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ANALYSIS OF ANEUPLOIDY IN HUMAN OOCYTES AND SPERM BY CYTOGENETICS AND MULTI-COLOUR FLUORESCENCE *IN SITU* HYBRIDIZATION

by

KATE W.K. CHAN

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

AUGUST, 1993

ISBN 0-315-88497-5

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Analysis of Aneuploidy in Human Oocytes and Sperm by Cytogenetics and Multi-Colour Fluorescence *in situ* Hybridization" submitted by Kate W.K. Chan in partial fulfillment of the requirements for the degree of Master of Science.

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Dr. Birgitte Roland,
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August , 1993

ABSTRACT

A technique for the retrieval and *in vitro* maturation of non-stimulated oocytes from ovarian tissue is presented. Oocyte chromosomes were analyzed for aneuploidy using cytogenetic and two-colour fluorescence *in situ* hybridization (FISH) techniques. Two metaphases were cytogenetically analyzable: a normal 23,X and an abnormal 24,X,+G. The two oocytes analyzed by FISH, unfortunately, had not matured. From this small sample it is impossible to determine the frequency of aneuploidy or the effect of maternal age on aneuploidy.

The frequency of X- and Y-chromosome aneuploidy in 11,548 sperm from a normal male was determined, using three-colour FISH and an internal autosome hybridization control. The frequency of sex chromosome aneuploidy was: 0.24% XX, 0.18% YY, 0.29% XY, and 0.37% with no sex chromosome. Multi-colour FISH is a sensitive and rapid technique. However, international guidelines for sample preparation, probes, and scoring criteria must be established before its reliability can be assessed.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Renée Martin, for her continual guidance and support throughout the course of my M.Sc program. Her knowledge in the field of human aneuploidy was an asset to my studies and source of inspiration for women in science. I would also like to thank the members of my supervisory committee, Drs. N. Torben Bech-Hansen, and J.B. Rattner, for their advice and participation. Dr. Birgitte Roland is gratefully acknowledged for her contribution as examiner.

Special thanks to the doctors, nurses, and staff in the Departments of Obstetrics and Gynecology and Pathology at the following hospitals: Foothills Hospital, Calgary General Hospital-Peter Lougheed Center, Calgary General Hospital-Bow Valley Center, and Grace Women's Hospital. My studies would not have been possible without their participation.

Many thanks to all members of Renée's laboratory, especially Leona Barclay for her technical expertise and her good vibes, and Evelyn Ko for her encouragement, patience, and careful reading of this manuscript. Many thanks also to Kathy Gratton for her technical help in probe preparation.

Thanks also to Beth, Nafisa, Judy, and everybody at the Alberta Children's Hospital Cytogenetics Lab for their advice and support.

DEDICATION

To my parents.

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LIST OF ABBREVIATIONS

cc	cubic centimeter
cm	centimeters
Cy3™	Cyanine-3
°C	degrees celsius
DAPI	4,6,diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dUTP	deoxyuridine triphosphate
DNA	deoyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FITC	fluorescein-isothiocyanate
g	gram
mg	milligram
μg	microgram
ng	nanogram
G	gravity
HCl	hydrochloric acid
IU	International Units
Kb	kilobase
LIS	3,5 diiodosalicyclic acid-lithium salt
l	liter
Mb	megabase
μC	microcurie
ml	milliliter
μl	microliter
mm	millimeter
μm	micrometer
mM	millimolar

M	molar
nm	nanometer
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
V	volt

I. INTRODUCTION

Chromosomal aneuploidy is a significant cause of pregnancy wastage, congenital malformations and mental retardation in liveborns. An aneuploid conceptus usually arises at fertilization from fusion between a normal haploid gamete and a gamete with an abnormal chromosome number. The abnormal gamete can be either a sperm or an oocyte, and the error can arise during either the first or second meiotic division.

The most common cause of aneuploidy is nondisjunction, which describes the movement of nondisjoined bivalents (meiosis I) or univalents (meiosis II) into one of the two daughter cells. The result is a bivalent or univalent loss in one daughter cell with a corresponding bivalent or univalent gain in the other daughter cell. Another cause of aneuploidy is anaphase lag where a univalent (anaphase I) or sister chromatid (anaphase II) is lost by exclusion from a daughter cell independent of the other daughter cell.

A. PREVALENCE OF ANEUPLOIDY

The effect of a change in chromosome number is a change in gene dosage and genetic imbalance. Phenotypic consequences of genetic imbalance are not as dependent on the total chromosome number as on the identity of the missing or extra chromosome. Since each chromosome contains a unique ensemble of genes, the consequences of the genetic imbalance will depend on which genes are extra or deficient. The degree of genetic imbalance will determine whether the conception will result in a livebirth, stillbirth, spontaneous abortion, or preimplantation loss.

Aneuploidy in liveborns is restricted primarily to trisomies of chromosomes 13, 18, 21, X, and Y, and monosomy of chromosome X. Generally, only trisomy 21 and sex chromosome aneuploidies survive beyond the perinatal period. The frequency of all aneuploidies in liveborns is approximately 0.32 percent, of which 0.14 percent involves the autosomes, and 0.18 percent involves the sex chromosomes (Bond and

Chandley, 1983). Among autosomal aneuploidies, a missing chromosome is rarely observed. However, sex chromosome aneuploidies can involve an extra chromosome, as in the trisomies XXX, XXY and XYY, or a missing chromosome, as in monosomy X.

The incidence of chromosomal abnormalities in stillbirths is not well-characterized. The overall frequency is approximately 4 percent (Hassold and Jacobs, 1984), which is an order of magnitude higher than in liveborns. The rate among macerated fetuses (10 percent) is much higher than among non-macerated fetuses (3.5 percent) (Jacobs and Hassold, 1987); therefore, this frequency is probably underestimated because of technical difficulties in the culture of macerated tissue. In stillborns as in liveborns, the most common autosomal aneuploidies are trisomies 13, 18 and 21, with XXX, XXY and XO sex aneuploidies also represented (Hassold and Jacobs, 1984; Alberman and Creasy, 1977).

A summary of numerous cytogenetic surveys indicates that approximately 50 percent of all spontaneous abortions show some karyotypic abnormality (Jacobs, 1992), which is one order of magnitude greater than found in stillborns and two orders of magnitudes greater than found in liveborns. Of these, 73 percent are aneuploid, with trisomies accounting for 55 percent and monosomies contributing the remaining 18 percent (Jacobs, 1992). Trisomies for all chromosomes except for chromosome 1 have been identified in spontaneous abortions (Bond and Chandley, 1983). Approximately 26 percent of spontaneous abortions are trisomic, but the incidence of the individual trisomies varies greatly (Hassold and Jacobs, 1984). Trisomy 16 occurs most frequently, accounting for almost one third of all trisomies seen in clinically-recognized pregnancies. Trisomies of the G-group chromosomes, 21 and 22, are observed the next most frequently; when taken together, trisomies 16, 21 and 22 constitute almost half of the trisomies seen in spontaneous abortions. Monosomy X accounts for almost 20 percent of the conceptions which have a chromosomal abnormality

(Hassold, 1986).

Cytogenetic abnormalities of preimplantation losses are difficult to characterize. The frequency can be expected to be quite high, since the percentage of chromosomal anomalies appears in increasing orders of magnitude as gestational age decreases. It has been reported that at least 25 percent of all pregnancies are lost prior to implantation, and that an additional 30 percent are lost in the early post-implantation period, before pregnancy is clinically recognized (Kline and Stein, 1985). The reasons for early pregnancy wastage are largely unknown as these preimplantation and early postimplantation stages are difficult to study. It is reasonable, however, to suppose that a significant percentage of these conceptions have cytogenetic abnormalities which do not allow the embryo to survive to the stage where the pregnancy is clinically recognizable.

B. Pregnancy Wastage Studies

Genetic studies of pregnancy wastage have some limitations: unintentional and intentional selection of specimens, unreliable assignment of gestational age to a specimen, the inability to study preimplantation and early postimplantation losses and nondetection of chromosomal abnormalities whose genetic consequences are mild. For example, although XYY trisomies account for 0.04 percent of all clinically-recognized pregnancies, they are seldom lethal and therefore are rarely represented in spontaneous abortions or stillborns (Hassold and Jacobs, 1984).

To better understand the etiology of human chromosomal abnormalities, attempts have been made to assign aneuploidies to the parent of origin. Karyotype analysis provides an overall genetic profile of the individual at the chromosome level; many technical strategies have been employed to determine whether the error originated in meiosis I or II, and whether the error occurred during spermatogenesis or oogenesis.

i. Techniques to Study Aneuploidy

Since nondisjunction involves failure of separation of homologous chromosomes or sister chromatids at the centromere, the centromere is crucial to all techniques studying the origin of meiotic error. Therefore, any marker employed for this purpose must segregate with the centromere. Chromosomal heteromorphisms, restriction fragment length polymorphism and microsatellite markers have been used to study aneuploidy.

Chromosomal heteromorphic markers are stable, heritable, cytologic differences in the shape or size of a chromosome, usually at the centromere or satellite region. These heteromorphisms, when Q-, G-, C-, or R-banded, have been used to determine the origin of the extra chromosome in autosomal aneuploidies (Hassold *et al.*, 1984; Juberg and Mowrey, 1983). Since heteromorphism analysis is dependent on cytogenetic differences that are regularly present on only 10 of the 22 autosomes, another technique is needed to determine the parent of origin in cases involving the remaining autosomes or the sex chromosomes. Furthermore, centromeric heteromorphisms cannot be used to distinguish between a nondisjunctional event occurring in the second meiotic division from one which occurs in an early post-zygotic division, when mosaicism is not observed.

Restriction fragment length polymorphisms (RFLPs) are heritable DNA sequence variations that are recognized in restriction enzyme digests of DNA. RFLP variants have been used to determine the parental origin of aneuploidy in both autosomal (Hassold *et al.*, 1987a; Bricarelli *et al.*, 1988) and sex chromosome (Cockwell *et al.*, 1991; May *et al.*, 1990) aneuploidies. However, RFLP analysis is limited by the degree and number of informative loci in the general population, and by the fact that the RFLP site must segregate with the centromere.

Microsatellite markers are tracts of variable number dinucleotide repeats (VNDR) (Weber and May, 1989) that are heritable and chromosome-

specific. VNDRs are highly polymorphic in the general population and can be quickly and accurately assayed using polymerase chain reaction amplification (Saiki *et al.*, 1985). They have been used to determine the parental origin of aneuploidy and the stage at which the meiotic error has occurred (Peterson *et al.*, 1992; Antonarakis *et al.*, 1992). There is much promise for the use of VNDRs, with the discovery of more VNDRs that are polymorphic and tightly-linked to the centromere.

ii. Parent of Origin

Parent of origin studies have consistently concluded that maternal error in meiosis I is responsible for most autosomal trisomies (Hassold and Jacobs, 1984), 90% of XXX trisomies (May *et al.*, 1991), and approximately 50% of XXY trisomies (Lorda-Sanchez *et al.* 1992). Paternal error in meiosis I is responsible for approximately 50% of the XXY trisomies (Lorda-Sanchez *et al.*, 1992) and paternal error in meiosis II is responsible for 10% of XXX trisomies (May *et al.* 1992) and for all of the XYY trisomies. The monosomy X condition arises most often from preferential absence of the paternal sex chromosome and may not always involve a nondisjunction process (Cockwell *et al.*, 1991). Monosomy X conceptions may be normal XX / monosomy X mosaics arising from chromosome mitotic loss during postfertilization rather than from meiotic error (Bond and Chandley, 1983).

C. Differences Between Spermatogenesis and Oogenesis

Parental origin and variation in the aneuploidy frequency may be due to the special nature of meiosis and to the differences between spermatogenesis and oogenesis. In spermatogenesis, meiosis begins post-natally. On average, spermatogenesis does not begin before age 13, and continues throughout the male lifetime. The meiotic cell cycle is continuous, from the diploid spermatogonia to the mature haploid sperm. An average of 60-90 days is required for sperm development (Mange and Mange, 1990) and possibly this short time period is advantageous in minimizing the effects of aneugenic mechanisms.

In oogenesis, meiosis begins prenatally, then is arrested at dictyotene of prophase I at birth. These immature oocytes are diploid, with bivalent chromosomes held together by chiasmata. In a female reproductive lifetime there are many ovulation cycles; the first cycle begins at menarche, while the last cycle may not begin until 40 years later. With each cycle a number of immature oocytes are stimulated to resume meiosis, but a hormonal feedback system usually allows only one of the recruited oocytes to complete meiosis I; the others atrophy and die. The mature haploid oocyte is ovulated and completes meiosis II only if fertilized. This discontinuous cell cycle, controlled by hormonal fluctuations, lasts between 13 and 50 years. It is possible that the long period of time between the commencement and completion of meiosis allows aneugenic mechanisms to have an effect.

E. Hypotheses on the Etiology of Aneuploidy

Despite the prevalence and significance of aneuploidy, predisposing factors and the mechanisms of aneuploidy are not well understood. Advanced maternal age is the only unequivocal factor implicated in the etiology of many trisomies, particularly in the most common trisomy, that of chromosome 21 (Bond and Chandley, 1983). There is a considerable variation in the magnitude of the effect of maternal age on the genesis of trisomic conceptions (Hassold and Jacobs, 1984). The incidence of trisomy for the larger A, B, and C group chromosomes show a mild maternal age effect. The incidence of trisomy for the smaller D, E, F, and G chromosomes show a more pronounced effect. The single exception is the E group chromosome 16, which occurs with a high frequency in all age groups. There is also an advanced maternal age effect for XXX trisomies where nondisjunction occurs in meiosis I (May *et al.*, 1990). There is no association between paternal age and the incidence of autosomal trisomies found in spontaneous abortions (Hatch *et al.*, 1990). The association between paternal age and sex chromosome trisomies is unclear; some studies report a significant increase (Lorda-

Sanchez *et al.*, 1992) while other studies report a decrease (Carothers and Filippi, 1988). Because some trisomies are age-dependent (trisomy 21) while others are age-independent (trisomy 16), it is probable that there are many different etiological factors in human aneuploidy.

Many hypotheses have been proposed to explain the strong association between the incidence of aneuploid conceptions and advanced maternal age. The main hypotheses are: the damaged cytoskeleton hypothesis (Penrose, 1965), the relaxed selection hypothesis (Ayme and Lippman-Hand, 1982), the production line hypothesis (Henderson and Edwards, 1968), the hormonal imbalance hypothesis (Rundle *et al.*, 1961), the altered cell cycle hypothesis (Hummler *et al.*, 1987) and the nucleolar persistence hypothesis (Polani *et al.*, 1960).

i. Damaged Cytoskeleton

Aneuploidy risk may be due to accumulated damage to the cytoskeleton components which mediate proper chromosome orientation, separation or migration to the spindle poles (Penrose, 1965). An understanding of the consequences of ageing on the formation and structure of the meiotic spindle may provide insight into the elements required for proper separation of the chromosomes. A number of structural studies have shown alterations in mammalian oocyte spindles due to pre- and postovulatory aging (Mikamo, 1968; Szollosi, 1971), but use of light microscopy prevented the examination of small spindles, individual microtubules and the finer details of spindle structure.

More recently, immunofluorescent techniques, tubulin antibodies and better cell-preparation techniques have permitted the cytoskeletal examination of oocytes (Wassarman and Fujiwara, 1978; Maro *et al.*, 1985; Eichenlaub-Ritter *et al.*, 1986). There was no age-related effect in spindle structure, chromosome orientation or chromosome positioning during the dirst meiotic divison studied in oocytes, matured in vitro, from CBA mice (Eichenlaub-Ritter *et al.*, 1988). The high incidence of chromosomally unbalanced gametes in aged females appears related to

disturbances in preovulatory development rather than damaged cytoskeleton and microtubule elements resulting in non-separation and lagging of chromosomes during late anaphase / telophase.

ii. Relaxed Selection

The "relaxed selection" hypothesis (Ayme and Lippman-Hand, 1982) proposes that *in utero* recognition and abortion of abnormal conceptions is compromised with increasing maternal age. This theory states that "current evidence favours similar rates of nondisjunction for all chromosomes"; using this assumption, data was pooled from all trisomy prenatal deaths. Due to the great variation in the frequency of spontaneous abortions for each of the chromosomes, and because only direct analysis of human sperm and oocytes can confirm whether all chromosomes are equally susceptible to nondisjunction, re-evaluation of the data has shown no consistent evidence for "relaxed selection" (Hook, 1983). There is no difference in the survival frequency of 46, X or Y, -14,+t(13q:14q) conceptions born to older or younger women who are all carriers of this Robertsonian translocation (Ferguson-Smith and Yates, 1984). This demonstrates that abortion of abnormal conceptions is not compromised with advancing maternal age.

iii. Production Line

The production line hypothesis proposed by Henderson and Edwards (1968) states that there are two aspects of oocyte development which may explain the increase in aneuploid conceptions in older females. Firstly, oocytes from older women can be suspended in dictyotene for up to 50-55 years and as menopause approaches, some of these oocytes may begin to show signs of atrophy. In these older oocytes, the bivalents are held together by fewer and or more terminally-located chiasmata, allowing them to behave like univalents, and predisposing the oocyte to nondisjunction. This loss of chiasmata is predicted to have a greater effect upon the small chromosome pairs than upon the large pairs, since small chromosomes normally have only 1-2 chiasmata (Hulten, 1990).

Secondly, the order in which the immature oocytes are formed determines the order of their release as mature ova during the female reproductive lifetime; oocytes formed late in fetal life would be ovulated towards the end of a woman's reproductive years. These late-entry oocytes are postulated to develop deeper in the tissues of the ovary where poor access to essential developmental nutrients may impede chiasma formation.

There have been many attempts to resolve the contention that bivalents in late-formed oocytes have fewer chiasmata holding the homologues together, resulting in increased malsegregation of the univalent pairs at metaphase I (Henderson and Edwards, 1968). As chiasmata are the physical evidence of crossing over, cytological studies have tried to relate the effect of aging on chiasma frequency in meiotic cells; results have been inconsistent. Beerman *et al.*, (1987) saw neither a reduced cross-over frequency nor a significantly-increased nondisjunction rate in oocytes from aged females. However, in a summary of five studies, Tease and Fisher (1989) note that while age appears to have an effect on reduced chiasma frequency, a corresponding increase in terminally-located chiasmata and univalency was only occasionally observed.

Cytogenetic studies do not demonstrate a higher incidence of metaphase I univalents with advanced maternal age in aged mice (Speed, 1977; Polani and Jagiello, 1976), Chinese hamsters (Sugawara and Mikamo, 1983) or Djungarian hamsters (Hummler *et al.*, 1987). It was noted that univalent formation may be artefactual (Polani and Jagiello, 1976; Sugawara and Mikamo, 1983); this was confirmed by Sugawara and Mikamo (1986) when more univalents were found in oocytes from aged hamsters using Tarkowski's (1966) air-drying technique compared to those fixed using the gradual fixation technique (Sugawara and Mikamo, 1986).

The associations between chiasma formation, recombination and nondisjunction risk is also unresolved. Analysis of the extra chromosome

21 in Down syndrome individuals (Warren *et al.*, 1987; Sherman *et al.*, 1991) and of the XY pseudoautosomal region in Klinefelter syndrome individuals (Hassold *et al.*, 1991; Lorda-Sanchez *et al.*, 1992) shows that recombination is significantly reduced or absent in chromosomes that have undergone nondisjunction. In individuals where trisomy 21 is a result of paternal error, nondisjunction is not due to failure to pair and/or recombine at meiosis I (Sherman *et al.*, 1991). There appears to be an association between reduced recombination and maternal meiosis I nondisjunction in some trisomy 21 individuals (Sherman *et al.*, 1991) and an absence of recombination in the XY pseudoautosomal region of paternally derived XXY individuals (Hassold *et al.*, 1991), but in their studies, the majority of cases were not informative. Preliminary evidence also suggests reduced recombination in the meiosis I X-chromosome tetrad in XXX and XXY trisomies of maternal origin, as well as an excess of recombination in the pericentromeric region (Morton *et al.*, 1990). Molecular DNA typing of aneuploid individuals suggests that nondisjunction due to maternal error is associated with abnormally low levels of recombination, but studies with more informative markers are needed.

In addition to indirect evidence for the production-line hypothesis obtained from cytogenetic and cytological investigations, direct evidence from studies of radioactively-labelled mouse ovaries also suggests a non-random maturation and release of ova (Polani and Crolla, 1991). Fetal mouse ovaries were cultured with a radioactive label to the premeiotic synthesis stage and transplanted to spayed adult females to complete meiosis I. The proportions of labelled first- and second- meiotic metaphases seen in these mature oocytes were compared to proportions of labelled prophase I oocytes at the pre-transplantation stage. Oocytes that incorporated the radiolabel during premeiotic DNA synthesis first, entered meiosis first *in fetu* and were released first at puberty; oocytes that incorporated less label (and entered meiosis

later *in fetu)* were not harvestable as metaphase II at puberty.

Polani and Crolla (1991) creatively demonstrated physical evidence for a production line of release of ova that is related to the time of entry into meiosis *in fetu*. Henderson and Edwards (1968) show, quite elegantly, experimental evidence for the effect of maternal age on chiasma frequency and univalent formation. However, interpretation of the relationship between chiasma frequency and univalent formation is unclear. Moreover, the effect of maternal age on the dynamics of meiosis I chromosomes remains an enigma.

iv. Hormonal Imbalance

Aneuploidy risk may be due to changes in the ovarian environment resulting from hormonal imbalances and cycle irregularities characteristic of a young reproductive system trying to establish a regular cycle or a reproductive system approaching menopause. Significantly higher levels of steroids are present in the urine of women who produce a trisomy 21 child when young, compared with older women producing such a child late in life (Rundle *et al.*, 1961), implying a hormonal imbalance as an aneuploidy risk.

The X chromosome in mammals appears to be important to germ cell development and survival. The total lifetime of reproductive competence and performance of 40,X mice is significantly shorter than 40,XX mice (Lyon and Hawker, 1973). Human females who are 45,X typically have streak gonads with no germ cells present (Singh and Carr, 1966). Possibly, the reproductive system of women approaching menopause, like 45,X females, is characterized by oocytes beginning to show signs of atrophy. Human monosomy X females who are able to conceive have increased fetal wastage, an increased risk for trisomy 21 conceptions and reach menopause in their late twenties (King *et al.*, 1978). Furthermore, when the reproductive systems of young mice were artificially aged by performing a unilateral oophorectomy, there was an earlier onset of irregular hormonal cyclicity and an earlier rise in

aneuploidy compared to sham-operated controls (Brook *et al.*, 1984). These results suggest that aneuploidy risk is determined by the hormonal profile of the biological rather than the chronological age of the maternal reproductive system.

v. Altered Cell Cycle

Hummler *et al.*, (1987) propose that an alteration in the meiosis rate rather than univalent formation is associated with meiosis I nondisjunction. They observed that meiosis I resumption is delayed in older female Djungarian hamsters, resulting in an increase in nondisjunction, but without a concomitant increase of univalents or a decrease in chiasmata. In contrast, kinetic studies of the maturation cell cycle of CBA mouse oocytes, aged in vitro, from old and young age groups revealed that oocytes from aged females proceed faster through the first maturation division than do those from younger animals (Eichenlaub-Ritter and Boll, 1989a). Specifically, a significant shortening of the time between germinal vesicle breakdown and polar body formation was observed, corresponding to the critical period when chromosomes are oriented at the metaphase plate and attach to the spindle. Furthermore, C-banding of these metaphase II-arrested oocytes showed that oocytes from aged females are more prone to nondisjunction, as shown by a significantly-higher incidence of aneuploidy. Further study is required to elucidate the relationship between an altered cell cycle and a predisposition to nondisjunction.

Eichenlaub-Ritter and Boll (1989b) isolated oocytes from both young and aged female CBA mice were that exposed to nocodazole, during *in vitro* maturation to test Kaufman's (1985) postulate that alterations in the immediate environment which disturb the final stages of oocyte maturation lead to aneuploidy. Previously, *in vitro* application of spindle disruptors such as colchicine (Tease and Fisher, 1986) and such as nocodazole (Generoso *et al.*, 1989) elevate the level of aneuploidy in mouse oocytes. However, in these experiments (Eichenlaub-Ritter and

Boll, 1989b), drugs were applied to the entire organism *in vivo* to test for sensitivity of the spindle apparatus of oocytes. Nocodazole was observed to cause rapid and complete microtubular depolymerization and chromosome scattering in oocytes from old and young mice. The recovered oocytes were able to complete meiosis I demonstrating that the nocodazole dosage was not lethal but just enough to disrupt the spindle apparatus. For both groups, the nocodazole treatment resulted in similar dramatic increases in hyperhaploid metaphase II oocytes. The spindle apparatus in oocytes from older females were neither more labile nor more susceptible to disturbances. Nocodazole also erased any temporal differences in the cell cycle between young and aged females; the critical period available for spindle formation before chromosome segregation was shortened in all oocytes. Since the oocytes isolated from their natural environment and cultured *in vitro* under identical conditions and were not hormonally-treated, Eichenlaub-Ritter and Boll (1989b) conclude that there are inherent cellular differences between young and aged oocytes.

Differences in the altered cell cycle and risk factors for nondisjunction appear to be intrinsic. Oocytes isolated from the ovaries of young females move through the different stages of meiosis at a different rate than oocytes isolated from ovaries of aged females.

vi. Nucleolar Persistence

Persistence of a nucleolus organizer in oocytes has been implicated in the origin of aneuploidies (Polani *et al.*, 1960), particularly since 40% of human lethal trisomies involve chromosomes with nucleolus organizing regions (NOR) (Boue and Boue, 1973). The nucleolus is composed of ribosomal genes and a silver-positive (Ag-positive) staining protein (Hofgartner *et al.*, 1979). Acrocentric chromosomes have NORs located at their satellites. Homologous and non-homologous acrocentric bivalents are often associated in a common nucleolus; up to 3 NOR-containing chromosomes have been observed in

juxtaposition to one another (Mirre *et al.*, 1980). The morphological relationships of nucleolar chromosomes remain unchanged throughout diplotene until their separation at metaphase I. However, a bridge of silver-positive-staining protein is often observed linking satellites of metaphase I acrocentrics.

In sperm, the opposite phenomenon is observed: the amount of Ag-positive-staining protein decreases during prophase and becomes absent during metaphase I and II. As the disaggregaton of the nucleolus organizer is believed to involve an enzyme which transforms or degrades the protein (Mirre *et al.*, 1980), oocytes maintained in diplotene for 30-40 years may have a deficiency or insufficiency of this enzyme. Subsequently, the presence of the nucleolar organizer could allow linking of the satellites of metaphase I acrocentrics and interfere in the separation process.

The relationship between maternal age and the frequency of satellite association in parents of trisomy 21 individuals is unclear. Some studies report an increased satellite association in parents of trisomy 21 individuals (Hansson, 1979), while others report no significant difference in satellite association between parents of trisomic individuals and a control group (Jacobs and Mayer, 1981). In a separate study, Risch *et al.* (1987) did not find any evidence for an age-dependent mechanism specific to acrocentrics.

There has been a similar lack of consistency in studies linking the presence of nucleolar organizing regions to an increased risk of trisomic conceptions. NOR variants are reported to be increased in parents of trisomy 21 individuals (Jackson-Cook *et al.*, 1985). Subsequent studies of couples who had a trisomic spontaneous abortus (Hassold *et al.*, 1987b) did not detect any significant difference in the frequency or type of NOR variants between parents of acrocentric trisomic conceptuses and parents of other trisomic conceptuses.

The nucleolus has also been observed to connect to a long segment

of a bivalent and/or to a synaptonemal complex (Garcia *et al.*, 1989), possibly disturbing homologous pairing. Non-nucleolar chromosomes also interact with nucleolar structures; these interacting regions may be in the heterochromatic regions of chromosomes 1, 9, and 16 (Stahl *et al.*, 1975). Nucleolus organizer-related nondisjunction may also be the consequence of disturbances in recombination involving the short arms of non-homologous acrocentric chromosomes (Schmikel *et al.*, 1985).

When all of the studies completed to date are reviewed, evidence suggests that the intrinsic properties of NORs do not increase the risk for nondisjunction. However, it is unclear at this time whether the maintenance of nucleoli in diplotene-stage oocytes does or does not, in fact, increase the risk for nondisjunction.

E. Hypotheses of the Mechanism of Aneuploidy

Results from animal models cannot be extrapolated to explain human conditions completely, but they can give indications of the mechanisms responsible for a predisposition to nondisjunction. As with humans, the CBA mouse strain shows a rise in hyperhaploid metaphase II oocytes (Martin *et al.*, 1976) and chromosomally-unbalanced preimplantation embryos with advanced maternal age (Gosden, 1973). Furthermore, both human and CBA mouse ovaries have lost most of their oocytes toward the end of their respective reproductive periods (Costoff and Mahesh, 1975; Faddy *et al.*, 1983). Species such as the hamster, which has a heterogeneous karyotype similar to that of humans, may be suitable for studying the response of specific classes of chromosomes to radiation (Mikamo, 1982), gonadotropins (Hansmann *et al.*, 1980) or cytoskeleton-disrupting substances (Hummler and Hansmann, 1985).

i. Premature Chromatid Separation

It is assumed that trisomy formation arises from nondisjunction of whole bivalents and their subsequent incorporation into a single daughter cell. However, the primary event may not be nondisjunction of the homologues in anaphase I but rather a premature division of

univalents into single chromatids (Rodman, 1971). It is suggested (Angell, 1991) that at metaphase I, these homologous univalents follow one of two main courses: (1) both univalents migrate to one pole and none to the other pole, resulting in nondisjunction (2) one of the univalents divides precociously into itstwo single chromatids. Cytogenetic examination of chromosomes at metaphase II shows single chromatid formation(s). In humans, three categories of chromosome errors involving single chromatids in oocytes were seen at metaphase II: (1) 22 whole chromosomes plus one single chromatid, (2) 22 whole chromosomes plus two single chromatids, (3) 23 whole chromosomes plus one single chromatid (Angell, 1991). Extra single chromatids, and additional whole chromosomes as a result of bivalent predivision have been found in a number of mammalian species (Polani and Jagiello, 1976; Hummler *et al.* 1987). Formation of single chromatids from malsegregated univalents may be challenged, particularly in the first two of these categories, as a technical artifact, as more univalents are found in oocytes from aged mice when using Tarkowski's (1966) air-drying technique rather than a gradual fixation technique (Sugawara and Mikamo, 1986).

ii. Asynchronous Segregation

Hummler *et al.*, (1987) proposes a mechanism to explain both the occurrence of specific trisomies at higher maternal ages and the phenomenon of a decreased frequency of aneuploidy with very old females that is seen in both CBA mice (Martin *et al.*, 1976) and humans (Ferguson-Smith and Yates, 1984). This model makes two assumptions: 1) there is asynchronous segregation of bivalents which differ between species and 2) there is variability in the kinetics of meiosis and/or cytoplasmic maturation, which in turn influences the quantity and effectiveness of factors controlling chromosome segregation (Hummler *et al.*, 1987). Physiological factors in the maturation milieu initiate the resumption of meiosis by acting on the pole kinetochore microtubules (pKMT). The pKMT of the various bivalents are sensitive to physiological

factors at different times of the cell cycle, resulting in asynchronous segregation of bivalents. Any alteration during oocyte maturation causing immobility, destabilization, or inverted direction of pKMT is proposed to induce nondisjunction of specific chromosomes during the limited time span when that particular bivalent's pKMT is sensitive to the maturation factors. This pattern of specific nondisjunction may explain why certain aneuploidies are more common at certain maternal ages. An alteration of conditions affecting the earlier stages of maturation during a period when no bivalent's pKMT is sensitive, combined with the observation that oocytes from aged females proceed faster through the first maturation division than oocytes from younger females (Eichenlaub-Ritter and Boll, 1986a) may explain the lower incidence of aneuploidy in females of very advanced age (Martin *et al.*, 1976).

Probability of involvement and risk for a chromosome to undergo nondisjunction may be influenced by alterations in the meiotic cell cycle. (Hummler *et al.*, 1987). In Djungarian hamsters, there is an increased incidence of hyperhaploid oocytes with increased maternal age. Interestingly, the middle-aged group does not show any irregularities in ovulation behaviour, yet has significantly more malsegregated bivalents per oocyte and the larger metacentric and submetacentric chromosomes are preferentially involved. There is no significant difference in mean chiasma frequency, nor in the number of univalents seen in young and aged females. The chromosomes involved in nondisjunction never appear as univalents. Moreover, in contrast to the faster progression through meiosis seen in CBA mice, there is considerable delay in the resumption of meiosis I in Djungarian hamsters. These observations provide evidence that in meiosis, chromosomes are not all equally susceptible to nondisjunction.

F. Aneuploidy in Gametes

Gamete chromosome studies have the potential to provide insight into the etiology of aneuploidy and the genetic factors that influence survival by accurately answering the following questions. What is the true frequency of aneuploid conceptions? What are the relative percentages of monosomic conceptions due to nondisjunction and to anaphase lag? Are all chromosomes equally susceptible to nondisjunction? Is there a parental age effect on the frequency of aneuploid gametes?

i. Sperm

At present, direct estimates of the levels of aneuploidy in gametes from normal, fertile individuals are based solely on studies using males. The technical problems associated with studying gametes are daunting. Only three laboratories (Mikamo *et al.*, 1990; Brandriff and Gordon, 1990; Martin *et al.*, 1991) have successfully amassed large amounts of data on the frequency of aneuploidy in sperm from chromosomally-normal males by using the technique of *in vitro* fertilization of zona-free hamster eggs pioneered by Rudak *et al.* (1978) Unfortunately, the fixation technique may result in artefactual chromosome loss when metaphases are scattered, resulting in spurious hypohaploid counts. It is therefore customary to report a conservative estimate of the frequency of aneuploidy, calculated by doubling the frequency of hyperhaploid complements.

A total of 17,377 sperm have been cytogenetically analyzed from 3 large studies of normal donors, 18-55 years of age (Mikamo *et al.*, 1990; Brandriff and Gordon, 1990; Martin *et al.*, 1991). From these worldwide studies, the overall mean frequency of numerical abnormalities is 2.7% with 0.7% hyperhaploid and 1.7% hypohaploid. The mean conservative estimate of aneuploidy is 1.4%, and the mean frequency of structural abnormalities is 10.5%. There is much agreement between the conservative estimate of aneuploidy in these three large surveys; it is also evident that numerical abnormalities are less common than structural

abnormalities in human sperm.

Using the assumption that all chromosomes are equally susceptible to nondisjunction, Martin *et al.* (1991) also examined the distribution of aneuploidy. The observed hyperhaploidy frequencies for individual chromosomes is not found to be significantly different from the expected frequencies, with the exception of chromosomes 1, 21 and the sex chromosomes. If, however, chromosome loss is assumed to be random, chromosomes 1, 2, 3, 4, 7 and 9 show significantly less hypohaploidy than expected, whereas chromosomes 18, 21, 22 and the sex chromosomes have significantly elevated levels of hypohaploidy. This suggests that chromosome loss preferentially affects small chromosomes, either through meiotic error or technical loss arising during fixation.

Martin and Rademaker (1987) analyzed the frequency of hyperhaploid and hypohaploid complements to determine if there was a relationship between paternal age and aneuploidy. A significant negative correlation between the frequency of hyperhaploid complements and age was observed: young men have the highest frequency of hyperhaploid sperm. This study concludes that there is no increase in the risk of trisomy with advancing paternal age.

ii. Oocytes

The study of oocyte chromosomes has been less successful, in part due to the extreme difficulty in obtaining oocytes from normal fertile females, and partly due to the technical difficulties of maturing these oocytes to obtain meiotic chromosomes. Therefore, the majority of observations have been made in small studies using oocytes donated from *in vitro* fertilization programs (reviewed by Pellestor, 1991 and Zenzes and Casper, 1992). Only 1498 oocytes yielded analyzable metaphases and even fewer of these metaphases have been completely analyzed, as individual chromosomes can be identified only in banded preparations. Aneuploidy is observed in all chromosome groups with a mean conservative estimate of aneuploidy of 15% and a range of 3% to 42%. The mean

frequency of numerical abnormalities is 26.5%, with 7.7% hyperhaploid and 21.6% hypohaploid.

The relationship between maternal age and the frequency of aneuploidy in oocytes is unresolved. Macas *et al.*, (1990) report a significant correlation between increasing rates of aneuploidy with increasing maternal age, while Djalali *et al.*, (1988) report similar incidences of aneuploidy among the different age groups. The only similarity between these studies is that numerical abnormalities are more common than structural abnormalities in oocytes, compared with sperm, where the reverse is true. These findings are consistent with data from spontaneous abortions: most numerical abnormalities are due to a maternal meiosis I error whereas most structural abnormalities are due to a paternal error (Tomar *et al.*, 1984).

There are some reservations about whether cytogenetic data from IVF oocytes are representative of aneuploidy in oocytes from normal, fertile women. The oocytes examined are "spares" or "rejects" from *in vitro* fertilization programs and are therefore, not morphologically "normal". The effect of hormonal stimulation used to retrieve large numbers of oocytes is unknown, but the frequency of aneuploidy (Van Blerkom and Henry, 1988) and the type of chromosomal aberrations (Macas *et al.*, 1990) do not appear to vary between different stimulation protocols. It is unknown whether a mechanism exists during natural oocyte maturation or ovulation which selects against abnormal oocytes. Nonetheless, it has been suggested that the hormonal stimulation used to mature larger numbers of oocytes, may also mature a disproportionally large number of abnormal oocytes which would otherwise atrophy (Wramsby *et al.*, 1987).

G. New Developments in Gamete Studies

The first example of retrieval and *in vitro* maturation of non-IVF human oocytes was reported by Edwards (1965). There have been a few inconclusive studies on the cytogenetics of oocytes from women not

involved in IVF programs (Jagiello *et al.*, 1976; Van Blerkom, 1991). More recently, Cha *et al.*, (1991) retrieved oocytes from ovaries which were surgically removed from premenopausal women. The oocytes were matured and fertilized *in vitro* and transplanted into a woman who had been hormonally prepared for pregnancy. Pregnancies resulting from the transplantation of these donated oocytes produced healthy children. Hence, oocytes matured *in vitro* are amenable for genetic studies into the etiology of aneuploidy.

Most gamete studies rely on cytogenetically analyzable chromosomes. The technique of chromosome preparation is prone to technical artefact producing an excess of hypohaploid complements, so the true frequency of monosomy is unknown. The cytogenetic techniques utilized are also very labour intensive and expensive. Furthermore, analyzable chromosomes are very difficult to obtain, especially from meiotic cells. Recently, Pinkel *et al.* (1986) have developed the fluorescent in situ hybridization (FISH) technique to detect aneuploidy in interphase cells (Cremer *et al.*, 1986). The FISH technique uses repetitive, centromeric, chromosome-specific DNA probes and does not rely upon the visualization of individual chromosomes, as the fluorescently-labelled centromere is clearly visible in any cell.

Alpha-satellite or satellite III centromere-specific probes are commonly used in FISH. Alpha-satellite sequences have a fundamental repeat monomer of approximately 171 base pairs in length (Willard and Waye, 1987). Each repeat array has a number of unique differences in: the primary nucleotide sequence of the higher-order repeat unit, the restriction enzyme used to visualize the higher-order repeat, the size of the higher-order repeat unit, and the estimated copy number of the units (Willard and Waye, 1987). Highly-ordered organization into long tandem arrays confers specificity to a particular chromosome's centromere. Satellite III DNA are repeat arrays that have a high frequency of *Hinf* 1 sites and a 9-base-pair repeat unit. Chromosome-

centromere specificity arises from differences in the restriction repeat length fragment and in the nucleotide sequence of the 9-base-pair repeat (Cooke and Hindley, 1979). Visualization of either of these satellite DNAs necessitates that the target sequence is sufficiently repeated. Intra-chromosome copy number is variable but inter-individual copy number is usually quite constant for each chromosome.

FISH is a rapid and inexpensive technique for the detection of aneuploidy, particularly as a replacement for cytogenetic studies of morphologically-poor meiotic chromosomes. Detection of aneuploidy using FISH involves counting coloured domains each of which represents a specific chromosome's centromere. As cells are intact, the possibility of introducing artefactual chromosomal losses are minimized and a more accurate ascertainment of the incidence of hypohaploidy is possible. FISH has the potential to be especially useful for detection of any nondisjunction and anaphase products in the matured haploid secondary oocyte and its corresponding haploid polar body.

The availabilty of multiple band pass fluorescent microscope filters permits simultaneous visualization of two differently-coloured fluorochromes. Two chromosome-specific sequences each labelled with a different colour fluorochrome provides a number of experimental controls. For example, a single chromosome-specific signal "A" with no chromosome-specific signal "B" would indicate that hybridization was successful but that the cell was nullisomic for chromosome A. Two "A" signals and one "B" signal would indicate that the cell is disomic for the "A" signal. Presence of two each of "A" and "B" signals would most likely indicate a diploid cell, which in the case of an oocyte is probably an immature cell.

As fluorescent signals can be seen in an intact interphase sperm cell, FISH permits rapid aneuploidy analysis of large numbers of sperm. Of particular interest is the frequency of X- and Y- chromosome aneuploidy because paternal meiotic error is responsible for a

significant number of XXY (Lorda-Sanchez *et al.*, 1992), and for all XYY aneuploid conceptions.

H. Strategy and Research Objectives

In the present study, a technique for oocyte retrieval from ovaries, *in vitro* maturation, single-cell fixation for cytogenetic and multi-colour FISH analysis will be described. Control for hybridization conditions and *in vitro* maturation will be accomplished by the use of two-colour FISH. It is hypothesized that in non-hormonally-stimulated oocytes from a healthy unselected population of females:

1. the incidence of aneuploidy will vary with each chromosome group.
2. the smaller G-group chromosomes will exhibit an increased susceptibility to nondisjunction.
3. the incidence of aneuploidy will increase with maternal age.

The technique and guidelines for analyzing multi-colour FISH in a large number of interphase cells will be established. The frequencies of X- and Y-chromosome aneuploidy in interphase sperm from a normal male will be determined using 3-colour FISH. The third probe will be autosome-specific to provide an internal control for hybridization, if a sperm is nullisomic for the sex chromosomes, and for diploidy if a sperm is disomic for the sex chromosomes. The data will be used to:

1. determine the frequency of X- and Y-chromsome aneuploidy in a large sample of sperm.
2. test the reliability and sensitivity of multi-colour FISH analysis as compared to cytogenetic analysis and single-colour FISH analysis.

II. Materials and Methods

A. Oocytes

i. Selection of Ovary Donors

Ovaries were obtained from premenopausal women undergoing oophorectomies for various gynecological reasons at one of three Calgary Hospitals: Foothills Hospital, Calgary General Hospital, and Grace Women's Hospital. Ethical approval was obtained from the University of Calgary and from each of the hospitals. Reproductive history, a brief medical history, radiation history, and use of cigarettes, alcohol and drugs were detailed on a donor information form which was completed for each specimen.

ii. Oocyte Retrieval

It was important that the ovarian specimen be as fresh as possible. An average of two hours was allowed to pass from surgical removal to culturing of the oocytes; the maximum time allowed was 4-5 hours. After removal, specimens were immersed in room-temperature sterile, commercially-prepared Ham's F10 with L-glutamine medium (Gibco), or phosphate-buffered saline at pH 7.3. Ovarian tissue which was not required for pathological examination was made available for this study. It was determined that the area of the ovary distal to the fallopian tube yielded the most oocytes. Whenever possible, cross-sectioning of the specimen was minimized to avoid breakage of the oocyte-containing follicles.

The specimen was cleaned of blood in a wash of F10 supplemented with penicillin (10^5 IU/ml-Sigma) streptomycin (5mg/ml-Sigma) and brought to pH 7.3. The ovary was finely minced in a sterile plastic petri dish containing 5 mm of wash medium. The specimen slurry was then filtered through two membranes (Lockertex) of 200 μm and 80 μm pore size, respectively. The transport medium and slurry were passed through the filter sets 8-10 times. After the final filtration, the 80 μm membrane was removed from the filter set and placed face-down in another

sterile petri dish. A sterile pasteur pipette containing approximately 3 ml of wash medium was forced through the membrane to dislodge the oocytes; this medium was passed through the membrane 2-3 times. The supernatant was carefully searched for oocytes under 150x magnification, using a dissecting stereomicroscope outfitted with an adjustable mirror (Wild Leitz). Using a mouth-controlled Pasteur micropipette, oocytes were transferred under 300x magnification from the wash medium to a petri dish containing 20 μl drops of culture medium; an overlay of immersion oil (Canlab) prevented evaporation of the medium. Handling of the oocytes was minimized to preserve the covering of cumulus cells around the oocytes.

iii. Oocyte Culture and Fixation

The culture medium was composed of equal parts of F10 (supplemented with 10^5 IU/ml penicillin and 5mg/ml streptomycin at pH 7.3), and fetal bovine serum (Flow). A single oocyte was placed in each drop of culture medium and was incubated at 37°C, with 5% CO_2, and 95% humidity. Oocyte culture time varied, dependent upon a visual estimation of maturity; germinal vesicle breakdown followed by polar body formation indicated oocyte maturity. Culture time varied from 4 to 52 hours; mean culture time was 36 hours. It was noted that not all oocytes would mature: some appeared healthy after 48 hours of incubation, yet yielded degraded chromosomes when fixed.

Oocytes were fixed directly onto glass slides using a modified version of the embryo fixation technique devised by Tarkowski (1966).

Four wells of a 9-well glass plate (Fisher) were filled with 600 μl of the following solutions: two wells of wash medium, one well of freshly-prepared protease type IV (10mg/ml-Sigma), and one well of hypotonic (1%) sodium citrate. All solutions were incubated for 2-3 minutes at 37°C. Fresh fixative (3 ml of 95% ethanol and 1 ml of glacial acetic acid) was prepared and stored in a closed vial. Slides were precleaned with isopropyl alcohol.

Using a mouth-controlled Pasteur micropipette, a single oocyte was transferred from the drop of culture medium to the first wash well. After wiping the pipette free of immersion oil, the oocyte was transferred briefly to the second wash well, and then into the protease solution. After 5 minutes in protease to weaken the zona pellucida, the oocyte was swelled for 5 minutes in hypotonic citrate. The oocyte, in a small drop of hypotonic solution, was then placed on a precleaned slide. Using an automatic pipette fitted with a small bore micropipette tip, 10 μl of fixative was immediately dropped directly over the oocyte. A breath of warm moist air helped to disperse the fixative so that the oocyte became visible to the naked eye, and a circle was etched around the oocyte on the underside of the slide using a diamond-tipped pencil. Before the fixative dried, a second drop was added from a height of 1-2 cm, was allowed to dry slightly, and a third drop was added. A total of 5-8 drops of fixative were added in a similar fashion, until the oocyte became visibly flattened. Gentle blowing after the last drop facilitated drying and spreading of the chromosomes.

B. Sperm

i. Selection of Sperm Donor

Sperm was obtained from a 33-year-old normal fertile male for whom previous cytogenetic sperm analysis of 415 karyotypes had detected an aneuploidy frequency of 2%, which is within normal limits (Martin *et al.*, 1991). This donor had also participated in previous FISH studies using centromere-specific probes for chromosomes 1, 12 and X (Holmes and Martin, 1993).

ii. Sperm Slide Preparation

A previously frozen 0.5 ml sperm sample was thawed, then washed by mixing with 5 - 10 mls of Tris / 0.9% NaCl and centrifuging at 600G for 6 minutes, removing the supernatant, and repeating twice. After the final wash, the sample was brought to a final concentration of approximately $6x10^7$ sperm/ml. Using a micropipette fitted with a small

bore micropipette tip, 3 μl of the sample was evenly smeared over a 1 cm^2 area on a clean glass slide and allowed to air dry.

The sperm heads were decondensed on the slides at room temperature in a series of solutions: 30 minutes in dithiothreitol (DTT) (10 mM DTT (Sigma) in 0.1M TRIS), 3 hours in 3,5 lithium diiodosalicyclic salt (LIS) (Sigma) / DTT solution (10mM LIS, 1mM DTT in 0.1 M TRIS), followed by a rinse in 2x SSC. Slides were air-dried and used immediately for FISH or stored at room temperature for future use.

C. DNA Probe Preparation

i. Probe Sequence Information

Four probes were used in this study. A chromosome 1-specific sequence of satellite III DNA, pUC1.77, (Cooke and Hindley, 1979) was provided by H.J. Cooke, Edinburgh. A chromosome 12-specific alpha satellite sequence, D12Z3, (Baldini *et al.*, 1990) was supplied by A. Baldini, Yale University. An X-chromosome-specifc sequence, XC, (Jabs *et al.*, 1989) was provided by E. Jabs, Johns Hopkins University. A Y-chromosome-specific sequence, DYZ3, (Wolfe *et al.*, 1985) was purchased from the American Type Culture Collection. Table 1 summarizes sequence length, restriction enzyme, and vector information for each of the chromosome-specific sequences.

ii. DNA Transformation

Plasmid DNAs from chromosomes 1 and 12 were transformed into competent JM109 cells (prepared as described by Hanahan (1983) and generously provided by Dr. T. Bech-Hansen's lab) before amplification. Transformation was carried out as described by Maniatis *et al.* (1982) with some modifications. To 100 μl of competent cells, DNA was added to a final concentration of 40 ng/μl, set on ice for 30 minutes and heat shocked at 42°C for 2 minutes. Bacteria were allowed to recover in 1 ml Luria broth (LB) (Gibco) (Sambrook *et al.*,, 1989) at 37°C for 30 minutes. After gentle mixing, the cells were streaked on LB plates containing 50 μg/ml ampicillin (Sigma). Two ampicillin-containing

control plates were also prepared: one plate containing only JM109 cells demonstrated that JM109 does not have inherent ampicillin resistence, and one plate containing insert-free blue script plasmid demonstrated that JM109 cells are competent and able to produce ampicillin-resistent colonies. All plates were incubated at 37°C for 16-24 hours and stored at 4°C until colonies were chosen.

Table 1. Summary of the sequence length, restriction enzyme and vector information for the chromosome-specific probes.

NAME	CHROMOSOME #	TYPE	INSERT SIZE (kb)	CUT SITE	VECTOR NAME	VECTOR SIZE (kb)
pUC1.77	1	sat III	1.77	EcoRI	pUC9	2.7
D12Z3	12	α-sat	0.68	HindIII	pBS	3.0
XC	X	α-sat	2.0	BamHI	pBR322	4.36
DYZ3	Y	α-sat	5.3	EcoRI	pUC13	2.7

iii. Rapid "mini" Plasmid Isolation

An overnight culture of 5 ml of LB containing 50 μg/ml ampicillin and a single colony was grown at 37°C with agitation. The following day, 100 μl of culture was reserved for the large-scale preparation, and the remainder was centrifuged at 12,000 G for two minutes. The pellet was resuspended in 300 μl STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris-Cl, pH 8.0) to which 25 μl lysozyme (10mg/ml-Boehringer-Mannheim) had been added, and was mixed by vortexing. After boiling for 1 minute, the pellet was centrifuged at 12,000 G for 15 minutes. The pellet was discarded and the supernatant was deproteinated using standard phenol:chloroform (1:1) and chloroform:isoamyl (24:1) extraction techniques (Sambrook *et al.*, 1989). Following precipitation with an equal volume of isopropanol at room temperature, the pellet was redissolved in 50 μl of low TE (10mM Tris-Cl, 1mM EDTA, pH 8.0). One microliter of RNase (2 g/l-Boeringher-Mannheim) was added, and the mixture was incubated for 40 minutes at 42°C. The DNA was ethanol-precipitated with 1/10 volume of 4M NH_4OAc and 2.5 volumes of freezer-cold 95% ethanol at -20°C for 1 hour. DNA was dissolved in 30-50 μl of low TE, then was rotated at 4°C for 24 hours to ensure complete dissolution. The following day, 5-15 μl of DNA was digested to release the DNA insert with the appropriate restriction enzyme(s) (according to manufacturer's specifications). The digestion products were loaded onto an electrophoresis gel (0.8% agarose and 0.5 mg/ml ethidium bromide). A control 5 μl of undigested DNA was loaded onto the same gel, and the gel was run at 60 - 80 V for 1-2 hours in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002M EDTA).

iv. Large Scale Plasmid Isolation

Using 5μl of the reserved aliquot from the rapid "mini" plasmid preparation, a 5 ml culture (5 ml LB-broth and 10 μl ampicillin) was innoculated. After growth overnight, the 5 ml culture was used to innoculate 500 ml of LB. The 500 ml culture was grown overnight at 37°C

with agitation. Bacterial cells were pelleted at 4220 G for 10 minutes at 4°C and the supernatant was discarded. The pellet was redissolved in 4 ml of lysis solution I (15% sucrose, 0.05M EDTA, 0.05M Tris-HCl, pH 8.0), 40 mg of lysozyme was added, the mixture was vortexed and cooled on ice for 20-30 minutes. Four milliliters of lysis solution II (1% TX-100, 0.05M EDTA, 0.05M Tris-HCl, pH 8.0) was added, and the mixture was inverted several times. If lysis was not seen immediately, the mixture was cooled on ice for a further 10-20 minutes. After lysis had occurred, the mixture was centrifuged at 34,500 G for 40 minutes at 4°C, the supernatant was collected, and high TE (100mM Tris-Cl, 40mM EDTA, pH 8.0) was added to bring the volume to 8.5 ml. This solution was used to dissolve 9.1 g of CsCl, 0.200 ml ethidium bromide (5mg/ml) was added, and paraffin oil was added if needed to completely fill TH1270 ultracentrifuge tubes (Sorvall). Tubes were balanced to within 0.50 g, the tops were crimped, the contents were mixed by inversion, and the cesium chloride gradients were centrifuged (Sorvall Ultracentrifuge OTD65B) at 131,000G for 40-70 hours. The plasmid DNA band was collected using a 21 gauge needle mounted on a 3 cc syringe, as described by Sambrook *et al.* (1989). Several extractions with water-saturated butanol removed ethidium bromide (Sambrook *et al.*, 1989). DNA samples were dialyzed overnight against low TE at 4°C using Type III dialysis tubing (maximum 3500 molecular weight-Spectra/Por), before being ethanol-precipitated. Purified DNA was dissolved in an appropiate volume of low TE.

v. DNA Electro-elution and Purification

Concentration and purity of each DNA sample was determined by measuring the optical density of the sample at 260 nm and 280 nm through a 1 cm light path cuvette (Beckman DU-65 spectrophotometer). Samples were stored in low TE at 4°C.

Restriction-enzyme digests were carried out on 60-100 μg of DNA, following the manufacturers' specifications; 100 μg of DNA was used if

the insert sequence accounted for less than 50% of the combined plasmid and insert weight. Digested samples were electrophoresed in a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide for 17-19 hours at 60-80 V, using circulated TAE buffer (40mM Tris, 20mM sodium acetate, 2mM EDTA, brought to pH 8.1 with acetic acid). Insert DNAs were eluted into troughs as described by Maniatis *et al.* (1982). Following a single water-saturated butanol extraction (Sambrook *et al.*, 1989), the DNA was ethanol-precipitated and dissolved in 40-80 μl of low TE.

vi. DNA Nick-Translation

DNA was labelled with either a hapten-conjugated nucleotide ((biotin-14-dATP) (Bethesda Research Laboratories) or digoxigenin-11-dUTP) (Boehringer-Mannheim)), or a fluorochrome-conjugated nucleotide ((rhodamine-4-dUTP) (Amersham), fluorescein (FITC)-11-dUTP (Amersham), or coumarin-4-dUTP (Amersham)). Hapten-conjugated nucleotides were incorporated into the DNA by nick-translation; fluorochrome-conjugated nucleotides were incorporated by nick-translation (manufacturer's specifications). All nick-translation reactions were terminated by adding 2.5 μl of 0.5M EDTA (0.012M) and 0.5 μl of 20% SDS (0.97M), and incubating 65°C for 15 minutes. The efficiency of incorporation of the hapten-conjugated nucleotide was determined by monitoring the reaction with 5 μCi of ^{32}P-dCTP; as the kinetics of fluorochrome-conjugated nucleotides are not well-understood, it was not possible to test the efficiency of incorporation of these nucleotides. The percentage incorporation of tritium into the DNAs was 5.67% to 6.37%, as determined by standard spin-column chromatography (Sambrook *et al.*, 1989) with a modified TES buffer (50mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% SDS), followed by precipitation of nucleic acids with trichloroacetic acid and adsorption on DE-81 filters (Sambrook *et al.*, 1989). Labelled DNAs were stored at 4°C.

D. Hybridization

i. Denaturation of Probe and Cell(s)

Fluorescent *in situ* hybridization with repetitive DNA probes was carried out as described by Eastmond and Pinkel (1990), with some modifications. Labelled DNAs, whether containing hapten-conjugated nucleotides or fluorochrome-conjugated nucleotides, were denatured in the same manner. Sperm were probed with biotinylated-DYZ3, digoxigeninylated-XC, and coumarin-pUC1.77 DNAs. Oocytes were hybridized with FITC-pUC1.77 and biotin-D12Z3 DNA probes. Probe specificity was confirmed using lymphocyte slides containing both metaphase and interphase cells. Hybridization mixtures were prepared by adding 30-50 ng of DNA and 500 ng of salmon testis carrier DNA (Sigma) to 7 μl MM2.1 (55% formamide, 1X SSC, 10% dextran sulfate). The probe mix was heated at 70°C for 5 minutes, then was snap-cooled in an ice-water bath for 5-10 minutes.

Slide DNA was denatured by immersion in a prehybridization bath (70% formamide, 2X SSC, pH 7.0) at 70°C for 2 minutes. The slides were snap-cooled and dehydrated in Coplin jars containing 70%, 85%, 95% ethanol at -20°C, then were air-dried at room temperature and prewarmed to 40°C on a slide warmer. Probe mix was applied to the slide. The sperm slide was covered with a 22mm^2 glass coverslip (#1 thickness, Canlab) and the edges were sealed with rubber cement to prevent evaporation. A wax pencil was used to circumscribe the oocyte: the probe mix remained within the circle and no coverslip was used.

ii. Hybridization Conditions

All slides were incubated in a dark, well-humidified chamber at 37°C. Oocyte slides were incubated for 1-2 hours; the sperm slide was incubated for 16.5 hours.

iii. Post-Hybridization and Probe Detection of Sperm Slide

After hybridization, the sperm slide was removed from the incubator and the coverslip was removed; care was taken to ensure that

the slide did not dry throughout the post-hybridization and probe detection steps. The slide was put through 3 two-minute post-hybridization washes (50% formamide, 2X SSC, pH 7.0) at 45°C, then was rinsed for 1 minute in PN buffer (0.1M $NaH_2PO_4 \cdot 7H_2O$, 0.1M $NaH_2PO_4 \cdot 2H_2O$, pH 8.0, 0.1% nonidet P-40 (Sigma)) at room temperature. Incubation for 3 minutes with 20 μl of PNM buffer (PN buffer plus 5% nonfat dry milk and 0.02% Na azide (Sigma)) blocked non-specific sites. The slide was briefly rinsed in PN buffer before proceeding through the detection steps.

To visualize the biotinylated-DYZ3 and digoxigeninylated-XC, avidin-Cy3™ (3.3 μg/μl (BioCan) in PNM buffer) and anti-dig-FITC (6.6% in 70% PNM buffer, 13 mg/ml normal anti-sheep serum (Sigma) and 0.7 mg/ml bovine serum albumin (Pharmacia)) were mixed 1:1, and 20 μl were applied to the slide under parafilm for 1 hour at 37°C. As coumarin was incorporated directly into the pUC1.77 DNA, no further detection steps were required in order to visualize the blue signal. After a vigorous wash in PN buffer at room temperature, 10 μl of propidium iodide (0.003 mg/ml-Sigma in antifade) was applied for 60 seconds, then was rinsed off by vigorous swishing in PN buffer. The slide was coverslipped under 8 μl of antifade.

On a pale-red propidium iodide background, the coumarin-pUC1.77 and digoxigenin-XC-anti-digoxigenin-FITC probes produced clear blue and pale yellow-green signals, respectively. The red biotinylated-DYZ3-avidin-Cy3™signal required amplification, and the yellow-green XC signal was considered to be incompletely detected, so the cover slip was removed, the slide was vigorously rinsed in PN buffer, and 20 μl goat anti-avidin (0.01 mg/ml-Vector in PNM buffer) was applied for 30 minutes under parafilm at room temperature. A vigorous wash in PN buffer was followed by application of 20 μl of avidin-Cy3:anti-dig-FITC (1:1) for 1 hour, at 37°C, under parafilm. After a vigorous wash in PN buffer, propidium iodide was applied as described above, and the slide was again

coverslipped in antifade.

iv. Post-Hybridization and Probe Detection of Oocyte Slides

After hybridization, slides were very carefully rinsed for 2 minutes in each of 3 post-hybridization washes (50% formamide, 2X SSC, pH 7.0) at 45°C. After a gentle 30-second rinse in PN buffer at room temperature, 10 μl of avidin-Cy3™ was applied to the slide, under parafilm, for 1 hour at 37°C. The biotinylated chromosome 12 signal was amplified with 10 μl goat anti-avidin followed by 10 μl avidin-Cy3, each for 30 minutes under parafilm at room temperature. As the nuclear DNA stain, propidium iodide, was taken up very poorly by oocyte DNA, DAPI (10 μl, 0.000125 μg/μl-Sigma in antifade) was applied to the slides for 60 seconds. After removal of the DAPI in two washes of PN buffer, 8 μl of antifade solution was applied, and the slide was coverslipped. The FITC-pUC1.77 and biotin-D12Z3-avidin-Cy3™ probes produced yellow and red signals, respectively, on DAPI-blue chromosomes.

v. Analysis of Hybridization

All hybridized slides were examined on a Zeiss Axiophot epifluorescent microscope fitted with two filter sets: a FITC/rhodamine dual band pass filter set (Zeiss 51004) and an AMCA single-colour filter set (Zeiss 487902). The excitation and emission wavelengths of the FITC, rhodamine and AMCA filters are as follows: 490 nm and 525 nm (FITC), 540-560 nm and 580 nm (rhodamine), and 345 nm and 425 nm (AMCA). The FITC / rhodamine filter set permitted simultaneous viewing of the yellow-green FITC signal, the red Cy3™ and rhodamine signals, and the red propidium iodide nuclear stain. The AMCA filter set allowed viewing of the blue coumarin signal and the DAPI nuclear stain, but could be visualized simultaneously with the yellow and red signals only when a doubly-exposed photograph was taken (Kodak Ektachrome 100HC film).

Black and white photographs of unstained oocyte chromosomes were taken before hybridization, using phase contrast and a Zeiss Photomite III at 250X magnification. Photographs were printed on Ilford Multigrade

III paper using an automatic Ilford printer. These photographs were useful both for identifying chromosomes lost during the hybridization procedure and for karyotyping, whenever the latter was possible. Unstained oocyte chromosomes could not be identified individually, but were assigned to the various chromosome groups according to the International System of Human Cytogenetic Nomenclature (Harnden *et al.*, 1985).

Use of two chromosome probes in the oocytes provided an internal control for oocyte maturation, oocyte fixation and hybridization. For example, one yellow-green (chromosome 1) signal and one red (chromosome 12) signal indicated a normal haploid cell, whereas one yellow-green and two red signals indicated a mature haploid cell disomic for chromosome 12. Due to the low probability that sister chromatids from two chromosomes will split independently during fixation, two yellow-green and two red signals were considered to indicate an immature cell.

One sperm slide with particuarly clear signals was used to collect all sperm data. Interobserver variability was not a concern after the following criteria were established for scoring the fluorescent domains: only intact, clearly-defined, non-overlapping nuclei were scored; sperm clumps were ignored. Sperm were considered disomic only if the duplicated signals were of the same size and intensity, were separated by a distance of half a domain, and were not on the cell periphery. Fine focus adjustment was required to visualize domains on different focal planes. Identification of single (as opposed to overlapped) cells was facilitated by the visualization of sperm tails, often present when using this sperm preparation.

Sperm nuclei were scored by identifying red and/or yellow-green domains within each sperm, and counting the number of nuclei which were nullisomic, unisomic or disomic for the X and Y chromosomes. A single fluorescent domain was expected within each sperm: a single red signal indicated a sperm unisomic for the Y chromosome and a single yellow-

green signal indicated a nucleus unisomic for the X chromosome. The blue chromosome 1 signal served as an internal control for hybridization and diploidy when deviations from the expected single signal were encountered. For example, when two signals were observed using the FITC/rhodamine filter, the nucleus was checked under the AMCA filter to determine if a single blue signal was present (indicating a sperm disomic for the sex chromosomes), or if two blue signals were present (indicating a diploid nucleus).

IV. RESULTS

A. Oocytes

i. Donor Profile

At the beginning of the study, all ovary specimens were accepted; many oocytes were required for experimentation to optimize the culture and fixation conditions. However, only oocytes from women who fulfilled the criteria of being premenopausal, free of cancer involving the reproductive organs, had not been exposed to radiation, and were not on hormone therapy were included in the data. A total of 73 ovaries were collected from women who ranged between the ages of 25 to 58 years. Of these women, 4 (5.5%) were in their 20s, 28 (38.3%) were in their 30s, 36 (49.9%) were in their 40s, and 5 (6.8%) were in their 50s. Most of the women were of proven fertility (76.7%). Of the women, 16.4% smoked cigarettes, 21.9% drank alcoholic beverages, and 28.8% did both. The most common reasons for oophorectomy were: chronic pelvic pain, dysmenorrhoea, menorrhagia, endometriosis and uterine fibroids. Not surprisingly, many women were taking painkillers. Aside from the gynecological problems leading to the oophorectomies, the women were generally healthy.

ii. Procurement of Specimen and Oocytes

It was observed that the portion of the ovary distal to the fallopian tubes yielded the greatest number of oocytes. Anatomically, oocytes are located in the cortex of the ovary. Shallow cuts through the cortex and parallel to the surface of the ovary often ruptured follicles, while transverse sections through the ovary with a minimal number of cuts yielded the greatest number of intact follicles.

The most common method of oocyte recovery involves the puncture and aspiration of follicles on the surface of an intact ovary. As the specimens available for the present study were generally portions or slices of ovaries, the procedure was modified as follows: puncture of visible follicles yielding a small number of oocytes, and the remainder

of the oocytes were recovered by fine mincing of the ovarian tissue. Differential filtration separated the oocytes from the cortex: a 200 μ membrane removed large debris while permitting the oocytes to pass through, then an 80 μ membrane retained the oocytes and allowed smaller debris to wash away. Cleaned oocytes were then back-washed into a sterile petri dish for observation.

A total of 313 oocytes were retrieved for culture. Recovery of oocytes was largely dependent upon the age of the donor: 65.2% of oocytes were recovered from women in their 30s, with smaller percentages retrieved from women in their 20s (2.6%), 40s (31.9%) and 50s (0.3%). The average number of oocytes recovered from women in their 20s, 30s, 40s and 50s were 2, 7.8, 2.7 and 0.2, respectively. The maximum numbers of oocytes recovered from women in their 20s, 30s, 40s and 50s were 6, 52, 21 and 1, respectively. A large percentage (41.1%) of specimens yielded no oocytes at all: 50% of the ovaries from women in their 20s, 28.6% from those in their 30s, 44.4% from those in their 40s, and 83.0% from women in their 50s were devoid of oocytes. In a number of cases, lack of success in oocyte retrieval was due to the miniscule amount of specimen available for processing.

iii. Oocyte Culture and Fixation

a. Description of Oocytes

Healthy oocytes were characterized by cytoplasm of uniform texture and a fine granularity, with a light-coloured, "glassy" appearance. A minimum of two layers of intact, slightly dispersed cumulus cells surrounding the oocyte proved to be an important indicator of healthiness and potential for *in vitro* maturation. In contrast, unhealthy oocytes were often irregular in shape and characterized by a cytoplasm of coarse granules which occasionally contained a small, dark organelle / vesicle cluster. Oocytes which were unlikely to survive were often denuded of cumulus cells, and in the worst cases had atrophied and pulled away from the zona pellucida.

b. Culture Time

At maturity, cumulus cells that once covered the oocyte dispersed and began monolayer growth. Observation of germinal vesicle breakdown (GVBD) and polar body formation (PBF) were further indications of oocyte maturity, but the disk-like polar body at the oocyte - zona pellicida interface was often difficult to see. Culture times ranged from 18-52 hours, depending on visualization of GVBD, PBF, and upon the general healthiness of the oocyte. Often oocytes without signs of maturation were fixed because they were beginning to atrophy. At other times, apparently-healthy oocytes were left to culture for the longer period of time waiting for signs of maturation, only to yield fragmented and degenerated chromosomes upon fixation. Possibly these oocytes were not programmed to mature.

c. Oocyte Fixation

Sugawara and Mikamo (1986) demonstrated that gradual fixation produced fewer artefactual univalents than Tarkowski's (1966) standard method of fixation by air-drying. In the present study, however, the gradual fixation method was not successful; oocytes tended to float away and, more seriously, would not break on the slide. The proteolytic action of trypsin, often used to remove zona pellucida from mammalian oocytes (Martin, 1991), did not remove the zona from human oocytes. While protease Type IV (Racowsky et al., 1992) did not remove the human oocyte's zona pellucida, it was weakened enough that fixation using Tarkowski's method was more gentle than usual. Although a greater number of drops of fixative were needed in order to fix the oocyte, the weakened zona pellucida helped to contain the cytoplasm and the chromosomes when the egg membrane ruptured, eliminating overspread chromosomes.

iv. Refinement of FISH for Single Cells

As FISH was being performed on single cells, it was of extreme importance to minimize cell losses inherent in the technique. The oocyte

could be lost during the denaturation, hybridization, or post-hybridization steps, so slides were always carefully handled. It appeared that the loss was most likely to occur during the hybridization or post-hybridization steps. In an attempt to reduce oocyte loss, oocytes were fixed onto 3-aminopropyltriethoxysilane-coated slides (Weimer et al., 1992) and were hybridized. However this resulted in great amounts of non-specific binding of the fluorochrome-labelled probes which obscured any analysis. Ageing the oocyte appeared to prevent its loss; however, excessive ageing prevented hybridization. Due to the limited numbers of oocytes available for this study, an optimal ageing time has not yet been determined. However, it was observed that the slide must be aged for more than one week for the oocyte to remain fixed on the slide during hybridization, and that hybridization is impeded after the oocyte slide has been aged for greater than two months.

In the standard FISH technique, a coverslip is placed over the denatured cell preparation to evenly spread and prevent evaporation of the hybridization mix. Removal of the coverslip for the post-hybridization washes was an opportunity for cell loss; therefore, a coverslip was not used during oocyte hybridizations. Instead, a wax circle was drawn around the oocyte to contain the hybridization mix in a "bead" over the cell. The reduced 1-2 hour hybridization time was sufficient for probe-substrate hybridization and minimized evaporation.

v. Aneuploidy Detection

As meiotic chromosomes have an extremely poor morphology, a combination of cytogenetic and FISH analysis was used to detect aneuploidy. Until single-cell FISH techniques were established, oocyte chromosomes were C- or Q-banded (Verma and Babu, 1989) and photographed to help identify the chromosome groups by their centromere position. Later, unbanded chromosomes were photographed and then used for FISH; the photographs were used for karyotyping and for identifying any

chromosomes lost during the FISH procedure.

a. Cytogenetic Analysis

A total of six oocyte metaphases was analyzable; hypohaploid complements of less than 20 chromosomes could possibly arise from technical artefact from chromosome fixation and were not analyzed. However, only two of the six oocyte metaphases were from women who fulfilled the donor criteria. One metaphase had 23 chromosomes (Figure 1a,b); the oocyte was from a 33 year old woman of proven fertility . The sister chromatids of all the chromosomes were split, but in close proximity to each other. The other metaphase had 24 chromosomes with an extra G group chromosome (Figure 2a,b); the oocyte was from a 44 year old woman of proven fertility. The sister chromatids of only two chromosomes were split, however, the extra G group chromosome showed pronounced chromatid separation. The remaining four metaphases were from women who were on hormone replacement therapy and could not be included in the data. In all four metaphases there were split sister chromatids. The karyotypes were: 22,X,-B,+Bcht, 22,X,-C,+Ccht,+Ccht, 21,X,-C,+Echt, 22,X,-C,+Gcht.

b. FISH Analysis

Single-cell FISH analysis was successful on only two oocyte metaphases; the other metaphases were either lost entirely or lost chromosomes during the hybridization or post-hybridization steps. The chromosome morphology of these two metaphases was very poor and unanalyzable but suitable for FISH analysis. However, both metaphases were at the meiosis I stage, that is, they had not matured (Figure 3). Immaturity was inferred from the two domains of each of the green chromosome 1 and the red chromosome 12 probes. The two domains of each chromosome represented sister homologues; it is very unlikely that they represented split sister chromatids because the chromosomes do not appear over-spread from fixation and the low probability that sister chromatids on two different chromosomes could be split.

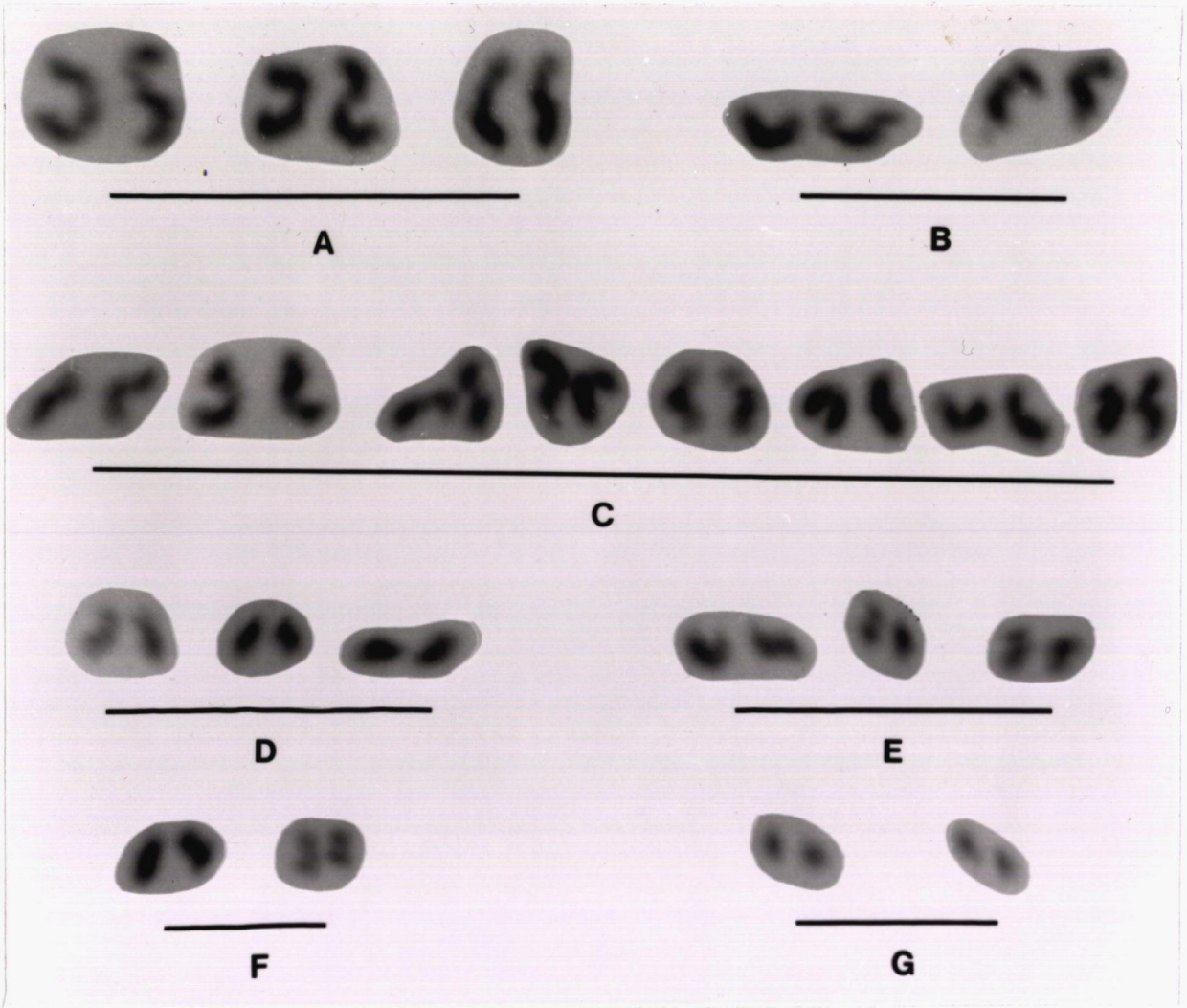

Figure 1a: Normal Oocyte Karyotype, 23,X

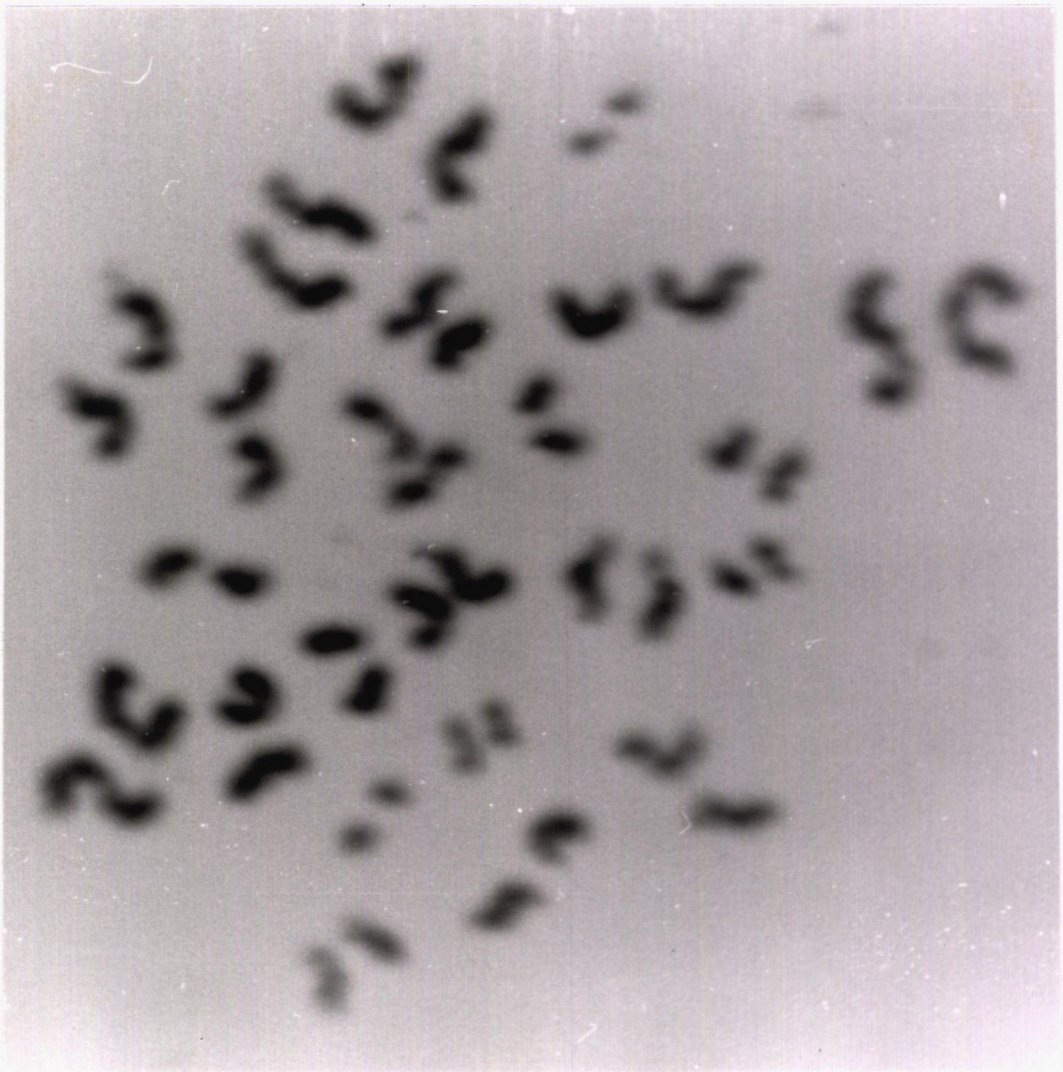

Figure 1b: Normal Oocyte Metaphase, 23,X

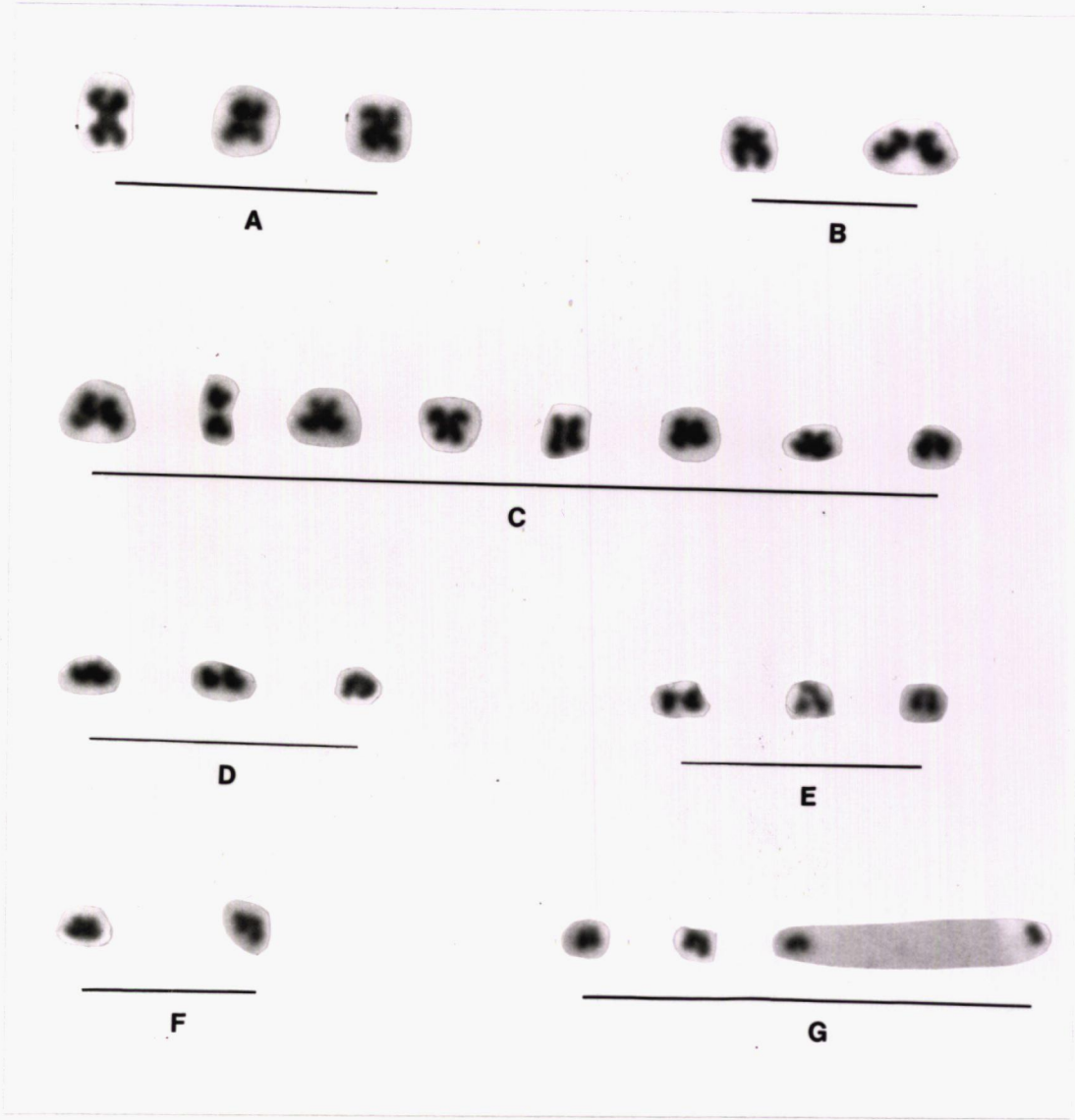

Figure 2a: Abnormal Oocyte Karyotype, 24,X,+G

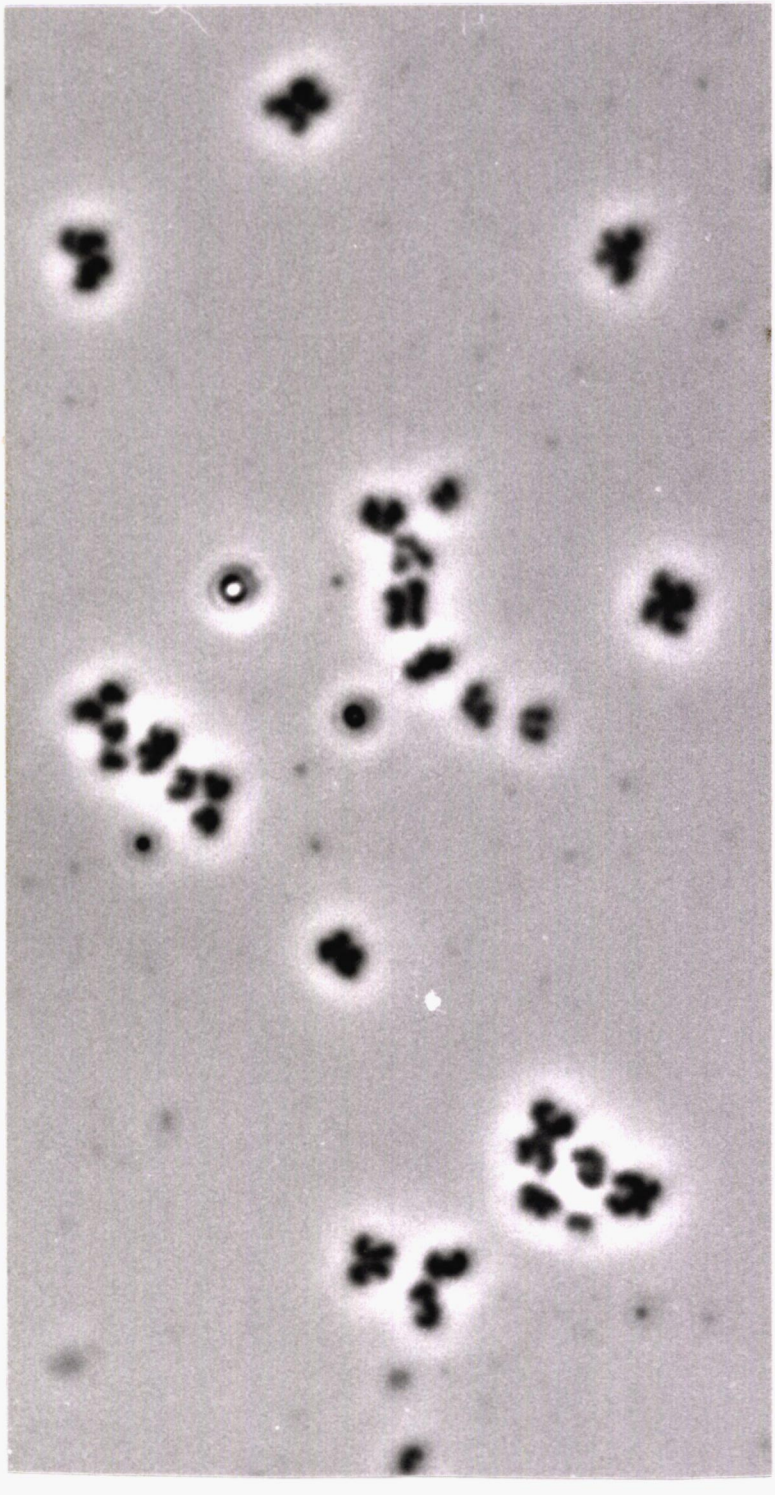

Figure 2b: Abnormal Oocyte Metaphase, 24,X,+G

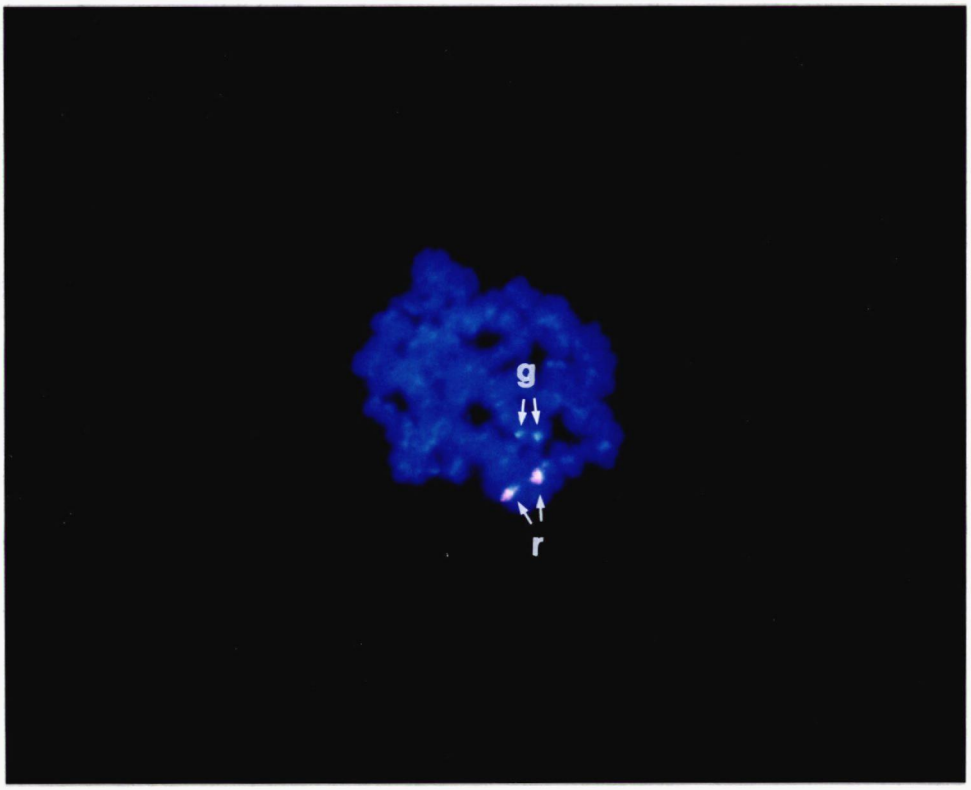

Figure 3: DAPI-stained immature oocyte metaphase, showing two red chromosome 12 signals (r) and two green chromosome 1 signals (g).

B. Sperm

i. Detection of Aneuploidy in Sperm with FISH Analysis

A total of 11,548 sperm was analyzed for X- and Y-chromosome aneuploidy (Table 2). Of the 11,423 (98.92%) monosomic sperm, 5720 (49.53%) were X-bearing and 5703 (49.39%) were Y-bearing. Diploid cells, as verified by the autosome internal control, were not scored. Disomy was observed in 82 sperm (0.71%), and was distributed as follows: 28 (0.24%) XX sperm, 21 (0.18%) YY sperm, and 33 (0.29%) XY sperm (Figure 4). There were 43 (0.37%) nullisomic sperm. Overall, X- and Y-chromosome aneuploidy accounted for 125 sperm (1.08%).

The occurrence of X- and Y-bearing sperm is not significantly different from the expected 1:1 ratio ($X^2_{d.f.=1}=0.025$, $p>0.5$). Both the XX and YY genotypes arise from a meiosis II error; there is no significant difference in the frequency of XX and YY aneuploidy ($X^2_{d.f.=1}=0.41$, $p>0.9$). The XY genotype arises from a meiosis I error, while the nullisomic genotype can arise from a meiosis I or II error.

C. Probe and Hybridization Quality Control

A control was used to determine the efficiency of probe labelling: a reaction mix containing a trace amount of ^{32}P-dCTP was nick-translated under identical conditions to those used for the hapten-labelled reaction mix. The efficiency of radioisotope-labelling was lower than manufacturer's specifications for both the biotin-dATP and digoxygenin-11-dUTP nucleotide reactions. However, when the probes were used in single-colour FISH experiments, there was a high efficiency (>97%) of hybridization, implying that the vast excess of probe relative to the

Table 2. Frequency of X- and Y-chromosome aneuploidy in 11,548 sperm from a normal male.

XX	X	Y	-	XY	YY	TOTAL
28 0.24%	5,720 49.53%	5,703 49.39%	43 0.37%	33 0.29%	21 0.18%	11,548 100.00%

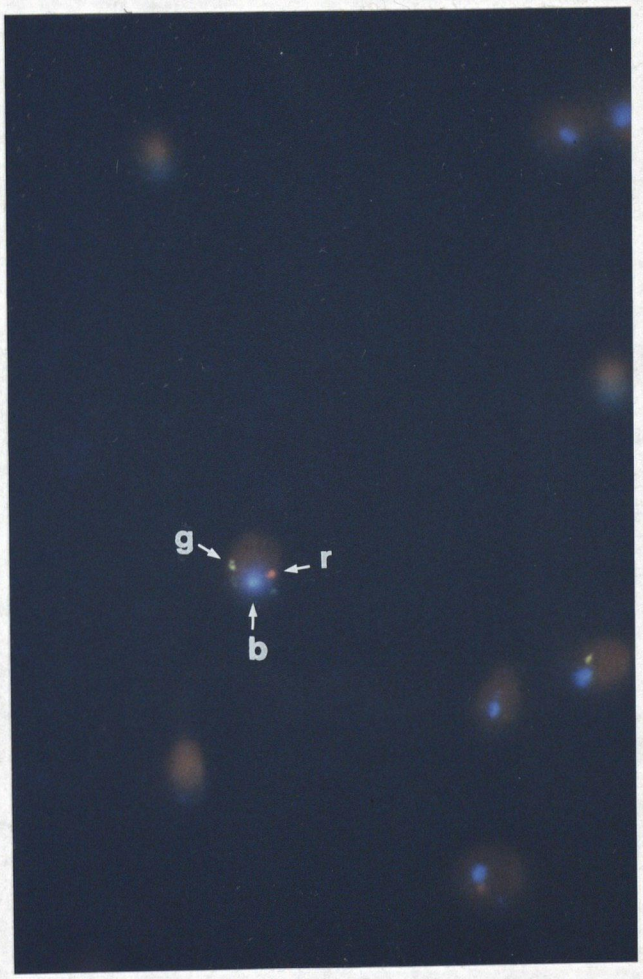

Figure 4: Abnormal sperm, bearing both X and Y chromosomes, as shown by three-colour FISH. Red signal (r) is Y chromosome, green signal (g) is X-chromosome, and blue signal (b) is chromosome 1.

number of target sequences is more significant to the success of FISH than the amount of hapten incorporated into the probes.

No significant differences have been found between signal domain analyses of two-colour FISH slides hybridizing a pair of hapten-conjugated nucleotides, two fluorochrome-conjugated nucleotides, or one hapten-conjugated and one fluorochrome-conjugated nucleotide (R.H. Martin *et al.*, unpublished observations). In addition, data obtained from hybridizations of different slides did not vary significantly (R. H. Martin *et al.*, unpublished observations).

V. DISCUSSION

A. Oocytes

In 1959, Lejeune *et al.* first documented the chromosomal nature of the most common aneuploid disorder, trisomy 21. After three decades of research, the etiology and mechanisms of congenital aneuploidy are just beginning to be understood. Studies of spontaneous abortions and liveborns clearly and consistently conclude that the majority of aneuploid conceptions are due to fertilization of an aneuploid oocyte arising from preovulatory maternal meiosis I error.

i. IVF Studies

To date, studies on maternal contribution to aneuploidy have used oocytes from IVF programs. However, several difficulties arise when an attempt is made to extrapolate IVF data to women in the general population. IVF information is from a selected group of women, many of whom have unexplained infertility. Oocytes are "spares" remaining after the most mature oocytes are chosen for fertilization, or they are those which are rejected after failing to fertilize. Superovulation hormones may interfere with the normal oocyte maturation process, resulting in a greater risk for nondisjunction, and/or may induce maturation of abnormal oocytes which would have atrophied without hormonal stimulation.

The use of rejected and spare oocytes may introduce a bias towards the collection of data from oocytes with inherent developmental problems, which in turn may be due to chromosome abnormalities. Spare oocytes are donated for cytogenetic studies precisely because they appear less mature than those chosen for fertilization, and failure of rejected oocytes to fertilize may be related to cytoplasmic immaturity. Cytoplasmic immaturity occurs when an oocyte remains arrested at metaphase II after sperm penetration, while the sperm chromosomes continue to condense. In a study of oocytes that failed to develop after fertilization, 29.5% were found to be cytoplasmically immature, and

58.7% had chromosomal abnormalities (Almeida and Bolton, 1993). It is possible that cytoplasmic immaturity may be associated with spindle progressive destabilization and detachment of chromosomes (Eichenlaub-Ritter *et al.*, 1988). The high incidence of hypohaploidy may then be due to chromosomes lost because of underdeveloped microtubules in an immature cytoplasm.

Van Blerkom and Henry (1992) analyzed newly-aspirated follicular oocytes unsuitable for insemination due to a dysmorphic appearance. Aneuploidy was found in 36% of these dysmorphic oocytes, with the acrocentric D- and G-group chromosomes over-represented and the A-, E-, and F-groups under-represented. The frequency of aneuploidy may also be correlated with the type of dysmorphic phenotype present; oocytes displaying dysmorphic features associated with the earlier stages of maturation also exhibit the highest frequency of aneuploidy. The incidence of aneuploidy does not appear to be related to maternal age.

Given the above evidence of the importance of cytoplasmic maturity in oocytes, it is clear that cytogenetic studies of oocytes from both stimulated and non-stimulated cycles must consider the potential influence of these non-genetic factors on the analysis of aneuploidy frequency.

Exogenous ovarian stimulation yields high numbers of mature oocytes for insemination. However, even under laboratory conditions and biochemical culture environments optimal for fertilization and embryo development, the probability of implantation and development of a viable fetus is about 15% (Society for Assisted Reproductive Technology and The American Fertility Society, 1993). In contrast, 28.3% of naturally-ovulated single oocytes result in conceptions (Short, 1976). Since the aneuploidy rate in superovulated oocytes from women participating in IVF programs is so much higher (11%-65%) (Van Blerkom, 1991) than the conservative aneuploidy frequency in superovulated oocytes from fertile women (3.6%) (Tarin *et al.*, 1991), their "unexplained infertility" may

be genetic in origin.

It has been claimed that the hormones which induce ovulation also increase the risk of genetically-abnormal oocytes (Boue and Boue, 1973; Alberman, 1978), but the evidence is still unclear (Plachot *et al.*, 1988, Wramsby and Fredga, 1987). It is likely that hormone-induced superovulation involves the recruitment of follicles in earlier stages of development rather than those undergoing atrophy. In experimental animals, gonadotropins do not cause degenerating follicles to return to the ovulatory pathway (Hirshfield, 1989). Thus, the assortment of superovulated oocytes are asynchronous with respect to cytoplasmic and chromosomal maturation, possibly resulting in cytogenetic anomalies that are related to induced maturation rather than a genetic defect in meiosis I. Van Blerkom (1991) notes that 2-3% of stimulated oocytes show a single, large, centrally-located "vacuole" that was observed in none of the 300 GV-stage oocytes obtained from unstimulated ovaries. The vacuolated oocytes rarely fertilized, suggesting that this aberration may be related to hyperstimulation (Van Blerkom, 1991). The type of hormonal stimulation protocol can affect fertilization and implantation rates (Testart *et al.*, 1989), but no difference in the frequency of aneuploidy between different regimes has been found (Pieters *et al.*, 1991).

ii. Non-stimulated Oocyte Studies

There are few studies examining the frequency of aneuploidy in non-stimulated oocytes matured in vitro: Jagiello *et al.* (1976) reported chromosome anomalies in 6/411 (1.5%) oocytes, and Van Blerkom (1991) found 4/157 (2.5%) oocytes to be aneuploid. While these studies of non-stimulated oocytes from non-IVF patients are very small, they suggest that hormonal stimulation and recruitment of multiple follicles may result in a significant elevation of aneuploidy. Gras *et al.* (1992) report no effect of advanced maternal age and no differences between the incidence of aneuploidy in non-stimulated oocytes from IVF patients

(4/20, 20%) as compared to superovulated uninseminated oocytes from IVF patients (23/68, 34%); however, these results are clearly based on very small numbers.

Genetic studies of oocytes from natural ovulation cycles have been hindered by two major obstacles: procurement of oocytes and the necessity for *in vitro* maturation. In the few studies involving non-stimulated immature oocytes, follicles were punctured or aspirated in order to retrieve the oocytes. These immature follicular oocytes were collected from whole excised ovaries or from ovarian biopsies (Edwards, 1965; Edwards *et al.*, 1969; Cha *et al.*, 1991); on average, whole-ovary specimens yielded almost twice as many oocytes as biopsy specimens (Edwards, 1965). In one study of mature non-stimulated oocytes, vaginal ultrasound was used to retrieve these oocytes in vivo from ovarian follicles, after the natural luteinizing hormone surge (Gras *et al.*, 1992). In contrast, less than 10% of the specimens obtained in the present study were whole ovaries, and intact follicles were rarely present in the biopsy specimens. Furthermore, the limited number of follicles in the biopsy specimens necessitated the mincing and differential filtration of the ovarian tissue, despite possible damage to the oocytes. As follicular fluid development indicates that an oocyte has been recruited for maturation, follicular oocytes are more likely to resume meiosis in vitro. The few follicular oocytes observed during this study were healthier in appearance than those recovered through mincing and filtration.

Since this study of human oocytes relied on successful *in vitro* maturation of unstimulated oocytes, much effort was expended in the definition of optimal conditions for maturation. *In vitro* maturation of non-stimulated human oocytes can be achieved by three methods: culture in serum-supplemented medium, culture in chemically-supplemented medium, and culture in follicular fluid-supplemented medium. Oocytes, isolated from Graafian follicles and cultured in a physiological medium

supplemented with serum, are capable of resuming meiosis and extruding a first polar body (Edwards, 1965; Edwards *et al.*, 1969; Racowsky *et al.*, 1992). Since few oocytes mature while still in the follicle (Kennedy and Donahue, 1969), it is hypothesized that the follicle itself may be exerting an inhibitory effect on oocyte maturation which is removed when the oocyte is liberated from the follicle (Edwards, 1965). It has been demonstrated that oocytes can be matured in a culture medium with the addition of pyruvate as a main energy source (Kennedy and Donahue, 1969), but the effect of the pyruvate on the maturation process is not clearly understood. Resumption of meiosis by oocytes cultured in a medium containing mature follicular fluid (Cha *et al.*, 1991; Racowsky *et al.*, 1992) may be due to similarities between this medium and the natural maturation milieu.

This study attempted to increase the probability of human oocyte survival and *in vitro* maturation by the addition of various supplements to standard F10 medium in a number of experiments. The scarcity of information on non-stimulated human oocytes resulted in the need to consider data from animal studies, despite the fact that laboratory animals are genetically homogeneous and selected for their good breeding success. The frequency of human oocyte survival decreased when hormonal supplements (follicle stimulating hormone, human chorionic gonadotropin and estradiol 17-β), similar to those which had been used in the successful maturation of bovine oocytes (Weimer *et al.*, 1992), were added to the culture medium. Supplementing the F10 culture medium with fluid from mature human ovarian follicles, (Cha *et al.*, 1991), was halted after concerns about the ill-defined, heterogeneous hormonal status of follicular fluid. The paucity of healthy oocytes in the present study precluded a controlled assessment of different culture media; culture in serum-supplemented medium was chosen to avoid introducing non-controllable variables from unknown constituents in other supplements. The modified final medium (50% FBS in F10 with

antibiotics) was adopted based on the success with human oocyte *in vitro* maturation described by Cha *et al.* (1991) and Racowsky *et al.*, (1992).

A great deal of effort was put into maximizing oocyte retrieval, minimizing manipulation of the oocytes, and determining optimal culture time for *in vitro* maturation. Racowsky *et al.*, (1992) had synchronized oocytes by exposure to 3-isobutyl-1-methyl-xanthine (Sigma), a synthetically derived inhibitor of adenosine 3':5-cyclic monophosphate phosphodiesterase which reversibly maintained meiotic arrest in hamster oocytes. However, the scarcity of oocytes available for the present study precluded experimentation with such a procedure.

The role of cumulus cells in maturation is unclear; they may provide an energy source (Donahue and Stern, 1968), or may produce hormones or factors capable of regulating maturation (Vanderhyden and Armstrong, 1989). That an intact cumulus cell layer is important to the maturation of the oocyte is well-documented in studies of oocytes from IVF programs (Testart *et al.*, 1983; Dandekar *et al.*, 1991), and from non-stimulated cycles (Kennedy and Donahue, 1969). The present study also noted that oocytes with a slightly-dispersed cumulus cell layer were more likely to survive and yield chromosomes.

iii. Analysis of Aneuploidy

a. Chromosome Analysis

From a total of 313 oocytes available for *in vitro* maturation, only six oocytes yielded meiosis II chromosomes which were cytogenetically analyzable; two oocytes, analyzable by FISH, were shown to contain meiosis I chromosomes.

Of the six metaphases obtained, two karyotypes could be included in the data because of donor characteristics. One karyotype of 23 chromosomes was obtained from a 33-year-old woman of proven fertility. A second oocyte, from a 44-year-old woman of proven fertility, yielded a metaphase of 24 chromosomes, with an extra G group chromosome split into sister chromatids. From this small sample, it is impossible to determine

either the frequency of aneuploidy in human oocytes or whether there is an age effect. However, it is notable that the aneuploid oocyte had an extra G group chromosome and is from a woman over 40 years of age.

The remaining four karyotypes could not be included in the study because the oocytes were from women who were on hormone therapy. The four karyotypes had split and scattered chromosomes; in contrast, chromosomes from the oocytes not exposed to hormones were less split and the sister chromatids were in close proximity to each other.

It has been suggested (Angell, 1991) that premature chromatid separation of meiosis I chromosomes is a major mechanism leading to trisomy formation. Precocious division of univalents in meiosis I is observed as single chromatids in meiosis II. The resultant oocytes can be divided into four categories. In the first instance, 22 whole chromosomes plus a single chromatid would result, after fertilization, in a 50% chance of a chromosomally-normal conceptus, and a 50% chance of a monosomic conceptus. After fertilization, the second group of oocytes, with 22 whole chromosomes and two single chromatids, would lead to frequencies of normal, trisomic, and monosomic conceptuses of 50%, 25%, and 25%, respectively. The third category would include those oocytes containing 23 whole chromosomes plus one single chromatid, and would result in 50% normal and 50% trisomic conceptuses after fertilization. Finally, 23 whole chromosomes plus two single chromatids would give rise, after fertilization, to conceptuses with a 50% frequency of trisomy, and 25% frequencies of normal chromosomes and of double trisomy.

In the present study, one metaphase fell into the final category, with two extra single chromatids from the G group. The separated single chromatids could arise from technical artefact during fixation. However, this is unlikely since other chromosomes, especially those in the vicinity of the split G-group chromatids, experienced the same assaults from fixation, yet did not display split chromatids. The four oocyte

karyotypes from the hormonally-treated women all had separated chromatids. Perhaps hormonal stimulation is recruiting oocytes that are more prone to centromere instability (Hansmann *et al.*, 1980).

Recently, Kamiguchi *et al.*, (1993) found that prematurely-separated chromatids accounted for one-third of the meiosis I malsegregation products; nondisjunction of whole chromosomes was the major mechanism in the meiotic error. They suggest that single chromatids may have prematurely separated after meiosis I chromosome segregation as a result of oocyte degeneration. More unstimulated oocytes from fertile women are needed to determine the frequency of aneuploidy and to determine whether premature chromatid separation is truly a mechanism of trisomy formation or is merely a technical artefact of oocyte degeneration and or of fixative assaulting the centromere.

b. FISH Analysis

Many oocytes were sacrificed to develop the procedure for FISH analysis: oocytes were used to determine which variables affect the adhesion of a single oocyte to the slide during the many hybridization and wash steps, as well as the conditions needed for probe hybridization.

Single cell FISH analysis was successfully performed on only two oocytes; unfortunately, neither had matured. Each oocyte had two green domains (chromosome 1) and two red domains (chromosome 12). Since the probability of a double aneuploidy involving chromosomes 1 and 12 is very low, as is the possibility of precocious separation of the sister chromatids, simultaneously affecting the two chromosomes under study, the two domains for each chromosome probe are assumed to represent homologous meiosis I chromosome pairs, slightly separated due to fixation problems.

FISH has been used to detect aneuploidy in neoplastic cells from which metaphases are difficult to analyze (Cremer *et al.* 1988). In a similar fashion, FISH analysis may allow aneuploidy detection in meiotic

chromosomes, which are also extremely difficult to analyze. However, FISH analysis with chromosome-specific probes may be limited to those cell stages where chromatin is condensed enough to produce discrete domains. Decondensed prometaphase chromosomes or degenerating cells may give ambiguous results, especially in the differentiation of signals from a pair of homologues or from two sister chromatids.

While it is desirable to continue studying aneuploidy in oocytes from non-stimulated women, the lack of follicular oocytes will continue to plague future researchers. In addition, premature chromatid separation may preclude informative results about aneuploidy in oocytes using either the cytogentics or FISH techniques.

B. Sperm

i. Assessment of the Three-Colour FISH Technique

The FISH technique is a rapid and sensitive method of detecting aneuploidy in interphase sperm. Repetitive probes are available commercially, or may be prepared using either classical molecular biology techniques or PCR amplification (Celeda *et al.*, 1992). After the acquisition of probes and fine-tuning of hybridization conditions, FISH analysis of 10,000 sperm takes approximately 1 week. The large sample size in itself permits detection of rare meiotic errors. FISH reliability is enhanced by the fact that selection is not a consideration in the hybridization process: lack of hybridization affects normal and abnormal sperm proportionally and equally. Hybridization efficiency is scored as 100% when using a chromosome 1 internal control for 2-colour FISH of the sex chromosomes, so only sperm that hybridize to one of the three probes are counted. Since domain sizes and intensities must be equal in XX and YY gametes, inter-observer perception variability is minimized. Furthermore, the requirement that two signals be separated by at least one-half domain ensures that a stretched segment of decondensed chromatin is not misinterpreted as two signals. Disomic XY-bearing sperm contain one domain of each colour and

are easy to visualize.

There are some disadvantages to the FISH technique. Assessment of aneuploidy is confined to the probes that are being hybridized: a simultaneous genetic profile of all chromosomes is not possible. Amplification of indirectly-labelled fluorescent signals may be required, leading to an increased risk for spurious signals. However, the greatest challenge to personnel is to maintain strict precision when scoring, as application of the scoring criteria to sperm is open to a certain amount of subjectivity.

ii. Frequency of X- and Y-Chromosome Aneuploidy

The data show that there is no bias towards X- or Y-bearing sperm and that an error in meiosis II is equally likely to affect the X- or the Y-chromosome. Each nullisomic sperm has a reciprocal XY, XX, or YY sperm, so the sum of the sperm disomic for the sex chromosomes would be expected to equal the number of nullisomic sperm. The fact that the number of nullisomic sperm (43) is fewer than the total of the XY, XX and YY gametes (82), can be explained by a number of factors. Variables inherent to the FISH technique can distort the data: inconsistencies in sperm decondensation, differences in signal sizes, and differing signal intensities can all lead to visualization problems. Clear signals are also dependent on the number of copies of the repetitive sequence and a low copy number can present a false negative result (Mizunoe and Young, 1992). The use of indirect probes, which can give rise to a problem with spurious background signals, may cause a nullisomic sperm to be falsely scored as monosomic. The net effect of the above factors could be an artificially-low frequency of nullisomic sperm.

The data also show the number of meiosis II (XX and YY) aneuploid gametes (49) to be greater than the number of meiosis I (XY) aneuploid gametes (33) (Table 2). The chromosome-specific probes for the non-homologous X- and Y-chromosomes act as markers, useful in determining whether an error has occurred in meiosis I or II. However, after

assessing only one donor, it is not possible to determine whether nondisjunction occurs at different frequencies for the two types of meiotic division. Data from a number of men will be required to ascertain whether this study's observation - that meiosis II errors are more common than meiosis I errors in sperm - is universal to all males or is an isolated case.

Paternal meiosis I error is responsible for 50% of 47,XXY liveborns (Lorda-Sanchez *et al.*, 1992) and the frequency of 47,XXY pregnancies is estimated to be 0.05% (Jacobs *et al.*, 1989). Hence, 0.025% of liveborns have 47,XXY karyotypes which are due to paternal meiosis I error. However, the frequency of XY-bearing sperm is tenfold greater at 0.29%. Paternal meiosis II error is responsible for 10% of 47,XXX (May *et al.*, 1990) and 100% of 47,XYY liveborns. The frequency of 47,XXX and 47,XYY pregnancies is estimated to be 0.06% and 0.04%, respectively (Jacobs *et al.*, 1989). Hence, paternal meiosis II error is responsible for 47,XXX and 47,XYY karyotypes in 0.006% and 0.04% of liveborns, respectively. Again, the frequency of XX- and YY-bearing sperm is much greater, at 0.24% and 0.18%. The discrepancy in aneuploidy frequency between gamete and pregnancy data may reflect the high rate of fetal wastage in humans, and emphasizes the need to examine gametes directly in order to determine the true frequency of aneuploidy in humans.

The prevalance of 47,XXY, 47,XXX, and 47,XYY liveborn is estimated to be 0.11%, 0.10% and 0.10%, respectively (Bond and Chandley, 1983). The FISH results may, in fact, over-estimate sex chromosome trisomies since there is a tenfold discepancy between gamete and liveborn data.

The mechanism of meiosis I nondisjunction is hypothesized to involve the 3 Mb pseudoautosomal region of the distal Xp and distal Yp (Ellis and Goodfellow, 1989). The pseudoautosomal region is important in the initiation of X- and Y-chromosome pairing (Mohandas *et al.*, 1992), recombination (Hassold *et al.*, 1991), and chromosome separation during

male meiosis I. A possible mechanism for meiosis I nondisjunction is that the single "obligate" chiasma (Hulten, 1992) does not "conjoin", resulting in XY univalency or asynapsis.

Meiosis II is an equatorial division of homologous chromosomes rather than a reductional division. Its mechanism of nondisjunction remains elusive.

iii. Comparison of Multi-Colour FISH and Cytogenetics Data

The donor in the present study has previously participated in sperm chromosome studies (Martin *et al.*, 1991). Of his 415 karyotypes, 53.4% are X-bearing and 46.6% are Y-bearing. The frequency of numerical abnormalities is 1.9%, but no sex chromosome anomalies have been detected. For this donor, the increased sensitivity of FISH was able to detect a low incidence of sex chromosome aneuploidy.

Table 3 summarizes the sperm karyotype data for the X- and Y-chromosomes. In 11,078 karyotypes of many donors, sex chromosome nullisomy was found to be 0.42%, and the incidences of XX, YY, and XY aneuploidy were 0.02%, 0.03% and 0.05%, respectively (R.H. Martin, unpublished data). From multi-centre, multi-donor studies of 16,237 karyotypes, XX, YY, and XY disomy averaged 0.01% (Brandriff and Gordon, 1990; R.H. Martin, unpublished data), 0.03% (Brandriff and Gordon, 1990; R.H. Martin, unpublished data), and 0.06% (Brandriff and Gordon, 1990; R.H. Martin, unpublished data), respectively. There is a 10-fold

Table 3. Sperm karyotype data of X- and Y-chromosome aneuploidy

GROUP	TOTAL	XX	YY	XY	no X, Y
Brandriff & Gordon, 1990	5159	-	2 0.04%	3 0.06%	11 0.21%
Martin, unpublished data	11,078	2 0.02%	3 0.03%	6 0.05%	47 0.42%

increase in the frequency of each of these disomies detected by FISH (Table 2). This discrepancy may either reflect the greater sensitivity of this method of analysis, both in detecting disomy and in minimizing artefactual hypohaploidy, or may reflect an artificially high incidence of disomy arising from the counting of spots that do not represent chromatin domains.

Direct examination of sperm using FISH analysis may more accurately reflect true aneuploidy levels. Human sperm chromosome studies are analyses of pronuclear chromosomes and it is unknown whether aneuploid sperm have reduced fertilizing capacity in the hamster oocyte system. Little is known about the effects of hamster oocyte cytoplasm on human sperm chromosomes. Just as fertilization of hamster oocytes has been shown to repair human sperm chromosome aberrations (Genesca *et al.*, 1992), sperm chromosomes may degenerate in an unhealthy oocyte cytoplasm.

In the future, large studies using both cytogenetic and FISH techniques will be needed to compare the frequencies of aneuploidy of specific chromosomes, and to compare the differences found in the frequencies of aneuploidy observed for the same chromosome using the two techniques.

iii. Comparison of FISH Data with Other Laboratories' Data

Table 4 compares the rates of X- and Y-chromosome aneuploidy in sperm obtained in the present study with those obtained by other laboratories. The literature data show a wide variation in the frequency of aneuploidy: XY-disomy ranges from 0.06% to 0.22%, XX-disomy ranges from 0.03% to 0.28%, YY-disomy ranges from 0.06% to 0.27%; sex

Table 4. Frequency of X- and Y-chromosome aneuploidy in sperm using FISH analysis.

GROUP	N	Y	YY	X	XX	XY	no X, Y
Joseph et al., 1984	6636	51.73%	0.18%	-	-	-	-
West et al., 1989	3900	46.60%	0.03%	-	-	-	-
Guttenbach & Schmid, 1990	8061	49.39%	0.27%	-	-	-	-
Wyrobek et al., 1992	10,000	-	0.06%	-	0.06%	0.06%	-
Han et al., 1992	13,396	-	-	48.22%	0.29%	-	-
Holmes and Martin, 1993	7,004	-	-	49.44%	0.03%	-	-
Han et al., 1993	12,507	47.31%	0.26%	47.93%	0.28%	0.22%	4.05%
Robbins et al., 1993	31,856	50.27%	0.06%	-	-	-	-
Chan and Martin, 1993	11,548	49.39%	0.18%	49.53%	0.24%	0.29%	0.37%

chromosome nullisomy is 4.05% in the only study which reports it. Data from the present study is within these ranges for XX-disomy and for YY-disomy. However, the XY-disomy frequency is slightly greater than reported, while sex chromosome nullisomy is tenfold less. Some of the disparity between aneuploidy frequencies can be explained by variations in the experimental conditions used by the laboratories. The main factors to be considered are the method of sperm decondensation used, and the presence of an autosomal internal control for hybridization when scoring the sex chromosomes.

The nuclear chromatin of mammalian sperm is highly condensed into a package of minimal volume (Wyrobek *et al.*, 1976) by a network of intra- and interprotamine disulfide bridges (Balhorn, 1982). Quinacrine dihydrochloride staining produces a single bright spot in some sperm, thought to represent the Y chromosome (Barlow and Vosa, 1970). However, using this technique, the proportion of single spots was less than the expected 50% (Wyrobek *et al.*, 1983), suggesting that not all sperm containing the Y chromosome were stained. Wyrobek *et al.*, (1990) demonstrated that the nuclear compaction of mature sperm interfered with DNA probe penetration and that decondensation and increased swelling of 50% could be achieved by treatment with lithium diiodosalicylate (LIS).

Studies undertaken to determine aneuploidy frequency in sperm using FISH have not always included a chromatin decondensation step (Joseph *et al.*, 1984; West *et al.*, 1989, Guttenbach and Schmid, 1990). The disulphide reducing agent, dithiothreitol (DTT), has been used to promote nuclear decondensation (Han *et al.*, 1993; Holmes and Martin, 1993). DTT treatment probably removes the nuclear membrane (Balhorn *et al.*, 1977), without a dramatic effect on chromatin decondensation, whereas LIS is able to extract membrane proteins and nuclear histones to produce nuclear swelling in somatic cells, resulting in better probe penetration (Mirkovitch *et al.*, 1984). Inconsistent or lack of chromatin decondensation could account for the wide range of sex chromosome

aneuploidy frequencies.

The present study, using the LIS treatment found the YY-disomy frequency to be 0.18%. The lower incidence of YY-disomy (0.06%) reported by Wyrobek *et al.* (1992) and Robbins *et al.* (1993), both of whom also used the LIS decondensation technique, could have two possible explanations. Robbins *et al.*, (1993) used a 3.4 kb Y-chromosome-specific non-alphoid repeat probe (Smith *et al.*, 1987) which may be insufficient to consistently detect the Y chromosome in sperm. The present study used a 5.5 kb alphoid repeat probe with an estimated 100 copies per genome, yet amplification of an indirectly-labelled fluorescent probe was required in order to consistently visualize the signal. In addition, YY-disomic sperm may not have been detected due to a lack of hybridization in the reported studies; an autosomal probe was not present as an internal control for hybridization. Han *et al.*, (1993) report a high frequency of nullisomic sperm; the nullisomic sperm greatly exceed the sum of the disomic sperm. Possibly DTT is an inadequate promoter of sperm decondensation: hence, poor probe access to the target does not allow visualization of a signal even though the chromosome is present. Again, an autosomal control is required to differentiate between sperm nullisomic for a sex chromosome and sperm which fail to hybridize.

To date, two methods have been used to decondense sperm: decondensation while the sperm are in suspension, or while they are fixed in place on the slide (*in situ*). The suspension method involves the decondensation and fixation of a suspension of sperm nuclei before storage at -20°C, and the same suspension is used for many experiments (Holmes and Martin, 1993, Han *et al.*, 1993). The *in situ* method involves smearing a washed sperm sample onto a clean slide, then decondensing and fixing the sperm in place on the slide (Wyrobek *et al.*, 1992; Robbins *et al.*, 1993). It has recently been discovered that the efficiency of hybridization decreases as the suspended sperm nuclei age in fix (Martin

et al., in press). This is possibly due to a hardening of the nuclear cell membrane after long periods in fixative, resulting in poor probe access to the target substrate. The *in situ* sperm decondensation method used in the present study has many advantages: in addition to being a less time-consuming protocol, more consistent decondensation of nuclei is obtained, and more quality control can be exercised (a poorly-decondensed slide can be discarded without disposing of the entire sperm preparation).

The present study used the same sperm donor and X-chromosome probe as were used by Holmes and Martin (1993) in their study of suspension-decondensed sperm. The percentage of X-bearing sperm is comparable between the two studies, but the XX-disomy frequency is increased eightfold in the present study. Two factors may explain this discrepancy. The DTT decondensation step used by Holmes and Martin (1993) may have been insufficient for adequate probe penetration, and the nuclear membranes in their sperm suspension may have begun to harden by the end of their study, again resulting in poor probe access.

Few other groups have used the multicolour FISH system to study aneuploidy. Using chromosome 17 as an autosome control, Han *et al.*, (1992) report the frequency of XX disomy to be 0.29%. In a later study of X- and Y-chromosome aneuploidy, Han *et al.*, (1993) report the frequency of XX, YY and XY disomy to be 0.28%, 0.26%, and 0.22%, respectively; an autosomal internal control was not used in this study. The frequency of XX and YY disomy reported in the present study is lower than that reported by Han and her colleagues. However, Han *et al.* 1993 report that they may have included a number of split signals in their disomic numbers, whereas the present study's strict criteria eliminate spurious disomic counts from split signals. This study's XY disomy frequency is greater than reported by Han *et al.* (1993), possibly due to better probe access, since Han *et al.* (1993) also report a large percentage of sex chromosome nullisomy. In the present study, it was

found that despite signal amplification, the X- and Y-chromosome probes were sometimes difficult to visualize. Nevertheless, if the internal autosome control probe produced a single signal and the sperm tail could be seen, true XY disomy was confirmed.

The present study is unique in its use of an autosome as an internal hybridization control; neither Wyrobek *et al.* (1992) nor Han *et al.* (1993) employed an internal autosomal control in their detection of X- and Y-chromosome disomy with multi-colour FISH. As only one of the sex chromosomes will normally be present in each sperm, simultaneous hybridization to an autosome is needed in order to provide a signal from the internal control in every cell. It is almost impossible for an observer to simultaneously score more than two different probes for the presence of aneuploidy due to the multitude of potential signal combinations. However, the use of multiple probes greatly improves the reliability of FISH by providing an internal control for hybridization. The nullisomy frequency in the present study is less than expected; spurious signals from indirectly-labelled fluorescent probes may have caused some nullisomic sperm to be scored as monosomic sperm. Nonetheless, the internal hybridization control (which, as a directly-labelled probe, is not susceptible to spurious background signals) does eliminate non-hybridized sperm from the nullisomic population.

There are a number of possible reasons for the inconsistencies reported by different laboratories in the sex chromosome aneuploidy frequencies. Han *et al.*, (1993) do not report any inter-donor variability for sex chromosome aneuploidy. In contrast, Robbins *et al.*, (1993) report significant inter-donor variability: in comparison to other donors, one male had an increased incidence of YY-bearing sperm detected by FISH analysis that was confirmed in an analysis of his sperm karyotypes.

Even when the same sperm preparation methods are used (Wyrobek *et al.*, 1992, Robbins *et al.*, 1993, the present study), there are

differences in the results obtained - yet within each research group, the results are consistent. Since there are so many factors which can influence the visualization and interpretation of signals, it is clear that in order to study aneuploidy using FISH, guidelines must be established for: 1) the type of cell preparation that allows the probe optimal access to the target, 2) the use of large, multiple-copy probes, and 3) the precise scoring of domains.

In summary, FISH is a rapid and sensitive technique for aneuploidy detection in sperm. Its reliability will be assessed when the guidelines for detecting aneuploidy in interphase cells established by the various laboratories yield reproducible results around the world.

CONCLUSION

Non-hormonally-stimulated oocytes were retrieved from ovarian tissue and were matured *in vitro* to complete meiosis I. The oocytes were analyzed for the presence of aneuploidy by karyotyping and by two-colour fluorescence *in situ* hybridization (FISH).

A total of 313 oocytes were cultured, yielding six analyzable metaphases. Two of these metaphases were included in the data, because they were obtained from women who fulfilled all donor criteria. One metaphase, from a 33-year-old woman had a normal 23,X karyotype. The other metaphase, from a 44-year-old woman, was an aneuploid 24,X,+G karyotype; the extra G-group chromosome displayed split chromatids which could have arisen from technical artefact or from premature chromatid separation.

Many oocytes were used to develop the FISH technique for single cells. FISH was successfully performed on two oocytes, but neither oocyte had matured to complete meiosis I. It was observed that ageing the cell for a minimum of one week prevented its loss during the post-hybrization wash steps. However, ageing the cell for more than two months prevented hybridization of the probe to its target.

From the small sample, it is not possible to determine either the frequency of aneuploidy in human oocytes or whether there is any maternal age effect on aneuploidy frequency. In the future, the paucity of follicular oocytes and the possibility of premature chromatid separation may preclude informative results about aneuploidy in oocytes using current cytogenetic or FISH techniques.

The technique and guidelines for analyzing multi-colour FISH in large numbers of interphase cells were established. Using three-colour FISH with probes for the X- and Y-chromosomes and an internal autosome hybridization control, the incidence of X- and Y-chromosome aneuploidy was determined in 11,548 sperm from a normal male of proven fertility. The aneuploidy frequency was 0.29% for XY-disomy, 0.24% for XX-disomy,

0.18% for YY-disomy, and 0.38% for sex chromosome nullisomy.

The frequency of each disomy detected by FISH is tenfold greater than was observed from cytogenetic studies of this donor, and from large cytogenetic studies of many normal donors, suggesting that increased sensitivity is possible using FISH analysis. The frequency of nullisomy detected by FISH is less than observed in cytogenetic data, suggesting that FISH is able to decrease spurious hypohaploidy arising from technical artefact.

The present study is the first to use an internal autosome probe to control for hybridization. There is much variability in the frequency of aneuploidy reported in the literature. Data from the present study is within the range reported for XX- and YY-disomy; the XY-disomy frequency is greater and the sex chromosome nullisomy frequency is much less than reported. This study demonstrates that multi-colour FISH is a rapid and sensitive technique for detecting aneuploidy in sperm. However, international guidelines for sample preparation and domain scoring must be established before its reliability can be assessed.

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