreads (those with an

extra chromosome present) than that found in the normal population (Juberg *et al.*, 1985). These results suggest that couples experiencing reproductive wastage may be predisposed to nondisjunction in mitotic cells; it is possible that this predisposition may also exist in meiotic cells, affecting gamete production. The abnormal number of chromosomes within an affected germ cell would lead to an aneuploid conceptus, which in most cases would be spontaneously aborted. In an attempt to verify these results, Rosenbusch and Sterzik (1991) investigated men with a normal 46,XY karyotype who had experienced recurrent reproductive losses. Analysis of sperm karyotypes, obtained using human sperm / hamster oocyte fusion analysis, did not reveal a significant difference in the aneuploidy rate from that seen in normal fertile donors. However, a significantly higher level of chromosome breaks and acentric fragments was observed in these men relative to a group of men with normal fertility. Rosenbusch and Sterzik (1991) suggested that increased levels of chromosome breaks and acentric fragments in the sperm from these men may contribute to fetal chromosomal abnormalities through the subsequent loss of chromosome pieces in early embryonic divisions.

B. Pairing Abnormalities During Meiosis

Proper chromosome segregation and distribution during meiosis is essential for producing viable gametes. Federley (1931) recognized, from a study of interspecific hybrids, that chromosome pairing was important and failure of the homologs to pair had detrimental consequences (Miklos, 1974). Subsequently, pairing of chromosomes during meiosis has been studied in a number of diverse organisms including

Drosophila melanogaster, grasshoppers, and mice (Grell, 1969; Cooper, 1964; Hewitt, 1973; Rathenberg and Muller, 1973). These organisms have given insight into the process and importance of chromosome pairing in humans. Two theories of chromosome pairing associated with infertility have been proposed: i) failure of the pairing of the sex chromosomes (Miklos, 1974) and/or the autosomes (Burgoyne and Baker, 1984) and ii) reactivation of the X chromosome (Lifschytz and Lindsley, 1972).

i. Homologous Chromosome Pairing

The pairing associated with the sex chromosomes during male meiosis was investigated in *Drosophila* by Sandler and Braver (1954), who carried out experiments in which the amount of heterochromatin on the X chromosome was varied using deletions of different sizes. They showed that as the size of the deleted segment increased, the frequency of X chromosome loss also increased, and that nullisomic gametes were recovered more frequently than the complementary disomic gametes. Furthermore, Y-bearing sperm were recovered less often than deleted-X-bearing sperm. In fact, with increases in the size of the deletion of the X chromosome, the recovery of the deleted X was more frequent. These results suggest that with reduced pairing of the sex chromosomes, a subsequent increase in the frequency of loss of the sex chromosomes occurs. Miklos (1974) proposed that sperm development in many species, including man, was dependent on pairing of chromosomes. During male meiosis, Miklos (1974) hypothesized that normal chromosome segregation and spermatogenic development required the sex chromosomes to pair for post-meiotic

development to be normal. If this interaction was incomplete and sites on the sex chromosomes remained unpaired, then nondisjunction was more likely to occur, increasing the probability of spermatogenic failure. It was suggested that this disruption within the unpaired regions of the sex chromosomes initiates one of two alternatives which ultimately leads to cell death: prevention of the normal condensation and subsequent packaging of chromatin in the sperm head, or activation of post-meiotic transcription which is not normally present.

Evidence for Miklos' theory came from prior investigations on infertile men. Chandley and Edmond (1971) had studied meiotic chromosomes from a sterile man and had found that in 86% of the cells at diakinesis there were 22 bivalents and two univalent sex chromosomes. In subsequent divisions, there was a large deficiency of cells suggesting that meiotic arrest had occurred, resulting in azoospermia. Further evidence for Miklos' theory came from the study of the 47,XYY condition in man. The fertility of these men is variable, but it has been reported that the extra Y chromosome is usually eliminated prior to the appearance of primary spermatocytes (Thompson *et al.*, 1967; Evans *et al.*, 1970; Tettenborn *et al.*, 1970; Chandley *et al.*, 1976). However, Hulten and Parsons (1971) found that up to 45% of the primary spermatocytes in a 47,XYY patient had a YY bivalent and an X univalent, whereas 49% contained simply an XY bivalent. These results have been confirmed by Berthelsen *et al.* (1981) who found in three out of four XYY patients studied, a significant portion of germinal cells retained an extra Y chromosome. Nonetheless, among fertile XYY men whose offspring have been karyotyped, normal XY sons and

XX daughters have been reported, and the risk of passing an extra Y chromosome to his sons appears to be negligible (Thompson *et al.*, 1967; Court Brown, 1968; Lisker *et al.*, 1968; Benet and Martin, 1988). Miklos suggests that infertility in this group arises mainly from the genetic imbalance resulting from the presence of two Y chromosomes.

As Miklos' theory is restricted to an explanation of the pairing of the sex chromosomes, it is unable to explain infertility seen in men with autosomal translocations. In structural rearrangements where pairing of the homologs is disrupted, for example with a ring chromosome, meiotic arrest of spermatocytes occurs regardless of whether the pairing failure involves the sex chromosomes (Chandley and Edmond, 1971) or an autosome (McIlree *et al.*, 1966). If Miklos' theory about sex chromosome pairing can be extrapolated to the autosomes, a similar dysfunction in the pairing of autosomal homologs will also lead to problems in spermatogenesis. Based on the hypothesis presented by Miklos (1974), Burgoyne and Baker (1984) proposed a mechanism by which gametogenic cells with meiotic pairing failure were removed. Their theory states that those meiotic pairing sites which remain unpaired in the homologous chromosomes become activated, setting in motion a process that eventually leads to destruction of the nucleus. In this theory, spermatogenic disruption depends on the number of unpaired sites: when a significant proportion of sites remain unpaired, inappropriate expression of genes occurs and leads to cell death. Thus, according to Burgoyne and Baker (1984), cell degeneration is the consequence of pairing failure.

It is unknown whether synaptic failure results in cell death, or if cell death occurs prior to pairing anomalies. Setterfield *et al.* (1988), while studying pairing errors in male and female mice, noted that spermatocytes exhibit a much lower frequency of autosomal synaptic failure than that seen in oocytes. They suggested that a major cause of such breakdown was the physiological conditions pertaining to oogenesis and argued that the pairing failure is a result of cell degeneration. These results have been supported in humans by Speed and Chandley (1990), who have determined that for both male and female chromosomally-abnormal individuals, there is severe germ cell degeneration even prior to meiotic prophase and before full pairing is established. However, further studies are required to determine whether cell death occurs prior to pairing and leads to pairing abnormalities.

Homologous chromosome pairing was further studied by Egozcue *et al.* (1983), who investigated meiotic chromosomes in 1100 infertile and sterile males. In 41 of the 1100 men studied (3.27%), there was a synaptic anomaly found: 35 were sterile, two patients had a normal child (paternity not investigated), two patients had repeated first-trimester abortions, one patient had a son with Down syndrome, and another had two mentally-retarded daughters by two different women. In these men, desynapsis was the most common chromosomal abnormality observed. These results suggest that not only is pairing important, but the maintenance of the paired state is critical. Subsequent studies by Navarro *et al.* (1990) on the sperm chromosomes of an oligoasthenoteratozoospermic infertile 46,XY man using the human sperm / hamster oocyte technique showed no difference in the frequency of chromosomal

abnormalities relative to that seen in their normal group. They suggested that those spermatogenic cells with pairing abnormalities do not continue through metaphase I and only normal germ cells, which display correct pairing between homologous chromosomes, continue spermatogenesis.

Further evidence to support the significance of desynapsis is provided by Speed and Chandley (1990), whose study of meiotic prophase in human sperm by electron microscopy determined that higher frequencies of synaptonemal complex fragmentation existed amongst the chromosomes in infertile men than in a normal control group. They also found an increased frequency of autosomal pairing anomalies in sperm obtained from infertile men as compared to control patients with normal spermatogenesis but unknown sperm counts.

The frequency of crossovers in humans was first estimated using cytological preparations of sperm and counting the number of chiasmata seen. A total of 52 crossovers was found, with larger chromosomes showing a greater number of chiasmata than smaller chromosomes (all chromosomes had at least one chiasma) (Morton *et al.*, 1982). Despite the dissimilarity between the X and Y chromosomes, it was assumed that a single obligatory recombination event took place in the pseudoautosomal region located on distal short arms (Chandley *et al.*, 1984; Cooke *et al.*, 1985; Simmler *et al.*, 1985). Though recent studies by Schmitt *et al.* (1994) and Rappold *et al.* (1994) suggest that double crossovers do occur, they are infrequent during male meiosis and thus in the majority of sperm there is usually only one crossover between the X and Y chromosomes. With the assumption of only one

obligatory chiasma, any disruption to the formation of this chiasma would result in an increased frequency of univalency and consequently nondisjunction (Hassold *et al.*, 1991) or sterility (Miklos, 1974).

A question remains to be answered about nondisjunction in male meiosis. Are the chromosomes of infertile men pairing and failing to recombine, or are the chromosomes not pairing at all, making recombination impossible? In an attempt to further investigate recombination, Hassold and associates (1991) studied XY chromosome nondisjunction in paternally-derived Klinefelter boys (47,XXY) and their parents. They were able to successfully detect crossing-over in only six of 39 cases studied. In addition, a genetic linkage map was generated based on meiosis involved in XY chromosome nondisjunction in these 47,XXY men. The map showed that the pseudoautosomal region is significantly shorter in these 47,XXY men than that found in 46,XY men. These results provide some evidence that failure of the sex chromosomes to recombine is associated with subsequent nondisjunction; however, these observations may also be a result of the inability of the sex chromosomes to form and maintain pairing, such that subsequent recombination of the sex chromosomes is hindered.

ii. XY-Autosome Association

Lifschytz and Lindsley (1972) attempted to explain spermatogenic breakdown in sterile heterozygous *Drosophila* males with X-autosome translocations. They suggested that the translocations between the X chromosome and an autosome may disrupt normal X-chromosome inactivation during meiotic prophase, resulting in

biochemical imbalances which would arrest spermatogenesis in *Drosophila*. Forejt (1974, 1979) extended Lifschytz and Lindsley's (1972) hypothesis after seeing autosomal translocations associating closely with the sex chromosomes in sterile mice. Forejt suggested that the incomplete pairing of rearranged chromosomes occurred at pachytene and subsequently resulted in an attraction between unpaired autosomal regions and unpaired parts of the X and Y chromosomes. With this association, X-chromosome inactivation was disrupted and transcription of nonpermissible gene products of the X chromosome occurred, thereby resulting in the final breakdown of spermatogenesis. As it is known that not all individuals carrying an autosomal translocations are sterile, Forejt (1979) suggested that the severity of spermatogenic breakdown depended on the genetic background. Carriers of the same reciprocal or Robertsonian translocation can thus show variable levels of spermatogenic breakdown. Meiotic preparations from various human translocations have shown associations between sex chromosomes and an autosome (Luciani *et al.*, 1984; Rosenmann *et al.*, 1985; Luciani *et al.*, 1987; Johannisson *et al.*, 1987; Guichaoua *et al.*, 1990; Guichaoua *et al.*, 1992). Several investigators have reported a correlation between the frequency of association of a translocation to the sex chromosomes with the degree of spermatogenic disruption (Johannisson *et al.*, 1987; Guichaoua *et al.*, 1990). The higher the frequency of association of the translocation with the sex chromosomes, the higher the degree of spermatogenic arrest.

Though the theories proposed by Miklos (1974) and Forejt (1979) suggest different mechanisms resulting in the final disruption of spermatogenesis, the two

mechanisms could interact simultaneously. Both theories rely on the inability of the homologous chromosomes to pair properly, and it has been suggested that initial pairing failures could result in asynapsed autosomal arms becoming secondarily attracted to the unpaired regions of the sex chromosomes (Speed and Chandley, 1990).

C. Potential Treatments for Male-Factor Infertility

Reproductive advances in artificial insemination have resulted in a variety of procedures aimed at treating female and male-factor infertility. Until the late 1980s, *in vitro* fertilization (IVF) was widely applied as the treatment of infertility. The frequency of successful fertilization after IVF when the infertility is due to the female is high (60-70% fertilization) but is significantly reduced (20-30% fertilization) in andrological cases (Tournaye *et al.*, 1992). To increase the fertilization rates in these cases, sperm have been treated with various agents (Palermo *et al.*, 1992) and attempts have also been made to re-inseminate an oocyte after an unsuccessful IVF treatment (Trounson and Webb, 1984; Boldt *et al.*, 1987; Pampiglione *et al.*, 1990; Fahmy *et al.*, 1991). Fertilization rates of up to 70% have been reported after a second insemination of mature oocytes (Boldt *et al.*, 1987); the same fertilization success rates, however, have not been reported by other investigators (Trounson and Webb, 1984; Pampiglione *et al.*, 1990; Fahmy *et al.*, 1991).

To facilitate re-insemination, Malter and Cohen (1989) employed the partial zona dissection (PZD) technique, where a hole is physically drilled through the zona pellucida using micromanipulation techniques. Fertilization rates were reported to have improved to 50% but high rates of polyspermy were also seen. Pregnancies and

births have been obtained after PZD (Cohen *et al.*, 1991). In an attempt to increase the fertilization rates obtained with PZD, Ng and associates (1988) inserted multiple sperm (3-7 sperm) directly into the perivitelline space of each oocyte. This technique, known as subzonal sperm injection (SUZI) has also been successful, resulting in pregnancies and live births (Levron *et al.*, 1992; Imoedemhe and Sigue, 1993; Soong *et al.*, 1993). However polyspermy is still a concern and for many infertile couples with male-factor infertility the rates of fertilization are still low (Sakkas *et al.*, 1992; Fishel, 1992).

Although some couples who experienced no success using IVF have subsequently had children using PZD or SUZI, fertilization rates have been low and polyspermy is frequent. Further advances in micromanipulation now allow the injection of a single immobilized spermatozoan into the ooplasm through intracytoplasmic sperm injection (ICSI), a technique first introduced by Palermo and associates (1992). Van Steirteghem *et al.* (1993) compared the success of ICSI with that of SUZI in couples unable to be helped by conventional IVF. A total of 2894 oocytes was injected; 37% (1073) underwent SUZI, while 63% (1821) had ICSI. After SUZI, 92.9% of injected oocytes remained intact, while 86.5% of the injected oocytes remained morphologically normal with ICSI. However, the fertilization rates following the procedures were dramatically different: 51% of ICSI oocytes were fertilized as compared to only 14.3% of SUZI oocytes. The number of embryos transferred varied: with SUZI, a single embryo was often transferred, while ICSI more than half of the transfers involved three embryos. A higher number of embryos

were transferred for the ICSI procedure because of the higher fertilization rates resulting in a greater number of embryos available for transfer, a definite advantage to this technique. Though normal, healthy liveborns have resulted because of SUZI and ICSI (Cohen *et al.*, 1994; Van Steirteghem *et al.*, 1994), couples undergoing this procedure are counselled to seek prenatal diagnosis, and further follow-up is being done to ensure the safety of the technique (Van Steirteghem *et al.*, 1993).

D. Research Objectives

The current study was undertaken to examine the chromosomal content of sperm from infertile men experiencing idiopathic infertility and compare the results to those obtained from normal fertile donors. Through the study of sperm karyotypes and aneuploidy detection by fluorescence *in situ* hybridization (FISH), it is hypothesized that in infertile males:

1) the frequency of structural abnormalities, as assessed by sperm karyotyping, will be increased compared to that of normal donors with proven fertility.

2) the frequency of numerical abnormalities, as assessed by sperm karyotyping, will be increased compared to that of normal donors with proven fertility.

3) the frequency of disomy for chromosomes 1, 12, X and Y, as assessed by FISH, will be increased compared to that observed in normal donors.

II. MATERIALS AND METHODS

A. Infertile Men

i. History and Patient Recruitment

Ten patients experiencing idiopathic infertility were recruited for this study from the University of Calgary Infertility Clinic in collaboration with Dr. H.A. Pattinson, and from the Calgary Urology Clinic in collaboration with Dr. M.D. Carter. The study was approved by the University of Calgary's ethics committee, and all donors gave informed consent.

A normal 46,XY karyotype was verified for each patient, through routine lymphocyte karyotyping by the Cytogenetics Laboratory at either the Alberta Children's Hospital or the Foothills Hospital. For each patient, a minimum of three semen samples were assessed to ensure consistently-abnormal semen characteristics prior to analysis by either sperm karyotyping or fluorescence *in situ* hybridization. All of the ten men were determined to be infertile with no known cause resulting in their condition. Information regarding childhood diseases, environmental exposures, substance abuse and prescription drug usage was determined to ensure that each patient's infertility was indeed of an unknown origin. All patients were determined to have a normal endocrinology profile, with normal levels of follicle stimulating hormone, leutinizing hormone and testosterone.

ii. Obtaining and Storing Human Sperm

Semen samples were collected in sterile specimen containers and were immediately liquefied at 37°C. As previous studies by Dr. Renée Martin's

laboratory have shown that cryopreservation of sperm does not significantly alter its chromosomal content (Chernos and Martin, 1989), samples which were not used immediately were cryopreserved for later use. Semen samples were either washed immediately and utilized for human sperm / hamster oocyte fusion analysis or fluorescence *in situ* hybridization, or else stored for future use by cryopreservation. To do so, the liquefied semen sample was measured into a mixing cone and an equal volume of Ackerman's cryoprotectant was added dropwise to the contents of the cone, with swirling. The resultant mixture was drawn into plastic freezing straws (0.50 ml) and the ends were plugged with polyvinylpyrrolidone (PVP) powder. A large dewar tank was filled to a depth of 2 inches with liquid nitrogen, the straws were suspended 30 inches above the surface for 25 minutes, and then were lowered to within 15 inches of the liquid nitrogen for another 25 minutes. The straws were finally plunged into the liquid nitrogen, and were stored in liquid nitrogen, at a temperature of -196°C, until needed.

B. Normal Donors

Normal donors were volunteers, recruited and studied in this laboratory during the past 12 years. The twelve donors whose sperm karyotype data have been used for comparison to the data from the present study are men of proven fertility, with more than 100 karyotypes analyzed per donor. FISH results from this study are compared to results obtained from the ten normal men who have been studied in this laboratory using fluorescence *in situ* hybridization analysis. Of these ten donors, the six men of proven fertility are also among the donors whose sperm karyotype data

are included.

C. Sperm Karyotyping

The human sperm / hamster oocyte fusion technique, introduced by Rudak *et al.* (1978), modified by Martin (1983), and further modified by Brandriff *et al.* (1985b), was utilized to obtain approximately 100 sperm karyotypes from each of the five patients. TES-Tris (TEST) yolk buffer, which enhances the sperm fertilization capability, was used to maximize the number of karyotypes obtained.

i. Media Preparation

The composition of all media is given in Appendix A; reagent sources are listed in Appendix B.

ii. Human Sperm Preparation

Fourteen to seventeen hours prior to the collection of oocytes, cryopreserved sperm was removed from liquid nitrogen, allowed to liquefy, and mixed with an equal volume of TEST yolk buffer. This TEST yolk buffer/cryopreservant/sperm mixture was sealed in a watertight vial, which in turn was placed in a jar containing water at room temperature. This jar was buried in crushed ice in an ice chest and stored at 4°C until experiment time. Fifteen minutes before collecting the oocytes, the TEST yolk buffer/cryopreservant/sperm mixture was retrieved, and diluted to 10 ml with BWW medium (if more than 5 mls of this mixture were present, then it was diluted to 20 ml). The sperm were washed by centrifugation (6 minutes at 600xG) at room temperature to remove TEST yolk buffer, cryopreservant, and seminal fluids. The supernatant was re-centrifuged in a separate tube to recover as many sperm as

possible, and the resultant supernatant was removed. Pellets from both centrifugations were combined and resuspended in 10 ml of BWW medium, and were centrifuged two more times. The final pellet was resuspended in BWW medium to give a final concentration between 10 and 46 x 10^6 sperm/ml. Four to six 20μl sperm drops were placed in plastic petri dishes, covered with light paraffin oil and kept at 37°C, 5% CO_2, and 95% humidity in the incubator until the oocytes were ready for co-incubation.

Gloves were worn when handling human semen, and preparations were conducted in a laminar flow cabinet. Waste containing sperm or seminal fluids was discarded in biohazard containers.

iii. Hamster Oocyte Preparation

Each experiment required eight female golden hamsters (*Mesocricetus auratus*) between the ages of two and five months. The hamsters were maintained on a controlled light/dark schedule (8am-10pm / 10pm-8am) for a minimum of one week prior to an experiment. The hamsters were injected intraperitoneally with 25 IU of pregnant mare's serum gonadotropin three days prior to the experiment. Sixteen hours prior to the collection of the oocytes, the hamsters were injected intraperitoneally with 25 IU of human chorionic gonadotropin.

At experiment time, hamsters were anesthetized and killed by cervical dislocation, then oviducts were dissected out and collected in BWW medium. Following a BWW wash, the distended section of each oviduct was punctured and the cumulus within was removed, using fine forceps, and released into a watchglass

containing medium. When all the cumulus cell mass was collected, hyaluronidase was added to a final concentration of 0.1%, resulting in the dispersion of the cumulus cells within a few minutes and release of the oocytes from the cumulus mass. The oocytes were separated from the cumulus cells using a micropipet, and were washed three times in BWW medium. The oocytes were treated with BWW medium containing 0.1% trypsin to dissolve the zona pellucida and release the first polar body. The zona-free oocytes were washed a further three times in BWW medium and approximately 30 oocytes were placed in each prepared sperm droplet. On the average, each hamster yielded 25 to 60 oocytes, resulting in a total of 200 to 500 oocytes per experiment. Oocytes and sperm were co-incubated at 37°C, 5% CO_2, and 95% humidity until fertilization occurred, or to a maximum of three hours.

To monitor the progress of fertilization, checks were performed on the oocytes, with the first fertilization check initiated after one-half hour of oocyte/sperm co-incubation. Microscope slides were prepared in advance: 4 small blobs of petroleum jelly were arranged on the slide to support the four corners of a cover slip. A drop of F10 medium containing five to 10 oocytes was placed into the prepared space, a coverslip was gently placed on the petroleum jelly blobs. The oocytes were flattened by pressing on the corners of the cover slip, then were visualized using a phase contrast microscope at 400x magnification. When at least one swollen sperm head was observed in half of the flattened oocytes, the remaining oocytes were washed free of sperm in F10 medium and transferred to F10 droplets (50 μl) under paraffin oil.

The oocytes from morning experiments were incubated in the F10 droplets for 5-7 hours (after which 0.4 μg/ml of colcemid was added); oocytes from the afternoon experiments were incubated for 12 hours in F10 (after which 0.8 μg/ml of colcemid was added). The oocytes were then incubated in F10/colcemid overnight (for morning experiments) or for an additional 5 hours (for afternoon experiments).

iv. Fixation of Oocytes

Oocytes were fixed using the method originally outlined by Tarkowski (1966), with some modifications. After incubation in colcemid, the oocytes were transferred to a watch glass containing a hypotonic solution of 1% sodium citrate at room temperature, for approximately 5 minutes. When the oocytes were swollen to $1^1/_3$ to $1^1/_2$ times their original size, five to ten oocytes were placed on glass slides which had been precleaned with alcohol. Four drops of fixative (3:1 ethanol:glacial acetic acid) were dropped onto the oocytes in order to fix the chromosomes onto the slide, as follows. The first drop of fixative was dropped from a height of 0.5 - 1 cm above the slide, followed by a warm, moist breath of air which fixed the oocytes onto the slide and prevented them from rolling. The position of the oocytes was then noted and the area encompassing all the oocytes was circled on the underside of the slide using a diamond pencil. Before the first drop dried, a second drop of fixative was applied, and the edges of the drop was wiped with lint-free tissue in a 2 cm square around the oocytes in order to spread the chromosomes. The third drop was added when a rainbow sheen appeared at the wiped edges of the second drop (just before the second drop dried), and the leading edges were wiped as for the second drop.

The fourth drop was applied and wiped in a similar manner, and when the rainbow sheen appeared, a warm, dry breath was gently blown onto the slide in order to smoothly evaporate the remainder of the fixative.

After fixation, slides were scanned with a Zeiss phase contrast microscope and all chromosome spreads were circled using a microscope-mounted diamond etcher. The slides were allowed to age at room temperature for a period of two weeks.

v. Q-banding and Photography of Chromosomes

After aging, the slides were stained with 0.5% quinacrine dihydrochloride (pH 4.4-4.5) for 25 minutes, and were rinsed three times in distilled water (pH 4.4-4.5) for a total of 10 minutes. The slides were then stored at 4°C until photographs were taken. The chromosome spreads were mounted under a drop of distilled water (pH 4.4-4.5), a coverslip was applied and sealed with paraffin wax. The circled chromosome spreads were photographed using transmitted fluorescence, on 35 mm Kodak technical pan film 2415, using a Zeiss WL100 microscope fitted with a 100 watt mercury bulb.

The exposed rolls of film were developed in Kodak D-19 solution for 4 minutes, rinsed in stop bath solution (1.5% acetic acid), fixed with Kodak Rapid Fixer solution for 2 minutes, rinsed with water, washed in Kodak Hypoclearing solution for 30 seconds, swished through a beaker of water containing a drop of Kodak Photo-Flo 200, and hung to dry.

vi. Analysis of Sperm Karyotypes

Photographs of the Q-banded sperm chromosomes were printed on Ilford Multigrade III paper using an Ilford 2150 RC print processor.

Chromosomes were solid stained before Q-banded karyotypes in the photographs were analyzed: coverslips were removed from the slides, oil and quinacrine were removed with a methanol rinse, and the slides then stained for five minutes in 6% Giemsa stain. The Q-banded chromosomes were identified on the prints using the Giemsa-stained slides and the chromosomes were karyotyped according to the International System of Human Cytogenetic Nomenclature (ISCN, 1985).

All human karyotypes were attempted to be analyzed and abnormal karyotypes were only inclued in the final data if certain conditions were met. Hypohaploid karyotypes were only kept if the karyotype was compact (with no scattered chromosomes) and the adjacent hamster karyotype complete. In karyotypes with an unidentifiable marker or acentric chromosome and a missing chromosome, the karyotype was listed as containing both a numerical and structural abnormality. However, if the marker could be identified as a portion of the missing chromosome, then the karyotype was listed as containing only a structural abnormality.

D. Fluorescence *in situ* Hybridization (FISH)

Aliquots of either fresh or frozen semen samples were washed three times in the manner described above for human sperm / hamster oocyte experiments. For the five donors for whom sperm karyotypes were obtained, the sperm remaining after the

hamster experiments was concentrated and used for FISH analysis. For the five remaining men, semen was collected, washed with 10 mM Tris/9% NaCl solution (pH 8), and concentrated (Martin and Ko, 1994). Because of low sperm counts in several patients, two or more semen samples were often combined to ensure that the final concentration of sperm was approximately 50 x 10^6 sperm/ml.

i. Media Preparation

The composition of all media is given in Appendix A.

ii. Sperm Slide Preparation and Decondensation

After washing and concentrating the semen sample(s) (frequently almost all of the supernatant was removed), between 2-7 μl of the sample was evenly smeared over a 1 cm^2 area on a clean glass slide. The drop was circled on the underside of the slide with a diamond etcher and allowed to dry for a minimum of 24 hours.

As sperm DNA is tightly compacted, the sperm were decondensed on the slides at room temperature in a series of solutions: 22-30 minutes in DTT, 2.5-3.0 hours in LIS / DTT solution followed by a rinse in 2x SSC. The time required for the decondensation varied depending on the sensitivity of the semen sample to the decondensation procedure: sperm samples from some of these men was hypersensitive to the decondensation, and the usual 30 minutes in DTT and 3 hours in LIS / DTT solution resulted in exploded sperm heads. Slides were air-dried and used immediately for FISH or stored for future use.

In order to investigate both autosomes and sex chromosomes, four chromosome-specific probes were utilized, two for the autosomes and two for the sex

chromosomes. The probes selected were known to show no cross-hybridization, were previously proven to be chromosome-specific using lymphocytes, and produced clearly-defined signals within sperm nuclei.

The four probes used in this study were specific for chromosomes 1, 12, and the X and Y chromosomes. A chromosome 1-specific satellite II sequence (pUC1.77), previously thought to be a satellite III sequence (Tagarro *et al.*, 1994), was kindly provided by H.J. Cooke, Edinburgh (Cooke and Hindley, 1979). A chromosome 12-specific alpha satellite sequence, D12Z3 was obtained from A. Baldini at Yale University (Baldini *et al.*, 1990), an X-specific alpha satellite probe, XC, provided by E. Jabs, John Hopkins University (Jabs *et al.*, 1989), and a Y-specific alpha satellite sequence, DYZ3 was obtained from the American Tissue Culture Collection (ATCC). The autosome-specific DNA probes and the X-specific sequence were labelled directly, using a fluorochrome-conjugated nucleotide (fluorescein-14-dUTP (fluorogreen™), rhodamine-4-dUTP (fluorored™), or coumarin-4-dUTP (fluoroblue™), Amersham), while the Y-specific probe was labelled indirectly with a hapten-conjugated nucleotide (biotin-14-dATP, Gibco) by nick translation.

iii. Denaturation of Probe and Sperm DNA

Fluorescence *in situ* hybridization (FISH) was performed on sperm nuclei using the technique originally described by Eastmond and Pinkel (1990). Some modifications have been made and are described by Martin and Ko (1994). Hybridization mixtures contained 7 μl MM2.1, 30-50 ng of each DNA and 500 ng of

salmon sperm DNA (Sigma). The probe mix was heated for 5 minutes at 70°C then snap-cooled in an ice-water bath for a minimum of 5 minutes.

The sperm DNA was denatured by placing the prepared slide in a pre-hybridization bath for 2 minutes. The slides were snap-cooled and dehydrated for two minutes in each of three pre-cooled (-20°C) coplin jars, containing 70%, 85%, and 95% ethanol. The slides were air-dried at room temperature, then prewarmed to 40°C on a slide warmer. The probe mix was applied to the sperm nuclei on the slide, and a coverslip was placed on top and sealed with rubber cement to prevent evaporation. The slides were incubated in a dark, humidified chamber at 37°C for 17-21 hours.

iv. Post-Hybridization and Probe Detection

After hybridization, the slide was removed from the incubator, dipped into the first post-hybridization wash and the coverslip carefully removed. The slide was then placed in each of three post-hybridization (45°C) washes for 2-5 minutes. (Because of the degree to which the semen samples were concentrated, there were large amounts of contaminants present in some samples. When elevated levels of contaminants were present in the concentrated sample, the length of the post-hybridization wash time was increased.) The slide was then rinsed in PN buffer at room temperature. For hybridizations containing a hapten-conjugated probe, a 20 μl drop of PNM buffer was applied to the slide for 5 minutes in the dark at room temperature to block nonspecific binding sites. The slide was then rinsed in PN buffer before proceeding through the detection steps. Care was taken to ensure that

the slide did not dry during the post-hybridization and detection steps.

When the biotinylated-DYZ3 probe was involved in a hybridization, avidin-Cy3™ (BioCan) was required in order to visualize the probe. Detection involved application of 15 μl of avidin-Cy3™ onto the slide under parafilm for 30 minutes at 37°C in a dark humidified chamber. At the end of this time, the slide was vigorously washed in PN buffer at room temperature. At this point, the protocol was the same for both hapten- and fluorochrome-conjugated probes. In order to visualize the sperm boundaries, sperm nuclei were stained with 10μl propidium iodide (Sigma) and 10 μl DAPI (Sigma), rinsed in PN buffer to remove any excess stain, and coverslipped under 8.5 μl of antifade. If the biotinylated Y probe did not have clear red signals, an amplification was attempted: the coverslip was removed gently, and the slide was rinsed vigorously with PN Buffer. Goat anti-avidin (15 μl) (Vector) was applied for 30 minutes under parafilm at room temperature. A vigourous wash in PN buffer was followed by application of 15 μl of avidin-Cy3™ for 30 minutes at 37°C under parafilm. After a vigorous wash in PN Buffer, propidium iodide was reapplied as before but the time was reduced to 30 seconds. DAPI was usually not reapplied unless the sperm boundaries were not distinct. The slide was washed again in PN buffer and coverslipped in antifade.

v. Analysis of Hybridization

All hybridization slides were examined using a Zeiss Axiophot epifluorescent microscope fitted with a FITC/rhodamine dual bandpass filter set (Zeiss 51004) and an AMCA single-colour filter set (Zeiss 487902) with the excitation and emission

wavelengths, respectively, at 490 nm and 525 nm for FITC, 540-560 nm and 580 nm for rhodamine and at 345 nm and 425 nm for AMCA. The FITC/rhodamine filter set allowed simultaneous visualization of pale-red propidium iodide-stained sperm with yellow-green signals (from either the pUC1.77 or the XC) and red signals (resulting from D12Z3 or DYZ3). The AMCA filter set allowed visualization of the DAPI nuclear stain (which often facilitated defining individual sperm outlines) and the fluoroblue™ 1 signal.

Hybridizations for the two autosomes investigated, chromosomes 1 and 12, were carried out simultaneously using a fluorogreen™ chromosome 1 probe and a fluorored™ chromosome 12 probe. The hybridizations for the sex chromosomes utilized three probe colours: a fluorogreen™ X chromosome probe, an avidin-Cy-3™ (red) Y probe, and a fluoroblue chromosome 1 probe for the internal autosomal control.

A minimum of 20,000 sperm was scored for each patient: 10,000 for chromosomes 1 and 12 and 10,000 for the sex chromosomes. The scoring data was only kept if the nullisomy frequency for each probe was less than 2 percent, indicating that the probe had a 98% hybridization efficiency. When scoring autosomes, normal haploid sperm contained one signal of each colour. Sperm missing one signal were considered nullisomic for that chromosome if the other signal was present. If neither signal was present, the hybridization was considered to have been unsuccessful in that particular sperm, and the sperm was not scored. Where there were two signals of one colour and one signal of the other, a sperm was

considered disomic, subject to the following criteria: the two signals had to be of equal intensity, at least one domain apart, and clearly within the sperm head. Sperm containing two signals of each colour were considered to be diploid (subject to the same criteria outlined above), and were not included in disomy values.

When scoring the sex chromosomes, normal sperm contained either a red (Y) or a green (X) signal, disomic sperm contained two signals of the same colour (XX or YY) or one signal of each colour (XY), and nullisomic sperm did not contain a green nor a red signal. Use of a fluoroblue™ chromosome 1 probe allowed the discrimination between diploidy and disomy, and between nullisomy and non-hybridized cells. The presence of two blue signals, a full domain apart and clearly within the sperm head, indicates a diploid sperm if two sex chromosomes are also seen within the sperm. However, one blue signal and two signals for a sex chromosome is a disomic sperm for the sex chromosomes seen. If a sperm was seen with only a blue signal and no signal present representing the sex chromosomes, then the sperm was scored as nullisomic.

E. Sample Size Considerations and Data Analysis

i. Sperm Karyotypes

In order to determine whether infertile men have an increased frequency of chromosomal abnormalities as compared to normal fertile donors, the two-sample chi square test (two-tailed test) was used to detect decreases as well as increases in abnormality rates. The numerical sample sizes required to detect a significant increase in the normal fertile male frequency of aneuploidy can be calculated using a

two sided, two binomial proportions test (Rosner, 1986). With a significance level of 0.05 and a power level of 0.80, the sample size (n=518) is large enough to detect a 3.9 fold increase in the frequency of aneuploidy in the infertile population.

ii. Fluorescence *in situ* Hybridization

Approximately 10,000 sperm nuclei were counted for each chromosome investigated by FISH and this larger sample size allows an increase of 1.4-1.7 times the normal fertile male frequency to detect a significant increase in the disomy frequency in the infertile men.

III. RESULTS

A. Infertile Patient Profiles

The patients recruited for this study were men who were undergoing fertility investigations after having experienced at least one year of infertility. These men either had low sperm counts (≤20 million sperm/ml), reduced motility (<40% motile) and/or poor morphology (<40% normal forms). Semen profiles for each of the ten patients investigated in this study are shown in Table 1.

All patients were between the ages of 28-46 years, with a mean age of 32.8 years. None had any children at the time of study, either naturally or through *in vitro* fertilization (IVF). The spouse of patient 10 had unsuccessfully attempted IVF, and those of patients 4 and 6 had both undergone intrauterine insemination (IUI).

Through routine lymphocyte karyotyping, all patients were found to have a normal 46,XY karyotype, with patient 3 carrying a normal variant for chromosome 15 (missing satellites).

B. Sperm Karyotypes

Sperm karyotypes were obtained for the first five patients (1-5), using the human sperm / hamster oocyte fusion assay. The mean age of these five patients was 31.6 years. A total of 30 human sperm / hamster oocyte fusion experiments was completed, resulting in 518 karyotypes. Since these men's sperm characteristics were suboptimal, the number of experiments required to obtain 100 karyotypes per patient was higher than that needed for normal donors: between 2 and 11 experiments were necessary, as compared to 2-5 experiments for a normal donor (Table 2).

Table 1: Semen parameters for ten infertile patients.

Patient	Age	Count (10^6/ml)	% Motile	% Normal Forms	Primary Abnormality
1	30	50	40	24	teratozoospermia
2	29	26	35	29	teratozoospermia
3	35	16	49	51	oligozoospermia
4	30	37	30	41	asthenozoospermia
5	34	13	36	40	oligozoospermia
6	36	10	9	38	oligoasthenozoo-spermia
7	28	20	40	59	oligozoospermia
8	46	7	26	38	oligoasthenozoo-spermia
9	28	3	37	27	oligoteratozoo-spermia
10	32	26	30	38	asthenozoo-spermia

Table 2: The number of experiments required, and the final number of karyotypes obtained for each of the five patients investigated using the human sperm / hamster oocyte fusion technique.

Patient	Diagnosis	# Experiments Required	# Karyotypes Obtained
1	teratozoospermia	4	107
2	teratozoospermia	11	91
3	oligozoospermia	2	138
4	asthenozoospermia	7	86
5	oligozoospermia	6	96

i. Normal Fertile Donors

The normal fertile group consisted of 12 normal, fertile donors, from each of whom at least 100 sperm karyotypes had been previously obtained using the human sperm / hamster oocyte fusion assay. A total of 3585 sperm karyotypes from these men was used to calculate the values for normal fertile donors. The mean age of this group of men was 31.2 years.

ii. Infertile Men

A total of 518 analyzable sperm chromosome complements was obtained from the five somatically-normal, infertile patients. The specific chromosomal abnormalities in the karyotypes of each patient are presented in Table 3. The frequencies of numerical and structural abnormalities for each of the five infertile patients studied are shown in Table 4. Conservative estimates of aneuploidy in each of the five patients are detailed in Table 5.

For patient 1, eight of the 107 karyotypes obtained were hypohaploid, with a small chromosome (chromosomes 16 to 22) being lost in more than half of the hypohaploid sperm complements. The single hyperhaploid complement contained an extra copy of chromosome 2. Structural abnormalities included marker chromosomes as well as acentrics, and two of the structurally-abnormal karyotypes also had a missing chromosome 14 interpreted to be structurally altered and not therefore a numerical abnormality. Other structurally-abnormal karyotypes contained either a chromosome break of the X chromosome or a chromatid gap of chromosome 6. A total of four sperm had multiple breaks and rearrangements.

Table 3: Chromosomal abnormalities in the sperm karyotypes from each of five infertile men.

Patient	Structural Abnormalities	Numerical & Structural Abnormalities	Numerical Abnormalities
1	22,X,-3,-4,-7,-14,-15,del(1)(q12),+dic, +3mar,+4ace 23,X,-14,+mar(?del(14)(q13or21) 23,X,csb(X)(q25or26or27) 23,X,ctg(6)(q1) _,Y,MB+R (1 sperm) _,_,MB+R (3 sperm)	22,_,-XorY,+ace 21,Y,-16,dic(1;8)(p;q)	22,X,-9 22,X,-11 22,Y,-13 22,X,-17 22,Y,-18 22,X,-20 22,X,-21 22,Y,-22 24,Y,+2
2	23,Y,+ace 23,X,csg(1)(q4) 23,X,del(9)(q21or22) 20,Y,ctg(14or15)(q2),cte(16;17;19;20) (complex),+ace 23,Y,-8,-9,+2mar(C),del(7)(q22or31), ctg(1)(q31),+2aces _,_,Y,MB+R (1 sperm)		22,_,-Xor-Y 22,X,-14 22,Y,-D 22,X,-21 24,Y,+11

3	23,Y,csb(9)(q3) 23,Y,csb(11)(p11.2) 23,X,csb(13)(p11) 23,Y,csb(20)(?p?q) 23,Y,ctb(20)(q12or13) 22,X,cte(9;16)(q12;q11.2)(qr,asy,complete) 23,Y,ctg(1)(q31) 23,Y,del(19)(q12) _,Y,MB (2 sperm) _,_,MB+R (5 sperm)	22,Y,-22,+ace 22,X,-14,-16,-22,+2mar,ctg(1)(q32), cte(19)(p;q) 22,X,-12,-14, -22,+2mar,ctg(3)(p21), +2or3ace	21,X,-2,-3 21,Y,-16,-21 22,X,-9 22,X,-10 22,Y,-C (6,7,9,or11) 22,X,-15 22,Y,-15 22,X,-18 22,X,-18 22,Y,-18 22,X,-20 22,Y,-20 24,Y,+20 48,XXYY 23,Y,-5,+3
4	23,X,csb(8or10)(q2) 23,XorY,csb(8)(q21or22) 22,Y,cte(8;19)(p12or21;?p13or?q13) (tr,incomplete) 22or23,X,ctg(1)(q4),ctg(19)(q12or13),?csg (22)(q11or 12),?cte(21;22)(p12;p13) _,X,MB _,_,MB+R		22,_,-Xor-Y 22,_,-Xor-Y 21,_,-Xor-Y,-17 21,X,-3,-21 21,Y,-8,-20 22,Y,-12 22,X,-19

5	23,Y,+ace 23,Y,csb(5)(p13or14) 23,X,csb(6)(q2),+ace 23,Y,csb(8)(cen),del(21)(p) 23,Y,csg(12)(q24) 23,Y,ctb(10)(q22) 23,Y,del(1)(q),15(q+)(?1q4>qter),+2ace 23,Y,del(17)(p12or13) 23,Y,del(18)(p11.2) 22,Y,-7,-10,-15,+mar(c)(?10),+dic(15;?)(pter>q2;?),del(3)(q22or23or24),del(5)(q31or32), del(6)(q24or25),+2or3ace _,_,MB _,X,MB+R _,_,MB+R (2 sperm)	20,Y,-9,-11,dic(1;16)(p31;p13), ctg(13)(q31),+2ace	

Table 4: Mean frequencies of chromosomal abnormalities as determined by sperm karyotyping for each of the five patients.

Patient	Structural (%)	Numerical (%)	Numerical & Structural (%)	Total (%)
1	8 (7.5)	9 (8.4)*	2 (1.9)	19 (17.8)
2	6 (6.6)	5 (5.5)	0 (0)	11 (12.1)
3	15 (10.9)	15^ (10.9)†	3 (2.2)	33 (23.9)†
4	7 (8.1)	7 (8.1)	0 (0)	14 (16.3)
5	14 (14.6)	10 (10.4)†	1 (1.0)	25 (26.0)†
Mean for Infertile Men	50 (9.6)	46 (8.9)†	6 (1.2)	102 (19.7)†
Mean for Normal Fertile Men	353 (9.8)	152 (4.2)	15 (0.4)	520 (14.5)

^ includes one double aneuploid karyotype

* frequency of abnormality is significantly higher than that in normal fertile donors at $p < 0.025$

† frequency of abnormality is significantly greater than that in normal fertile donors at $p < 0.005$

Table 5: Conservative estimate of aneuploidy for each of the five infertile men as determined by sperm karyotypes.

Infertile Patient	Karyotypes Obtained	Hypohaploid Spreads	Hyperhaploid Spreads	Conservative Estimate
1	107	10	1	1.87%
2	91	4	1	2.20%
3	138	15	3^	4.35%
4	86	7	0	0
5	96	8	3	6.25%*
Infertile Men	518	44	8	3.1%*
Men With Proven Fertility	3585	152	15	0.84%

^ includes one double aneuploid spread

*$\chi^2 < 8.5$, $p < 0.005$

Both numerical and structural abnormalities were observed in two karyotypes. In one, the sex chromosome was not discernible, and an acentric fragment was present. In patient 2, hypohaploid spreads accounted for four of five numerically-abnormal karyotypes. One of these hypohaploid karyotypes was missing a sex chromosome, two were missing a chromosome from the D Denver group, and one karyotype was missing chromosome 21. The hyperhaploid spread contained an extra chromosome 11. Both simple and complex structural abnormalities were found in the sperm karyotypes obtained from patient 2, with one karyotype showing multiple breaks and rearrangements.

In patient 3, known to carry a normal variant of chromosome 15, hypohaploid karyotypes found showed spreads missing chromosomes 9, 10, and an unidentifiable C chromosome (6, 7, 9 or 11). In addition, one karyotype was missing both chromosomes 2 and 3 and another both chromosomes 16 and 21. Two karyotypes obtained were missing chromosome 15, three karyotypes were missing chromosome 18 and two others lacked chromosome 20. Two hyperhaploid spreads were found: one contained an extra chromosome 20 and the other karyotype, 48,XXYY. This 48, XXYY karyotype could result from a number of possibilities, including a diploid sperm with nondisjunction of the sex chromosomes, two aneuploid sperm fertilizing one egg, or one sperm trisomic for the sex chromosome and one euploid sperm fertilizing the same egg. In addition, one double aneuploid complement was obtained, which contained an extra chromosome 3 and was missing chromosome 5. Chromosome breaks, chromatid breaks, exchanges and gaps accounted for a great

number of the structural abnormalities, seven karyotypes had multiple breaks and rearrangements, and three complements contained both numerical and structural abnormalities.

Numerical abnormalities in the sperm karyotypes from patient 4 consisted solely of hypohaploid complements, with 3 of the 7 hypohaploid karyotypes missing a sex chromosome. Three sperm complements were obtained with multiple breaks and rearrangements; other structural abnormalities observed were chromosome breaks, chromatid exchanges, and gaps.

Of the 96 karyotypes obtained for Patient 5, 26% (25 karyotypes) were abnormal. Of the 10 numerically-abnormal karyotypes, seven were hypohaploid: two karyotypes were missing a copy of one of the larger chromosomes (chromosomes 4 and 7), one was missing 3 small chromosomes (chromosomes 16, 17, and 22), two were missing a D-group chromosome, one karyotype was missing chromosome 19, and one was missing both a sex chromosome and a chromosome 3. The three remaining numerical aneuploidies had extra chromosomes: one had an extra sex chromosome (Figure 1), one contained an extra chromosome 2 and the third showed an additional D-group chromosome. Structural abnormalities observed included simple and complex abnormalities involving chromatids and/or chromosomes. Three sperm were found with multiple breaks and rearrangements, and one karyotype had multiple breaks with no rearrangement of the chromosome material. One abnormal karyotype contained both numerical and structural abnormalities.

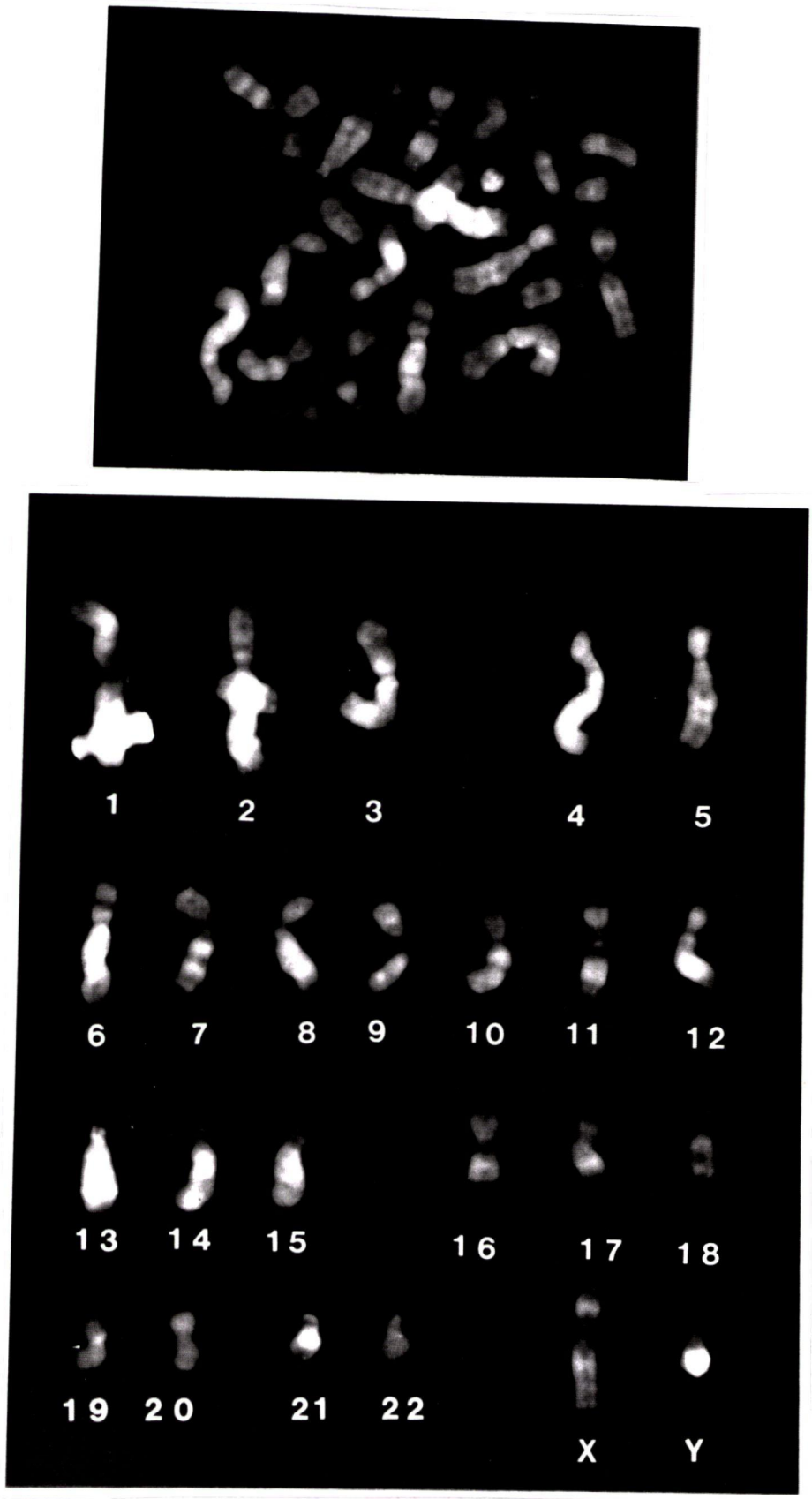

Figure 1: Q-banded karyotype from patient 5, containing an extra sex chromosome.

Considering each patient individually, the frequencies of abnormality as determined by sperm karyotypes were compared to those from normal men with proven fertility. Patient 1, a teratozoospermic man, showed an increased frequency only in numerical abnormalities (χ^2= 5.98, p <0.025). For patients 2 and 4, the sperm karyotype results show no significant differences in the frequency of chromosomal abnormalities relative to that obtained from men with proven fertility. For both oligozoospermic patients (patients 3 and 5), there is a highly significant increase in both numerical abnormalities (χ^2=18.0, p < 0.001 for patient 3; χ^2=8.0, p<0.005 for patient 5) and total abnormalities (χ^2=13.4, p <0.001 for patient 3; χ^2=9.0, p <0.005 for patient 5), as determined by sperm karyotyping. Three of the patients in this study, patients 1, 3 and 5, show an elevated frequency of numerical abnormalities. An elevated aneuploidy frequency was caused not only by hypohaploid spreads, but also by hyperhaploid karyotypes (lost and extra chromosomes are listed individually in Table 3). Since chromosome loss using Tarkowski's fixation method does contribute to an elevated frequency of hypohaploid spreads (Tarkowski, 1966), a high frequency of hypohaploidy may be due to technical artefact. To avoid including artefactual hypohaploidies, a conservative estimate for the frequency of aneuploidy was obtained by doubling the observed hyperhaploidy frequency (Table 5).

The conservative estimate of aneuploidy for men with proven fertility was determined to be 0.84% (15 hyperhaploid / 3585 karyotypes x 2). Relative to the fertile donors, patients 1, 2, 3 and 5 have a higher conservative estimate of

aneuploidy, but only for patient 5 is the increase statistically significant (χ^2=9.0, $p<0.005$).

iii. Infertile Patients as a Group

An overall chromosomal abnormality frequency of 19.7% (102/518) was observed for the five infertile men studied by sperm karyotypes; the frequency of abnormalities in normal men of proven fertility was significantly lower, at 14.5% (χ^2= 9.0, $p < 0.005$). When comparing the total of all numerical abnormalities found (including karyotypes from the numerical & structural category) in the infertile group (52/518, or 10%) relative to the total seen in the fertile group (4.7%), the infertile group shows a significantly elevated frequency of numerical abnormalities (χ^2 = 26.6, $p<0.001$). The hypohaploid frequency was determined to be significantly increased in the group of infertile men relative to that obtained from normal fertile men karyotypes (χ^2=17.1, $p<0.001$). In the infertile group, 8 of the 518 karyotypes obtained were hyperhaploid, yielding a conservative estimate of aneuploidy of 3.1%, which is significantly higher (χ^2= 8.56, $p<0.005$) than that calculated for the fertile group (0.84%).

The frequency of structural abnormalities in karyotypes from infertile men is not significantly different from the frequency found in karyotypes from normal men with proven fertility.

iv. Teratozoospermic Patients

When considering only teratozoospermic patients, 30 of 198 karyotypes obtained from Patients 1 and 2 through 15 human sperm / hamster egg experiments were abnormal. These karyotypes do not show a significant increase in either total abnormalities, numerical abnormalities or structural abnormalities relative to that observed in the men with proven fertility.

v. Oligozoospermic Patients

The two oligozoospermic patients in this study, Patients 3 and 5, are of interest. Of the 234 complements observed, 58 (24.8%) of the karyotypes were abnormal, with numerical abnormalities accounting for 29 karyotypes (12.4%), and structural abnormalities in 33 karyotypes (14.1%); four karyotypes with both numerical and structural abnormalities are included both in numerical and structural categories. When these results were compared to those obtained from men with proven fertility, a significant increase is found in the frequencies of both numerical abnormalities ($\chi^2=25.4$, $p<0.001$) and total abnormalities ($\chi^2=17.3$, $p<0.001$). The six hyperhaploid spreads observed in the 234 karyotypes obtained from the two oligozoospermic patients yielded a conservative estimate of aneuploidy of 5.1% for these patients. This frequency of conservative estimate of aneuploidy is significantly higher than that seen in fertile donors ($\chi^2=14.77$, $p<0.001$).

vi. Sex Ratios

No significant deviation from a 1:1 ratio was observed in the sex chromosome frequency in any of the 5 patients studied by sperm karyotyping (Table 6). Because of the relatively small number of karyotypes of obtained for each patient, minor deviations from the 1:1 ratio are statistically insignificant. Considering the infertile men as a group, 495 karyotypes were informative for the sex chromosomal content (sex chromosome content could not be identified in 23 karyotypes). Of these 495 karyotypes, 243 were X-bearing and 252 were Y-bearing. This ratio was not significantly different from the expected 1:1 ratio.

vii. Sex Chromosomal Aneuploidy

In total, 8 hyperhaploid spreads were obtained from the five infertile patients, two of which had an extra copy of the sex chromosome: a double karyotype with two extra sex chromosomes (48,XXYY) obtained from patient 3, and a 24,XY complement, obtained from patient 5. In normal, fertile donors, 15 of 3585 karyotypes obtained were hyperhaploid, and three of these contained an extra copy of a sex chromosome: 24,YY, 26,XYY,+9, and 24,XX. Because the number of karyotypes with sex chromosomal aneuploidy is so small, a statistical analysis is not meaningful.

Table 6: Frequency of X- and Y- bearing sperm karyotypes obtained in the five infertile men studied.

Patient	X-bearing	Y-bearing
1	46.6%	53.4%
2	53.3%	46.7%
3	50.8%	49.2%
4	48.1%	51.9%
5	46.2%	53.8%
Mean For Infertile Men	49.1%	50.9%
Mean for Normal Men With Proven Fertility	50.8%	49.2%

C. Fluorescence in situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) analysis was completed on a total of ten infertile men (the first five men in the FISH study were the same patients whose sperm karyotypes were studied using the human sperm / hamster oocyte fusion assay).

i. Normal Donors

Results from FISH analysis for infertile men were compared to those obtained from normal donors on whom FISH analysis had been completed for chromosomes 1, 12, X and Y. This normal group consists of 10 men (mean age = 36.3 years): six of proven fertility and four of unproven fertility, all of whom had had sperm karyotypes studied previously. Sperm karyotypes from the six men with proven fertility are among those in the normal group for the sperm karyotypes portion of this study. A total of 225,494 sperm nuclei from the 10 donors was counted for the four chromosomes of interest in this study. For chromosomes 1 and 12, a total of 122,803 sperm nuclei were analyzed, and an additional 102,691 sperm nuclei were analyzed for the sex chromosomes. The disomy frequency determined in normal men of proven fertility as compared to normal men with unproven fertility was not significantly different, with the exception of the YY disomy frequency, which was found to be significantly higher in the men with proven fertility ($\chi^2=4.2$, $p<0.05$). The increase in YY disomy frequency was due to a single donor of proven fertility who had a much higher frequency of YY disomy than any of the other males studied. Disomy and diploidy results from these control donors are shown in Tables 7 and 8.

Table 7: Disomy frequencies (%) as determined by FISH for chromosomes 1, 12, X, and Y in sperm nuclei from 10 normal donors.

Donor	Disomy 1	Disomy 12	XX Disomy	YY Disomy	XY Disomy
1	0.09	0.15	0.17	0.14	0.22
2	0.06	0.15	0.03	0.13	0.16
3	0.15	0.20	0.05	0.23	0.17
4	0.15	0.10	0.10	0.43	0.18
5	0.05	0.17	0.05	0.10	0.08
6	0.08	0.12	0.10	0.21	0.11
7	0.16	0.25	0.07	0.14	0.24
8	0.09	0.16	0.08	0.13	0.19
9	0.18	0.13	0.04	0.22	0.16
10	0.06	0.15	0.04	0.11	0.11
Mean	0.10	0.16	0.07	0.19	0.16
Range	0.05-0.18	0.10-0.25	0.03-0.17	0.10-0.43	0.08-0.24

Table 8: The frequency of diploidy (%) determined by FISH analysis for chromosomes 1 and 12 and the sex chromosomes in 10 normal donors.

Donor	Chromosome 1/12 Analysis	Sex Chromosome Analysis
1	0.15	0.11
2	0.06	0.05
3	0.05	0.11
4	0.08	0.10
5	0.12	0.05
6	0.17	0.17
7	0.51	0.33
8	0.17	0.31
9	0.04	0.09
10	0.31	0.07
Mean	0.13	0.14
Range	0.04-0.51	0.05-0.33

ii. Ten Infertile Men

a. Disomy Frequency Obtained for Investigated Chromosomes

For the ten infertile men studied by FISH, a total of 205,213 sperm nuclei have been analyzed: a total of 102,656 were studied for chromosomes 1 and 12, and 102,557 for the sex chromosomes. The results are presented in Tables 9 and 10.

To determine whether the results obtained for individual patients were significantly different from that obtained for normal donors, the Chi-square test was applied. These results are presented in Table 10.

It can be seen in Table 10 that FISH results for Patients 7, 8 and 10 show no significant increase in the frequency of disomy for any of the chromosomes investigated, but do show a significant decrease in the frequency of YY disomy. Patients 4 and 9 show a significant increase only in the frequency of XY disomy. Only patient 1 has all disomy frequencies in the normal range.

Of the 10 infertile men investigated, six show a highly significant increase in the frequency of XY disomy and four show a highly significant increase in the frequency of disomy for chromosome 1. Only patient 5 shows a significant increase in the frequency of disomy for chromosome 12. Interestingly, a significant decrease in the frequency of disomy for chromosome 12 was seen for patient 8, while patients 2, 4, 5, 7, 8 and 10 all show a highly significant decrease in the frequency of YY disomy. Though in the normal men studied there is one anomalous male (donor 4)

Table 9: Disomy frequencies (%) for chromosomes 1, 12, and the sex chromosomes as determined by FISH analysis of sperm nuclei from infertile men.

Patient	Disomy 1	Disomy 12	XX Disomy	YY Disomy	XY Disomy
1	0.16	0.12	0.10	0.10	0.18
2	0.19	0.22	0.10	0.09	0.36
3	0.25	0.19	0.11	0.12	0.31
4	0.08	0.16	0.05	0.08	0.47
5	0.22	0.28	0.05	0.06	0.34
6	0.18	0.20	0.09	0.15	0.65
7	0.12	0.21	0.10	0.07	0.20
8	0.06	0.05	0.03	0.05	0.13
9	0.12	0.23	0.11	0.17	1.39
10	0.04	0.13	0.02	0.07	0.13
Mean for Infertile Men	0.14	0.18	0.08	0.10	0.42
Range for Infertile Men	0.04-0.25	0.05-0.28	0.02-0.11	0.05-0.17	0.13-1.39
Mean for Normal men	0.10	0.16	0.07	0.19	0.16

Table 10: Chi-square analysis of the disomy frequency obtained for each of the infertile patients as compared to the values obtained from 10 normal donors.

Patient	Disomy 1 χ^2 value	Disomy 12 χ^2 value	XX Disomy χ^2 value	YY Disomy χ^2 value	XY Disomy χ^2 value
1	2.53	0.59	1.97	3.40	0.08
2	**6.28**	1.82	0.40	4.73*	**18.84**
3	**8.36**	0.536	1.06	2.03	**10.03**
4	0.26	0.003	0.28	5.70*	**47.70**
5	**11.60**	**7.83**	0.41	7.68*	**15.10**
6	**4.67**	1.11	0.12	0.56	**104.52**
7	0.18	1.15	0.52	6.46*	0.55
8	1.20	6.71*	1.95	10.82*	0.48
9	0.36	0.26	1.10	0.08	**269.13**
10	2.81	0.28	3.03	6.37*	0.40
All Infertile Men	**8.02**	1.53	0.07	29.80*	**87.11**

*frequency is significantly below that seen in the results obtained from normal men.
Bold values indicate a significant increase in the frequency of disomy as compared to results obtained from 10 normal men.

with an unusually high YY disomy frequency of 0.43, patients 2, 4, 5, 7, 8 and 10 all fall below the low end of the disomy range values seen in the normal group. No patients showed a significant change in the frequency of XX disomy.

As a group, the infertile men's FISH results show a significant decrease in the frequency of YY disomy (χ^2=29.80, $p<0.001$), and also a highly significant increase in the frequency of disomy for chromosome 1 (χ^2 =8.02, $p<0.005$), and XY disomy (χ^2 =87.11, $p<0.001$).

b. Sex Ratios

When analyzing the frequency of X-bearing versus Y-bearing sperm nuclei for each patient, four of the ten patients (patients 4, 5, 6 and 9) investigated showed a significant deviation from the expected 1:1 ratio (Table 11). The frequency of X- and Y-bearing sperm does not deviate from the expected ratio when the infertile men are taken as a group.

c. Diploidy Frequency

The frequncy of diploidy as determined by FISH for the investigated chromosomes is shown in Table 12. The diploidy frequency for each patient, as determined during the analysis of both the autosomes and the sex chromosomes showed slight variations. The mean diploidy frequency for the infertile patients was found to be 0.16 during both autosomal and sex chromosomal content analysis.

Table 11: Sex ratio in the sperm of 10 infertile patients, as determined by FISH.

Patient	Total Sperm Nuclei Analyzed	Total-X Bearing Sperm Nuclei	Total-Y Bearing Sperm Nuclei
1	10072	5100 (50.60%)	4882 (48.47%)
2	10389	5141 (49.48%)	5145 (49.52%)
3	10183	5085 (49.94%)	4973 (48.84%)
4*	11103	5677 (51.13%)	5271 (47.47%)
5*	10062	5363 (53.30%)	4622 (45.93%)
6*	10184	5164 (50.71%)	4846 (47.58%)
7	10079	4981 (49.42%)	5028 (49.89%)
8	10181	4969 (48.81%)	5086 (49.96%)
9*	10127	5129 (50.65%)	4728 (46.60%)
10	10015	5089 (50.81%)	4858 (48.51%)
Mean for All Infertile Men	102395	52698 (51.46%)	49439 (48.28%)

* The frequency of X- and Y- bearing sperm which is significantly different from the expected 1:1 ratio ($\chi^2 \geq 10.10$, $p < 0.01$).

Table 12. Diploidy frequency (%) as determined by FISH analysis of chromosomes 1 and 12 and the sex chromosomes in 10 infertile men.

Patient	Chromosome 1/12 Analysis	Sex Chromosome Analysis
1	0.05	0.09
2	0.20	0.36
3	0.15	0.12
4	0.18	0.08
5	0.23	0.04
6	0.06	0.20
7	0.24	0.28
8	0.04	0.01
9	0.34	0.32
10	0.11	0.09
Mean for Infertile Men	0.16	0.16
Range for Infertile Men	0.04-0.34	0.01-0.36
Normal Donor Range (Mean)	0.04-0.51 (0.13)	0.05-0.33 (0.14)

IV. DISCUSSION

A. Sperm Karyotypes

To date, a number of investigators have utilized sperm karyotypes to determine the incidence of sperm chromosomal abnormalities in normal men. The frequency of chromosomal abnormalities in sperm from normal men has been determined to range from 5% (Rudak *et al.*, 1978) to 15.9% (Mikamo *et al.*, 1990). Only three laboratories have analyzed greater than 5,000 karyotypes for normal men (Brandriff and Gordon, 1990; Mikamo *et al.*, 1990; Martin and Rademaker, 1990) (Table 13).

Examination of sperm karyotypes obtained from human sperm / hamster oocyte fusion allows the visualization of both structural and numerical abnormalities from an individual sperm. Similar total chromosomal abnormality frequencies have been found by groups which investigate sperm karyotypes. The frequencies of structural abnormalities reported are similar amongst all three groups. The frequency of numerical abnormalities varies amongst investigators - a result which is not altogether surprising, since technical artefact is implicated in the loss of chromosomes during chromosome fixation. However, the conservative estimate of aneuploidy, determined by doubling the hyperhaploid frequency, is similar amongst the three laboratories ranging from 1.14-1.96%. A major limitation of this technique is that relatively small numbers of sperm can be studied because of the enormous investments of time (and funds) required. In addition, the necessity for the sperm to successfully fertilize eggs limits the technique's usefulness in studying suboptimal sperm, so few studies have investigated the chromosomal content of sperm from men

Table 13. Comparison of results obtained from the three largest studies utilizing the human sperm / hamster oocyte fusion assay to analyze the sperm of normal men.

STUDY	# Donors	Total Karyo-types	% Abnormal	% Numerical	% C.E.[a]	% Structural
Brandriff & Gordon, 1990	24	5997	~9.4	2.3	1.96	~8
Mikamo *et al.*, 1990	26	9280	15.9	1.3	1.36	14.6
Martin & Rademaker, 1990+ unpublished	87	7191	13.2	3.9	1.14	8.8

[a] conservative estimate of numerical abnormalities (2 x % hyperhaploidy)

with reproductive problems. Poor semen characteristics reduces the proportion of hamster oocytes penetrated by the sperm of infertile men, making sperm karyotypes more difficult to obtain.

i. Sperm Chromosomal Abnormalities

The frequency of chromosomal abnormalities, as determined by sperm karyotyping for each of the five 46,XY men with idiopathic infertility, was compared to the frequency of chromosomal abnormalities obtained from normal men of proven fertility. The frequency of hypohaploid karyotypes obtained from infertile patients was significantly higher than that found in normal fertile donors ($\chi^2=17.1$, $p<0.001$). An excess of hypohaploid karyotypes has been observed by a number of investigators analyzing sperm karyotypes (Martin *et al.*, 1987; Martin and Rademaker, 1990) and human lymphocyte karyotypes (Ford *et al.*, 1988). Chromosome loss during the fixation process is believed to account for a large proportion of lost chromosomes; Ford *et al.* (1988), however, have shown anaphase lag to be a mechanism by which chromosome loss occurs in human lymphocyte cultures. The lagging of chromosomes or chromatids during anaphase, and their subsequent elimination through micronuclei, results in two hypoploid cells or one normal and one hypoploid cell, respectively. Hence it is possible to have a greater number of cells missing a chromosome than displaying an extra chromosome. Because it is difficult to determine whether a hypohaploid karyotype is the result of anaphase lag or technical artefact, researchers question the validity of using hypohaploid karyotypes in evaluating the chromosomal aneuploidy of sperm. Instead, the frequency of

hyperhaploidy is doubled to arrive at a conservative estimate of aneuploidy. In this study, the frequency of hyperhaploidy was significantly elevated in the karyotypes obtained from the five infertile patients ($\chi^2 = 8.37$, $p < 0.005$) giving a conservative estimate of aneuploidy of 3.1% ($\chi^2 = 8.56$, $p < 0.005$). Analysis of sperm karyotypes from each of the five infertile patients, particularly those from the oligozoospermic men (patients 3 and 5), showed increased frequencies for both total abnormalities and numerical abnormalities relative to normal fertile donors (Table 4).

Oligozoospermia and its more severe form, azoospermia, have been the focus for many investigators studying male factor infertility. Pairing abnormalities in the autosomes and the sex chromosomes have been implicated in the disruption of spermatogenesis in infertile men. Egozcue *et al.* (1983) reported that in infertile males, there is an increased frequency of pairing disruptions during meiosis, resulting in meiotic arrest during spermatogenesis, causing these cells to fail to continue through spermatogenesis and leading to subsequent oligo- or azoospermia.

The present study shows that chromosomally-abnormal sperm are present in the ejaculate of infertile men, particularly the oligozoospermic men. The sperm karyotypes obtained from the two oligozoospermic males (patients 3 and 5) show an increased frequency of hyperhaploidy (Table 5). A total of six hyperhaploid spreads was obtained from these two men; four of the hyperhaploid spreads contained an extra autosome (chromosomes 2, 3, D and 20), and two contained a sex chromosome anomaly (24,XY and 48,XXYY), indicating that nondisjunction can occur in both autosomes and sex chromosomes. Several researchers (Egozcue *et al.*, 1983; Navarro

et al., 1990) have speculated that pairing abnormalities result in reduced sperm counts as the resultant cells undergo meiotic arrest, suggesting that spermatogenesis is completed only in those cells in which meiotic pairing and segregation of chromosomes is normal and correct. Although it is impossible to determine from an examination of sperm karyotypes whether a pairing abnormality has occurred during spermatogenesis, the elevated frequency of hyperhaploidy observed in this study may give credence to the possibility that some cells which experienced nondisjunction, perhaps due to pairing abnormalities in meiosis, are arrested during meiosis resulting in oligozoospermia while other aneuploid cells are capable of completing spermatogenesis.

ii. Comparative Results from Studies on Men Experiencing Reproductive Problems

a. Habitual Spontaneous Aborters

Rosenbusch and Sterzik (1991) studied sperm chromosomes of ten constitutionally-normal men whose partners had experienced recurrent spontaneous abortions. Prior sperm analysis for each man had shown that the sperm were morphologically normal and the semen parameters were generally normal. A total of 308 sperm chromosome complements was analyzed for the ten men in the habitual abortion group. These results were compared to 413 karyotypes obtained from a control group which consisted of 15 fertile donors whose partners had no prior history of spontaneous abortions. Comparison of these karyotypes to those obtained from normal men showed some interesting trends. The frequency of numerical

abnormalities found in the study group (3.6%) was not statistically higher than that determined for the normal group (1.9%). However, the frequency of structural abnormalities obtained from the karyotypes for men whose partners experienced reproductive wastage was found to be significantly higher (14.6%) than that determined for normal men (7.0%). The structural abnormalities found included higher levels of chromosome breaks (5.8% versus 2.4% in normal donors) and acentric fragments (8.1% versus 2.4% in the normal group).

In our investigations of men with idiopathic infertility who have not knowingly experienced reproductive wastage, the frequencies of structural abnormalities in the sperm chromosomes are not significantly different from those seen in normal men with proven fertility. However, the frequency of numerical abnormalities, particularly hyperhaploidy, is significantly greater than that found in the normal group.

Although at first glance the results obtained by Rosenbush and Sterzik (1991) differ from those obtained in this study, it is important to take several factors into account before comparing the data. First, the number of karyotypes for both the control group and the habitual aborters were relatively small in the Rosenbush and Sterzik study, which may explain that study's elevated frequency of structural abnormalities, found in both spontaneous aborters and controls. Second, when considering only the habitual aborters in the Rosenbush and Sterzik study, 5 (1.6%) of the 308 karyotypes obtained were hyperhaploid. Doubling this percentage to obtain a conservative estimate of aneuploidy gives a value of 3.2%, which is very close to the significanty-elevated conservative estimate of aneuploidy (3.1%) observed for the

infertile men in this study.

b. Infertile man with Oligoasthenoteratozoospermia

Navarro and associates (1990) studied the sperm chromosomes of one infertile man using the human sperm / hamster oocyte fusion system. The 46,XY male was characterized with oligoasthenoteratozoospermia: a sperm count of 1.4 million sperm/ml, 66% of the sperm immotile and only 15% with normal motility. The percentage of abnormal forms is not specified. Although the man was an alcoholic, etiology for the infertility was listed as unknown. Meiotic preparations showed abnormal pairing of the bivalents from the beginning of prophase I in a proportion of metaphase I cell preparations, and absence of metaphase II figures. Surprisingly, with such poor sperm characteristics, Navarro *et al.* (1990) were able to obtain 30 sperm chromosome karyotypes. Twenty-four of the 30 karyotypes were normal. The six abnormal karyotypes obtained included: one karyotype with structural abnormalities, one hypohaploid karyotype with structural abnormalities, and four hypohaploid karyotypes. The frequency of structural abnormalities is not significantly different from that found in normal men. The frequency of total abnormalities (6/30, or 20% of the karyotypes) is high relative to those obtained by other groups investigating sperm chromosomes (Table 13), but due to the possibility of technical artefact artificially inflating the rate of hypohaploidy, the significance of this value is questionable.

Before comparing our results to those of Navarro *et al.* (1990), it is important to recognize that although the diagnosis was one of idiopathic infertility, the role of

alcohol as a cause of infertility cannot be eliminated. In addition, the small sample size (30 karyotypes) makes it difficult to accurately ascertain the frequency of chromosomal abnormalities in the sperm of this oligoasthenoteratozoospermic patient. Although meiotic pairing abnormalities were found in the sperm of the infertile patient, chromosomally-normal sperm cells were found in the ejaculate. Navarro and associates (1990) suggested that cells experiencing pairing problems do not proceed beyond metaphase I and that only normal germ cells continue through spermatogenesis. The results of the present study indicate that in the infertile men studied, the sperm present in the ejaculate does contain a higher frequency of chromosomal abnormalities as compared to men of proven fertility. Thus, if a spermatocyte experiences pairing difficulties resulting in subsequent chromosomal abnormalities, the spermatocyte may still be able to continue through spermatogenesis and be present in the ejaculate.

iii. Sex Ratios

Deviations from the Mendelian expectation in human sex ratios have been noted. Specifically in livebirths resulting from IVF, several reports have documented excessive numbers of male births (Al-Shawaf and Craft, 1989; Steer *et al.*, 1989; Thatcher *et al.*, 1989), with a sex ratio as high as 1.83 reported by Thatcher *et al.* (1989). Though the sex ratios observed in this study of sperm karyotypes obtained from five infertile men do show a slightly greater number of Y-bearing sperm (243 X-bearing: 252 Y-bearing) the difference is not significantly different from the expected 1:1 ratio (Table 6).

iv. Sperm Selection

Within the female genital tract, only a portion of the sperm in an ejaculate are successful in reaching the site of fertilization (Mortimer, 1979; Nestor and Handel, 1984; Redi *et al.*, 1984). Highly motile, morphologically-normal sperm have a greater chance of passing the cervix than morphologically-abnormal and/or nonmotile sperm. Several studies (Foldesy *et al.*, 1984; Redi *et al.*, 1984; Brandriff *et al.*, 1986a; Estop *et al.*, 1988) have investigated the genetic content of the two groups of sperm to determine whether genetically-abnormal sperm are able to fertilize an egg. Utilization of the human sperm / hamster oocyte fusion system allows an *in vitro* system where both motile and nonmotile sperm are placed in proximity to oocytes. Brandriff *et al.* (1986a) showed that the chromosomal content of highly-motile sperm did not differ from the chromosomal content of unselected sperm. Similarly, Martin and Rademaker (1988) found no significant relationship between the frequency of morphologically-abnormal sperm and the frequency of chromosomally-abnormal sperm. These results suggest that there is no selection based on chromosomal content in the human sperm / hamster oocyte fusion technique, and that (with allowances for artefactually-induced hypohaploidy) the technique should reflect the chromosomal content of sperm *in vivo*.

When comparing semen characteristics of the infertile patients in this study (Table 1) to the number of experiments required to obtain approximately 100 karyotypes (Table 2), sperm selection does not appear to interfere with the results. For example, the sperm from patient 2 is primarily defined as teratozoospermic, with

normal morphology in only 29% of the sperm present. However, 11 human sperm / hamster oocyte fusion experiments were required to obtain a total of 91 karyotypes. Although it was extremely difficult to obtain karyotypes for this patient, the sperm karyotypes proved to have no significant increase in the frequency of chromosomal abnormalities. A similar evaluation of the sperm from patient 3, a mildly oligozoospermic patient, shows that only two experiments were required to obtain 138 karyotypes. Interestingly, it is also patient 3 that shows a significant increase in the frequencies of chromosomal abnormalities relative to the normal group. Though the results here suggest that sperm selection does not play a role in human sperm / hamster oocyte fusion analysis, further studies are needed to determine whether sperm selection does exist both *in vitro* and *in vivo*.

B. Fluorescence *in situ* Hybridization (FISH) Studies

Analysis of sperm from infertile men using both sperm karyotyping and fluorescence *in situ* hybridization (FISH) permits us to take advantage of the strengths inherent in each of the techniques. Obtaining large numbers of sperm karyotypes for each man investigated was impractical because of the enormous technical time required in the human sperm / hamster oocyte fusion assay, whereas FISH allowed analysis of thousands of sperm in a short period of time. Reduced sperm motility, poor morphology and low sperm counts - which wreak havoc with the fusion assay - were of no consequence when using FISH. Conversely, although only numerical abnormalities for the investigated chromosome pairs can be ascertained when using FISH, the utilization of sperm karyotypes in our study allowed the determination of

the overall frequency of both structural and numerical abnormalities. The FISH results were then used to confirm the numerical abnormalities observed in the karyotypes on a much larger sample size. The utilization of FISH to determine aneuploidy in sperm cells allowed the elimination of any questions regarding sperm selection, as washed sperm were fixed in place on a slide for the hybridization, and both normal and abnormal sperm were treated, side by side, under identical conditions. In addition, the inherent internal control present when using two-colour hybridizations for autosomal investigation and three-colour hybridizations for investigations of the sex chromosomes allowed the differentiation between non-hybridization and nullisomy, and between disomy and diploidy. As such, when using two- or three-colour FISH a sperm must have at least one signal to be considered efficiently hybridized. Finally, strict criteria are utilized to determine whether a sperm nucleus contains two like-coloured signals so as to minimize inter-observer variability. Two like-coloured signals must be at least one-domain apart to ensure that a stretched segment of decondensed chromatin is not misinterpreted as two signals. All signals must also be clearly within the sperm nuclei. However, the major disadvantages of this technique stem from the subjectivity inevitable as different observers score sperm nuclei and the difficulty in not being able to directly view the chromosome being analyzed.

i. One-colour versus Multicolour FISH

FISH has been now utilized to study the disomy frequencies of chromosomes 1, 3, 4, 6, 7, 8, 11, 12, 15, 16, 17, 18, X and Y (Guttenbach and Schmid, 1990;

Guttenbach *et al.*, 1994; Bischoff *et al.*,1994; Spriggs *et al.*, in press). However, the majority of these chromosomes have been studied using one-colour FISH. One-colour FISH studies helped provide preliminary information on sperm aneuploidy and allowed the exploration of a new method from which limited amounts of less costly information as to the chromosomal content of sperm could be extracted, but increased availability of multicoloured probes and dual/triple bandpass filters mean that much more accurate information can now be obtained using multicolour FISH. Since a single chromosome-specific probe is used in each hybridization, one-colour FISH does not allow the differentiation between non-hybridized and nullisomic sperm (neither of which would contain a signal) nor between disomic sperm and diploid sperm (both of which would contain two signals). Indeed, two-colour FISH studies now show that diploid sperm can account for a significant portion of sperm nuclei that contain two like-coloured signals (Bischoff *et al.*, 1994; Spriggs *et al.*, in press). Furthermore, the frequency of diploid sperm is highly variable amongst donors so the value obtained for one donor cannot be extrapolated to other donors.

ii. Disomy Frequencies Obtained by Other Laboratories Using Multicolour FISH

A number of investigators have studied sperm aneuploidy in normal men using FISH but only three other groups have utilized two- and three-colour FISH to determine the disomy frequency of various chromosomes (Table 14). The four groups report similar frequencies of disomy for the sex chromosomes. With the exception of Bischoff *et al.* (1994), all the values for XX disomy fall within a narrow range (0.04-

0.07%). The generally-higher values of disomy reported by Bischoff *et al.*, (1994) are probably due to the omission of a criterion included by all other groups: a minimal distance of one domain between two like-coloured signals, which would lead to overestimation of disomy frequencies (Martin and Rademaker, in press). Similarly, YY disomy frequencies fall within a narrow range with the exception of Spriggs *et al.* (in press) who observed an unusually-high incidence of YY disomy (0.43%) in one of five donors analyzed. If this donor is ignored, the mean frequency for YY disomy decreases to 0.14%. The XY disomy frequencies determined by all four investigators range from 0.09% to 0.15%.

iii. Frequency of Autosomal Aneuploidy in Infertile Men

Autosomal aneuploidy was investigated using two-colour FISH for chromosomes 1 and 12. The disomy frequency for chromosome 1 was significantly elevated in four of the ten infertile men studied (Table 10). Of the four patients (Patients 2, 3, 5 and 6) with a significant increase in the frequency of chromosome 1 disomy in their semen sample, three are patients who have reduced sperm counts (Patients 3, 5, and 6) as determined by semen analysis (Table 1). These results suggest that chromosome 1 may have a predisposition to improper disjunction in these infertile patients. Considered as a group, the ten infertile men also show a significant increase in the disomy frequency for chromosome 1, suggesting that in men with

Table 14. Disomy frequencies (%) reported by other laboratories on sperm nuclei from normal men, utilizing two-colour FISH for investigation of chromosomes 1 and 12, and three-colour FISH to investigate the sex chromosomes.

STUDY	Disomy 1	Disomy 12	XX Disomy	YY Disomy	XY Disomy
Wyrobek *et al.* 1993	-	-	0.04	0.04	0.09
Williams *et al.** 1993	-	-	0.04	0.06	0.09
Bischoff *et al.* 1994	-	0.30	0.38	0.08	0.13
Spriggs *et al.* (in press)	0.10	0.16	0.07	0.21	0.15

* Disomy frequency of XX and YY determined using two-colour FISH; frequency of XY disomy was determined using three-colour FISH.

idiopathic infertility, sperm chromosomes may contain more abnormalities than are seen in normal men.

Disomy frequency of chromosome 12 was significantly increased only for donor 5 and the infertile men studied as a group do not show an increased frequency of disomy for chromosome 12 when compared to the values obtained for normal men investigated by FISH in our laboratory. Once again, the mean chromosome 12 frequency value reported by Bischoff *et al.*, (1994) (0.30%) is higher than the disomy 12 frequency obtained for Patient 5 (0.28%) - and as explained above, the higher value is probably due to the lack of a one-domain criterion in the Bischoff laboratory.

iv. Frequency of X- and Y-Chromosome Aneuploidy in Infertile Men

In human males, it is possible to differentiate meiosis I (MI) and meiosis II (MII) nondisjunction because of the heterogametic nature of the sex chromosomes. The presence of both an X chromosome and a Y chromosome within a sperm nucleus indicates that the sex chromosome segregation error occurred during MI. In contrast, MII nondisjunction results in gametes containing two X chromosomes or two Y chromosomes. In six of the ten infertile men investigated, the frequency of MI error (represented by XY disomic sperm nuclei) was significantly elevated when compared to the frequency found in normal donors. In addition, when results from all ten infertile men were grouped together, the mean XY disomy frequency was significantly higher than that seen in normal donors. In fact, these results indicate that meiosis I error occurs more frequently than meiosis II error in infertile men, and that the frequency of nondisjunction in meiosis I is significantly higher than that reported

previously in normal men.

When considering MII error, the frequency of XX disomy is not significantly different from that seen previously in normal men. However, the frequency of YY disomy is significantly decreased in the infertile men as a group. When the mean frequency of YY disomy obtained for the ten infertile men (0.10%) is compared to values reported by other groups (Table 14), it becomes evident that the frequency obtained in this study is within the range of values obtained for normal men.

The frequency of sperm nuclei containing a sex chromosomal abnormality associated with meiosis I or II error differs from the frequency of these events in liveborns and aneuploid fetuses. Aneuploid fetuses and liveborns have been examined to determine the parent of origin and the meiotic stage during which the nondisjunction occurred. While paternal origins of autosomal aneuploidies account for 5-10% of trisomic conceptuses (Antonarakis, 1991; Sherman *et al.*, 1991; Nothen *et al.*, 1993; Fisher *et al.*, 1993), a greater number of sex chromosomal aneuploidies result from paternal nondisjunction. Although only 7% of 47,XXX liveborn females result from paternal meiosis II errors (May *et al.*, 1990), paternal errors account for 100% of 47,XYY males, 50% of 47,XXY males (Lorda-Sanchez *et al.*, 1992), and 80% of 45,XO fetuses and liveborns (Hassold *et al.*, 1992). The frequency of 47,XXY sex chromosomal aneuploidies in pregnancies is estimated to be 0.05% (Jacobs *et al.*, 1989). Since 50% of these are of paternal origin, 0.025% are caused by paternal meiosis I nondisjunction. As expected, the frequency of XY-bearing sperm in our ten normal donors studied by FISH was found to be higher (0.16%) (Table 7), reflecting

the high rate of fetal wastage, and possibly the postzygotic loss of extra chromosomal material. Conversely, chromosomally-abnormal sperm may be unable to penetrate the oocyte.

When assessing the sex chromosomal aneuploidy frequency in infertile men, of primary significance is the increased frequency of meiosis I error. As there may exist an increased frequency of XY disomic gametes and of the complementary nullisomic gametes in the sperm of infertile men, the frequency of paternal origin of 47,XXY and 45,XO fetuses would be expected to be higher in the idiopathic infertile male population. The actual prevalence of these sex chromosomal aneuploidies in children born to couples experiencing infertility is difficult to determine since some infertile couples have success spontaneously, with intrauterine insemination (IUI), through IVF or using other procedures. An examination of IVF pregnancy outcomes is not an accurate source of information about the children of infertile men, as a majority of these patients have causes of infertility other than idiopathic male infertility. Furthermore, IVF offers limited success to patients with male-factor infertility (De Krester *et al.*, 1985). In the future, a more relevant study would determine the frequency of 47,XXY and 45,XO in liveborns resulting from either sub-zonal sperm insertion (SUZI) or intracytoplasmic sperm injection (ICSI). Both techniques are offered to couples for whom conventional IVF has failed because of the inability of the sperm to fertilize oocytes or for those who have been previously excluded from assisted reproductive technologies because of suboptimal semen profiles. Although both of these advances are new and relatively few children have been born, a recent

study reported by Cohen *et al.* (1994) found that in 647 cycles in which microsurgical fertilization procedures were preformed via either (SUZI) or partial zona dissection (PZD), 127 patients delivered a total of 162 babies. In a questionnaire sent out to these patients, of the 116 patients who participated in the study (accounting for 148 babies), eight babies were found to have major malformations (5.4%). This is higher than that reported in the general population (3.7%), but statistically insignificant because of the small sample size. It is interesting to note that one of the eight major abnormalities was a sex chromosomal aneuploidy (47,XXY) whose father had been diagnosed with oligoasthenoteratozoospermia.

Van Steirteghem and associates (1994) have now reported results of 600 treatment cycles of SUZI and ICSI performed on couples who had failed standard IVF or had insufficient sperm present for IVF. A total of 421 embryos was transferred, resulting in 63 pregnancies. At the time of publication, 22 babies were born from these 63 pregnancies and only one major congenital abnormality was found in one child of a set of twins: cheilopalatoschisis (cleft lip and palate) and duplication of the renal calyces. Further evaluation is still needed, and though centers such as the one directed by Van Steirteghem in Brussels require each patient to sign a consent form stating that they will have prenatal diagnosis, some patients do not comply because of the small risk of losing the pregnancy that they have worked so hard to achieve. As a result, the possibility exists that other seemingly-normal 47,XXY boys or 45,X girls may be born who do not have their chromosomal abnormalities detected until much later in life.

The most unusual pairing of human chromosomes is that between the sex chromosomes during male meiosis. Despite morphological dissimilarities, the X and Y chromosomes recombine as a result of a single obligatory crossover in the pseudoautosomal region (Rouyer *et al.*, 1986). The pseudoautosomal region seems to be responsible for the initiation of pairing and the formation of the synaptonemal complex between sex chromosomes (Hassold *et al.*, 1991; Mohandas *et al.*, 1992). Because of the unique features associated with crossing over in the sex chromosomes, studies have focussed on abnormalities known to generate anomalous sex chromosome disjunction. Speed and Chandley (1990) studied infertile men with a normal 46,XY karyotype who had reduced sperm counts, by investigating meiotic prophase using electron microscopy. They noted that there appeared to be a reduced extent of XY synapsis in these infertile men relative to normal men studied in a similar manner. Further studies by Hassold *et al.* (1991) of men with a 47,XXY karyotype determined that there was a significant reduction in the recombination normally seen in the XY bivalent. Hassold's group concluded that this reduced recombination leads to an increased frequency of nondisjunction of the sex chromosomes. Similarly, the XY bivalent in the infertile man may have an increased tendency to non-disjoin, perhaps due to failure of the sex chromosomes to recombine. An increased frequency of chromosomally-abnormal sperm containing both an X and a Y chromosome could be expected, and our investigation of the sperm nuclei of infertile men confirms an elevated frequency of XY disomy.

Structural rearrangements or deletions in the XY pairing region have been associated with the breakdown of spermatogenesis between MI and MII (Chandley and Edmond, 1971; Gabriel-Robez *et al.*, 1990). However, in chromosomally-normal infertile men, Speed and Chandley (1990) observed cells where the X and Y chromosome failed to remain paired during MI. They suggested that initial pairing failures may be brought on by poor physiological conditions in spermatocytes (Setterfield *et al.*, 1988), resulting in sperm degeneration. Speed and Chandley (1990) also noted the presence of an enlarged proteinaceous stalked body on the Y long arm during late zygotene in oligozoospermic patients. The significance of this is as yet undetermined; however, the enlarged stalk is located close to the gene for spermatogenesis, AZF, (Tiepolo and Zuffardi, 1976) and may thus be implicated. In *Drosophila hydei* (Vogt and Henning, 1986) and *Drosophila melanogaster* (Bonaccorsi *et al.*, 1988), proteins are found to bind to newly-formed lampbrush loops following RNA transcription for the repetitive DNA sequences of male fertility factors on the Y chromosome. Speed and Chandley (1990) speculated that during transcription of AZF during spermatogenesis, protein binding may also be occurring and accumulation of protein could arise when maturation of the germ cells is impaired.

Although Miharu and associates (1994) have investigated the disomy frequency in infertile men utilizing FISH, it is difficult to compare their results to those obtained in the present study due to major differences in the structure of the studies. Miharu's group used one-colour FISH to determine the disomy frequency of chromosomes 1, 16, X and Y (so XY disomy could not be observed), and two-colour FISH of

chromosomes 17 and 18 was used to determine the diploidy frequency in order to extrapolate from these probes the incidence of diploidy for chromosomes 1, 16, X and Y. The infertile men studied by Miharu's group show more heterogeneity than the infertile patients in the present study, with 5 oligozoospermic men, 6 men with unexplained infertility and 1 with antisperm antibodies. In addition, a relatively small number of sperm nuclei were studied per donor (mean of 4291 sperm nuclei per donor for each of the investigated chromosomes). Given the above factors, it is perhaps not unexpected that there was no significant difference between the frequencies of aneuploidy found in the study's infertile men and fertile donors, and the authors concluded that aneuploidy was not a major contributor to infertility. One factor in particular is noteworthy: an adequate sample of sperm nuclei per donor must be studied in order to accurately evaluate the frequency of disomy, given that on average (for each chromosome analyzed), a single disomic sperm is observed per thousand sperm examined.

v. Sex Ratios

The sex ratio, as determined by FISH on the sperm of ten infertile men, showed that there were significantly more X-bearing sperm than Y-bearing sperm in three of the ten patients studied (patients 4, 5, and 6) (Table 11). Although the X- and Y-bearing sperm are expected to be in a 1:1 ratio, Spriggs *et al.* (in press) also report a normal donor in whom the frequency of X-bearing sperm is significantly higher than that of Y-bearing sperm. When considering the infertile men as a group, the frequency of X-bearing sperm is slightly higher than that of Y-bearing sperm

(Table 11), but this difference is statistically insignificant.

vi. Frequency of Diploidy

Utilizing two- and three-colour FISH allows the differentiation between diploid cells and disomic sperm. Other investigators have emphasized the importance of determining the diploidy frequency (Williams *et al.*, 1993; Bischoff *et al.*, 1994; Miharu *et al.*, 1994), and the mean frequencies of diploidy reported by these groups range from 0.15% to 0.45%. In the present investigation, diploid sperm were noted during analysis of both autosomes (chromosomes 1 and 12) and sex chromosomes, for all ten infertile men (Table 12). The mean frequency of diploidy for these men was determined to be 0.16%, which is within the range reported for normal men.

Individual frequencies of diploidy were fairly consistent, whether measured during the analysis of chromosomes 1 and 12, or during X- and Y-chromosome analysis. However, inter-patient variability in diploidy frequencies was observed, emphasizing the need to utilize multicolour-FISH in each sperm sample investigated to distinguish between diploid and disomic cells.

By using sex chromosome-specific probes, the proportion of diploid sperm resulting from complete failure of MII can be calculated. Williams *et al.*, (1993) determined that 75% of diploid cells were due to MII nondisjunction. In contrast, Spriggs *et al.*, (in press) found that only 20% of diploid cells were due to MII error, and 33% of diploid cells (45/138) were due to complete failure of MII division in the present study, suggesting that failure of MI rather than MII may be responsible for the majority of diploidy in sperm cells.

C. Comparison of Multicolour FISH and Cytogenetic Data

As stated previously, a study of sperm karyotypes allows the examination of all chromosomes present in a sperm, for both structural and numerical abnormalities. However, only a small sample size can be studied because of financial and time constraints, and although results indicate that sperm selection is not a factor in penetration of oocytes, it is difficult to prove conclusively. The inclusion of fluorescence *in situ* hybridization (FISH) allows a large sample size to be analyzed for numerical abnormalities in selected chromosome pairs, and completely eliminates the possibility of a selection bias in the sperm assessed.

A few trends become evident when comparing sperm karyotype and FISH results from the five patients (patients 1-5) studied using both methods. Of the 518 karyotypes obtained, sex chromosome content was analyzable in 495 karyotypes: 243 were X-bearing and 252 were Y-bearing giving the expected 1:1 ratio (Table 6). Eight (1.5%) of the 518 karyotypes contained an extra chromosome, a frequency which was significantly elevated over the incidence in normal fertile donors. Two of the eight hyperhaploid karyotypes contained a sex chromosome anomaly.

Table 15 shows the results obtained for men with idiopathic infertility compared to those obtained in our laboratory from normal men with proven fertility, to all the karyotypes obtained in our laboratory from normal men, and to the results obtained by Brandriff and Gordon (1990) for sex chromosomal aneuploidy.

Table 15. Sperm karyotype data for X- and Y-chromosome aneuploidy

Study	# Karyo-types	# XX (%)	# YY (%)	# XY (%)	Other	# Missing X or Y (%)
Infertile men in this study	518	0	0	1 (0.2)	48,XXYY	6 (1.16)
Fertile, normal donors in this study	3585	1 (0.03)	1 (0.03)	0	26, XYY,+9	12 (0.33)
Brandriff & Gordon, 1990	5997	0	2 (0.04)	3 (0.06)	-	11 (0.21)
Martin and Rademaker, 1990 and unpublished data	7191	2 (0.03)	1 (0.01)	2 (0.03)	26, XYY, +9	19 (0.26)

In the present study of infertile men, despite the relatively small sample size, the frequency of sex chromosomal aneuploidy in sperm karyotypes is high: the frequencies of both XY disomy (0.2%) and the corresponding nullisomic gamete (1.16%) are elevated. An analysis of the results obtained from FISH on the same five men shows the average frequency of XX disomy, YY disomy and XY disomy to be 0.08%, 0.09% and 0.33%, respectively. The frequency of XY disomy is elevated significantly from that seen in the normal group, as is the corresponding sperm karyotype value.

Sperm karyotype and FISH results are in general agreement: both show an increased frequency of sperm containing an extra autosome or sex chromosome. The increased aneuploidy frequency found in sperm karyotypes and the increase in chromosome 1's disomy frequency as obtained by FISH supports the hypothesis that pairing disruptions may be generalized beyond the sex chromosomes, affecting all chromosomes (Egozcue *et al.*, 1983).

D. Conclusions

Investigation of men with idiopathic infertility resulting in oligo-, astheno- or teratozoospermia suggests that their sperm contain an elevated frequency of numerical chromosomal abnormalities associated with both the autosomes and the sex chromosomes as determined by sperm karyotyping. These results are supported by further studies on a larger sample size of sperm nuclei from 10 infertile men using fluorescence *in situ* hybridization. The frequencies of chromosome 1 and XY disomy was significantly elevated in these men with idiopathic infertility. Pairing

abnormalities during spermatogenesis have been implicated in several studies of sperm from infertile men. The sperm of infertile men in this study show an increased frequency of aneuploidy; whether this is a consequence of abnormal meiotic pairing of homologous chromosomes remains to be determined. These chromosomally-abnormal sperm are present in the ejaculate, and may increase the risk to these men of fathering offspring with trisomies and sex chromosomal abnormalities. Fetal selection against autosomal abnormalities is high, so the frequency of liveborns with autosomal trisomies may remain low. However, selection against sex chromosomal abnormality in the fetus appears to be less stringent, resulting in a significantly-decreased frequency of sex chromosomal aneuploidy in spontaneously aborted fetuses (Hassold, 1986). With the increased frequency of sex chromosomal aneuploidy in the sperm of men with idiopathic infertility observed in this study, there is a concern that there may be an elevated occurrence of sex chromosomal abnormalities seen in liveborns fathered by these men. Because of this, couples with male-factor infertility may benefit from prenatal counselling coupled with prenatal or preimplantation diagnosis.

VI. REFERENCES

Ahmed, Ali. Al-Qur'an. New Jersey. Princeton University Press, 1990.

Al-Shawaf, T., and I. Craft. 1989. Sex ratio and assisted conception. Lancet 8653:53.

Andersson, M., Page, D.C., Brown, L.G., Elfving, K., and A. de la Chapelle. 1988. Characterization of a (Y;4) Translocation by DNA Hybridization. Hum Genet 78:377-381.

Antonarakis, S.E. 1991. Down syndrome collaborative group. Parental origin of the extra chromosome in trisomy 21 as indicated by analysis of DNA polymorphisms. New Engl J Med 324:872-876.

Baldini, A., Rocchi, M., Archidiacono, N., Miller, O.J., and D.A. Miller. 1990. A human alpha satellite DNA subset specific for chromosomes 12. Am J Hum Genet 46:784-788.

Bischoff, F.Z., Nguyen, D.D., Burt, K.J., and L.G. Shaffer. 1994. Estimates of aneuploidy using multicolor fluorescence in situ hybridization on human sperm. Cytogenet Cell Genet 66:237-243.

Benet, J. and R.H. Martin. 1988. Sperm chromosome complements in a 47,XYY man. Hum Genet 78:313-315.

Berthelsen, J.G., Skakkebaek, N.E., Perboll, O. and J. Neilsen. 1981. Electron microscopical demonstration of the extra Y chromosome in spermatocytes from human XYY males. In: Development and Function of Reproductive Organs, edited by A. G. Byskov and H. Peters. International Congress Series No. 559. Elsevier/North Holland, Amsterdam.

Boldt, J., Howe, A.M., Butler, W.J., Mcdonough, P.G. and S.L. Padilla. 1987. The value of oocyte reinsemination of human oocytes in vitro. Fertil Steril 45: 58-62.

Bonaccorsi, S., Pisano, C., Puoti, F., and M. Gatti. (1988). Y chromosome loops in

Drosophila melanogaster. Genetics 120:1015-1034.

Bourrouillou, G., Dastungue, N., and P. Colombies. 1985. Chromosome studies in 953 infertile males with a sperm count below 10 million/ml. Hum Genet 71: 366-367.

Brandriff, B., Gordon, L., Ashworth, L., Watchmaker, G., Moore II, D., Wyrobek, A.J., and A.V. Carrano. 1985a. Chromosomes of human sperm: Variability among normal individuals. Hum Genet 70:18-24.

Brandriff, B., Gordon, L., and G. Watchmaker. 1985b. Human sperm chromosomes obtained from hamster eggs after sperm capacitation in TEST-yolk buffer. Gamete Res 11:253-259.

Brandriff, B. and L.A. Gordon. 1990. Human sperm cytogenetics and the one-cell zygote. In: Mutation Induction and Heritability in Mammalian Germ Cells (Banbury Report 34). Allen J.W. (ed). Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. Pages 183-194.

Burgoyne, P.S. and T.G. Baker. 1984. Meiotic pairing and gametogenic failure. In Controlling Events in Meiosis (eds C.W. Evans and H.G. Dickinson), The Company of Biologists, Cambridge, pp. 349-362.

Chandley, A. C. 1979. The chromosomal basis of human infertility. Br Med Bull 35:181-186.

Chandley, A.C. and P.E. Edmond. 1971. Meiotic studies on a subfertile patient with a ring Y chromosome. Cytogenetics 10:295-304.

Chandley, A. C., Edmond, P., Christie, S., Gowans, L., Fletcher, J., Frackiewicz, A., and M. Newton. 1975. Cytogenetics and infertility in man I. Karyotype and seminal analysis. Results of a five year survey of men attending a subfertility clinic. Ann Hum Genet 39:231-253.

Chandley A.C., Maclean N., Edmond P., Fletcher J., and G.S. Watson . 1976. Cytogenetics and infertility in man. II. Testicular histology and meiosis. Results of a five year survey of men attending a subfertility clinic. Ann Hum Genet 40:165-176.

Chandley, A.C., Goetz, P., Hargreave, T.B., Joseph, A.M., and R.M. Speed. 1984. On the nature and extent of XY pairing at meiotic prophase in man. Cytogenet Cell Genet 38:241-247.

Chandley, A.C., Speed, R.M., McBeath, S., and T.B. Hargreave. 1986. A human 9;20 reciprocal translocation associated with male infertility analyzed at prophase and metaphase I of meiosis. Cytogenet Cell Genet 41:145-153.

Chernos, J.E. and Renee H. Martin. 1989. A Cytogenetic investigation of the effects of cryopreservation on human sperm. Am J Hum Genet 45:766-777.

Cohen, J., Talansky, B.E., Malter, H., Alikani, M., Adler, A., Reing, A., Berkeley, A., Graf, M., Davis, O. and H. Liu. 1991. Microsurgical fertilization and teratozoospermia. Hum Reprod 6:118-123.

Cohen, J., Schattman, G., Suzman, M., Adler, A., Alikani, M. and Z. Rosenwaks. 1994. Micromanipulating human gametes. Reprod Fertil Dev 6:69-83.

Cooke, H.J., and J. Hindley. 1979. Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 6:3177-3197.

Cooke, H.J., Brown, W.R.A. and G.A. Rappold. 1985. Hypervariable telomeric sequences from the human sex chromosomes are pseudoautosomal. Nature 317:687-692.

Cooper, K.W. 1964. Meiotic conjunctive elements not involving chiasmata. Proc Nat Acad Sci, Wash. 52:1248-1255.

Court Brown, W.M. 1968. Development and knowledge about males with an XYY sex chromosome complement. J Med Genet 5:341-359.

De Kretser, D.M., Yates, C.A., McDonald, J., Leeton, J.F., Southwick, G., Temple-Smith, P.D., Trounson, A.O., and E.C. Wood. 1985. The Use of in vitro Fertilization in the management of male infertility. In: Gamete Quality and Fertility Regulation, (ed) Rolland R., Heineman, M.J., Hiller, S.G., Verner, H., Amsterdam, Excertpa Medica, p 213-223, 1985.

Eastmond, D.A., and D. Pinkel. 1990. Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in situ hybridization with chromosome-specific probes. Mut Res 234:303-318.

Egozcue J., Templado C., Vidal F., Navarro J., Morer-Fargas F., and S. Marina. 1983. Meiotic studies in a series of 1100 infertile and sterile males. Hum Genet 65:185-188.

Eiben, B., Leipoldt, M., Rammelsberg, O., Krause, W., and W. Engel. 1987. High incidence of minor chromosomal variants in teratozoospermic males. Andrologia 19(6):684-687.

Estop, A., Catala, V., Santalo, J., and K. Garver. 1988. The chromosome constitution of highly motile mouse sperm. Am J Hum Genet 43 (Suppl.):A107.

Evans, E.P., Chaganti, R.S.K., Blank, C.E., and H. Hunter. 1970. XY spermatocytes in an XYY male. Lancet i, 719-720.

Fahmy, N.W., Benoit, J., Bissonnette, F., Duchesne, C., Girard, Y. and R. Sullivan. 1991. Impact of a second insemination on the results of an in vitro fertilization-embryo transfer (IVF-ET) program. J In Vitro Fertil Embryo Transfer 8:80-83.

Ferguson-Smith, M.A., Lennox, B., Mack, W.S., and Stewart J.S.S. 1957. Klinefelter's syndrome: frequency and testicular morphology in relation to nuclear sex. Lancet 2:167-169.

Fishel, S. Sperm-egg manipulation for the treatment of male factor infertility. In: Infertility (ed, A.A. Templeton and J.O. Drife). Oxford (UK): Springer-Verlag, 1992, 115-132.

Fisher, J.M., Harvey, J.F., Lindenbaum, R.H., Boyd, P.A., and P.A. Jacobs. 1993. Molecular studies of trisomy 18. Am J Hum Genet 52:1139-1144.

Foldesy, R.G., Bedford, J.M., and M.C. Orgebin-Crist. 1984. Fertilizing rabbit spermatozoa are not selected as a special population by the female tract. J Reprod Fert 70:75-82.

Ford, J.H., Schultz, C.J. and A.T. Correll. 1988. Chromosome elimination in micronuclei: a common cause of hypoploidy. Am J Hum Genet 43:733-740.

Forejt, J. 1974. Non-random association between a specific autosome and the X-chromosome in meiosis of the male mouse: possible consequence of homologous centromeres separation. Cytogenet Cell Cenet 13:369-383.

Forejt, J. 1979. Meiotic studies of translocations causing male sterility in the mouse. II. Double heterozygotes for Robertsonian translocations. Cytogenet Cell Genet 23:163-170.

Foss, G.L. and F.J.W. Lewis. 1971. A study of four cases with Klinefelter's syndrome, showing motile spermatozoa in their ejaculates. J Reprod Fert 25:401-408.

Gabriel-Robez, O., Ratomponirina, C., Dutrillauz, B., Carre-Pigeon, F., and Y. Rumpler. 1986. Meiotic Association Between the XY Chromosomes and the Autosomal Quadrivalent of a Reciprocal Translocation in Two Infertile Men, 46XY,t(19;22) and 46,XY,t(17;21). Cytogenet Cell Genet 43:154-160.

Gabriel-Robez, O., Rumpler, Y., Ratomponirina, C., Petit, C., Croquette, M.F. and J. Couturier. 1990. Deletion of the pseudoautosomal region and lack of sex-chromosome pairing at pachytene in two infertile men carrying an X;Y translocation. Cytogenet Cell Genet 54:38-42.

Grell, R.F. 1969. Meiotic and somatic pairing. In: Genetic Organization (ed, E.W. Caspari and A.W. Ravin) Academic Press, London, pg 361-493.

Guichaoua, M.R., Quack, B., Speed, R.M., Noel, B., Chandley, A.C. and J. M. Luciani. 1990. Infertility in Human Males with Autosomal Translocations: Meiotic Study of a 14;22 Robertsonian Translocations. Hum Genet 86:162-166.

Guichaoua, M.R., Speed, R.M., Luciani, J.M., Delafontaine, D., and A.C. Chandley. 1992. Infertility in Human Males With Autosomal Translocations II. Meiotic Studies in Three Reciprocal Rearrangements, One Showing Tertiary Monosomy in a 45-Chromosome Individual and His Father. Cytogenet Cell Genet 60:96-101.

Guttenbach, M. and M. Schmid. 1991. Non-isotopic detection of chromosome 1 in

human meiosis and demonstration of disomic sperm nuclei. Hum Genet 87:261-265.

Guttenbach, M., Schakowski, R. and M. Schmid. 1994. Incidence of chromosome 3, 7, 10, 11, 17, and X disomy in mature human sperm nuclei as determined by nonradioactive in situ hybridization. Hum Genet 93:7-12.

Hassold, T.J. 1986. Chromosome abnormalities in human reproductive wastage. Trends Genet 2:105-110.

Hassold, T.J., Sherman, S.L., Pettay, D., Page, D.C. and P.A. Jacobs. 1991. XY Chromosome nondisjuction in man is associated with diminished recombination in the pseudoautosomal region. Am J Hum Genet 49:253-260.

Hassold, T., Pettay, D., Robinson, A. and I Uchida. 1992. Molecular studies of parental origin and mosaicism in 45,X conceptuses. Hum Genet 89:647-652.

Hendry W.F., Polani P.E., Pugh R.C.B., Sommerville I.F. and D.M. Wallace. 1976. 200 Infertile males: correlation of chromosome, histological, endocrine and clinical studies. Br J of Urology 47:899-908.

Hewitt, G. 1973. Variable transmission rates of a B-chromosome in *Myrmeleotettix maculatus*. Chromosoma 40:83-106.

Hojager, B., Peters, H., Byskov, A.G. and M. Faber. 1978. Follicular development in ovaries of children with Down's syndrome. Acta Paediat Scand 67:637-643.

Hulten, M. And P.L. Pearson. 1971. Fluorescent evidence for spermatocytes with two Y chromosomes in an XYY males. Ann Hum Genet 34:273-276.

Imoedemhe , D.A.G., and A.B. Sigue. 1993. Subzonal multiple sperm injection in the treatment of previous failed human in vitro fertilization. Fert Steril 59(1):172-176.

I.S.C.N. 1985. An international system for human cytogenetic nomenclature. Birth Defects: Original Article Series 21(1). S. Karger, Basel.

Jabs, E.W., Goble, C.A. and G.R. Cutting. 1989. Macromolecular organization of

human centromeric regions reveals high-frequency, polymorphic macro DNA repeats. Proc Natl Acad Sci USA 86:202-206.

Jacobs, P.A. Population surveillance: A cytogenetic approach. In: Morton N.E. and C.S. Chung. "Genetic Epidemiology". New York, Academic Press, 1978. p 463.

Jacobs, P.A., Baikie, A.G., Court Brown, W.M., Macgregor, T.N., Maclean, N. and D.G. Harnden 1959. Evidence for the existence of the human 'super female'. Lancet ii, 423-425.

Jacobs, P.A., Hassold, T., Harvey, J. and K. May. The origin of sex chromosome aneuploidy. In: Hassold, T.J. and C.J. Epstein (ed.). Molecular and Cytogenetic Studies of Nondisjunction. Alan R. Liss, N. Y. 1989. p 130-144.

Johannisson R., Lohrs, U., Wolff, H.H. and E. Schwinger. 1987. Two different XY-quadrivalent associations and impairment of fertility in men. Cytogenet Cell Genet 45:222-230.

Johnston, A.W., Ferguson-Smith, M.A., Handmaker, S.D., Jones, H.W. and G.S. Jones. 1961. The triple-X syndrome. Br Med J ii, 1046-1052.

Juberg, R.C., Knops J. and P.N. Mowrey. 1985. Increased frequency of lymphocytic mitotic non-disjunction in recurrent spontaneous aborters. J Med Genet 22:32-35.

Levron, J., Brandes, J.M., Lightman, A., Itskovitz-Eldor, J. and D.W. Stein. 1992. Pregnancy after subzonal insertion of cryopreserved spermatozoa from patient with testicular seminoma. Fert Steril 58(4):839-840.

Lifschytz, E. and D.L. Lindsley. 1972. The role of X-chromosome inactivation during spermatogenesis. Proc Nat Acad Sci USA. 69:182-186.

Lindsten, J. 1963. The nature and origin of X chromosome aberrations in Turner's syndrome. A cytogenetical and clinical study of 57 patients. Almquist and Wiksell, Uppsala.

Lisker, R., M.T. Zenzes and M.T. Fonesca. 1968. YY syndrome in a Mexican. Lancet ii, 635.

Lorda-Sanchez, I., Binkert, F., Maechler, M., Robinson, W.P. and Schinzel, A.A. 1992. Reduced recombination and paternal age effect in Klinefelter syndrome. Hum Genet 89: 524-530.

Luciani J.M., Guichaoua M.R., Delafontaine D., North M.O., Gabriel-Robez O. and Y. Rumpler. 1987. Pachytene analysis in a 17;21 reciprocal translocation carrier: role of the acrocentric chromosomes in male sterility. Hum Genet 77:246-250.

Malter, H.E. and J. Cohen. 1989. Partial zona dissection of the human oocyte: a nontraumatic method using micromanipulation to assist zona pellucida penetration. Fertil Steril 51:139-148.

Matsuda, T., Nonomura, M., Yamamoto, S., Hayashi, K. and O. Yoshida. 1991. Sperm morphology in subfertile carriers of autosomal translocations. Int J Fertil 36:178-182.

Martin, R.H. 1983. A detailed method for obtaining preparations of human sperm chromosomes. Cytogenet Cell Genet 35:252-256.

Martin, R.H. and E. Ko. Fluorescence in situ hybridization in human sperm. In: Verma R.S. and Babu A., editors. Human chromosomes: manual of basic techniques. New York, NY: McGraw-Hill, 1994:223-231.

Martin, R.H., Rademaker, A.W., Hildebrand, K., Long-Simspon, L., Peterson, D., and Y. Yamamoto. 1987. Variation in the frequency and type of sperm chromosomal abnormalities among normal men. Hum Genet 77:108-114.

Martin R.H. and Rademaker, A. 1988. The relationship between sperm chromosomal abnormalities and sperm morphology in humans. Mut Res 207:159-164.

Martin, R.H. and A. Rademaker. 1990. The frequency of aneuploidy among individual chromosomes in 6,821 human sperm chromosome complements. Cytogenet Cell Genet 53:103-107.

Martin, R.H. and A.W. Rademaker. 1995. Reliability of aneuploidy estimates in human sperm: results of fluorescence in situ hybridization studies using two different scoring criteria. Molecular Reproduction and Development (in press).

May, K.M., Jacobs, P.A., Lee, M., Ratcliffe, S., Robinson, A., Nielsen, J. and T.J. Hassold. 1990. The parental origin of the extra X chromosome in 47,XXX females. Am J Hum Genet 46:754-761.

McIlree, P.E., Price, W.H., Court-Brown, W.M., Tulloch, W.S., Newsan, J.E. and N. MacLean. 1966. Chromosome studies on testicular cells from 50 subfertile men. Lancet 2:69-71.

Micic, M., Nikolis, J. and S. Micic. 1992. Clinical and meiotic studies in an infertile man with Y:13 translocation. Hum Repro 7(8):1118-1120.

Miharu, N., Best, R.G. and S.R. Young. 1994. Numerical chromosome abnormalities of fertile and infertile men detected by fluorescence in situ hybridization. Hum Genet 93:502-506.

Mikamo, K., Kamiguchi, Y., and H. Tateno. 1990. Spontaneous and in vitro radiation-induced chromosome aberrations in human spermatozoa: application of a new method. In: Mutation and The Environment, Part B: metabolism, testing methods and chromosomes. Proceedings of the Fifth International Conference on Environmental Mutagens, Cleaveland, Ohio, July 10-15, 1898, (ed. M.L. Mendelsohn and R.J. Albertini) pp. 447-456. Wiley-Liss Inc., New York.

Miklos, G.L.G. 1974. Sex-Chromosome Pairing and Male Fertility. Cytogenet Cell Genet 13:558-577.

Mohandas, T.K., Speed, R.M., Passage, M.B., Yen, P.H., Chandley, A.C. and L.J. Shapiro. 1992. Role of the pseudoautosomal region in sex chromosome pairing during male meiosis: meiotic studies in a man with a deletion of distal Xp. Am J Hum Genet 51:526-533.

Mortimer, D. 1979. Differential motility of diploid rabbit spermatozoa. Arch Androl 2:41-47.

Morton, N.E., Lindsten, J., Iselius, L. and S. Yee. 1982. Data and theory for a revised chiasma map of man. Hum Genet 62:266-270.

Navarro, J., Templado, C., Benet, J., Lange, R., Rajmil, O. and J. Egozcue. 1990. Sperm chromosome studies in an infertile man with partial, complete asynapsis of

meiotic bivalents. Hum Repro 5:227-229.

Ng, S.C., Bongso, A., Ratnam, S.S., Sathananthan, H., Chan, C.L., Wong, P.C., Hagglund, L., Anandakumar, C., Wong, Y.C. and V.H. Goh. 1988. Pregnancy after transfer of sperm under zona. Lancet 2(8614):790.

Ng, S.C., Bongso, A. and S.S. Ratnam. 1991. Microinjection of human oocytes: a technique for severe oligoasthenoteratozoospermia. Fert Steril 56:1117-1123.

Neilsen, J. and I Sillesen. 1975. Incidence of chromosome aberrations among 11,148 newborn children. Humangenetik 30:1-12.

Nestor, A. and M.A. Handel. 1984. The transport of morphological abnormal sperm in the female reproductive tract of mice. Gamete Res 10:119-125.

Nothen, M.M., Eggermann, T., Erdmann, J., Eiben, B., Hofmann, D., Propping, P. and G. Schwanitz. 1993. Retrospective study of the parental origin of the extra chromosome in trisomy 18 (Edwards syndrome). Hum Genet 92:347-349.

Palermo, L.G., Joris, H., Devroey, P. and A.C. Van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340:17-18.

Pampiglione, J.S., Mills, C., Campbell, S., Steer, C., Kingsland, C. and B.A. Mason. 1990. The clinical outcome of reinsemination of human oocytes fertilized in vitro. Fertil Steril 53:306-310.

Rappold, G.A., Klink, A., Weiss, B. and C. Fisher. 1994. Double crossover in the human Xp/Yp pseudoautosomal region and its bearing on interference. Hum Mol Genet 3(8):1337-1340.

Rathenberg, G. and D. Muller. 1973. X and Y chromosome pairing and disjunction in the male mouse with an XYY sex-chromosome constitution. Cytogenet Cell Genet 12:82-92.

Redi, C.A., Garagna, S., Pellicciari, C., Manfredi Romanin, M.G., Capanna, E., Winking, H. and A. Gropp. 1984. Spermatozoa of chromosomally heterozygous

mice and their fate in male and female genital tracts. Gamete Res 9:273-286.

Retief, A.E., Van Zyl, J.A., Menkveld, R., Fox, M.F., Kotze, G.M. and J. Bursnicky. 1984. Chromosome studies in 496 infertile males with a sperm count below 10 million/ml. Hum Genet 66:162-164.

Rosenbusch, B. and K. Sterzik. 1991. Sperm chromosomes and habitual abortion. Fertil Steril 56:370-372.

Rosenmann, A., Wahrman, J., Richler, C., Voss, R., Persitz, A. and B. Goldman. 1985. Meiotic association between the XY chromosomes and unpaired autosomal elements as a cause of human male sterility. Cytogenet Cell Genet 39:19-29.

Rosner, B. 1986. Fundamentals of biostatistics. 2nd ed. Duxbury, Boston.

Rouyer, F., Simmler, M.C., Johnsson, C., Vergnaud, G., Cooke, H.J., and J. Weissenbach. 1986. A gradient of sex linkage in the pseudoautosomal region of the human sex chromosomes. Nature 319:291-295.

Royal Commission on New Reproductive Technologies. The Prevalence of Infertility in Canada. Volume 6 of the Research Studies. Canada Communications Group, 1993.

Rudak, E., Jacobs, P.A. and R. Yanagimachi. 1978. Direct analysis of the chromosome constitution of human spermatozoa. Nature 274:911-913.

Sakkas, D., Gianaroli, L., Diotallevi, L., Wagner, I., Ferraretti, A.P. and A. Campana. 1992. IVF treatment of moderate male factor infertility: a comparison of mini-Percoll, partial zona dissection and sub-zonal insertion techniques. Hum Repro 8(4):587-591.

Sandler, L. and G. Braver. 1954. A meiotic loss of unpaired chromosomes in *Drosophila melanogaster*. Genetics 39:365-377.

Schmitt, K., Lazzeroni, L.C., S. Foote, D. Vollarath, E.M.C. Fisher, Goradia, T.M., Lange, K., Page, D.C. and N. Arnheim. 1994. Multipoint linkage map of the human pseudoautosomal region, based on single sperm typing: Do double crossovers

occur during male meiosis? Am J Hum Genet 55:423-430.

Setterfield, L.A., Mahadevaiah, S. and U. Mittwoch. 1988. Chromosome pairing and germ cell loss in male and female mice carrying a reciprocal translocation. J. Reprod Fert 82:369-379.

Sherman, S.L., Takaesu, N., Freeman, S.B., Grantham, M., Phillips, C., Blackston, R.D., Jacobs, P.A., Cockwell, A.E., Freeman, V., Uchida, I., Mikkelsen, M., Kurnit, D.M., Buraczynska, M., Keats, B.J.B. and T.J. Hassold. 1991. Trisomy 21: Association between reduced recombination and nondisjunction. Am J Hum Genet 49:608-620.

Simmler M.C., Rouyer, F., Vergnaud, G., Nystrom-Bahti, M., Ngo, K.Y., de la Chapelle, A. and J. Weissenbach. 1985. Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes. Nature 317:692-297.

Skakkebaek, N.E., Hulten, M., and J. Philip. 1969. Meiotic chromosomes in Kelinefleter's syndrome. Nature, London 221:1075-1076.

Skakkebaek, N.E., Hulten, M., Jacobsen, P. and M. Mikkelsen. 1973. Quantification of human seminiferous epithelium. II. Histological studies in eight 47,XYY men. J Repro Fert 32:391-401.

Smith, G.F. and J.M. Berg. 1976. Down's anomaly, 2nd edition. Churchill Livinstone, Edinburgh.

Soong, U.K., Lai, Y.M. Chang, S.Y., Wang, M.L., Chang, M.Y. and Lee, C.L. 1993. A successful pregnancy after subzonal insertion with epidiymal sperm and coculture on Vero cell monolayer. Fert Steril 59(6):1308-1310.

Spriggs, E.L., Rademaker, A.W. and R.H. Martin. Aneuploidy in human sperm: Results of two- and three-colour fluorescence in situ hybridization using centromeric probes for chromosomes 1, 12, 15, 18, X and Y. Cytogenet Cell Genet (in press).

Steer, C., Tan, S.L., Rizk, B., Bringden, P., Mason, B.A., and S. Campbell. 1989. Sex ratio and in vitro fertilization. Lancet 8667:863.

Stern, C. 1960. Addendum to Stewart-Mechanisms of meiotic nondisjunction in man. Nature, Lond. 187:805.

Stewart, J.S.S. and A.R. Sanderson. 1960. Fertility and oligophrenia in an apparent triple-X female. Lancet ii, 21-23.

Speed, R.M. and A.C. Chandley. 1990. Prophase of meiosis in human spermatocytes analyzed by EM microspreading in infertile men and their controls and comparisons with human oocytes. Hum Genet 84:547-554.

Tagarro, I., Fernandez-Peralta, A.M. and J.J. Gonzalez-Aguilera. 1994. Chromosomal localization of human satellites 2 and 3 by a FISH method using oligonucleotides as probes. Hum Genet 93(4):383-388.

Tarkowski, A.K. 1966. An air-drying method for chromosome preparations from mouse eggs. Cytogenet 5:394-400.

Tettenborn U, Gropp, A., Murken, J.D., Tinnefeld, W., Fuhrmann, W. and E. Schwinger. 1970. Meiosis and testicular histology in XYY males. Lancet ii, 267-268.

Thatcher, S., Restrepo, U., Lavy, G. and A.H. DeCherney. 1989. Invitro fertilization and sex ratio. Lancet 8645:1025-1026.

Thompson, H., Melnyk, J. and F. Hecht. 1967. Reproduction and meiosis in XYY. Lancet ii, 831.

Tiepolo L. and O. Zuffardi. 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Genet 34:119-124.

Tournaye, H., P. Devroey, M. Camus, C. Staessen, B. Nico, Smitz, J. and A.C. Van Steirteghem. 1992. Comparison of in-vitro fertilization in male and tubal infertility: a 3 year survey. Hum Repro 7:218-222.

Trounson, A. and J. Webb. 1984. Fertilization of human oocytes following reinsemination in vitro. Fertil Steril 41:816-819.

Van Steirteghem A.C., Nagy Z., Joris H., Liu J., Staessen C., Smitz J., Wisanto A. and P. Devroey. 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum Repro 8(7):1061-1066.

Van Steirteghem, A.C., Liu, J., Joris, H., Nagy, Z., Staessen, C., Camus, M., Wisanto, A., Van Assche, E. and P. Devroey. 1994. Assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. Reprod Fertil Dev 6:85-91.

Vogt, P. and W. Henning. 1986. Molecular structure of the lampbrush loop nooses of the Y chromosome of *Drosophila hydei*. I. The Y chromosome-specific repetitive DNA sequence family ay I is dispersed in the loop DNA. Chromosoma 94:449-458.

Williams, B.J., Ballenger, C.A., Malter, H.E., Bishop, F., Tucker, M., Zwingman, T.A. and T.J. Hassold. 1993. Non-disjunction in human sperm:results of fluorescence in situ hybridization studies using two and three probes. Hum Mol Genet 2:1929-1936.

Wyrobek, A.J., Robbins, W.A., Tang, C., Kobayashi, A., Baulch, J., Weier, H.U. and D. Pinkel. 1993. Hierarchical organization of human sperm chromatin is a critical factor in the detection of chromosomal aneuploidy by fluorescence in situ hybridization. Am J Hum Genet 53(Suppl.):130.

VII. APPENDICES

A. Media and Solutions for the Human Sperm / Hamster Oocyte Fusion Assay

BWW Stock Solution

	g/L
NaCl	5.540
KCl	0.356
$CaCl_2$ (pellets)	0.189
KH_2PO_4	0.162
$MgSO_4.7H_2O$	0.294
Pyruvic acid, Na salt	0.028
Dextrose	1.000

	ml/L
Antibiotic Stock Solution	1.0
0.5% Phenol Red	0.5
Acid Hepes	9.5
Base Hepes	10.5
Distilled H_2O	To raise to 100 ml

Maintain solution at 4°C for a maximum of two weeks.

BWW Working Solution

$NaHCO_3$	0.2106 g
DL-Lactic Acid, Na Salt (60% syrup)	0.37 ml
Human Serum Albumin (Fraction V)	0.5 g
BWW Stock Solution	100 ml

Mix and dissolve crystals, then filter-sterilize through a cellulose acetate/nitrate membrane (pore size 0.22 um).

Antibiotic Stock Solution For BWW

Penicillin-G, Na Salt	10^5 IU/ml
Streptomycin sulfate	50 mg/ml

Freeze in 1 ml aliquots.

Acid Hepes (2M Hepes in distilled H_2O)

Hepes	47.66 g
Distilled H_2O	To raise to 100 ml

Base Hepes (2M Hepes in 3M NaOH)

NaOH	12 g
Hepes	47.66 g
Distilled H_2O	To raise to 100 ml

F10 Working Solution (15% Fetal Bovine Serum)

Ham's F10	42.5 ml
Fetal Bovine Serum (heat inactivated at 56°C for 30 minutes)	7.5 ml
Antibiotic Stock Solution	0.5 ml

Antibiotic Stock Solution for F10 Working Solution

Penicillin-G, Na Salt	10^5 IU/ml
Streptomycin Sulfate	5 mg/ml

Hyaluronidase Solution (0.2%)

Hyaluronidase (Type 1-S)	3 mg
BWW Working Solution	1.5 ml

Working concentration will be halved as it is added to an equivalent volume of BWW Working Solution.

Trypsin Solution (0.1%)

Trypsin (Type XII)	3 mg
BWW Working Solution	3 ml

Colcemid Solutions

For Afternoon Experiments (0.8 ug/ml)

Colcemid (Gibco, 10 ug/ml)	0.2 ml
F10 Working Solution	2.3 ml

For Morning Experiments (0.4 ug/ml)

Colcemid (Gibco, 10 ug/ml)	0.1 ml
F10 Working Solution	2.4 ml

Both are added to equivalent amounts of F10 Working Solution, however, the concentration of the colcemid is halved.

Sperm Count Diluent

$NaHCO_3$	5 g
35% Formaldehyde	1 ml
0.9% NaCl	100 ml

Mix and store at 4°C.

Ackerman's Cryopreservant

Bacto Egg Yolk Enrichment-50%	40 ml
Glycerol	30 ml
Glycine	2.0 g
Glucose	2.6 g
Sodium Citrate	2.3 g
Distilled H_2O	98.7 ml

Heat inactivate at 56°C for 30 minutes. Adjust pH and store at -20°C in aliquots.

Test Yolk Buffer Salt Stock

TES	2.1629 g
Tris	0.5135 g
Dextrose	0.1000 g
Streptomycin Sulfate	0.0125 g
Penicillin-G, Na Salt	0.0075 g
Distilled H_2O	Raise to 50 ml

Freeze in 10 ml aliquots.

TEST Yolk Buffer

Fresh Hen's Yolk	2.5 ml
TEST Yolk Buffer Salt Stock	10 ml

Mix and centrifuge (1200xG) for 10 minutes. Decant the supernatant and adjust the pH with solid Tris. Store 1 ml aliquots at -20°C.

Giemsa Solid Stain (5-6% n Gurr Buffer)

Harleco Gimesa Stain	3 ml
Gurr Buffer	50 ml

Skim off oxidized surface prior to use. Needs to be made fresh daily.

Gurr Buffer

1 Gurr® Buffer Tablet (pH 6.8) in 1 litre distilled H_2O. Adjust the pH to 4.4 and store at 4°C.

Quinacrine Dihyrochloride

Quinacrine Dihyrochloride	0.25 g
Distilled H_2O	50 ml

May be maintained at 4°C for up to one week. The container is wrapped in foil to protect from the light.

B. Media and Solutions for Fluorescence *in situ* hybridization

Dithiothreitol (DTT)	10 mM DTT in 0.1M TRIS
Lithium salt (LIS) / DTT solution	10mM LIS, 1mM DTT in 0.1 M TRIS
MM2.1	55% formamide, 1x SSC, 10% dextran sulfate
Pre-hybridization bath	70% formamide, 2x SSC (pH 7.0)
Post-hybridization wash	50% formamide, 2x SSC

	(pH 7.0)
PN buffer	0.1 m $NaH_2PO_47H_2O$, 0.1 M $NaH_2PO_42H_2O$, pH 8, 0.1% nonidet P-40
PNM buffer	PN Buffer plus 5% nonfat dry milk and 0.02% Na azide
Avidin-Cy3™	3.3 $\mu g/\mu l$ in PNM buffer
Propidium iodide	25 ng/ ml in PN buffer
DAPI	0.000125 $\mu g/\mu l$ in PN buffer
Goat anti-avidin	0.01 mg/ml in PNM buffer

C. Reagents

Bacto Egg Yolk Enrichment (50%)	Difco 3347-73-8
$CaCl_2$ (pellets)	Fisher C-614
Colcemid (10 ug/ml)	Gibco G.D. 1024
Formaldehyde	BDH B10113
D-Glucose (Dextrose)	Fisher D-16
DL-Lactic Acid, Na Salt (60% Syrup)	Sigma L4263

Ethanol	
Fetal Bovine Serum (Cellect Silver)	Flow 29-161-49
Giemsa Stain	Harleco 620
Glacial Acetic Acid	Fisher A38-4
Glycerol	
Gurr® Buffer Tablets (pH 6.8)	Hopkins and Williams 065568
Glycine	
1X Ham's F10 with L-Glutamine	Flow 12-403-54
Hepes	Sigma H3375
Human Choronic Gonadotropin	A.P.L. Ayerst
Human Serum Albumin (Fraction V)	Sigma A2386
Hyaluronidase (Type 1-S)	Sigma 3506
KH_2PO_4	Fisher P-382
$MgSO_4.7H_2O$	Fisher M-63
NaCl	Fisher S671-500
Na Citrate	Fisher S279

$NaHCO_3$	Fisher S233
NaOH	Fisher S318B
Penicillin-G, Na Salt	Sigma P-3032
Phenol Red	EM PX0530-3
Polyvinylpyrollidone	
Pregnant Mares' Serum Gonadotropin	Sigma G4877
Pyruvic Acid, Na Salt (Type II)	Calbiochem 5510
Quinacrine Dihydrochloride	Sigma Q-0250
Streptomycin Sulfate	Sigma S-6501
TES (N-tris [Hydromethyl]methyl-2-aminoethanesulfonic acid)	Sigma T-1375
Tris (Trizma® Base-Tris [Hydroxymethyl]aminomethane)	Sigma T-1503
Trypsin (Type XII)	Sigma 2884