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The use of fluorescence *in situ* hybridization on human sperm: the effect of donor age on the frequency of aneuploidy

by

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Abstract

The purpose of this study was to determine if a donor age effect exists for the frequency of aneuploidy and other chromosome abnormalities in human sperm. Sperm samples were collected from 18 healthy men from the general population. Each individual belonged to one of 6 age groups (20-24, 25-29, 30-34, 35-39, 40-44, 45+) with 3 men in each group. A series of multicolor fluorescence in situ hybridizations was performed on sperm from each donor using probes for the sex chromosomes and chromosomes 13 and 21. In addition, two chromosome 1-specific probes were chosen to allow for detection of duplications and deletions within chromosome 1. The abnormality frequencies and the Pearson correlation coefficients were calculated to determine if a relationship exists between donor age and the frequency of chromosome abnormalities in sperm. A statistically significant association with donor age was detected for the frequency of YY disomy and acentric fragments of chromosome 1.

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List of Abbreviations

°C	degrees Celsius
µL	microlitre
cen	centromere
cm	centimeter
DAPI	4,6 diamidino-2-phenylindole
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
FITC	fluoroisothiocyanate
LIS	lithium diiodosalicylate
M	molar
mM	millimolar
NaCl	sodium chloride
ng/mL	nanograms per millilitre
NP-40	nonionic detergent P-40
p	short arm of a chromosome
PVP	polyvinylpyrollidone
q	long arm of a chromosome
s	seconds
SSC	standard saline citrate solution

I. Introduction

> "There is of necessity a resemblance in some part to each of the parents, if indeed the sperm passes into the child from both bodies and whichever [parent] contributes more to the likeness, i.e. [contributes] from more parts of the body, that is the one that the child resembles in more parts. It happens sometimes that a daughter born resembles the father in more parts than the mother and a boy born looks more like his mother than his father." – Aristotle from *On Semen* (Morsink, 1979)

A. Aneuploidy

The concepts of inheritance and reproduction have aroused the curiosity of both ancient and modern scientists. Although some of the theories created by Aristotle and others seem almost laughable today, a number of their observations have withstood the test of time. Namely, children may resemble either parent or both parents or even relatives that are no longer living and the health of newborns can be influenced by the age of the parents. In fact, Aristotle recommended that men become fathers between thirty-seven and fifty-four years of age and women become mothers between eighteen and thirty-six years of age (Cline Horowitz, 1976). Although these findings would later be proven to be correct, many beliefs were rooted in superstition and fear. The smell of a freshly extinguished lamp was thought to cause deformed children and fright, malnutrition or lovingly staring at statues during pregnancy was thought to give rise to children who do not resemble their parents (Stubbe, 1972). Although the more irrational components of these hypotheses have long since been excluded, our comprehension of genetics and reproduction is still a relatively recent achievement.

Aneuploidy is a significant cause of reproductive wastage in humans. It is defined

as "an abnormal chromosome number due to an extra or missing chromosome" (Thompson and Thompson, 1991, pg. 202) and nondisjunction, or failure of chromosomes to separate properly during cell division, is the main cause of aneuploidy (Griffin *et al.*, 1996). Estimates of the incidence of aneuploidy in human conceptions range from 8% to 50% (Bond and Chandley, 1983) and this frequency is thought to be higher than that of any other mammal. Quite a large proportion of these conceptions are unable to survive beyond the first trimester and the reasons for this tremendous reproductive loss remain elusive. Human beings invest a significant portion of their lifetime raising their offspring and ensuring the success of subsequent generations. Perhaps a high rate of early pregnancy loss is an evolutionary strategy to allow for adequate time to elapse between live births (Vogel and Motulsky, 1986). Whatever the explanation, the causes of aneuploidy in humans remain enigmatic, despite significant efforts to unravel the etiologies.

The only factor that has been unequivocally associated with an increasing frequency of aneuploidy is that of maternal age. Over 100 years ago, it was noted that children with Down syndrome tended to be the youngest sibling in families and therefore, the greater a woman's age, the greater her likelihood of giving birth to a child with a chromosome imbalance (Bond and Chandley, 1983). Very few researchers would dispute the phenomenon of a maternal age effect, but the question of a paternal age effect has been controversial, as is shown by the conflicting results in the literature.

B. Epidemiological Studies

Through the use of cytogenetic registers, clinic records and data from prenatal

diagnoses and spontaneous abortions, numerous studies have been performed with the intention of proving or refuting the existence of a paternal age effect for chromosome abnormalities. In a case-control study of spontaneous abortions, no evidence of a paternal age effect was detected with over 461 aneuploid conceptuses studied (Hatch *et al.*, 1990). Cases were defined as chromosomally normal and abnormal spontaneous abortions that occurred before 28 weeks gestation and controls were defined as those pregnancies that were delivered at or after 28 weeks gestation. Chromosomally abnormal cases were categorized according to karyotype and no cases of trisomy 1, 11 or 19 were observed. Data analysis was performed to determine if there was a difference in paternal age between categorized aneuploid losses and chromosomally normal losses when compared with controls, while adjusting for maternal age. There was no significant difference in paternal age between chromosomally normal losses and controls. Although a slight age effect was detected for paternal age and trisomy 7, 9, 18 and 21, an inverse paternal age effect was detected for trisomy 13, 20 and 22 with the inverse effect of paternal age being statistically significant for trisomy 22 only. The authors concluded that these results are unlikely to reflect a true age effect for two reasons. First, given the structural similarities between chromosomes 21 and 22, it would be expected that both chromosomes would demonstrate similar mechanisms of nondisjunction and would therefore be affected similarly by factors that may influence nondisjunction, such as age. Second, the possibility that one of the more than 15 statistical tests performed in the analysis has led to a false positive result cannot be excluded, as no correction for multiple comparisons was used.

Further evidence to dispute the presence of a paternal age effect is provided by a

case-control study of over 300 Down syndrome children and teenagers by de Michelena *et al.*, (1993). A minimum of 3 controls was assigned to each case and all were matched for date of birth, sex and maternal age with the uncontrolled variable being paternal age. The mean paternal ages were calculated for each group and compared with each other using both parametric and nonparametric methods and no paternal age effect was detected. Yet, an investigation of prenatal diagnosis data by Stene *et al.*, (1987) suggests that a positive paternal age effect for Down syndrome is present in two distinct age groups: those individuals who are 40 years of age or older and those individuals up to 33 years of age. In each of these groups, the observed number of trisomy 21 cases was significantly greater than the expected number, but the fathers between the ages of 34 and 39 revealed a lower than expected number of trisomy 21 cases, although this was not statistically significant. Consequently, the authors suggested that genetic counseling should perhaps be based on a more individualized risk.

Carothers *et al.*, (1978) investigated a series of XXX, XYY and XXY individuals selected from endocrine/fertility clinics, mental institutions or hospitals, general practice, hospital wards and penal institutions. These affected individuals were matched with liveborn full siblings who were assumed to have a normal karyotype. Statistical models were derived to estimate the relative risk of a chromosomally abnormal child at age 40 vs. age 30. The results suggested a maternal age effect for 47, XXY and 47, XXX individuals and an inverse parental age effect for 47,XYY. The same authors also reported a case-control study of 290 Klinefelter individuals (47, XXY) and no evidence of an independent paternal age effect was detected (Carothers *et al.*, 1984). A similar method of

ascertainment for cases was used as in the previous study, however controls were selected from a birth registry and matched for sex, legitimacy and home/hospital birth. Additional statistical models were created to calculate relative risks and the results suggested that any age effect detected was due to maternal age alone.

In an investigation of over 50 000 pregnancies with mothers 35 years or older (Ferguson-Smith and Yates, 1984), study subjects were selected on the basis of maternal age alone and any pregnancies with a history of a previous chromosome abnormality or a parental chromosomal translocation were excluded. Using regression models, no paternal age effect was detected for trisomy 21, trisomy 18 and 47, XXX, however the results did suggest a paternal age effect for 47, XXY. There were insufficient numbers of cases of trisomy 13, 47, XYY and 45, X to allow for appropriate data analysis and the authors do state that the results for Klinefelter syndrome must be interpreted with caution, as there were only 23 cases in their sample population.

Numerous problems are encountered in interpreting the results of these epidemiological studies. First is the concern of selection bias of the sample population. Some studies include only livebirths (de Michelena *et al.*, 1993) and the selection process used by Carothers *et al.*, (1978) in their study of 47, XYY individuals probably resulted in a sample unrepresentative of the true population. Second, although several variables have been taken into account when conducting these types of studies, (for example, socioeconomic class, season of birth, geographic location and maternal age effect), it is still possible that the results have been influenced by a variable that has not yet been considered. Finally, paternally derived cases have not been isolated from maternally

derived cases. It is known through parent of origin studies using molecular DNA polymorphisms, the majority of nondisjunction occurs in female meiosis and as a result, the number of cases most likely to be influenced by paternal age is substantially smaller than the sample size in these studies, thus reducing the ability to detect a paternal age effect. Overall, although there are numerous epidemiological studies, conflicting results and differences in experimental design render it very difficult to assign a definitive role for paternal age in aneuploidy from these studies.

C. Parent-of-Origin Studies

i. autosomal trisomies

In recognized pregnancies, numerous clinical studies have been carried out to determine the parent-of-origin of the nondisjunctional event leading to autosomal aneuploidy. Antonarakis *et al.*, (1991) studied 200 families; each with a single case of trisomy 21. The parental origin of nondisjunction could be determined for 193 of the 200 families. Using DNA polymorphisms, it was shown that 184 children (95%) had inherited two maternal chromosomes 21 and only 9 children (5%) demonstrated paternal nondisjunction. This distribution of paternal vs. maternal errors was significantly different from those trisomy studies using cytogenetic heteromorphisms to determine the origin of the extra chromosome. A summary of these cytogenetic investigations by Hassold and Jacobs (1984) showed that 20% of trisomy 21 liveborns were due to paternal nondisjunction and 80% were due to maternal nondisjunction. Because of the discrepancy between cytogenetic and molecular studies, Antonarakis *et al.* also analyzed 31 of their families using both techniques. With cytogenetic techniques, 26 cases (84%) were

attributed to maternal nondisjunction and the remainder (16%) to paternal nondisjunction. The molecular analysis, however, indicated that three of these families had been incorrectly classified as paternal in origin and that the frequency of paternal nondisjunction in cases of trisomy 21 was therefore only 6% with the remaining 94% of cases arising from maternal nondisjunction. This distribution of paternal and maternal errors for trisomy 21 have been borne out by numerous investigators. Sherman *et al.*, (1991) demonstrated that 94 of 100 cases were maternal in origin and 6 were of paternal origin. Twenty-four of 27 (89%) Down syndrome families indicated maternal nondisjunction in a study by Howard *et al.*, (1993) and similar results have been reported by Lorber *et al.*, (1992) (89%) and Yoon *et al.*, (1996) (86%).

Trisomy 18, or Edward's syndrome, is the second most common autosomal trisomy next to Down syndrome with an incidence of 0.18% in all clinically recognized pregnancies (Kupke and Müller, 1989). Using molecular techniques, Babu and Verma, (1986) studied one case of trisomy 18 and determined that the error occurred at paternal meiosis I. Kondoh *et al.*, (1988) investigated 5 newborns with trisomy 18 and found 3 were due to maternal nondisjunction and the remaining 2 were uninformative. A study of 63 trisomy 18 cases, including livebirths, stillbirths and fetuses ascertained through prenatal diagnosis, revealed that only 2 were of paternal origin and due to postzygotic mitotic errors rather than paternal nondisjunction (Fisher *et al.*, 1995). In 39 cases of trisomy 18, only one was found to be of paternal origin (Bugge *et al.*, 1994). This relatively small contribution of paternally derived cases relative to maternally derived cases of trisomy 18 has also been reported by Nothen *et al.*, (1993) (4/30 or 13.3%), Kupke and

Müller, (1989) (1/20 or 5%) and Ya-gang *et al.*, (1993) (5/22 cases or 23%). If the data from all of these studies is pooled, the distribution of maternal and paternal errors approaches that of Down syndrome i.e. out of a total of 178 informative trisomy 18 cases fourteen, or 7.9%, are due to paternal errors. Nothen *et al.*, suggested that a paternal age effect may be present as the mean paternal age of their 4 paternally derived trisomy 18 cases was higher than the mean paternal age in the 26 maternally derived cases. An increased paternal age was also found in 5 paternally derived cases by Ya-gang *et al.*, (1993), however this did not reach statistical significance due to the small sample size.

Trisomy 13 or Patau syndrome, is the least common autosomal trisomy with a frequency of 1/25 000 births (Thompson and Thompson, 1991). A summary of parent-of-origin cytogenetic studies by Ishikiriyama and Niikawa, (1984) revealed that of the 17 liveborns and abortuses studied, 3 (17.6%) were due to paternal nondisjunction. Using molecular techniques, a study of trisomy 13 or Patau syndrome, by Hassold *et al.*, (1987) demonstrated that 3 of 20 informative cases were due to paternal nondisjunction. The authors state that although their data is limited, there is strong evidence of a maternal age effect and no evidence of a paternal age effect, however the majority of cases are due to maternal nondisjunction and it would be impossible to accept or reject a paternal age effect on the basis of 3 cases.

Although trisomy 15 is not seen in liveborn children, nondisjunction of this chromosome can result in uniparental disomy for chromosome 15 in offspring as a result of gamete complementation or loss of the extra chromosome in a conceptus with trisomy 15. The syndromes that are associated with uniparental disomy (UPD) for chromosome

15, namely Prader-Willi and Angelman syndrome, have allowed for the study of the parental origin of nondisjunction. Robinson *et al.*, (1993) investigated 32 cases of uniparental disomy 15, 27 cases of Prader Willi syndrome due to maternal UPD and 5 cases of Angelman syndrome due to paternal UPD. Through the use of molecular markers, the authors found that all 5 cases of paternal UPD 15 were derived from meiosis II errors or mitotic errors. Further analysis of two Angelman syndrome cases by Mutirangura *et al.*, (1993) revealed complete isodisomy for the chromosome 15 markers, suggesting a mitotic origin. The possibility of a paternal age effect for nondisjunction of chromosome 15 has been raised in a case-control study by Robinson *et al.*, (1993). A significantly increased paternal age was detected in 4 paternal UPD Angelman syndrome cases when the mean paternal age was compared with 20 non UPD Angelman syndrome cases. Again, the majority of the paternally derived UPD cases were due to a mitotic origin (3/4 or 75%).

A survey of 432 trisomic fetuses and liveborns by Zaragoza et al., (1994) examined the parent-of-origin for a number of autosomal trisomies including twenty-seven cases of trisomy 13, twelve cases of trisomy 14, seventeen cases of trisomy 15, three hundred and fifty-two cases of trisomy 21 and twenty-four cases of trisomy 22. Molecular markers were used to determine if the nondisjunctional event occurred at paternal or maternal meiosis I or II or mitosis and the study population consisted of spontaneous abortions, stillbirths, therapeutic abortions and livebirths (trisomy 13 and 21 only). Approximately 12% (3/25) of cases of trisomy 13 were due to paternal nondisjunction for, 17% (2/12) of trisomy 14, 12% (2/17) of trisomy 15, 9% (32/352) of trisomy 21 and 11% (2/19) of

trisomy 22. Approximately half of these errors were due to nondisjunction at paternal meiosis II (10/19) in contrast to the maternally derived cases, where the majority of errors occurred at meiosis I. All paternally derived cases of trisomy 21 were limited to liveborns, however the fact that no paternally derived spontaneous abortion cases were observed was shown to be statistically insignificant. These data suggest that the parent-of-origin of the extra chromosome does not affect the survivability of the fetus. No differences in parental ages were detected between maternally derived cases and paternally derived cases, whether the stage of the error was meiosis I or II or mitosis.

ii. sex chromosome aneuploidies

Klinefelter syndrome is an entity with a clearly defined phenotype, which is less severe when compared with the autosomal trisomies and occurs with a frequency of approximately 1/600 liveborn males (Lorda-Sanchez *et al.*, 1992). A number of studies have investigated the parental origin of nondisjunction in these individuals. MacDonald *et al.*, (1994) demonstrated that of one hundred and forty-two 47, XXY males, 66 (46%) arose due to paternal nondisjunction during meiosis I, but with no evidence of increased paternal age in this group. Jacobs *et al.*, (1988) reported similar results with 20 of 35 informative families showing paternal nondisjunction and no age effect. Conversely, Lorda-Sanchez *et al.*, (1992) found that 23 of 47 cases of Klinefelter syndrome were due to paternal nondisjunction, and a significantly increased paternal age was detected in this group when compared with maternally derived cases.

Turner syndrome, or monosomy X, is an extremely common sex chromosome aneuploidy which affects 1-2% of all clinically recognized pregnancies, however only 1%

of these fetuses survive to become livebirths (Hassold, 1986). A number of studies have demonstrated that the paternal X is preferentially lost in a majority of cases (Jacobs *et al.*, 1990; Mathur *et al.*, 1991; Loughlin *et al.*, 1991; Hassold *et al.*, 1988, 1991a; Cockwell *et al.*, 1991). For example, of 66 families with a single case of monosomy X, only 13 were shown to retain the paternal X (Hassold *et al.*, 1991a). Hassold *et al.*, (1991a) suggest that this is unlikely to be due to paternal nondisjunction because the reciprocal product of a nullisomic gamete is a disomic gamete containing an extra X or Y chromosome. If monosomy X occurs through paternal nondisjunction, one would expect the total frequency of 47, XXX, 47, XXY and 47, XYY to exceed the frequency of Turner syndrome as maternal errors would also contribute to the occurrence of 47, XXX and 47, XXY in addition to paternal errors,. Yet, the combined frequency of these sex chromosome trisomies is less than 10% of the frequency of Turner syndrome. Therefore, the mechanism by which the paternal X is lost giving rise to a 45, XO conceptus must be due to a mechanism other than nondisjunction, such as anaphase lag.

The occurrence of trisomy X is approximately 1/2000 births and because of a broad range in phenotype, many of these individuals are likely undiagnosed (Thompson and Thompson, 1991). A study of 47, XXX females revealed that 26 of 28 cases arose due to nondisjunction of the X chromosome during maternal meiosis (May *et al.*, 1990) and similar results have been obtained by Hassold *et al.*, (1991a) and MacDonald *et al.*, (1994).

The only sex chromosome aneuploidy that is exclusively paternal in origin is also the most difficult to study clinically. A survey of forty-three 47, XYY males has shown

that although there may be an increased occurrence of minor congenital anomalies and a slight decrease in IQ scores, no distinct syndrome is evident in individuals with this karyotype (Robinson *et al.*, 1979). Parental ages were recorded and only 5 fathers were over the age of 35, leading to the conclusion that paternal age was not a factor in these cases.

iii. summary of parent-of-origin studies

The molecular studies suggest that the majority of aneuploidy cases are attributed to errors during maternal meiosis. As a result, the sample size of paternally derived cases is relatively small and it is difficult to gather conclusive evidence that paternal age plays a role in nondisjunction.

A possible exception to this situation is Klinefelter syndrome where nearly 50% of cases are paternally derived. Although over 200 cases have been reviewed in three studies, the results are contradictory: two of the three studies refute the presence of a paternal age effect (MacDonald *et al.*, 1994; Jacobs *et al.*, 1988) and one suggests that an age effect is present (Lorda-Sanchez *et al.*, 1992).

Virtually all of the clinical studies included patients who were ascertained by a variety of methods including newborn screening, clinical referral, prenatal diagnosis for advanced maternal age, spontaneous and therapeutic abortions as well as stillbirths, however a significant proportion of aneuploid conceptions are not available to investigators. Practically all monosomies and most trisomies do not survive to a gestational age which can be clinically recognized and as a result, only certain aneuploidies are amenable to large-scale clinical studies.

D. Gamete Studies

i. zona pelucida-free hamster egg technique

One way to circumvent the problems encountered in evaluating a paternal age effect with epidemiological and clinical studies is to examine sperm directly: problems with sample size, accuracy of records, assigning parental origin, selection bias and the confounding factor of maternal age are no longer an issue. The fertilization of zona pelucida-free hamster eggs by human sperm permits the karyotype of the sperm to be analyzed for abnormalities. Martin and Rademaker , (1987) examined the correlation between donor age and sperm chromosome abnormality frequencies using this technique (Martin and Rademaker, 1987). A minimum of 30 sperm karyotypes was analyzed from each of thirty men belonging to one of six age groups (20-24, 25-29, 30-34, 35-39, 40-44, 45+), with 5 men in each group. Results showed that there was no correlation between the frequency of hypohaploid complements and donor age, but there was a significant negative correlation between hyperhaploid complements and donor age. In addition, a significant positive correlation was found between donor age and the frequency of structural abnormalities. Although this technique allows for the analysis of several gametes from numerous individuals, it is time consuming, expensive and extremely labor intensive. Thus the overall sample size of sperm analyzed per male is limited.

ii. fluorescence in situ hybridization (FISH)

A relatively new technique of analyzing aneuploidy in human sperm involves the use of fluorescent labelled, chromosome-specific DNA probes. Although this approach does not detect structural rearrangements of chromosomes, there are a number of

advantages to using FISH analysis. Compared to the hamster procedure, FISH analysis is faster, simpler and less expensive and the disomy frequencies are calculated from tens of thousands of sperm as opposed to a few hundred sperm karyotypes. In addition, the disomy frequencies obtained from FISH have been shown to be comparable with the data obtained from the hamster technique (Holmes *et al.*, 1993; Robbins *et al.*, 1993; Wyrobek *et al.*, 1994) and as a result, several laboratories are now using FISH for analysis of selected sperm chromosome abnormalities (Williams *et al.*, 1993; Bischoff *et al.*, 1994; Miharu *et al.*, 1993; Robbins *et al.*, 1993; Wyrobek *et al.*, 1994).

Three previous studies from one laboratory have evaluated the association between age and disomy frequency using one-color FISH in human sperm. Guttenbach *et al.*, (1994b) determined that there was no age effect evident for chromosomes 3, 7, 10, 11, 17 and X in seven men ranging from 23 to 57 years of age. A further investigation of chromosome 18 (Guttenbach *et al.*, 1994a) and the Y chromosome (Guttenbach and Schmid, 1990) also revealed no evidence of an age effect for disomy frequency. Despite the information for several chromosomes, there are a number of drawbacks to these studies that may have affected the results. First, only 1000 to 2000 sperm were scored per chromosome per individual which is a small sample size when only 3 to 4 disomic sperm were found per 1000. Second, the use of one-color FISH does not distinguish between disomic sperm and diploid cells, therefore the interpretation of two signals as disomy results in inflated disomy frequencies. Through the simultaneous use of different colored probes (multi-color FISH), the observer can distinguish disomic sperm from diploid cells and collect data for more than one chromosome at a time.

Three studies have utilized multi-color FISH on human sperm to determine if there is evidence of an age effect for aneuploidy in males. An investigation of 10 normal men ranging in age from 21 to 52 years determined disomy frequencies for chromosomes 1, 12, X and Y (Martin *et al.*, 1995). A minimum of 10 000 sperm were counted per donor and the results revealed a significant positive correlation with age for YY disomy and disomy 1 only ($p=0.04$ and $p=0.01$ respectively). A study of the sex chromosomes and chromosome 8 in over 200 000 sperm from 14 healthy men in 2 age groups (mean ages 28.9 and 46.8 years) revealed an age effect for disomy X and Y only (Robbins *et al.*, 1995). Of particular interest are results from 3 men over 40 years of age from which 3 samples were collected over a time period of 4 months to 4.5 years. Analysis of disomy frequencies for each sample revealed that these numbers did not appreciably change for each of the three individuals over the various time periods. Griffin *et al.*, (1995) used probes for chromosomes X, Y and 18 to investigate the question of an age effect in approximately 400 000 sperm from 24 men ranging in age from 18 to 60 years. Data analysis was accomplished by both weighted linear regression and the grouping of the 24 men into two age categories (less than 36 years and 36 years and older) followed by comparison of disomy frequencies between the two groups. Evidence of a donor age effect was seen for XX, YY and XY disomy only, but XY disomy demonstrated a significant association with both linear regression and group comparison. Although the specifics of these three studies do differ, there is some suggestion that disomy frequency increases with age, particularly with respect to the sex chromosomes.

To date, no reported FISH studies have been specifically designed for detection of

a donor age effect on disomy frequency; the decisions regarding the statistical analyses were made following data collection. By contrast, in the study reported here donors were recruited from specific pre-designated age groups and the method of data analysis was established prior to data collection.

II. Materials and Methods

A. Donor Recruitment

Eighteen men were recruited from the general population for this study: three donors from each of the following six age groups, 20-24, 25-29, 30-34, 35-39, 40-44 and 45+ years of age. All donors gave informed consent and provided general information regarding their age, previous drug and radiation exposures, reproductive history and current health. Only those men who had no history of radiotherapy, chemotherapy or chronic illnesses were accepted into the study. Each donor provided the investigator with one sperm sample. This research was granted ethics approval by our institutional ethics committee.

B. Solutions, Buffers and Media

The constituents of buffers, solutions and media as well as commercial sources are available in the Appendices.

C. Cryopreservation of Sperm Samples

Specimens were collected in sterile containers and placed in an incubator to liquefy for 15 to 30 minutes at 37°C. A wet mount of each sample was prepared and the semen was analyzed for viscosity and contaminants, sperm motility, forward progression and morphology. A sperm count was obtained by combining 20 μL of semen to 380 μL of sperm count diluent and counting the diluted sperm using a hemocytometer slide. The volume of the sample was recorded and prepared for cryopreservation by dropwise addition of an equal volume of Ackerman's cryoprotectant to the semen in a mixing cone,

with continuous swirling. The mixture was drawn into 0.5 mL plastic freezing straws and the straws were sealed with polyvinylpyrollidone powder. Straws were placed in labelled plastic tubes and placed onto canes. Controlled freezing was achieved by filling a cryogenics tank with liquid nitrogen to a depth of approximately 5 cm. The canes were placed in a metal basket 75 cm above the liquid for 25 minutes and then lowered to a basket about 37 cm above the liquid for another 25 minutes. The canes were then immersed into liquid nitrogen and stored in a Dewar flask until needed. All specimens were processed and frozen in this manner; a previous investigation has shown that the freezing and storage procedure does not alter the chromosome constitution of sperm (Martin *et al.*, 1991).

D. Investigator Blinding

As all the data collection was to be done by the author, a system was developed to conceal the donor's identity and age while the data was being accumulated. Random numbers were assigned to each donor and recorded by a laboratory technician. Frozen straws were thawed as needed by laboratory members other than the author and were labelled with the assigned random number. Donor name and age were therefore unavailable to the author until after data collection was completed.

E. Sperm Preparation

Frozen semen was thawed as required and washed once in a 50% Percoll solution or 0.01M Tris-HCl-0.9% NaCl pH=8.0 followed by a second wash in 0.01 M Tris-HCl-0.9% NaCl, pH=8.0. Sperm were centrifuged at 600×g after each wash, and were

resuspended to a concentration of approximately 60×10^6 sperm per mL before refrigeration up to a maximum of 6 months. To prepare slides, between 0.5 μL and 2 μL of the washed sperm were smeared over a 1 cm^2 area on each slide, which was marked with a diamond-tipped scribe and allowed to air dry. The optimal distribution of sperm on the slide was such that only one layer of nonoverlapping sperm was visible under the phase contrast microscope, and this was assessed empirically for each donor. Sperm were decondensed by immersing the dried slides in 10 mM dithiothreitol (DTT)/0.1 M Tris-HCl, pH=8.0 for 5 to 30 minutes at room temperature, followed by immersion in 1 mM DTT /10 mM LIS/0.1M Tris-HCl, pH=8.0 for 30 minutes to 2.5 hours at room temperature. Sperm were optimally decondensed when the cells were 1.5-2 times their original size. The slides were then briefly rinsed in a solution of 2×SSC and allowed to air dry.

F. Selection of Probes

A series of three multicolor hybridizations were performed on the sperm of each donor. The probes were chosen on the basis of clinical significance in human conceptuses with regard to chromosomal aneuploidy and to allow for detection of duplications and deletions in the largest chromosome. The first hybridization was used to analyze aneuploidy for the sex chromosomes. A commercial probe for the Y chromosome labelled with SpectrumOrange™ was used in combination with an X chromosome fluorogreen™ probe and a chromosome 1 fluoroblue™ probe which allows the observer to distinguish a sperm which is disomic for the sex chromosomes from a diploid cell.. The second

hybridization utilized a commercial dual probe mixture containing a SpectrumGreen™ labelled probe for chromosome 13 and a SpectrumOrange™ labelled probe for chromosome 21. Finally, the third hybridization was used to analyze deletions and duplications in chromosome 1. This was accomplished by using a fluorored™ chromosome 1 centromeric probe in conjunction with a commercial chromosome 1 midisatellite probe which hybridizes near the telomere of the short arm of chromosome 1. In addition, a fluoroblue™ chromosome 12 probe was used as a control to distinguish between a sperm that is disomic for chromosome 1 from a diploid cell. A complete list of the probes and their sources is provided in Table 1.

Table 1: DNA probes and sources

Probe	Source
1 satellite III	Cooke and Hindley (1979)
1p midisatellite (1p36)	Oncor
X alpha satellite	Jabs *et al.*, (1989)
13q14/21q22.13-22.2	Vysis
12 alpha satellite	Baldini *et al.*, (1990)
Yq satellite III probe	Vysis

G. Fluorescence *in situ* Hybridization (FISH)

The procedures for these hybridizations were adapted from Martin and Ko, (1995) and altered somewhat according to the manufacturer's directions for the specific probes. Because the commercial probes have been developed for use on lymphocyte slides only, further adjustments had to be made to allow for optimal hybridization on sperm slides.

i. X and Y chromosomes

To denature the sperm DNA, the slide containing the washed and decondensed

sperm was immersed in a coplin jar containing 70% formamide/2×SSC (pH 7.5) at 68 to 73°C for 2 minutes. The slide was subsequently snap cooled in 70%, 85% and 95% ethanol baths at -20°C, and left to air dry. The slide was then placed on a slide warmer at 37°C. To prepare the probe, a mixture of 7 μL of MM 2.1 buffer, 1 μL of 500μg/mL salmon sperm DNA, 1 μL of fluoroblue™ chromosome 1 probe, 1 μL of fluorogreen™ X chromosome probe and 0.25 μL of SpectrumOrange™ Y chromosome probe was vortexed and centrifuged for 5 seconds and placed in a water bath at 69 to 74°C for 5 minutes. The denatured probe mixture was then snap cooled in an ice water bath for a minimum of 5 minutes, vortexed and centrifuged once more, placed onto the slide and allowed to warm for a few seconds. A coverslip was carefully applied to the slide to avoid any air bubbles, sealed with rubber cement and the slide was incubated overnight at 37°C in a humidified chamber. To loosen the coverslip, slide was rinsed briefly in the first post-hybridization bath containing 50% formamide/2×SSC at 43 to 47°C and then the rubber cement and the coverslip were removed. The slide was then immersed in a series of three 50% formamide/2×SSC (pH 7.0) baths for 2 minutes each. DAPI counterstain was applied (5ng/mL, 10s) and rinsed off in PN buffer. Antifade was applied followed by a coverslip and the slide was stored in the dark at room temperature until viewing.

ii. chromosomes 13 and 21

Each slide was denatured in a coplin jar containing 70% formamide/2×SSC (pH 7.5) at 70 to 74°C for 5 to 8 minutes. The slide was immersed in a 70%, 85% and 95% ethanol series at -20°C for 1 minute each and placed on a slide warmer at 45°C. The

probe was supplied premixed and predenatured so 10 μL of the thawed probe mix was applied directly to the slide, coverslipped and sealed with rubber cement. Hybridization occurred overnight in a dark humidified container at 37°C. The coverslip was removed and the slide was immersed into a post hybridization wash of 2×SSC (pH 7.0) at 69 to 72°C for 2 minutes followed by a brief rinse in 2×SSC/0.1% NP-40 (pH 7.0-7.5) at room temperature and allowed to air dry in darkness. The slide was coverslipped following the application of DAPI II counterstain (Vysis) or 10 ng/mL DAPI for 12 s and antifade. Slides were stored in the dark at room temperature until viewing.

iii. chromosome 1

Three different forms of the midisatellite probe were used, biotin and digoxigenin labelled as well as fluorescein labelled. The slide was immersed in a coplin jar containing 70% formamide/2×SSC at 69 to 72° for 5 minutes followed by snap cooling in a 70%, 85%, 95% ethanol series for 2 minutes each. The probe was prepared by combining 7 μL of MM 2.1 buffer, 1 μL of salmon sperm DNA, 1 μL of chromosome 1p probe, 1 μL of fluorored™ chromosome 1 cen probe and 1 μL of fluoroblue™ chromosome 12 probe and denatured at a temperature of 70 to 73°C for 5 minutes followed by snap-cooling in an ice water bath for a minimum of 5 minutes. Probe was applied to the etched area on the slide, coverslipped, sealed with rubber cement and hybridized overnight. The post hybridization protocol was dependent upon which type of 1p probe was used. For the direct-labelled fluorescein probe, the post hybridization wash was 2×SSC (pH 7.0) at 71°C for 5 minutes followed by DAPI counterstaining (5ng/mL, 10 s) and application of

antifade. The biotin and digoxigenin-labelled probes required 3 washes of 2 minutes each in 50% formamide/2×SSC (pH 7.0) post hybridization solution at 44 to 47°C. As these 1p probes are indirectly labelled, a detection step was required following the three post hybridization washes. For the digoxigenin labelled probe, 20 μL of PNM was applied to the hybridization area of the slide and left to incubate in the dark at room temperature for 3 to 5 minutes. The slide was rinsed in PN buffer and 15 μL of antidigoxigenin-FITC was applied to the slide, covered with Parafilm and incubated at 37 °C for 30 minutes. The slide was then rinsed in PN buffer, counterstained with 5 ng/mL DAPI for 12 s, and rinsed followed by the application of antifade and a coverslip. If the green signal was faint, an amplification step was performed. The coverslip was removed by a PN rinse and then 20 μL of Rabbit Anti-Sheep Antibody (Oncor) was applied to the slide, covered with Parafilm and incubated at 37°C for 15 minutes. The slide was rinsed in PN buffer and 20 μL of FITC/Anti-Rabbit antibody (Oncor) was applied to the slide, covered with Parafilm and incubated at 37°C for a further 15 minutes. Counterstain and antifade were reapplied as described previously. For the biotin labelled probe, 15 μL of avidin-FITC was applied to the slide and incubated at 37°C for 15-20 minutes following the post hybridization washes. The signal was amplified by applying 15 μL of anti-avidin to the slide and incubating at 37°C for 15 minutes, followed by 15 μL of avidin-FITC and incubating at 37°C for 15 minutes. Propidium iodide counterstain (10 ng/mL, 45 to 60s) and antifade were applied to the slide prior to viewing.

H. Data Collection

All slides were viewed under a Zeiss Axiophot epifluorescence microscope equipped with FITC and AMCA (to view DAPI-stained sperm) single bandpass filters, an FITC/rhodamine dual bandpass filter, a FITC/DAPI/rhodamine triple bandpass filter and a 100 watt power source and bulb. This combination of filters allows for the viewing of red, green and blue signals as well as detection of the sperm heads. Only those slides with a hybridization efficiency of at least 98% were scored. Cells were scored according to the number and color of the signals present and strict scoring criteria were followed to minimize the influence of observer variability on the calculated frequencies of chromosome abnormalities and aneuploidy (Martin *et al.*, 1995). To avoid scoring sperm heads that have been overdecondensed, no sperm more than twice the size of a nondecondensed sperm were scored. In addition, only sperm heads that were intact, nonoverlapping with other cells and had a clear border were scored. Duplicate signals were scored as such only if they were clearly within the sperm cell, of similar size, shape and intensity and a minimum of one signal diameter (domain) apart in the case of all probes except for the Yq chromosome probe. Because of the extremely large size of the signal produced by the Yq probe, it was agreed that the one-half domain separation between duplicate Yq signals would be interpreted as two signals, as a significant reduction in disomy frequency for the Y chromosome was observed when a full domain between like signals was used as a disomy criterion (Martin, 1996 unpublished data). A minimum of 10,000 sperm was scored per hybridization, thus a total of 30,000 sperm was counted per donor and a grand

total of over 540,000 sperm was counted for this study.

I. Data Analysis

Disomy and nullisomy frequencies were calculated for each chromosome, in addition to duplication and deletion frequencies for chromosome 1 and diploid frequencies for each donor. The interpretation of the number and color of the signals seen per cell is provided in Table 2. If a sperm contained no signals whatsoever, hybridization for that particular cell was considered to be unsuccessful and the sperm was not included in the data. Any sperm that were monosomic for the chromosomes being investigated were classified as normal. Sperm that had multiple numerical abnormalities or were triploid or tetraploid were scored as "other" and not included in the final totals.

Scatter plots of disomy frequencies for each chromosome and duplications and deletions for chromosome 1 were created and a Pearson correlation coefficient with its corresponding p-value was calculated. Inter-donor heterogeneity within the age groups was calculated for each abnormality using a χ^2 test with 2 degrees of freedom.

Table 2: Interpretation of Fluorescent Signals

Hybridization	Signals Observed within One Cell	Interpretation
1/X/Yq	1B 1R	monosomy Y
	1B 1G	monosomy X
	1B 1G 1R	XY disomy
	1B 2G	XX disomy
	1B 2R	YY disomy
	2B 2R or 2B 2G or 2B 1R 1G	diploid cell
	1B 0R 0G	nullisomy
13/21	1R 1G	monosomy 13/21
	1R 2G	disomy 13
	2R 1G	disomy 21
	0R 1G	nullisomy 21
	1R 0G	nullisomy 13
	2R 2G	diploid cell
1/1p/12	1R 1G 1B	monosomy 1
	2R 2G 1B	disomy 1
	0R 0G 1B	nullisomy 1
	0R 1G 1B	deletion 1 cen
	1R 0G 1B	deletion 1p
	2R 1G 1B	duplication 1 cen
	1R 2G 1B	duplication 1p
	2R 2G 2B	diploid cell

R=red, G=green, B=blue

III. Results

A. Donors

A total of 20 donors were recruited for this investigation and 2 donors were excluded, as they did not fit the study criteria. One donor had a chronic illness (epilepsy) and was on prescription medication (Dilantin) and the other had a sperm concentration of less than 20 million/mL, which is the lower limit of the normal concentration range for fertility as defined by the World Health Organization (1992). For the remaining 18 donors, all parameters such as morphology, motility, forward progression, contaminants and viscosity were within the normal range. Sperm concentrations ranged from 21×10^6/mL to 325×10^6/mL. Donor ages ranged from 23 to 58 years of age with a mean age of 35.6 years. Seven out of the 18 donors did not have children and those individuals were aged, 24, 25, 28, 30, 35 and 41 (2), therefore no age group was exclusively composed of unproven donors. Four donors aged 24, 25, 28, 30 and 37 were smokers and one individual, age 34, chewed tobacco. No individuals reported any exposures to chemotherapy or radiotherapy and all were in good health.

B. X/Y Hybridizations

A total of 181 562 sperm was scored for X and Y unisomy, XY, XX and YY disomy with an average of 10 087 sperm scored per donor. The most common type of sperm sex chromosome aneuploidy was XY disomy (mean frequency 0.30%, standard deviation 0.26%). The mean frequencies and standard deviations for disomy X were 0.05% and 0.03% respectively, and 0.06% and 0.03% respectively for disomy Y; thus

meiosis I errors were more common than meiosis II errors. The total mean frequency of sex chromosome disomy was 0.42%. Sex ratios for each donor were calculated and a 1×2 χ^2 test was carried out to determine if the ratio differed significantly from the expected 1:1 distribution of X-bearing sperm to Y-bearing sperm. Only one individual demonstrated a significant excess of X bearing sperm (donor #0236, p=0.025). Frequencies of unisomy and disomy plus sex ratios for individual donors are presented in table 3. To determine if disomy for the sex chromosomes or sex ratio was associated with donor age, scatter plots were produced (figures 1 to 4) and Pearson correlation coefficients were calculated. There was no significant correlation between donor age and XY disomy ($r=-0.190$, $p>0.20$), disomy X ($r=-0.107$, $p>0.20$) or sex ratio ($r=0.297$, $p>0.20$), however a statistically significant positive correlation was detected between donor age and disomy Y ($r=0.584$, $0.01<p<0.02$).

C. 13/21 Hybridizations

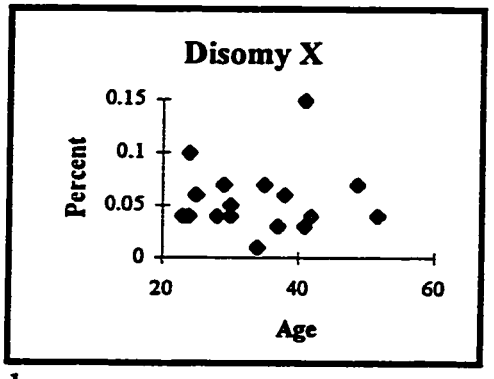
A total of 181 595 sperm was scored for unisomy or disomy of chromosomes 13 and 21 with an average of 10 089 sperm scored per donor. Unisomy and disomy frequencies for individual donors are given in table 4. In all cases but one (donor #0492), the disomy frequency of chromosome 21 was greater than the disomy frequency for chromosome 13. The mean frequency and standard deviation of disomy for chromosome 21 was 0.37 and 0.39 respectively, and 0.13 and 0.11 respectively, for chromosome 13. Scatter plots of disomy frequency versus age are shown in figures 5 and 6. Calculation of the Pearson correlation coefficients revealed no significant association between disomy

frequency and age for either chromosome 13 ($r=-0.070$, $p>0,20$) or chromosome 21 ($r=0.036$, $p>0.20$).

Table 3. Sex Chromosome Data

DONOR		Total	Unisomy X		Unisomy Y		Sex	Disomy X		Disomy Y		XY Disomy	
ID#	AGE	Scored	#	%	#	%	Ratio	#	%	#	%	#	%
2237	23	10069	4990	49.56	5006	49.72	1.00	4	0.04	4	0.04	32	0.32
1396	24	10090	5098	50.53	4944	49.00	0.97	4	0.04	5	0.05	22	0.22
7547	24	10090	5061	50.16	4982	49.38	0.98	10	0.10	4	0.04	27	0.27
2318	25	10090	5050	50.05	4948	49.04	0.98	6	0.06	7	0.07	62	0.61
2272	28	10089	5009	49.65	5037	49.93	1.01	4	0.04	4	0.04	16	0.16
8449	29	10089	4970	49.26	4867	48.24	0.98	7	0.07	8	0.08	124	1.23
0457	30	10090	5013	49.68	5038	49.93	1.00	4	0.04	2	0.02	15	0.15
0188	30	10049	4939	49.15	5077	50.52	1.03	5	0.05	2	0.02	20	0.20
8200	34	10090	5091	50.46	4966	49.22	0.98	1	0.01	3	0.03	12	0.12
0492	35	10090	5041	49.96	4933	48.89	0.98	7	0.07	11	0.11	31	0.31
1518	37	10090	4981	49.37	5062	50.17	1.02	3	0.03	9	0.09	24	0.24
3460	38	10090	5030	49.85	4987	49.43	0.99	6	0.06	6	0.06	22	0.22
0236	41	10096	5108	50.59	4884	48.38	0.96	15	0.15	9	0.09	13	0.13
8884	41	10090	5100	50.55	4940	48.96	0.97	3	0.03	8	0.08	20	0.20
7101	42	10090	5006	49.61	5048	50.03	1.01	4	0.04	3	0.03	11	0.11
0689	49	10090	4940	48.96	5090	50.45	1.03	7	0.07	9	0.09	29	0.29
5089	52	10090	4963	49.19	5059	50.14	1.02	4	0.04	12	0.12	36	0.36
3767	58	10090	5007	49.62	5025	49.80	1.00	2	0.02	9	0.09	27	0.27
mean	35.6	10087	5022	49.79	4994	49.51	0.99	5.3	0.05	6.4	0.06	30.2	0.30

Figures 1-6: Scatter Plots of Disomy Frequencies and Sex Ratio vs. Donor Age



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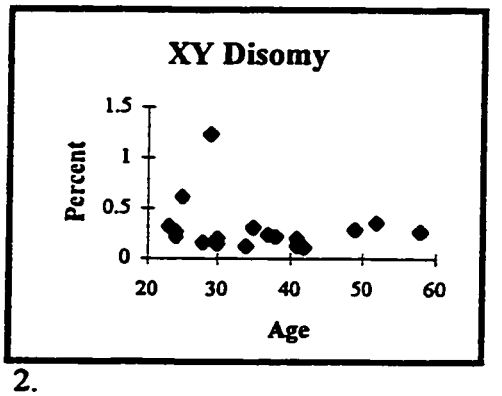

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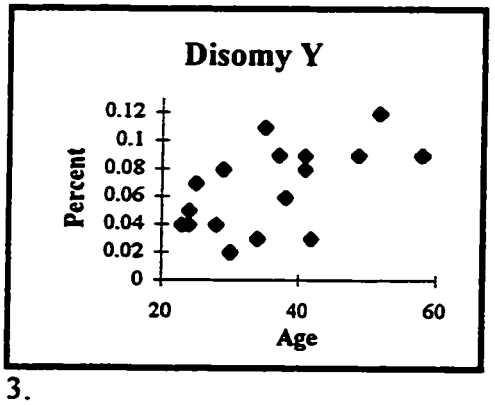

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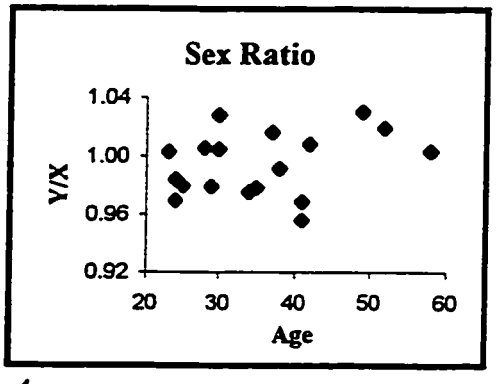

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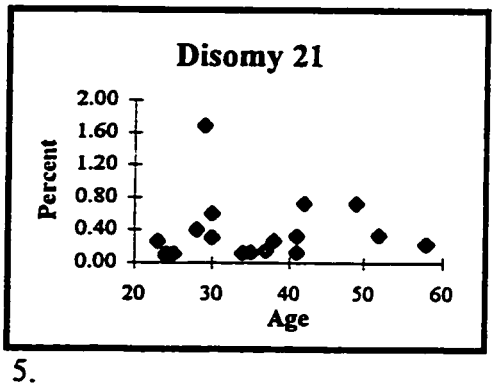

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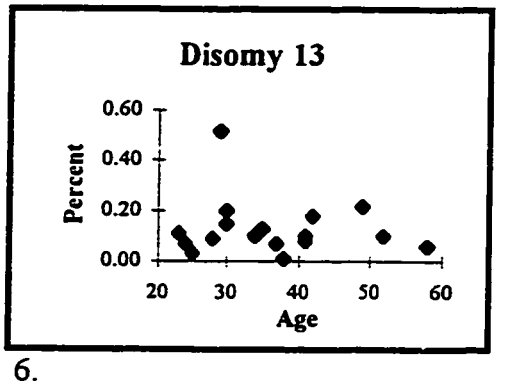


6.

Table 4: Chromosomes 13 and 21 Data

DONOR		Total	Unisomy 21		Unisomy 13		Disomy 21		Disomy 13	
ID#	AGE	Scored	#	%	#	%	#	%	#	%
2237	23	10090	10050	99.60	10050	99.60	26	0.26	11	0.11
1396	24	10091	10070	99.79	10077	99.86	11	0.11	7	0.07
7547	24	10090	10071	99.81	10076	99.86	8	0.08	7	0.07
2318	25	10090	10033	99.44	10052	99.62	11	0.11	3	0.03
2272	28	10071	10017	99.46	10036	99.65	41	0.41	9	0.09
8449	29	10090	9813	97.25	9974	98.85	170	1.68	52	0.52
0188	30	10090	10024	99.35	10063	99.73	61	0.60	20	0.20
0457	30	10090	10007	99.18	10040	99.50	31	0.31	15	0.15
8200	34	10090	10064	99.74	10074	99.84	12	0.12	10	0.10
0492	35	10090	10068	99.78	10061	99.71	13	0.13	13	0.13
1518	37	10092	10065	99.73	10039	99.47	15	0.15	7	0.07
3460	38	10090	10056	99.66	10076	99.86	27	0.27	1	0.01
0236	41	10090	10030	99.41	10072	99.82	13	0.13	8	0.08
8884	41	10085	10000	99.16	9978	98.94	33	0.33	10	0.10
7101	42	10090	9964	98.75	10042	99.52	73	0.72	18	0.18
0689	49	10086	9943	98.58	10026	99.41	73	0.72	22	0.22
5089	52	10090	10044	99.54	10059	99.69	34	0.34	10	0.10
3767	58	10090	10056	99.66	10077	99.87	23	0.23	6	0.06
mean	35.6	10089	10021	99.33	10048	99.60	37.5	0.37	13	0.13

D. 1p/1cen/12 hybridization

A total of 181 602 sperm was scored with an average of 10 089 sperm scored per donor. Sperm were classified as unisomic, disomic, deleted for the centromere or region of the p-arm of chromosome 1 or duplicated for the centromere or region of the p-arm of chromosome 1. These frequencies are given by individual donor in table 6 in addition to the type of chromosome 1 midisatellite probe used for that particular hybridization. Means and standard deviations for each abnormality are given in table 5 below.

Table 5: Mean, Standard Deviation and Pearson Correlation Coefficients for Chromosome 1 Abnormalities

	disomy 1	del'n 1p	del 1cen	dup 1p	dup 1cen
mean	0.09	0.22	0.04	0.21	0.08
st deviation	0.05	0.14	0.03	0.37	0.06
corr coeff	0.195	-0.143	0.504	-0.093	0.083

All correlation coefficients have a p-value >0.20 with the exception of deletions of the centromere region of chromosome 1 ($0.05>p>0.02$).

Theoretically, the type of label on the chromosome 1p midisatellite probe should have no effect on abnormality frequency, however to test this hypothesis, the Kruskal-Wallis test was used to determine if there was a significant difference between the 3 different probe types. Only abnormalities that were exclusively dependent on the presence or absence of the 1p signal were tested i.e. 1p duplications and 1p deletions. A significant p-value ($p=0.027$) was obtained when 1p duplications were tested. To further determine the origin of this difference, pairwise comparisons were performed using the Mann-Whitney test with the Bonferroni correction ($p<0.017$). The comparison between 1p duplication frequencies from biotin-labelled and digoxigenin-labelled probes produced

Table 6: Chromosome 1 Data

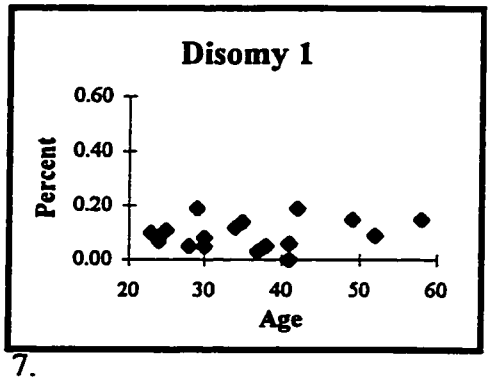
				Duplications				Deletions				Numerical			
DONOR		Total	Probe	1p		1 cen		1p		1 cen		Unisomy		Disomy	
ID#	Age	Scored	Type	#	%	#	%	#	%	#	%	#	%	#	%
2237	23	10090	dir	14	0.14	7	0.07	39	0.39	4	0.04	10016	99.27	10	0.10
1396	24	10090	dig	3	0.03	11	0.11	16	0.16	5	0.05	10039	99.49	8	0.08
7547	24	10090	dig	7	0.07	4	0.04	26	0.26	2	0.02	10044	99.54	7	0.07
2318	25	10090	dir/dig	8	0.08	12	0.12	22	0.22	1	0.01	10036	99.46	11	0.11
2272	28	10105	dir	17	0.17	1	0.01	25	0.25	0	0.00	10057	99.52	5	0.05
8449	29	10041	bio	168	1.67	17	0.17	56	0.56	0	0.00	9781	97.41	19	0.19
0188	30	10098	bio	14	0.14	4	0.04	2	0.02	1	0.01	10072	99.74	5	0.05
0457	30	10090	bio	17	0.17	4	0.04	23	0.23	5	0.05	10033	99.44	8	0.08
8200	34	10090	dir	3	0.03	6	0.06	12	0.12	2	0.02	10054	99.64	12	0.12
0492	35	10090	dig	10	0.10	6	0.06	11	0.11	0	0.00	10035	99.45	14	0.14
1518	37	10090	dig	24	0.24	6	0.06	10	0.10	7	0.07	10032	99.43	3	0.03
3460	38	10090	dig	6	0.06	27	0.27	6	0.06	4	0.04	10034	99.44	5	0.05
0236	41	10089	dig	2	0.02	4	0.04	37	0.37	8	0.08	10020	99.32	6	0.06
8884	41	10090	dig	8	0.08	5	0.05	20	0.20	1	0.01	10054	99.64	0	0.00
7101	42	10090	bio	28	0.28	9	0.09	24	0.24	7	0.07	10001	99.12	19	0.19
0689	49	10091	bio	31	0.31	12	0.12	27	0.27	5	0.05	10001	99.11	15	0.15
5089	52	10098	dig	9	0.09	12	0.12	35	0.35	6	0.06	10021	99.24	9	0.09
3767	58	10090	dig	15	0.15	5	0.05	7	0.07	6	0.06	10037	99.47	15	0.15
mean	35.6	10089		21	0.21	8.4	0.08	22.1	0.22	3.6	0.04	10020.4	99.32	9.5	0.0942

dig=digoxigenin labelled

bio=biotin labelled

dir=fluoroscein labelled

Figures 7-11: Scatter Plots of Chromosome 1 Abnormalities vs. Donor Age



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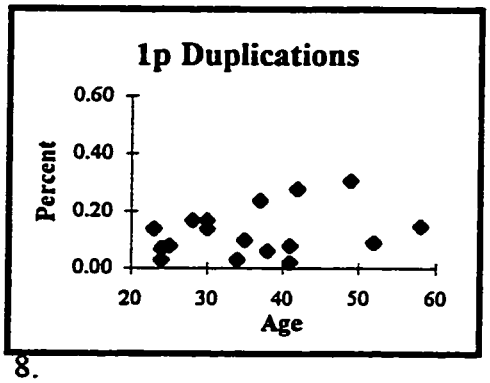

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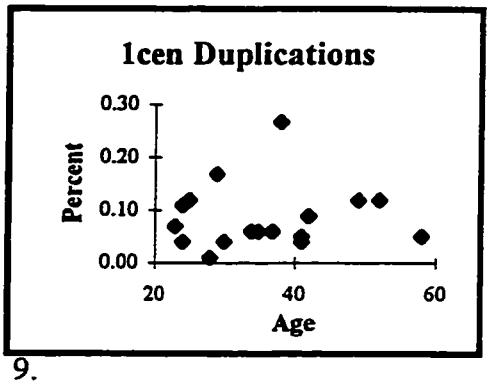

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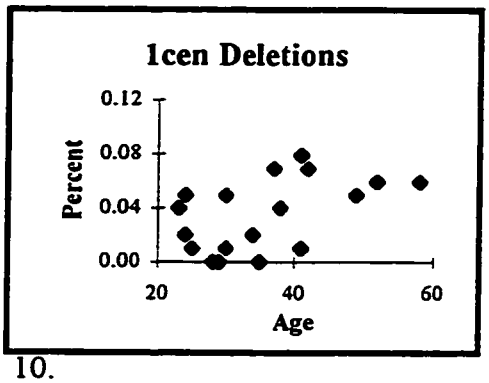

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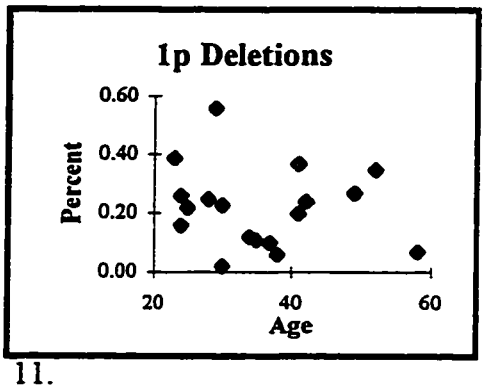


11.

a significant result (p=0.0093), while the comparisons between the direct-labelled probe and the indirect labelled probes yielded p-values of 0.579 (digoxigenin) and 0.101 (biotin). To determine if abnormality frequencies varied within each different probe type, a χ^2 test across donors within each probe group was performed and there was evidence of significant donor heterogeneity within probe types for both 1p deletions and duplications

E. Diploids

Diploid sperm were scored for each of the three hybridizations for each donor and the calculated frequencies for each individual as well as a p-value for comparison of the three frequencies are given in table 8. Mean diploid frequencies, standard deviations and Pearson correlation coefficients are given in Table 7 and no association between diploid frequency and donor age was detected in either of the three data sets (see scatter plots figures 12-14).

Table7: Mean, Standard Deviation and Pearson Correlation Coefficients of Diploid Data Sets

	X/Y	13/21	1p/1cen/12
mean	0.32	0.65	0.23
st. deviation	0.33	1.14	0.19
correl coeff	-0.188	-0.136	-0.236

To detect the presence of variation in diploid frequencies within each donor between the three data sets, a 3×2 χ^2 test was conducted on each donor with a Bonferroni correction for multiple comparisons ($p<0.0028$) (see Table 8). Results indicate that of 18 donors, 7 did not demonstrate significant heterogeneity for the three diploid frequencies.

F. Nullisomy

Sperm in which signals were noted to be absent were scored as nullisomic for the missing probe signal, e.g. 13/21 hybridization with a sperm containing a green signal (chromosome 13) only is scored as nullisomic for chromosome 21. Nullisomy frequencies for each hybridization are given in Table 9. From the nullisomy frequency, the hybridization efficiency can be calculated as 100 minus %nullisomy. In general, the laboratory standard is to maintain hybridization efficiencies between 98 and 100% (nullisomy frequencies between 0 and 2%) and all frequencies are well within this range. Average nullisomy frequency and standard deviations for the sex chromosomes are 0.29% and 0.27% respectively, 0.03% and 0.04% for chromosome 1, 0.27% and 0.24% for chromosome 13 and 0.30% and 0.28% for chromosome 21. No association between nullisomy frequency and donor age was detected as indicated by the correlation coefficients (X/Y $r=-0.070$, $p>0.20$; 1 $r=0.283$, $p>0.20$; 13 $r=0.018$, $p>0.20$; 21 $r=0.052$, $p>0.20$) and the scatter plots (figures 17-20). Sperm that were completely devoid of signals were not scored as this is assumed to reflect technical failure of hybridization as opposed to multiple missing chromosomes.

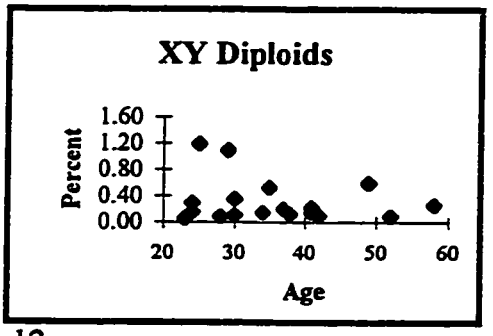
G. Donor Heterogeneity

The presence of donor heterogeneity within each of the 6 age groups for each abnormality was detected by a 3×2 χ^2 test. The calculated p-values for each type of chromosome abnormality are given in Tables 10-13. Those age groups which demonstrated significant donor heterogeneity ($p<0.05$) are indicated by the shaded areas.

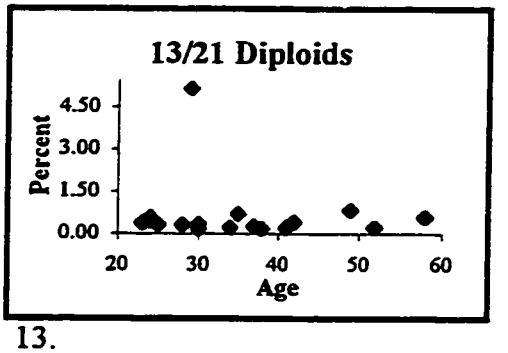
Table 8: Diploid Frequencies for Three Hybridizations on 18 Donors

DONOR		XY			13/21			1			Chi square
ID#	AGE	TOTAL	#	%	TOTAL	#	%	TOTAL	#	%	p-value
2237	23	10075	6	0.06	10130	40	0.39	10116	26	0.26	5.51E-06
1396	24	10106	16	0.16	10137	46	0.45	10103	13	0.13	1.72E-06
7547	24	10120	30	0.30	10151	61	0.60	10122	32	0.32	6.70E-04
2318	25	10212	122	1.19	10122	32	0.32	10127	37	0.37	5.09E-18
2272	28	10098	9	0.09	10104	33	0.33	10112	7	0.07	2.65E-06
8449	29	10200	111	1.09	10638	548	5.15	10125	84	0.83	8.19E-115
0188	30	10060	11	0.11	10109	19	0.19	10107	9	0.09	0.12
0457	30	10126	36	0.36	10125	35	0.35	10094	4	0.04	1.81E-06
8200	34	10105	15	0.15	10112	22	0.22	10129	39	0.39	2.49E-03
0492	35	10143	53	0.52	10162	72	0.71	10137	47	0.46	0.05
1518	37	10111	21	0.21	10120	28	0.28	10105	15	0.15	0.14
3460	38	10103	13	0.13	10108	18	0.18	10097	7	0.07	0.09
0236	41	10111	15	0.15	10112	22	0.22	10104	15	0.15	0.39
8884	41	10113	23	0.23	10105	20	0.20	10103	13	0.13	0.24
7101	42	10100	10	0.10	10132	42	0.41	10103	13	0.13	5.74E-07
0689	49	10150	60	0.59	10171	85	0.84	10117	26	0.26	2.21E-07
5089	52	10099	9	0.09	10112	22	0.22	10114	16	0.16	0.07
3767	58	10117	27	0.27	10149	59	0.58	10104	14	0.14	1.10E-07
mean	35.6	10119.4	32.6	0.3	10155.5	66.9	0.65	10112.2	23.2	0.23	

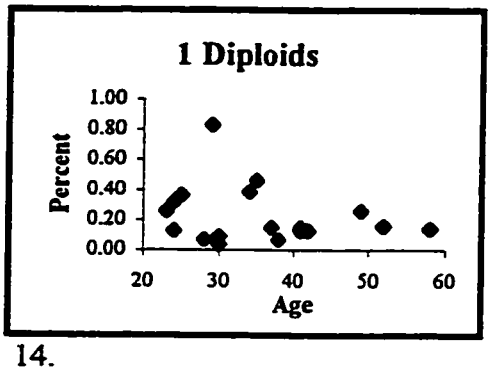
Figures 12-14: Scatterplots of Diploid Frequencies vs. Donor Age



12.

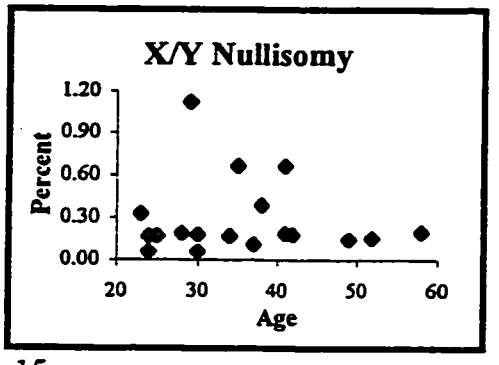


13.

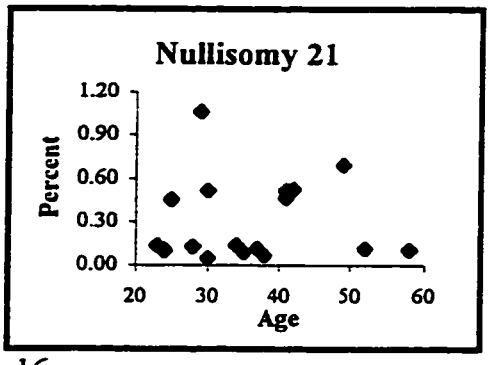


14.

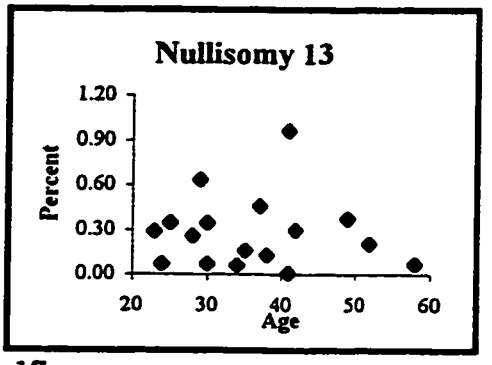
Figures 15-18: Nullisomy Frequencies vs. Donor Age



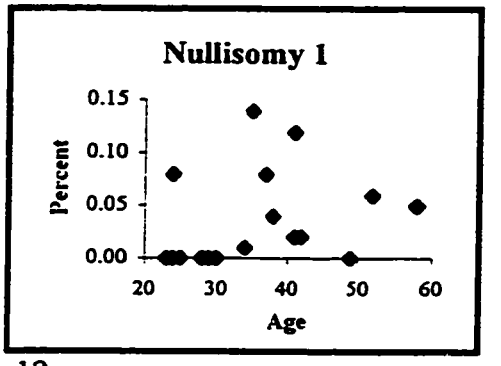
15.



16.



17.



18.

Table 9: Nullisomy Data

		XY hybridization			1/12 hybridization			13/21 hybridization				
DONOR		Total	XY		Total	1		Total	13		21	
ID#	AGE	exc dip	#	%	exc dip	#	%	exc dip	#	%	#	%
2237	23	10069	33	0.33	10090	0	0.00	10090	29	0.29	14	0.14
1396	24	10090	17	0.17	10090	8	0.08	10091	7	0.07	10	0.10
7547	24	10090	6	0.06	10090	0	0.00	10090	7	0.07	11	0.11
2318	25	10090	17	0.17	10090	0	0.00	10090	35	0.35	46	0.46
2272	28	10089	19	0.19	10105	0	0.00	10071	26	0.26	13	0.13
8449	29	10089	113	1.12	10041	0	0.00	10090	64	0.63	107	1.06
0188	30	10049	6	0.06	10098	0	0.00	10090	7	0.07	5	0.05
0457	30	10090	18	0.18	10090	0	0.00	10090	35	0.35	52	0.52
8200	34	10090	17	0.17	10090	1	0.01	10090	6	0.06	14	0.14
0492	35	10090	67	0.66	10090	14	0.14	10090	16	0.16	9	0.09
1518	37	10090	11	0.11	10090	8	0.08	10092	46	0.46	12	0.12
3460	38	10090	39	0.39	10090	4	0.04	10090	13	0.13	7	0.07
0236	41	10090	67	0.66	10089	12	0.12	10090	1	0.01	47	0.47
8884	41	10090	19	0.19	10090	2	0.02	10085	97	0.96	52	0.52
7101	42	10090	18	0.18	10090	2	0.02	10090	30	0.30	53	0.53
0689	49	10090	15	0.15	10091	0	0.00	10086	38	0.38	70	0.69
5089	52	10090	16	0.16	10098	6	0.06	10090	21	0.21	12	0.12
3767	58	10090	20	0.20	10090	5	0.05	10090	7	0.07	11	0.11
mean	35.6	10086	28.8	0.29	10089	3.4	0.03	10089	26.9	0.3	30.3	0.30

Table 10: P-values For 3×2 χ^2 Test of Disomy Frequencies within Age Groups

Age Group	XY	XX	YY	13	21	1
I (20-24)	0.39	0.14	0.93	0.53	2.0e-3	0.76
II (25-29)	8.4e-20	0.66	0.50	2.8e-15	7.1e-43	0.01
III (30-34)	0.35	0.27	0.87	0.19	2.1e-8	0.23
IV (35-39)	0.42	0.44	0.48	5.0e-3	0.04	9.0e-3
V (40-44)	0.22	2.0e-3	0.21	0.10	5.5e-11	1.2e-5
VI (45+)	0.10	0.23	0.74	4.0e-3	1.1e-7	0.40

Table 11: P-values For 3×2 χ^2 Test of Duplication and Deletion Frequencies for Chromosome 1 Within Age Groups

Age Group	Duplications		Deletions	
	1p	1cen	1p	1cen
I (20-24)	0.02	0.19	7.0e-3	0.53
II (25-29)	5.6e-56	1.1e-3	0.83	0.37
III (30-34)	8.0e-3	0.75	1.3e-4	0.20
IV (35-39)	1.0e-3	1.2e-5	0.46	0.03
V (40-44)	4.3e-7	0.31	0.05	0.07
VI (45+)	8.0e-4	0.18	1.2e-4	0.94

Table 12: P-values For 3×2 χ^2 Test of Diploid Frequencies Within Age Groups

Age Group	X/Y	13/21	1
I (20-24)	2.4e-4	0.09	0.02
II (25-29)	1.6e-21	4.0e-182	4.2e-16
III (30-34)	1.7e-4	0.06	1.1e-9
IV (35-39)	2.1e-7	8.6e-10	3.7e-9
V (40-44)	0.07	5.0e-3	0.91
VI (45+)	9.0e-10	1.5e-8	0.11

Table 13: P-values For 3×2 χ^2 for Nullisomy Frequencies Within Age Groups

Age Group	X/Y	1	13	21
I (20-24)	4.9e-5	3.3e-4	1.3e-5	0.70
II (25-29)	3.6e-27	1.00	1.2e-4	1.2e-18
III (30-34)	0.04	0.37	4.3e-8	3.6e-12
IV (35-39)	1.7e-9	0.05	1.6e-6	0.51
V (40-44)	2.8e-10	1.9e-3	3.1e-20	0.81
VI (45+)	0.66	0.06	1.7e-5	2.2e-16

Figure 19: Photograph of sperm disomic for chromosome 21. R=chromosome 21, G=chromosome 13

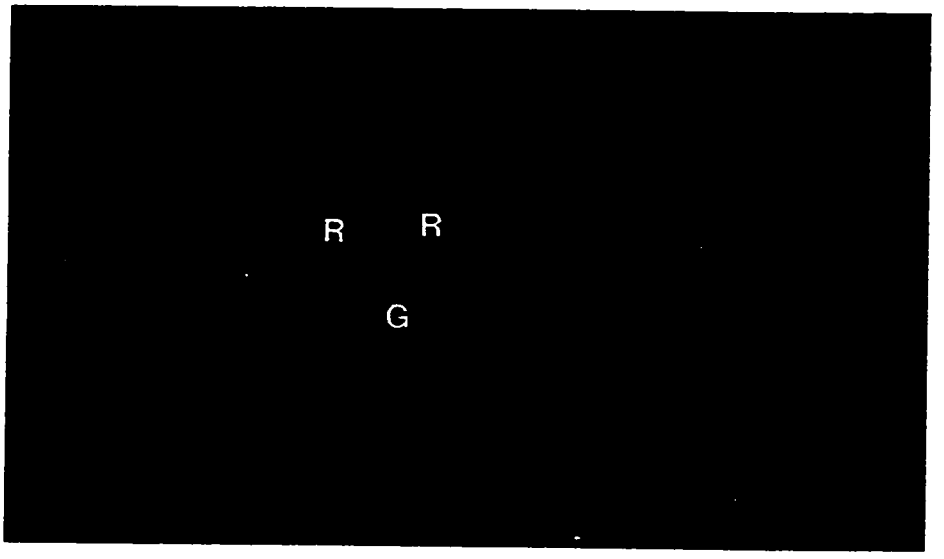
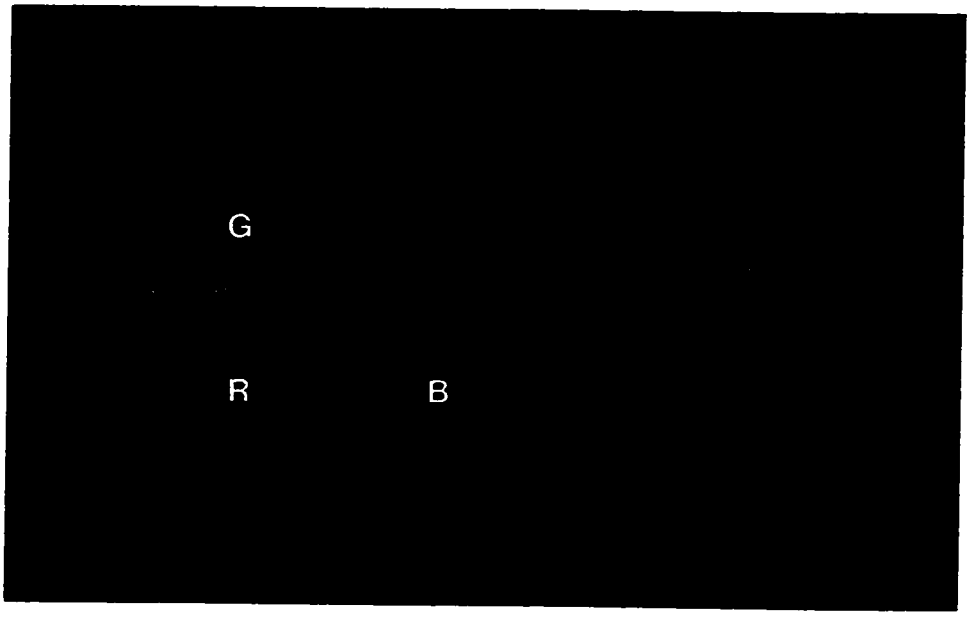


Figure 20: Photograph of a sperm that is heterodisomic for the sex chromosomes.
R=Y chromosome, G=X chromosome, B=chromosome 1.



IV. Discussion

The purpose of this investigation was to determine if aneuploidy frequencies in human sperm are influenced by donor age. Three other studies have examined donor age and disomy frequencies utilizing multicolor FISH (Robbins *et al.*, 1995; Griffin *et al.*, 1995, Martin *et al.*, 1995), however, this is the first study specifically designed to examine the relationship between donor age and the frequency of aneuploidy in human sperm as assessed by multicolor FISH analysis. The chromosomes that have been investigated in the three previous studies include the X and Y chromosomes and chromosomes 1, 8, 12, and 18. An age effect was detected for the sex chromosomes in all three investigations, however no such relationship was demonstrated by the autosomes with the exception of chromosome 1 (Martin *et al.*, 1995). This study is the first to report on the relationship between age and disomy frequencies for chromosomes 13 and 21, as well as duplications and deletions of chromosome 1. Our results indicate that disomy frequencies for the two acrocentric chromosomes and chromosome 1 are not influenced by donor age, however a significant association between donor age and the occurrence of acentric fragments of chromosome 1 was detected as well as a significant association with disomy frequency for the Y chromosome.

A. Sex Chromosomes

All investigations performed to date have shown some evidence of an effect of paternal age on the frequency of disomy of the sex chromosomes, but the results do vary from study to study. This particular investigation detected a paternal age effect for disomy Y only, which is the same result obtained by Martin *et al.*, (1995) on 10 men aged

21 to 52 years. The study by Robbins *et al.*, (1995) detected a paternal age effect for both meiosis II errors, namely disomy X and Y, by comparing the average disomy frequencies between two groups of men of different mean ages (Group A mean age was 46.8 years and Group B mean age was 28.9 years). The study by Griffin *et al.*, (1995) indicated a paternal age effect for disomy X and Y as well as XY disomy, suggesting that both meiosis I and meiosis II are affected by an age dependent mechanism of nondisjunction. That investigation contained a larger sample size for number of donors and number of sperm scored per individual (mean 16,000 per donor), in addition to a larger proportion of donors over the age of 40 (9/24 donors) when compared with the other three (Martin *et al.*, 3/10 donors); (present study, 6/18 donors); (Robbins *et al.*, 4/14 donors). As a result, a larger proportion of older donors may have been instrumental in detecting an age effect, particularly if such an effect influences disomy frequencies only in men in their 50's and 60's. In addition, two different types of statistical analyses were utilized to detect an age effect, a linear regression analysis, which the authors stated as being inherently conservative, and a comparison of young donors (<36 years) vs. old donors (≥ 36 years). The detection of a paternal age effect for meiosis II errors was only detected by the latter statistical analysis while an age effect for meiosis I errors was detected by both analyses. If the other 3 studies are indicative that meiosis II is more susceptible to an age effect than meiosis I, one would anticipate an age effect for meiosis II errors to be more readily detected than an age effect for the meiosis I errors, thus the results obtained by Griffin *et al.*, are somewhat unexpected. Sex ratio showed no relationship with paternal age in our study and this has been reported previously by Martin *et al.*, (1995) and Griffin *et al.*,

(1996). In addition, one individual was shown to have a statistically significant excess of X-bearing sperm which was also observed in a study by Spriggs *et al.*, (1995) and Griffin *et al.*, (1996). Although the studies to date do differ somewhat with respect to the relationship between age and the type of disomy for the sex chromosomes, it seems reasonable to conclude that a minor paternal age effect is present, however it is of no practical clinical significance.

Because these age studies contain a large number of donors and all used multi-color FISH, a comparison of disomy frequencies for the sex chromosomes is given in Table 14. Good agreement amongst the four studies is observed for disomy X, however considerable discrepancy is seen for disomy Y and XY disomy. One possible explanation for the variation in disomy Y frequencies is that the three studies with the lower disomy Y frequencies utilized a different Y chromosome probe than the study by Martin *et al.*. The signal size for the alpha satellite probe used by Martin *et al.*, (1995) is much smaller when compared with the satellite III probe used by the others and as a result, the distance between like-colored signals is smaller as defined by the domain criteria. Of particular interest, is the remarkable agreement in disomy frequencies for the Y chromosome between the studies by Griffin *et al.*, (1995), Robbins *et al.*, (1995) and this investigation, as different scoring criteria were used in all three. A one-half domain criterion was used for this investigation, the study by Griffin *et al.* used one full domain and the investigation by Robbins *et al.* stated that the distance between the two Y signals had to be the same absolute distance apart as two X or chromosome 8 signals on the slide. This may be due to the relatively large size of the Y chromosome satellite III probe signal as there is

probably little doubt about whether two signals are present within a sperm head, regardless of how far apart they are. The increased frequency of XY disomy in this study relative to the others can be explained by donor heterogeneity or the failure to observe more than one autosomal signal. Donor heterogeneity is evident with donor #8449 who had a frequency of XY disomy of 1.23%. If this individual is removed from the data, the mean XY disomy frequency drops to 0.24% for this study. Because the scoring of XY disomy is dependent upon the observation of two signals of different colors, differences in scoring criteria will only affect the autosomal control probe, thus a sperm may be classified as heterodisomic for the sex chromosomes when it is actually diploid. A general trend is apparent in most of these studies and that is the frequency of XY disomy is substantially higher than the disomy frequencies for the X and Y chromosomes, implying that the XY bivalent is particularly susceptible to nondisjunction. During meiosis I, normal pairing and recombination occurs between the X and Y chromosomes in the pseudoautosomal region. Because of this small region of homology, one obligatory cross over is thought to occur between the sex chromosomes during meiosis and this exchange between chromosomes is thought to be necessary for their proper disjunction (Burgoyne, 1982). If crossing over and hence, chiasma formation, fails to occur between the X and Y chromosomes, the sole mechanism by which the sex homolog is held together is absent, which may increase the risk of nondisjunction (Hassold *et al.*, 1991b). Through the use of DNA markers in the pseudoautosomal region, Klinefelter individuals and their parents have been studied to determine if changes in recombination are associated with sex chromosome nondisjunction (Hassold *et al.*, 1991b; Lorda-Sanchez *et al.*, 1992). In 35

out of 41 cases of 47,XXY individuals, no cross-over was detected between the paternally inherited sex chromosomes whereas one would expect to find 50% recombination, thus the results are significantly below the expected number of 19-20 (Hassold *et al.)*. Further comparison with a pseudoautosomal genetic map derived from normal 46,XY individuals revealed that the Klinefelter genetic map was significantly shorter, suggesting a significant decrease in recombination within this area for this population. Similar results were obtained by Lorda-Sanchez *et al.* in which 9 paternally derived 47,XXY cases failed to show any evidence of recombination within the pseudoautosomal region. Further support for this theory could be obtained by isolating and testing XY disomic sperm for recombination and comparing these results with normal X and Y bearing sperm; however this has not been accomplished as of yet.

Table 14: Comparison of Sex Chromosome Disomy Frequencies with Other Donor Age FISH Studies

	# of donors	disomy Y	disomy X	XY disomy
Robbins *et al.*, (1995)	14	0.031	0.031	0.095
Griffin *et al.*, (1995)	24	0.027	0.021	0.104
Martin *et al.*, (1995)	10	0.18	0.07	0.16
present study	18	0.06	0.05	0.30

B. Acrocentric Chromosomes

This is the first study of donor age to report on the disomy frequencies for both chromosomes 13 and 21. No significant age effect was detected for either of these chromosomes. Additional autosomes that have been investigated in other age studies include chromosome 18 (Griffin *et al.*, 1995), chromosome 8 (Robbins *et al.*, 1995) and chromosomes 1 and 12 (Martin *et al.*, 1995). With the exception of chromosome 1,

which showed higher disomy frequencies with increasing age, no age effect was detected for any of the autosomes.

One report from this laboratory has previously reported a mean disomy frequency for chromosome 21 of 0.29% in 5 men (Spriggs *et al.*, 1996) which compares reasonably well with the mean disomy frequency of 0.37% in this study. Additional reports include disomy 21 frequencies of 0.38% in a survey of 9 men (Blanco *et al.*, 1996) and 0.17% in 9 men (Griffin *et al.*, 1996). In all of the investigations, a comparison of disomy 21 frequency with other autosomes demonstrates that the frequency of disomy 21 is significantly higher, suggesting that this chromosome is particularly susceptible to nondisjunction (Spriggs *et al.*; Griffin *et al.*; Blanco *et al.*).

A recent report by Pellestor *et al.*, (1996a) provides disomy frequencies for chromosomes 13 and 21 using an alternative technique referred to as the primed in situ labeling technique or PRINS. This procedure involves the use of DNA primers for specific chromosome sequences which anneal to the corresponding DNA of undecondensed sperm cells. A polymerase chain reaction with digoxigenin or biotin-labelled nucleotides is then performed which generates multiple copies of the DNA sequences of interest. Following this, a detection step, similar to that of the FISH protocols, is performed to generate fluorescent signals which represent the DNA sequences of the chromosomes of interest. Using this procedure, the mean disomy frequencies for chromosomes 13 and 21 in 3 donors were 0.28% and 0.32% respectively. Although the disomy frequencies for chromosome 21 between this study and the study by Pellestor *et al.* are similar, there is a significant difference between disomy frequencies for

chromosome 13 (0.13% - this study vs. 0.28%). In fact, the results obtained for the other autosomes (chromosomes 8, 9 and 16) in the PRINS study are almost as high as the disomy frequency of chromosome 21 (0.31%, 0.28%, 0.26% respectively), thus the PRINS results do not agree with the FISH results (Spriggs *et al.*, 1996; Blanco et al., 1996, Griffin et al., 1996) and sperm karyotyping results (Martin and Rademaker, 1990). It would be of interest to perform PRINS for the X and Y chromosomes to determine if the relatively increased frequency of XY disomy compared to disomy X and disomy Y is still detected with this alternate technique. The authors did encounter some technical difficulties when performing this procedure, including occasional splitting of the fluorescent signals and significant variation in the size of the fluorescent spots from experiment to experiment. Both of these factors can influence the disomy frequencies and if they occur more frequently in PRINS than in FISH, this may be an explanation for the large discrepancy in results between the two methods. An additional cause for this difference could be due to the primers (20 to 30 nucleotides in length) annealing at two locations far enough apart along the sperm DNA to be interpreted as two separate signals. Whatever the explanation, it is apparent that any comparison between disomy frequencies obtained by these two methods must be made with caution.

C. Structural Abnormalities

The method used to detect abnormalities in chromosome structure in this study is quite new and provides a way to use the FISH technique to detect more than just aneuploid cells. Through the simultaneous use of a centromeric and a telomeric probe for a single chromosome, duplications and deletions within that chromosome can be detected

by the fluorescent signals. A centromeric signal in the absence of a telomeric signal implies loss of the telomeric region and the presence of two centromeric signals with one telomeric signal implies the duplication of the centromere region. This technique has been employed to establish abnormality frequencies in a series of healthy individuals (Van Hummelen *et al.*, 1996) and to compare frequencies between healthy men and men with Hodgkin's disease undergoing chemotherapy and several years after chemotherapy (Van Hummelen *et al.*, 1995). Telomeric and centromeric probes for chromosome 1 were chosen for these particular studies due to the large size of the chromosome (Mendelsohn *et al.*, 1973) as the larger the span of DNA, the higher the probability of duplications or deletions. The reasons for incorporating this technique into the paternal age study include the evidence for a donor age effect for structural abnormalities in human sperm, using the zona pellucida-free hamster egg assay (Martin and Rademaker, 1987), as well as the preponderance of paternally derived de novo structural rearrangements and point mutations in offspring (Chandley, 1991).

Out of the five abnormalities that were scored for chromosome number 1 (including disomy), a positive correlation with age was detected for centromeric deletions only. No significant age relationship was detected for centromeric or telomeric duplications, telomeric deletions or disomy of chromosome 1. The previous study by Martin *et al.*, (1995) did detect a statistically significant donor age effect for disomy 1 and our study also indicates a positive correlation, however it is not statistically significant. How the effect of age influences the frequency of acentric fragments is purely speculative. Perhaps as a male ages, the risk of improper pairing between homologs or between the

short and long arms of one chromosome increases. If unequal crossing over occurs between improperly paired homologs , one would also expect the production of dicentric chromosomes which should also increase with age, unless they are preferentially lost from the sperm cell. Chromosomes have been shown to form a hairpin structure in sperm cells (Zalensky *et al.*, 1995), so the opportunity does exist for illegitimate pairing within a chromosome to occur and if followed by a cross over, an acentric fragment and a small fragment of centromeric DNA would result. Again, the fragment containing the centromeric DNA would also need to be lost from the sperm cell in order for the model to conform with the results obtained here. In addition, this hairpin configuration could also permit a break on both sides of the centromere resulting in a deletion of the centromere.

If the data for all four structural abnormalities for chromosome 1 are combined, the Pearson correlation coefficient is -0.073, indicating no effect of donor age on chromosome 1 structural abnormalities. To get a clearer picture of how donor age affects structural abnormalities, the simultaneous use of centromeric and telomeric probes needs to be applied to several chromosomes. Although the sperm karyotyping study by Martin and Rademaker (1987) detected an age effect, the FISH results may not demonstrate the same, as this technique only detects gain or loss of material and not the rearrangement of chromosomes through balanced translocations or chromosome breaks or gaps that are readily visible on a karyotype.

Comparison of the results for chromosome 1 between this study and the publication of Van Hummelen et al., (1996) reveals some striking differences (Table 15).

Table 15: Comparison of Chromosome 1 Data

	1p del	1cen del	1p dup	1cen dup	disomy	nullisomy
Van Hummelen *et al.*	0.029	0.007	0.032	0.021	0.017	0.003
present study	0.219	0.035	0.212	0.084	0.094	0.034

Large differences are observed for deletions for the centromere, nullisomy and deletions and duplications of the p-arm with this study having consistently higher abnormality frequencies. Some of these differences may be explained by the use of two different chromosome 1 centromeric probes in the two studies, the different hybridization procedures and the difference in the number of donors. In addition, the decondensation protocols are different for the two investigations with a range of a 30 minute to 3 hour decondensation protocol for this study and a one hour decondensation protocol for the other. The higher frequency of abnormalities with duplicate signals may reflect splitting of signals, or the longer decondensation time may allow easier access of the multiple probe copies to hybridize with the DNA in the cell, however this does not explain the discrepancy in deletion frequencies.

Possible mechanisms of how duplications and deletions of both the centromere and the telomere of chromosome 1 are also provided in the investigation by Van Hummelen *et al.*, (1996). Unequal cross-overs during meiosis, chromosome and chromatid breakage may generate acentric fragments and deleted regions of chromosomes. If the fragments are lost, one would expect the frequency of sperm with deletions of 1p to be more frequent than those sperm with duplications of 1p, however in both studies, these abnormalities occur with approximately equal frequency. Also, the frequency of centromeric deletions would be less than the frequency of centromeric duplications by this

mechanism, which is the case for both studies. Another mechanism suggested by Van Hummelen *et al.* (1996) includes a balanced translocation between chromosome 1 and another chromosome that originates in the spermatogonia. As the stem cell proceeds through meiosis, unbalanced gametes are formed by adjacent I and II segregation. Adjacent I segregation would produce two types of gametes, one with duplications of 1p and one with deletions of 1p and these would occur in a 1:1 ratio. Adjacent II segregation would produce gametes with deletions of the centromere for chromosome 1 and duplications of the centromere for chromosome 1 in a 1:1 ratio. Rousseaux *et al.*, (1995) have used multi-color FISH to demonstrate that adjacent I occurs more frequently than adjacent II in balanced translocation heterozygotes which is in agreement with data from sperm karyotypes from translocation carriers (Martin, 1991) The result of the study by Van Hummelen *et al.* do not support this theory; however the overall results obtained in this investigation are in partial agreement with a possible balanced translocation mechanism. That is, deletions and duplications of the telomere occurred with greater frequency than duplications or deletions of the centromere. In addition, the mean frequency of telomeric duplications is approximately equal to the mean frequency of telomeric deletions, however the mean frequency of centromeric duplications is approximately twice that of centromeric deletions. Although the overall results may support the existence of a certain mechanism producing structural rearrangements, analysis of the data on an individual basis may not. Closer inspection of the data presented here reveals that although the mean frequency of duplications of 1p is approximately the same as the mean frequency of deletions of 1p, this is not true for most individuals.

Clearly, the results of these studies suggest that several mechanisms may be causing structural abnormalities and certain individuals may be more prone to particular mechanisms, depending on their genetic background or exposure history.

Because three different chromosome 1p probe types were used in this study, a comparison of biotin, digoxigenin and fluorescein-labelled probes was possible to determine if the different types of labels had an influence on abnormality frequency. The results indicate a significant difference in 1p duplication frequencies for the biotin-labelled and the digoxigenin-labelled probes with the biotin-labelled probes being the higher of the two. This is somewhat unexpected as both of these probes are indirect-labelled and both undergo a detection step, with the only difference between them being the immunological reagents that are used. It is entirely possible that anti-avidin FITC may give more background signals than anti-digoxigenin FITC and this prospect cannot be excluded. Further inspection of the data reveals that the biotin-labelled group also contains the highest frequency of 1p duplications for the entire study (donor #8449. 1.67%). This particular donor also has an increased frequency of XY disomy, disomy 13 and disomy 21; therefore, this increased frequency of abnormalities for chromosome 1 is not likely to be due to technical problems exclusive to the biotin-labelled probe. If this donor is excluded from the data analysis, the results of the Kruskal-Wallis test are no longer significant ($p=0.061$) which suggests that the use of different labels on the same probe has no influence on the frequency of duplications or deletions for 1p.

D. Nullisomy

The occurrence of nullisomy for specific chromosomes within a sperm cell has two

interpretations. One can assume that the absence of a signal arose due to technical problems which led to the inability of the probe to anneal to the sperm DNA, or alternatively, the absent signal reflects genuine loss of the chromosome. The policy in the past has been to interpret nullisomy as a measurement of hybridization efficiency, rather than genuine aneuploidy and there are arguments for and against this approach. In support of the genuine aneuploidy argument, the reciprocal product of nullisomy is disomy, therefore individuals with a high frequency of disomy would also have a high frequency of nullisomy. In fact, this is the case for donor #8449 who had the highest frequency of XY disomy (1.23%) and disomy 21 (1.68%), as well as the highest frequency of nullisomy for the sex chromosomes (1.12%) and chromosome 21 (1.06%). With the chromosome 1 hybridization, scoring a sperm as nullisomic requires that two signals be absent, the centromeric signal as well as the telomeric signal. One could say that the likelihood of both of these probes being absent due to technical artifact is very small and therefore, is more likely to indicate a sperm with no chromosome 1, rather than two probes failing to hybridize. In support of the technical artifact argument, the product description of the 1p midisatellite probe provided by Oncor states "A diploid sample should show a fluorescent signal near the telomere of the p arm of both chromosome 1 homologues in 70-90% of cells analyzed." Although the frequency of 1p deletions did not approach 10% in this study, the manufacturer has given a relatively wide range for the hybridization efficiency of this probe despite the fact that this statistic is based on analysis of metaphase spreads where both homologues can be visualized. Also, if technical artifact plays a significant role in hybridization failure, one would expect the nullisomy frequency

to at least approximate or exceed the disomy frequency. This is true in 39 out of 72 cases (54%) where disomy and nullisomy frequencies were calculated for a particular chromosome in this investigation (18 cases for each of chromosomes 13, 21 and 1 and the sex chromosomes). Possible explanations as to why nullisomy frequencies are lower than disomy frequencies include failure of visualizing the fluoroblue™ control probe (chromosome 1 for the sex chromosome hybridization and chromosome 12 for the chromosome 1 hybridization) and splitting of signals leading to an overestimation of disomy frequencies. Despite these arguments, the fact remains that nullisomy frequencies should be interpreted with caution, as these results can be influenced by a number of factors.

E. Diploids

Diploid frequencies were measured on three separate occasions for each individual and a wide range in frequencies was observed between donors (X/Y, 0.06%-1.19; 13/21, 0.18%-5.15%; 1p/1cen, 0.04%-0.83%) and no age effect was detected for any of the data sets. Several studies have scored diploid cells on a number of men, using a variety of different probes including the sex chromosomes, chromosomes 1, 12, 8, 15, 16 and 18 as well as the use of centromeric and telomeric probes for chromosome 1 (Griffin *et al.*, 1995; Martin *et al.*, 1996; Chevret *et al.*, 1995; Williams *et al.*, 1993; Robbins *et al.*, 1995; Van Hummelen *et al.*, 1996). Wide ranges in diploid frequencies have been reported (Griffin *et al.*, 1995, 0.03%-0.70%; Rademaker *et al.*, 1997, 0.05%-0.33%; Chevret *et al.*, 1995, 0.09%-0.21%; Williams *et al.*, 1993, 0.15%-0.66%; Robbins *et al.*, 1995, 0.03%-0.25%; Van Hummelen *et al.*, 1996, 0.04%-0.09%). The upper and lower

limits for the X/Y data in this study are broader than in previous reports, however this can be attributed to two donors (donor #8449 and #2318) who both had diploid frequencies over 1% which are the highest reported thus far.

The use of multi-color probes for the sex chromosomes and an autosome allow the observer to distinguish between failure of meiosis I (46, XY) and failure of meiosis II (46, XX or 46, YY) and Table 15 provides a summary of those studies that have used this technique. The study by Williams *et al.*, demonstrates the majority of diploid cells are due to failure of MII, however other studies indicate that a majority of diploid cells arise due to failure of meiosis I.

Table 16: Percent of Diploid Cells Attributed to Failure of MI and MII

	# of donors	meiosis I	meiosis II
Williams *et al.*, (1993)	6	0.12	0.36
Robbins *et al.*, (1995)	14	0.11	0.04
Chevret *et al.*, (1995)	4	0.13	0.03
present study	17	0.26	0.05

Although the diploid frequencies in this study are derived from a single sperm sample for each male, the majority of donors (11/18) demonstrated significant deviation between the three frequencies. One possible explanation for this is variability within the sample which was reported by Van Hummelen *et al.* in two out of four donors using the same probes; however a study of 10 men by Rademaker *et al.*, (1997) showed no variability in diploid frequencies in 9 donors using two hybridizations and two different sets of probes. Further inspection of our data reveals that in most cases (14/18), the diploid frequency obtained by the 13/21 hybridization is consistently higher than the X/Y

or 1p/1cen hybridization. This is likely due to a technical factor unique to the 13/21 probe mixture and the manufacturer (Vysis) states that "depending on the stage of the cell cycle and the coiling of the DNA each of the signals may appear split or diffuse." If this splitting is significant and the signals are far enough apart, one signal will be scored as two. For this to inflate diploid frequency, both signals would have to have an equal likelihood of splitting within one sperm and given that the decondensation within any given sperm appears to be uniform, this is likely a reasonable assumption. The difference between the X/Y data set and the chromosome 1 data set is not as marked, however a number of donors appear to have a lower diploid frequency with the chromosome 1 hybridization. This was suspected to be due to overlapping signals as a number of cells were observed that had two like-colored chromosome 1 signals, either centromeric and telomeric and the corresponding unlike-colored chromosome 1 signal, with two autosomal control signals (chromosome 12) e.g. for donor #8449, 30 of these cells were observed and not included as part of the diploid total. As a result, the chromosome 1 hybridization may lead to an underestimation of diploid frequency when like-colored signals overlap. Some of the variability encountered between donors in the diploid data sets is likely not due to the reasons described above, but due to genuine variability, e.g. in some individuals, the 13/21 frequency was lower than the other frequencies e.g. donor #2318, and the chromosome 1 frequency was higher than the others e.g. donor #8200. Thus, diploid frequency does vary within donors, however possible technical sources of this variation must be considered.

F. Donor Heterogeneity

As shown in the results, significant donor heterogeneity was found across all age groups and because of this variation within each strata, the data were analyzed on an individual basis. The variability in frequencies between donors have been reported for disomy 1 and Y in three donors (Robbins *et al.*, 1993), XY disomy and YY disomy in 14 donors (Robbins *et al.*, 1995), disomy 1, disomy 15, YY disomy and XY disomy in 5 men (Spriggs *et al.*, 1995), disomy 1, XX disomy, YY disomy and diploid frequencies in 10 men (Martin *et al.*, 1996) and in deletions of 1p in 3 men (Van Hummelen *et al.*, 1996). Donor variation in diploid frequencies has also been reported and is reflected in the wide range of frequencies as discussed previously. In this study, the variation observed could not be attributed to any one individual within each age group, however donor #8449 consistently produced high frequencies for diploidy, XY disomy, disomy 13, 21 and 1 as well as duplications of 1p. This individual is of proven fertility and has had no known exposures, although he is a smoker. There has been some suggestion that heavy smoking may increase the frequency of disomy in human sperm (Wyrobek *et al.*, 1995) however, other smokers in the study did not demonstrate the same increases in abnormality frequencies. Clearly, there is significant variation in chromosomes abnormality frequencies in the sperm of the normal male population, making it difficult to detect trends in these abnormality frequencies within a group of individuals. The source of this diversity is unknown, however it would be of great interest to clarify if it is due to genetic background or due to varying levels of exposures such as chemicals, pollutants or illness.

V. Conclusions

The results of this study and previous investigations indicate a small donor age effect is present for aneuploidy of the sex chromosomes, however there is no evidence of such an age effect for disomy of selected autosomes, diploidy, sex ratio, nullisomy or duplications and deletions of chromosome 1.

Although FISH is a quicker, inexpensive method of analyzing chromosome abnormalities in human sperm, the karyotype still provides more information with regard to structural abnormalities in sperm chromosomes. In addition, the technical aspects of FISH must be taken into consideration as the possibility that these factors will influence the results cannot be excluded.

The pattern of aneuploidy frequency in human sperm suggests that certain homologs are more susceptible to nondisjunction than others, as the sex chromosomes and chromosome 21 have higher disomy frequencies than other autosomes. The absence of recombination has been associated with the occurrence of nondisjunction of the sex chromosomes in individuals with Klinefelter syndrome (Macdonald *et al.*, 1994; Lorda-Sanchez *et al.*, 1992; Hassold *et al.*, 1991), however this has not yet been shown in XY disomic sperm. These investigations are indicative of chromosome-dependent mechanisms by which aneuploid sperm are generated and thus far, the data implies that age may play only a minor role in the production of chromosome abnormalities in human sperm.

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VI. Appendices

A. Solutions and Buffers

modified Ackerman's cryoprotectant

40 mL Bacto Egg Yolk Enrichment - 50%

30 mL glycerol

2.0 g glycine

2.6 g glucose

2.3 g sodium citrate

98.7 mL distilled water

Mix reagents and heat inactivate at 56°C for 30 minutes. Adjust pH to 7.2 - 7.4 and store at -20°C in 2.5 mL aliquots.

sperm count diluent

5 g sodium bicarbonate

1 mL 35% formaldehyde

100 mL 0.9% sodium chloride solution

Mix reagents and store at 4°C.

1 M Tris-HCl, pH=8.0

121.1 g Tris base

800 mL distilled water

≅42 mL concentrated HCl

Dissolve Tris in water, add concentrated HCl. Allow the solution to cool to room temperature and adjust the pH to 8.0. Bring the total volume to 1 litre with distilled water and autoclave. Store at room temperature.

0.01 M Tris /0.9% NaCl

9 g NaCl

100 mL 0.1 M Tris-HCl, pH=8.0

≅900 mL distilled water

Dissolve NaCl in Tris and add water to a total volume of 1 litre. Autoclave and store

at room temperature.

1 M Dithiothrietol (DTT)

3.08 g DTT

20 mL sterile distilled water

Dissolve DTT in water and store in 500 µL aliquots at -20°C.

20 mM lithium diiodosalicylate (LIS)

0.792 g LIS

100 mL sterile distilled water

Dissolve LIS in sterile distilled water and store at room temperature.

10mM DTT/0.1M Tris

0.4 mL 10mM DTT

40 mL 0.1M Tris-HCl, pH=8.0

Mix and use immediately for sperm decondensation.

1mM DTT/10mM LIS/0.1M Tris

40 µL 10mM DTT

20 mL 20mM LIS

20 mL 0.1M Tris-HCl, pH=8.0

Mix and use immediately for sperm decondensation.

20 × standard saline citrate (SSC)

175.2 g NaCl

88.2 g Na citrate

distilled water

Dissolve sodium chloride and sodium citrate in water. Adjust to pH 7.4 with concentrated NaOH solution and bring total volume to 1 litre with water. Autoclave and store at room temperature.

Formamide purification

1 g Amberlite® MB-1 resin

100 mL formamide

filter paper (Whatman #1004 150)

Mix resin beads with the formamide. Filter and use immediately.

70% formamide / 2×SSC (pre-hybridization solution)

35 mL ultrapure formamide

5 mL 20×SSC

sterile distilled water

Mix the formamide and SSC, and bring the pH to 7.5 with 1 M HCl. Adjust volume to 50 ml with water. Use for a maximum of 20 hybridizations or 1 month.

Store at 4°C between uses.

50% formamide / 2×SSC (post-hybridization solution)

75 mL ultrapure formamide

15 mL 20×SSC

sterile distilled water

Mix the formamide and SSC. Adjust the pH to 7 with 1 M HCl and bring volume to 150 mL with water. Divide solution into 3 Coplin jars (50 mL each) and store at 4°C. Use for a maximum of one month or 20 hybridizations.

MM2.1 High Stringency Hybridization Buffer

5.5 mL ultrapure formamide

0.5 mL 20×SSC

1 g dextran sulfate

Mix formamide with SSC and add dextran sulfate. Heat at 70°C to dissolve and cool. Adjust to pH 7 and dilute to 7 mL with sterile, distilled water. Store 1 mL aliquots at -20°C.

Salmon Sperm DNA

10 µL DNA from salmon testes (10 mg/mL)

190 µL sterile distilled water

Dilute salmon sperm DNA in distilled water and store at 4°C.

Sodium phosphate, dibasic

107.2 g $Na_2HPO_4.7H_2O$

distilled water

Dissolve sodium phosphate in distilled water to a total volume of 4 litres.

Sodium phosphate, monobasic

3.9 g $NaH_2PO_4 \cdot 2H_2O$

distilled water

Dissolve sodium phosphate in distilled water to a total volume of 250 mL.

PN Buffer

3800 mL sodium phosphate, dibasic solution

≅200 mL sodium phosphate, monobasic solution

≅4 mL Nonidet™ P-40

While monitoring the pH, add the sodium phosphate, monobasic solution to the sodium phosphate, dibasic solution until the combined solution reaches a pH of 8.0. Measure the final solution volume and add 1 ml Nonidet™ P-40 for each litre of solution.

2×SSC/0.1% NP-40

50 mL 20×SSC

425 mL sterile distilled water

0.5 mL Nonidet™ P-40

Mix SSC with 200 mL water. Adjust pH to 7.0-7.5 with NaOH and bring volume to 500 mL with remaining water. Add NP-40 and mix.

PNM Buffer

100 mL PN buffer

5 g powdered non-fat milk

0.02 g Na azide

Dissolve the milk powder and sodium azide in PN buffer, then centrifuge. Remove the supernatant and store at 4°C.

Anti-digoxigenin-fluorescein (FITC) stock solution (200 µg/mL)

0.2 g anti-digoxigenin-fluorescein, Fab fragments (Boehringer-Mannheim)

1 mL sterile distilled water

Mix anti-digoxigenin-FITC with water and store in 15 µL aliquots at -20°C.

Anti-digoxigenin-FITC working solution (13 µg/mL)

15 mL anti-digoxigenin-FITC stock solution

150 mL PNM buffer

50 mL normal sheep serum

15 mL BSA (10 mg/mL)

Mix and store at 4°C for a maximum of one month.

Biotinylated anti-avidin stock solution (0.5 mg/mL)

0.5 mg biotinylated anti-avidin D from goat (Vector Laboratories)

1.0 mL sterile distilled water

Mix biotinylated anti-avidin with water and store in 20 µL aliquots at -20°C.

Biotinylated anti--avidin working solution (10 µg/ml)

20 µL biotinylated anti--avidin stock solution

980 µL PNM buffer

Mix biotinylated anti-avidin stock solution and PNM buffer.

4',6-diamidino-2-phenylindole (DAPI)

1 mg DAPI

1 mL sterile distilled water

stock: Dissolve DAPI in water (1 mg/ml).

working: dissolve stock solution in an appropriate amount of PN buffer to obtain the concentration (5ng/mL or 10ng/mL).

Antifade

5 mg p-phenylenediamine

10 mL PN buffer

glycerol

stock: Dissolve p-phenylenediamine in PN buffer and mix final solution with an equal volume of glycerol.

working: dissolve stock solution in an appropriate amount of PN buffer to obtain the desired concentration (50% or 75%)

Propidium iodide

stock: 1 mg/mL in 50% EtOH

working: dilute above with an appropriate amount of PN buffer for the desired concentration

B. Reagents

Amberlite®	ICN Biomedicals 150330
bovine serum albumin	Gibco 15561-012
4,6-diamidino-2-phenylindole (DAPI)	Sigma D-9542
Dithiothreitol (DTT)	Sigma D-9779
3,5 diiodosalicylate – Lithium salt (LIS)	Sigma D-3635
egg yolk	Difco 3347-73-8
Formamide, ultrapure	ICN Biomedicals 800686
formaldehyde	BDH B10113
glycerol	BDH B10118
glycine	Fisher G-46
Nonidet P-40	Sigma N-6507
normal sheep serum	Sigma S-2382
Percoll	Pharmacia
Propidium Iodide	Sigma
Polyvinypyrollidone	Pharmascience
salmon testes DNA	Sigma D7656
sodium azide	Fisher S-227
sodium bicarbonate	Fisher S-233
sodium chloride	BDH B10241
sodium citrate	Fisher S-279
sodium phosphate dibasic	BDH ACS810
sodium phosphate monobasic	Fisher S-381
Trizma® Base (tris[Hydroxymethyl]aminomethane)	Sigma T-8524