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Human Sperm Aneuploidy After Exposure to Pesticides

by

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CEP	- chromosome enumeration probe
CEPH	- Centre d'Etude du Polymorphisme Humain
DNA	- deoxyribonucleic acid
DTT	- dithiothreitol
F-body	- fluorescent body
FISH	- fluorescence *in situ* hybridization
g	- grams
HCl	- hydrochloric acid
HOS	- hypo-osmotic assay
ICSI	- intra cytoplasmic sperm injection
IVF	- *in vitro* fertilization
L	- litre
LIS	- lithium diiodosalicylate
LSI	- locus specific identifier
M	- molar (solution concentration of moles / litre)
MI	- meiosis I
MII	- meiosis II
mL	- millilitre (10^{-3} litres)
mM	- millimolar (10^{-3} moles / litre)
n	- haploid chromosome number (23 in humans)
NaCl	- sodium chloride
NaOH	- sodium hydroxide
nm	- nanometre (10^{-9} metres)
p	- short arm of a chromosome
PVP	- polyvinylpyrollidone
q	- long arm of a chromosome
sec	- second
SSC	- standard saline citrate
TE	- Tris-EDTA (ethylene diamine tetra acetate)
Tris	- Trizma base
°C	- degrees Celsius
µL	- microlitre (10^{-6} litres)
µm	- micrometre (10^{-6} metres)
WHO	- World Health Organization

1. Introduction

Early ideas of heredity included the notion that a mother's and father's characteristics were blended together like two colours of paint and their children displayed the new colour. According to this theory of blended inheritance, the mating of a white mouse and a black mouse would produce grey offspring. Gregor Mendel's work with garden peas (*Pisum sativum*) led him to believe that traits were actually passed from parent to child in discrete units, and in 1865 he published his research describing a particulate theory of inheritance. It had been determined earlier in the century that all living things were made up of cells, but the chromosomes within the nuclei of cells were not described until about 1875. The duplication and separation of chromosomes coinciding with the processes of mitosis and meiosis were well known by the end of the 19th century, before the importance of chromosomes in heredity was completely understood (Crow, 1966).

The specialized divisions of meiosis reduce the diploid chromosome number, producing gametes with half the genetic complement of somatic cells. Therefore, combination of male and female gametes restores the diploid chromosome complement in the somatic cells of offspring, which is necessary to prevent doubling of the chromosome number in each successive generation. Since the early 1900s when the hereditary importance of chromosomes was first understood, scientists have studied meiosis, and have begun to elucidate the mechanisms of this complicated process. Even more interesting has been the

study of when meiosis goes awry and gametes without the proper complement of chromosomes are produced, resulting in a wide range of effects on the next generation including: infertility, embryonic and fetal loss, and a variety of genetic syndromes (Therman & Susman, 1993). Recently, research has begun to look at these anomalies, the reasons behind them, and the contribution of environmental agents (Mailhes, 1995; Parry *et al.*, 1996).

Damage that environmental exposures can inflict on DNA is of great concern as it can lead to cancer and other illnesses, and damage which can be transmitted to our offspring is of particular interest as it can lead to a number of reproductive difficulties including birth defects and infertility, therefore much research focuses on harm caused during germ cell development. Therefore, a brief review of cell division, aneuploidy, the consequences of chromosomal errors, and some potential mechanisms that lead to meiotic errors are presented. Research on the occurrence and distribution of chromosomal abnormalities provides data about the baseline of aberration frequencies in the human population.

a. Cell division

Replication of cells is fundamental to growth, healing, and procreation of living organisms, and accurate cell division is necessary to the very existence of human beings. An important part of this cell division is the precise division of the nucleus, which produces an exact replicate through mitosis or reduces the genetic complement to precisely half in meiosis.

i. Mitosis

Mitosis is a precise multi-step mechanism that ensures exactly equal division of the chromosomes, resulting in two daughter cells, each with an exact copy of the parental cell's DNA complement. Errors in the mitotic process generally lead to cells with abnormal chromosome complements and depending upon the degree of abnormality, the cells may not survive or may be present in a mosaic individual, who has both normal and abnormal cell lines.

ii. Meiosis

The products of meiosis are specialized for sexual reproduction, and are generated through two serial divisions preceded by one round of DNA replication. Meiosis yields four spermatozoa with haploid chromosomal complements in males, and a single haploid oocyte and three polar bodies in females. Union of a sperm and oocyte restores the normal diploid chromosome complement in the next generation. There are marked differences between the two meiotic divisions, with the first division (MI) having a number of specialized events, while meiosis II (MII) is analogous to a 'haploid mitosis' (Lamb *et al.*, 1996). Errors in meiotic divisions generally produce chromosomally abnormal gametes which can lead to infertility due to germ cell death, fertilization or implantation failure, miscarriage, or stillbirth. Liveborns can be affected by abnormalities in gametes, and are generally constitutionally abnormal individuals characterized by the presence of the chromosome anomaly in all somatic cells.

(1) Meiotic stages

DNA synthesis occurs during S phase of the cell cycle, and the genetic complement of the cell is doubled. Meiosis I (MI), the first division, reduces the chromosome number by half and is designated a reductional division. The second division, MII, is an equational division because the centromeres adhering sister chromatids divide, but the chromosome number remains the same. Each meiotic division is composed of four distinct stages: prophase, metaphase, anaphase and telophase.

In prophase of meiosis I chromosomes begin to condense, homologous chromosomes synapse, and recombination occurs between non-sister chromatids allowing the exchange of material between homologous chromosomes to increase genetic variety. Metaphase I is marked by the disappearance of the nuclear membrane. Homologous pairs line up at the cellular equator, and spindle fibres from each cellular pole attach to the chromosomes at their centromeres. Chromosomes are moved to opposite poles by spindle fibres in anaphase I, and because paired sister chromatids segregate together, centromeres remain intact in this reductional division. During telophase the chromosomes reach either end of the cell, two new nuclear membranes may form, and the cellular membrane invaginates to divide the cell into two daughter cells.

During interkinesis, the brief stage between MI and MII, the chromosomes may

decondense slightly. Prophase II follows and chromosomes return to (or retain) their condensed state. During metaphase II the sister chromatid pairs align at the equatorial plate and spindle fibres attach at the centromeres, which divide in half as paired chromatids move to opposite poles in anaphase II. Telophase II is identified by the re-formation of nuclear membranes and cleavage of the two cells into four. Each of the resulting gametes (four spermatozoa in males, and one oocyte in females) has one half of the genetic complement of the original parental cell (Griffiths *et al.*, 1993).

b. Aneuploidy

In humans normal somatic cells are diploid, with two copies of each of the 22 autosomes, and a pair of sex chromosomes, for a total of 46 chromosomes. Aneuploidy is an aberrant condition in which the chromosome number is not an exact multiple of the haploid number (n=23), and usually occurs when an individual chromosome has been gained or lost. Somatic cells that have gained a chromosome are trisomic (2n+1), whereas monosomic cells have only 45 chromosomes (2n-1). Among liveborn individuals, the most common example of a constitutional trisomy is Down syndrome, where individuals carry an extra copy of chromosome 21 in each of their cells. Generally, the only monosomy which is viable is 45,X resulting in females with Turner syndrome. Another type of numerical abnormality is polyploidy, which exists when there is an extra complete haploid (n) set of chromosomes, for example, triploidy in somatic cells (3n=69), or diploidy in germ cells (2n=46).

Human gametes (sperm and egg cells) are haploid (n), with a single copy of each of the 23 chromosomes. Because only one copy of each chromosome is present in germ cells, aneuploid gametes with a missing chromosome are nullisomic for that chromosome, while disomic sperm or eggs have a duplicate copy of an individual chromosome. Polyploid sex cells contain multiple haploid sets of chromosomes: for example, diploid gametes (2n), have an extra copy of each of the 23 chromosomes for a total of two haploid sets. The combination of a normal and an aneuploid (or polyploid) germ cell results in an abnormal conceptus, with deleterious reproductive results.

i. Outcomes of aneuploidy

Aneuploidy has widespread reproductive consequences and accounts for a large proportion of pregnancy wastage and abnormal births. Research indicates that approximately 26% of spontaneous abortions, 4% of still births, and 0.3% of live births have an extra chromosome, and that trisomies alone constitute over 4% of all clinically recognized pregnancies (Jacobs, 1992). When all types of numerically abnormal chromosome complements are considered (monosomy, trisomy, polyploidy, etc.), an estimated half of all spontaneous abortions are affected (Abruzzo & Hassold, 1995) and these aneuploidies occur for all chromosomes (Warburton & Kinney, 1996; Hanna *et al.*, 1997). Since at least 4% of recognized pregnancies are aneuploid, but only 0.3% are affected at term, over 90% of aneuploid conceptuses must be lost before birth. Very few chromosomal aneuploidies are compatible with survival to term, and even fewer

persist beyond the perinatal period. At term only trisomies of chromosomes 13, 18, 21, and certain sex chromosome aneuploidies, (ex. 47,XXX; 47,XXY; 47XYY; and 45,X) are commonly observed.

While the majority of trisomies of chromosomes 13 and 18 are lost prenatally, they are observed in liveborns, but they rarely survive beyond the first year of life. Patau syndrome (trisomy 13) is very rare, affecting only 1/20,000 live births (Jacobs, 1992). Neonates with and extra chromosome 13 have severe mental retardation and extensive physical malformations. Abnormalities of the eyes, cleft lip and palate, polydactyly, deafness, and complex heart anomalies are common. Patau infants rarely survive more than six months, with nearly half succumbing within one month of birth (Therman & Susman, 1993). While children with trisomy 18 (Edward syndrome) show less severe physical malformations than those with Patau syndrome, their condition is no less critical. Edward syndrome is characterized by hypertonia, clenched fists with overlapping fingers, cardiac malformations, low-set malformed ears, and an average existence of only a few months with only occasional cases of infants living up to one year (de Grouchy & Turleau, 1984). Approximately 1/10,000 births are afflicted with Edward syndrome (Jacobs, 1992).

(1) Viable aneuploid live births

Aneuploidies that survive beyond the perinatal period are primarily trisomy 21 (Down syndrome) which accounts for 0.12% of live births (Jacobs, 1992), and

various numerical abnormalities of the sex chromosomes such as: XYY, Klinefelter syndrome (47,XXY), 47,XXX, and Turner syndrome (45,X), with numerical sex chromosome anomalies occurring in approximately 1/600 livebirths (Jacobs, 1992).

(a) Down syndrome

Down syndrome was the first trisomy whose frequency was linked to maternal age: women at the age of 20 have a risk of approximately 1/1500 of having a trisomy 21 child, while the risk to women over the age of 40 years is nearly 1/100 (Eichenlaub-Ritter *et al.*, 1996). While it has become increasingly apparent over the last few decades that with appropriate interventions, education and training, individuals with Down syndrome are capable of contributing to society and living fulfilling lives, afflicted individuals still have many physical and mental challenges to overcome. Characteristics of this disorder include: hypotonia, cardiac malformations, broad hands and feet with short digits, distinctive facial dysmorphisms, and IQs ranging from <40 to 80. Down syndrome individuals have varied levels of mental deficiency and are most challenged by abstract reasoning, but generally have adequate social abilities (de Grouchy & Turleau, 1984).

(b) Sex chromosome aneuploidies

The characteristics of sex chromosome abnormalities vary extensively, and

karyotypically abnormal individuals do not necessarily stand out, as their physical appearance may not be obviously affected. In many cases, these conditions are not diagnosed until later in life (de Grouchy & Turleau, 1984).

XYY men have features including: tall stature (generally over six feet), muscle weakness, poor coordination, and diminished intelligence (Gotz *et al.*, 1999; de Grouchy & Turleau, 1984). Early studies reported that men carrying an extra Y chromosome (47,XYY individuals) were predisposed to abnormally aggressive and violent behaviour, as XYY men were found in increased frequencies in prisons and mental hospitals (Jacobs *et al.*, 1965). A great uproar occurred in the media, and the findings were sensationalised as evidence that the presence of an extra Y chromosome was responsible for crime. However, this perspective was eventually corrected when it was determined that these men were not necessarily prone to violent criminal behaviour, but due to their lowered intelligence and lack of emotional control they were more likely to display antisocial behaviours (Hunter, 1977). And while it appears that XYY men are more likely to commit an offence, their offences are generally crimes against property and not against people (Gotz *et al.*, 1999).

Klinefelter (47,XXY) syndrome occurs in 1/2000 live births (Jacobs, 1992), but affected men are often not diagnosed until puberty when they frequently demonstrate breast development, testicular atrophy, sparse facial hair and altered body proportions with abnormally long lower extremities (de Grouchy &

Turleau, 1984). At one time it was believed that these men were completely sterile, but aberrant spermatogenesis has been observed in Klinefelter men and many mosaics produce sperm but are generally oligo- or azoo-spermic. These men can reproduce with assistance (usually via intra-cytoplasmic sperm injection) and a handful of normal children have been born to XXY men, but care must be taken as chromosomal abnormalities have been noted in their offspring (Shi & Martin, 2001).

Females are born with an extra X chromosome (47,XXX) in approximately 1/2000 live births (Jacobs, 1992). There is no distinct phenotype associated with the extra sex chromosome, as these females can demonstrate a number of traits including: amenorrhoea, mild developmental delays, reduced intelligence, and / or a tendency to psychosis, but none are consistently observed (de Grouchy & Turleau, 1984).

Just as a genetic excess is problematic, so too is genetic insufficiency. The absence of an entire autosome is not a viable genetic complement as the deficit is too great, therefore constitutional autosomal monosomies do not survive to term. However, loss of a sex chromosome resulting in Turner syndrome (45,X), is seen in approximately 1/10,000 livebirths (Jacobs, 1992). However, survival of fetuses with X chromosome monosomy is very rare as an estimated 99% of 45,X conceptuses are lost before birth (Hassold *et al.*, 1996). The severity of Turner syndrome can vary markedly, as can the physical characteristics that these

women demonstrate. Females with this syndrome are generally short in stature and fail to develop secondary sexual characteristics, they can also have short webbed necks, cardiac and renal abnormalities, ovarian agenesis (streak gonads), and are generally infertile, although not all of these characteristics are consistently present (de Grouchy & Turleau, 1984).

c. Mechanisms of error

Aneuploidy is a product of meiotic errors that results in the loss or gain of a chromosome. Such errors yield abnormal oocytes and sperm which may lead to offspring with constitutional chromosomal abnormalities. Gametic studies show that all chromosomes (or chromosome groups) are represented in these deficits and excesses, however they are not represented equally. In a review by Martin *et al.* (1991b) of research conducted on more than 11,500 human sperm and nearly 775 human oocytes, karyotype analysis showed that individual chromosomes (or chromosome groups) did not demonstrate equivalent frequencies of nondisjunction. In oocytes, chromosomes in the D and G groups (chromosomes 13-15 and 21-22), were present in higher than expected percentages while C and F group chromosomes (chromosomes 6-12 and 19-20) were not observed as frequently as anticipated. Statistically significant increases in chromosome 21 aneuploidy frequencies were observed in oocytes and sperm, while the sex chromosomes also appear to have a greater tendency to be affected by meiotic errors in sperm (Martin *et al.*, 1991b). The reasons for the disparities noted between abnormality frequencies of individual chromosomes,

and the mechanisms responsible for the errors are just beginning to be elucidated. It has been proposed that different meiotic processes could go awry resulting in nondisjunction, failure of chromosome pairing, chromosome lag, or defective centromere division, and that each of these could play a role in the varied numerical abnormality frequencies observed between chromosomes.

i. Nondisjunction

Nondisjunction is the failure of chromosomes or chromatids to segregate properly from the equatorial plate to opposite cellular poles during nuclear division (Griffiths *et al.*, 1993), and can theoretically occur in either MI or MII, however chromosomes often display a tendency to nondisjoin at one meiotic division more so than at the other. For example, trisomy 21 in females results primarily from errors in meiosis I, which predominate (3:1) over MII errors; while paternal nondisjunction of chromosome 21 occurs in approximately a 1:1 ratio between the two meiotic divisions (Savage *et al.*, 1998). As a general rule, errors in maternal meiosis occur primarily in meiosis I, while errors in male meiosis are largely during the second division (Abruzzo & Hassold, 1995). The mechanisms and etiologies of nondisjunction remain unclear after several decades of investigation, but recent research indicates that maternal age and chromosomal recombination and pairing may play important roles.

(1) Maternal age

Maternal age has long been recognized as a risk factor for human aneuploidy. Although first associated with trisomy 21 (Down syndrome) by Penrose (1933), maternal age has since been linked to the other common trisomies (Hassold & Jacobs, 1984), and today increasing maternal age is believed associated with nearly all human trisomies (Hassold *et al.*, 1995). While the mechanism that couples increasing maternal age with increasing aneuploidy frequencies is not known for certain, a number of models have been proposed (Hassold *et al.*, 1995). Recent research suggests that recombination frequency may contribute to this association, as reduced recombination (Hassold *et al.*, 1995), and absent recombination (Robinson *et al.*, 1998) have been observed in conjunction with trisomy in cases of advanced maternal age.

(2) Altered recombination frequencies

The exchange of genetic information occurs when homologous chromosomes pair during prophase of meiosis I, facilitating recombination between non-sister chromatids. The observation of altered numbers and / or positions of chiasmata in cases of chromosome malsegregation at meiosis I has led to various investigations of recombination and nondisjunction (Hassold *et al.*, 2000). Reduced frequencies of recombination have been noted in trisomies originating in both sexes. Maternal MI trisomies of chromosomes 15 (Robinson *et al.*, 1998), 16 (Hassold *et al.*, 1995), 21 (Lamb *et al.*, 1997a) and XXY (MacDonald

et al., 1994) are all associated with an overall reduction in recombination frequency (with some displaying a complete lack of recombination); and abnormalities originating from the male parent have shown diminished chiasmata frequencies in cases of paternal (MI) trisomy 21 (Savage *et al.*, 1998) and trisomies of the sex chromosomes (Thomas *et al.*, 2000). Altered exchange has also been noted among the sex chromosomes in human sperm, where a significantly decreased recombination frequency was observed in XY disomic sperm compared with normal (23,X and 23,Y) sperm within the same individual (Shi *et al.*, 2001b). While the specific consequences of reduced numbers of chiasmata in MI are not understood, the relationship between chromosome pairings with no crossovers (nullichiasmate) and increased nondisjunction appears more obvious: with no contact between chromosome arms, pairing of homologous chromosomes is weakened, increasing the chances of incorrect segregation in meiosis. This idea is supported by research showing that large proportions of the MI errors which lead to the nondisjunction of various chromosomes have a complete lack of chiasmata, including: 21% of chromosome 15 maternal errors (Robinson *et al.*, 1998), 30% of chromosome 18 maternal errors (Bugge *et al.*, 1998), nearly 50% of all maternal chromosome 21 errors (Lamb *et al.*, 1997a), and approximately 70% of paternal sex chromosome errors (Hassold *et al.*, 1991). Based on the results of these and many other studies, recombination during MI appears to play an important role in proper chromosomal segregation. Conversely, MII errors are not expected to display

varied recombination frequencies as genetic exchange occurs exclusively in meiosis I. Research by Bugge *et al.* (1998) supported this position, showing that maternal trisomy 18 (due primarily to MII errors) showed no significant relationship between altered recombination and increased nondisjunction frequencies. However, Lamb *et al.* (1996) did observe significantly increased recombination frequencies in cases of maternal MII trisomy 21, and of further interest, chiasmata positions were altered in these cases.

(3) Altered recombination position

(a) Maternal origin trisomy

The modified chiasmata placement noted by Lamb *et al.* (1996) showed recombination sites primarily in the proximal region of 21q in maternal MII cases of trisomy 21. Another study by the same research group (Lamb *et al.*, 1997b) examined 292 non-mosaic trisomy 21 conceptuses of maternal origin to determine the relationship between meiotic errors and chiasmata configurations. Genetic linkage maps were constructed with selected chromosome 21 loci from various regions of the q arm. Analysis of the trisomy 21 progeny was used to determine if the heterozygosity (or homozygosity) of the mother's loci was maintained in the offspring or if there was a alteration, indicating a genetic exchange between markers. Chromosome 21 maps were generated for both MI and MII trisomy, using lengths based on recombination frequencies between the selected markers and these maps were compared with a genetic map of normal

female meiosis based on 40 CEPH families (Lamb *et al.*, 1997b). From this study is was determined that the majority of nondisjunction events showed altered recombination: nearly 50% of maternal MI errors were nullichiasmate, and the bulk of remaining MI errors displayed exchanges primarily clustered at the telomere. Maternal MII cases of trisomy 21 showed a stark contrast, as exchanges were grouped predominantly in the pericentromeric region (Lamb *et al.*, 1997a). One suggested interpretation of these findings was that chiasmata in close proximity to the centromere could lead to entanglement of chromosomes and prevent the bivalent from separating, which would result in both homologous chromosomes to segregating to the same pole in meiosis I. A reductional division of these entangled chromosomes would produce disomic gametes (see Figure 1). This altered first meiotic division would have a gametic outcome associated with a meiosis II error since the extra chromosomes would have the same centromeres. The implication based on this model is that all cases of maternal trisomy 21 arise from meiosis I, because even those appearing to be due to meiosis II errors would actually have originated from alterations of meiosis I (Lamb *et al.*, 1996). Other maternal trisomies have also revealed altered placement of chiasmata, for example, trisomy 16 shows a pronounced modification of crossover placement with exchange points primarily distal on both chromosome arms (Hassold *et al.*, 2000), and meiosis I errors of chromosome 18 predominantly display chiasmata at distal 18q (Bugge *et al.*, 1998).

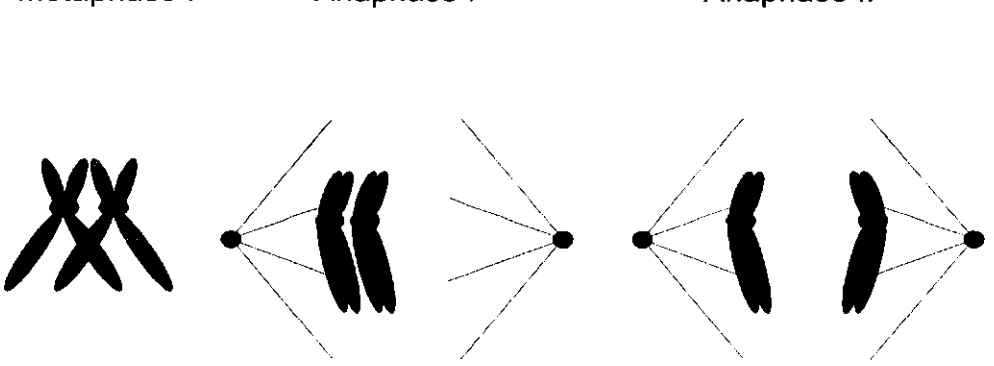


Figure 1: Proximal exchange could entangle chromosomes and prevent correct segregation at meiosis I. This would lead to disomic gametes with identical centromeres - indication of a meiosis II error. (Adapted from Lamb *et al.*, 1996).

(b) Paternal origin trisomy

Trisomies arising from paternal meiotic errors have not been scrutinized as closely for modified exchange configurations as maternal cases, but a potential change has been observed for chromosome 21. Hassold *et al.*(2000), noted a possible increase in proximal exchanges in paternal MII errors. This would agree with maternal data from the trisomy 21 study by Lamb *et al.* (1996), significantly linking increased proximal chiasmata to MII errors. However, more studies are required to understand the relationship of altered chiasmata frequencies and placements to increased meiotic nondisjunction in both males and females.

ii. Failure to pair

Just as reduced or absent recombination appears to have a negative effect on

chromosome segregation in meiosis, unpaired homologous chromosomes are also unlikely to separate correctly. Pairing of homologues in prophase unites chromosomes in preparation for metaphase when chromosomes align along the cell's equator to facilitate chromosome segregation and ensure that each resulting daughter cell will receive a complete matched set of DNA. When this coupling process fails, unpaired chromosomes will likely segregate randomly, potentially producing disomic and nullisomic gametes. It should be noted that while pairing failure and absent chiasmata are different mechanisms of chromosome malsegregation, the aneuploid products of the two are indistinguishable from one another. Therefore unless the stages of meiosis are observed, outcomes that could potentially be due to failed pairing may often be labelled generally as nondisjunction. It has been postulated that chromosomes that do not pair correctly are likely to align themselves with other chromosomes of similar size, and that this distributive pairing may influence the segregation of the chromosomes that neglected to pair correctly (Bond & Chandley, 1983). As mentioned above, a lack of recombination (which could result from failure to pair) has been associated with mis-segregation at maternal MI for chromosomes 15 (Robinson *et al.*,1998), 18 (Bugge *et al.*, 1998), 21 (Lamb *et al.*, 1997a), and the sex chromosomes (MacDonald *et al.*, 1994). Misdivision of the sex chromosomes at paternal MI has also been linked to such events (MacDonald *et al.*, 1994).

iii. Chromosome loss

When a chromosome is slower to move to the pole in anaphase than the rest of the chromosomes or if spindle fibres fail to attach to the centromere, a chromosome can be lost by essentially lagging behind. Chromosomes that do not segregate completely to the pole before the cell membrane begins to invaginate in telophase will not be located within either of the two newly forming nuclear membranes. This type of divisional error is termed anaphase lag, and generally results in a single cell that is missing a chromosome, rather than producing reciprocal aneuploid products (ex. disomic and nullisomic cells). The trailing chromosome may be lost in the cytoplasm of the cell, and a separate small nuclear membrane may enclose it, forming a micronucleus.

iv. Defective centromere division

Meiosis II is an equational division because it is the stage when centromeres adhering sister chromatids cleave during the second meiotic division (Griffiths *et al.*, 1993). Failure of chromatid separation, which occurs principally in MII, can be the result of centromeric flaws that prevent chromatids from breaking apart, or due to defective spindle fibres that neglect to draw the chromatids to each pole (Bond & Chandley, 1983). Premature division (when centromeres divide in MI instead of MII) is another type of faulty division. Single chromatids have been observed in research on human MII oocytes, suggesting that the centromeres precociously split prior to the second meiotic division (Wolstenholme & Angell,

2000). The authors suggest that the bivalent is weakened over time, and as maternal age increases premature centromeric division is more likely, leading to increased frequencies of aneuploidy.

d. Frequency and distribution of chromosome abnormalities

Regardless of the particular mechanism or divisional error that has produced aneuploid offspring, scientists have long been interested in learning more about affected individuals. Cytogenetic study of genetic anomalies relied upon research on liveborns, which produced useful information about the chromosomes involved and differing parental contributions (including the role of parental age). However, as most genetic abnormalities do not survive to term, liveborn studies have serious ascertainment biases, therefore other types of research have been employed to glean more information about the overall effects of aneuploidy on human reproduction. Research on spontaneous abortions and stillbirths has provided a wealth of data about aneuploid progeny that don't make it to the delivery room, and gamete studies have uncovered more information about meiotic error frequencies, indicating that many abnormalities are lost before or shortly following implantation.

i. Liveborn studies

Aneuploid births are rare occurrences representing only 0.3% of all livebirths (Abruzzo & Hassold, 1995), and only a few chromosomes are represented:

chromosomes 13, 18, 21, and the sex chromosomes account for nearly all newborns with numerical chromosomal abnormalities (Hassold *et al.*, 1996). Early studies of newborns utilized karyotypes to discern the frequencies of chromosomal abnormalities such as: trisomies, monosomy X, structural abnormalities, and mosaicism in various human populations (Hook & Hamerton, 1977). More recently, liveborn research has shifted to a molecular approach to identify more detailed information about chromosomal abnormalities that survive to term, such as: parent of origin, meiotic division in which the error occurred, and frequencies of meiotic recombination associated with these anomalies (Hassold *et al.*, 1991; MacDonald *et al.*, 1994; Robinson *et al.*, 1998). To date, we know that aneuploidy of chromosome 21 and the sex chromosomes contribute the majority of numerical chromosomal anomalies seen at birth. Chromosome 21 aneuploidy arises primarily from maternal nondisjunction, and specifically from errors occurring in MI (Lamb *et al.*, 1996), and sex chromosome aneuploidies result predominantly from MI errors in both parents but the females contribute the extra chromosome more often (MacDonald *et al.*, 1994).

Liveborn studies continue to provide a wealth of information about aneuploidy in living individuals, but their ascertainment bias limits their ability to address the overall effects of aneuploidy on human reproduction as they cannot take into account the vast majority of numerical chromosomal abnormalities which are incompatible with survival to term. Bond & Chandley (1983), compare the study of aneuploidy to an iceberg. Discussing the effects of aneuploidy on human

reproduction based solely on liveborn research would be akin to studying and iceberg without close observation and considering only the portion above the water. Much potential knowledge would be missed as the liveborn portion represents only a small fraction of the whole impact of chromosomal errors in human reproduction. Liveborn research has been complemented through different approaches which helped to determine that the bulk of aneuploid conceptuses are lost long before birth, and these chromosomal abnormalities are the single greatest cause of pregnancy wastage in humans (Abruzzo & Hassold, 1995).

ii. Spontaneous abortion / stillbirth studies

While only a few aneuploid conditions survive to term, spontaneous abortion and stillbirth research has revealed that all human chromosomes are affected by aneuploidy (Abruzzo & Hassold, 1995; Hanna *et al.*, 1997). Roughly 26% of spontaneous abortions are aneuploid, while a only 4% of stillbirths are estimated to be aneuploid, and even fewer (0.3%) of livebirths have a missing or extra chromosome (Jacobs, 1992). The percentage of aneuploid conceptuses apparent at each of these reproductive 'windows' decreases by an order of magnitude, demonstrating the large numbers of chromosomally abnormal fetuses lost throughout the various stages of pregnancy. Data from karyotype studies of spontaneous abortions demonstrate that trisomies of the various individual chromosomes are not present in equal frequencies. Chromosome 16 is observed the most often, being present in approximately 30% of cases;

followed by chromosome 2 and the acrocentrics (chromosomes 13-15, 21 and 22), which each account for 5-10% of all trisomies in spontaneous abortions. The remaining chromosomes each account for either 1-5% (chromosomes 4, 7, 8, 9, 10, 18 and 20) or <1% (1, 3, 5, 6, 11, 12, 17 and 19) of all trisomies observed (Warburton & Kinney, 1996). Based on the theoretical products of meiotic nondisjunction, trisomies and monosomies of each chromosome should occur equally, however monosomies are rarely observed. It is likely that the genetic paucity of whole chromosome monosomic conditions is so severe, that such zygotes are lost even before implantation.

Research on spontaneous abortions and stillbirths has provided a great wealth of knowledge compared to the information available solely from liveborn studies, but still does not provide a complete picture of the reproductive effects of aneuploidy. Spontaneous abortion and liveborn studies cannot ascertain the effects of aneuploidy on pre-implantation nor early gestational losses, and therefore knowledge is limited for the first 6-8 weeks of gestation (Jacobs, 1992). As well, not all spontaneous abortions are ascertained, and tissue culture difficulties also contribute to our incomplete view of this 'iceberg'. In order to observe all the numerical chromosomal abnormalities that arise from meiosis, the products of those cellular divisions must be studied directly.

iii. Gamete studies

Sperm and oocyte research permits the analysis of all meiotic products that

could potentially combine to form a conceptus. When the first gamete studies were conducted, oocytes were much more difficult to obtain than sperm, so male gametes became the specimen of choice. However, genetic study of sperm had special technical challenges as chromatin in male gametes is highly condensed in the sperm head which initially, made direct chromosomal analysis impossible. Eventually, oocytes bacome more readily available for research (but are still relatively rare when compared with the numbers of sperm easily obtained). Oocytes used in research are generally spare or discarded from *in vitro* fertilization (IVF) programmes and therefore the results of this research must be carefully considered as these gametes may not accurately represent 'normal' oocytes.

(1) Sperm

Due to the unsuitable nature of sperm chromatin for direct chromosomal analysis, indirect approaches were originally used to measure aneuploidy frequencies. Differential staining techniques based upon recognized staining variations of specific chromosomes in humans were among the first used in sperm aneuploidy studies (Barlow & Vosa, 1970; Pearson, 1972). Later research utilized direct techniques after the problem of the highly condensed chromatin in the sperm head was overcome by fusing human sperm with hamster oocytes permitting karyotyping of sperm chromosomes (Rudak *et al.*, 1978). Most recently, indirect methods are returning, with chromosome-specific DNA labels being applied to interphase sperm to enumerate chromosomes.

(a) Differential staining

A fluorescent stain specific to the large heterochromatic region of the Y chromosome was used to detect the 'F-body' by (Barlow & Vosa, 1970). Aneuploidy of the Y chromosome was determined by scoring the presence of this fluorescent spot in interphase sperm. A single visible 'body' suggested normal Y chromosome segregation, a sperm without any visible bodies was interpreted to be X bearing, and two fluorescing bodies were interpreted as an indication of Y chromosome nondisjunction. Frequencies of YY disomy reported from this technique were much higher than those noted in karyotype data. The technique was abandoned as unreliable when it was demonstrated that the bodies could be artefactual (Beatty, 1977). Other comparable techniques that stained the secondary constrictions of chromosomes 1 and 9 had similar difficulties (Pearson, 1972).

(b) Karyotype studies

Karyotyping techniques could not be applied directly to sperm due to the extremely condensed nature of sperm chromatin, as chromosomes from male gametes are visible only after fertilization takes place. Rudak *et al.* (1978) first described a technique that allowed direct analysis of sperm chromosomes by fertilizing (*in vitro*) a zona-free golden hamster oocyte with a capacitated human sperm. Following fertilization, the sperm and oocyte DNA condensed, forming pronuclear chromosomes that could be karyotyped. Hamster chromosomes

have distinct banding from those of humans, permitting the sperm chromosomes to be distinguished for analysis. This technique was a great breakthrough, allowing the first direct examination of human sperm chromosomes.

Information collected from karyotypic studies of human sperm have produced valuable information about abnormality frequencies in normal men that can be used as a baseline for comparison with results achieved using different research approaches and for studies of selected groups of men (ex. infertile men, men undergoing medical treatment, or men with specific environmental exposures). As with other study techniques, karyotyping also has its limitations: the volume of data generated, the success of tissue culture, and the quality of metaphase preparations (chromosome spread and banding) play a role in how much information can be gathered. Since a karyotype with a missing chromosome could be the result of a monosomic cell, or be a technical artefact, numerical abnormalities are generally only considered when there is an excess of chromosomes present. A conservative estimation of total aneuploidy in human sperm of 3-4% has been determined by doubling the hyperhaploid frequency (Martin *et al.*, 1991b). Overall chromosomal abnormality estimates in human sperm karyotypes (including aneuploid and structural anomalies) is approximately 10% (Martin *et al.*, 1991b).

Human sperm karyotyping provides detailed information about both structural and numerical abnormalities for all chromosomes, but is technically challenging

and only a handful of laboratories world-wide have been successful in obtaining karyotypes. Technical difficulties include preparations in which chromosomes are clumped together or are too dispersed, making chromosome identification difficult and leading to questionable aneuploidy estimates. In addition, the human / hamster technique is very expensive, time consuming and provides relatively few cells for analysis. Twenty years after the technique was introduced, information from only about 20,000 sperm has been collected however, it remains the 'gold standard' for comparison of other techniques.

(c) Fluorescence *in situ* hybridization (FISH)

More recently a new indirect technique has become available that allows simple, rapid measurement of chromosomal aneuploidy in sperm and generates large amounts of data on numerical abnormalities. FISH analysis was previously used to enumerate chromosomes in lymphocytes and has been adapted for use in sperm. The advantages of this technique include that it: can be used on interphase cells, generates a large amount of data in a short period of time and, is relatively simple and inexpensive. FISH analysis utilizes DNA probes complementary to chromosome specific sequences, which are hybridised to a specimen and viewed with a fluorescence microscope. Under the microscope fluorescent spots of colour can be seen in sperm heads, with each colour representing a different chromosome. Enumeration is carried out by scoring the number of fluorescing spots in each cell using defined criteria. Initially, there was concern about the validity of FISH results in spermatozoa due to previous

difficulties with other indirect methods of detecting aneuploidy (discussed earlier), and because the first FISH research produced aneuploidy frequencies wildly divergent from karyotype data. With refined techniques, more stringent scoring criteria, and the application of multicolour FISH to discern between diploid and disomic cells, improved results were observed. Estimated frequencies of aneuploidy from later FISH studies are very similar to those determined from karyotypes. Martin *et al.* (1993), published data based on FISH analysis with >10,000 sperm scored per sample for each chromosome, and reviewed a number of FISH studies comparing aneuploidy frequencies with those from karyotype data for chromosomes 1, 12, 15, 16, X and Y. The FISH research yielded aneuploidy frequencies comparable to those produced from karyotype research (Martin *et al.*, 1993). The number of cells that can be analysed rapidly and cheaply with FISH makes it a simple, quick and accurate technique for the study of aneuploidy.

FISH analysis was initially very limited in the type of information it provided when compared with karyotype analysis which affords complete information about structural and numerical abnormalities for all of the chromosomes. Initially, FISH was only used to determine numerical chromosomal abnormalities, however, in the last decade a number of researchers have combined multiple FISH probes targeted to different regions of the same chromosome to measure structural abnormalities as well (Van Hummelen *et al.*, 1996; McInnes *et al.*, 1998b; Baumgartner *et al.*, 1999). As with all FISH analyses, the use of this technique

to ascertain structural damage to chromosomes is indirect and requires assumptions.

(2) Oocytes

With the advent of *in vitro* fertilization (IVF), oocytes became more readily available for research, however, oocytes are still scarce so the number studied is relatively low. As most cells available for study have been rejected for IVF use, there are questions about how representative these data are of 'normal' female oocytes. Since most women undergoing IVF are infertile and may be of advanced age, and all of them undergo artificially stimulated ovulation, we may not be able to extrapolate information from these studies to 'normal' female oocytes.

(a) Karyotype studies

Data from oocyte karyotypes is limited as few oocytes are available and useful metaphase spreads can be exceedingly difficult to obtain, making it tough to produce good quality, informative karyotypes. Very often individual chromosomes cannot be identified because oocyte chromosomes are fuzzy and labourious to band, limiting identification to categorization by Denver group alone. Martin *et al.* (1991b), reviewed data collected from 772 karyotyped human oocytes studied by a number of different research groups, and concluded that aneuploidy frequencies were greatest for the D and G group chromosomes.

The overall aneuploidy frequency for oocytes was conservatively estimated (2X hyperhaploidy) to be 18-19% (Martin *et al.*, 1991b). In a similar review that included unpublished work from his research group, Pellestor (1991) compiled oocyte karyotype studies from 1984 - 1989 and calculated aneuploidy frequencies from the 1559 chromosomal complements. Overall, aneuploidy (estimated as 2X hyperhaploidy) was 22.8%, and Denver groups A, C, D, and G displayed significantly more hyperploidy ($p<0.05$) than expected if aneuploidy had been equally distributed among all chromosomes. The G group alone (chromosomes 21 and 22), displayed a highly significant difference between expected and observed frequencies of extra chromosomes. Pellestor notes, that the estimate of 22.8% overall aneuploidy in human oocytes may be an underestimate as it does not take into account errors of the second meiotic division.

(b) Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization studies on oocytes are limited, likely because oocytes are so difficult to acquire that karyotyping is preferred as much more information can be gained from each germ cell. In the last few years, a handful of researchers have attempted to use multi-colour FISH analysis in oocytes and polar bodies to aid in the selection of chromosomally normal oocytes for IVF and intra-cytoplasmic sperm injection (ICSI) (Vollmer *et al.*, 2000; Martini *et al.*, 2000).

(3) Embryos

Similar to the information from oocyte research, data generated from embryo studies must be considered with caution as it may have inflated abnormality frequencies since embryos are generally only available for research after they are prepared for assisted reproduction techniques and deemed unsuitable, or are no longer needed. Generally individuals seeking IVF or ICSI have increased gametic abnormality frequencies and are of advanced age, so embryos donated from such subjects may not produce anomaly estimates representative of the general population. A number of embryo studies have found high aneuploidy frequencies: Jamieson *et al.* (1994) found 24% of 178 diploid embryos to be non-mosaic aneuploid, and Munne *et al.* (1995) found 17.4% of diploid embryos with normal morphology were non-mosaic aneuploid. It is interesting to note that because meiotic nondisjunction theoretically leads to balanced trisomic and monosomic gametes, it has always been assumed that trisomy and monosomy occur in equivalent frequencies. However, monosomies are rarely noted in livebirths, still births or spontaneous abortions. Jamieson *et al.* (1994) noted that of the aneuploid embryos observed, approximately half were hyperploid and half hypoploid, which supports the notion that the two occur in equal frequencies but embryos with missing chromosomes are lost very early in pregnancy.

e. Environmental agents and male mediated effects

Beyond the baseline frequencies of chromosomal abnormalities observed at

various reproductive stages, there is growing concern about chemical and other exposures in our environment and the impact that they can have on transmissible genetic damage. Recently it has been recognized that male exposures can potentially cause heritable damage affecting offspring, and researchers are attempting to identify potential environmental hazards and their mechanisms of action. Garry *et al.* (1996) noted a significantly increased frequency ($p<0.001$) of birth abnormalities in the offspring of licensed pesticide appliers compared to that of the general population, when births in the state of Minnesota between 1989 and 1992 were evaluated (Garry *et al.*, 1996). Curious about the mechanisms of this apparently male mediated reproductive damage, a number of researchers began a collaboration to investigate the potential effects of pesticide exposure on endocrine disruption, spermatotoxicity, germ cell aneuploidy, and structural chromosomal aberrations in somatic cells, to try to determine what role this paternal pesticide exposure played in the observed birth anomalies. The research presented here is one aspect of this investigation and examined the relationship between male pesticide exposure and aneuploidy frequencies in human sperm.

f. Research Plan

In this research project, multi-colour fluorescence *in situ* hybridization was used to measure aneuploidy frequencies in human sperm for chromosomes 13, 21, X and Y. Disomy and diploidy frequencies in the sperm of twenty pesticide

appliers were compared to those of twenty control donors from the same region of Minnesota, to determine if spermatogenesis was adversely affected by exposure to agricultural pesticides.

2. Methods and Materials

a. Media and solutions

Instructions for the preparation of all media and solutions utilized in this study that were produced in the laboratory are available in Appendix A. All commercially available products used have been noted and details are in Appendix B.

b. Donors

Donors were recruited by researchers at the University of Minnesota, and were healthy males with no chronic disease and no chronic use of medication. A total of 144 men volunteered to take part in the study, 95 agreed to donate both semen and blood samples, of which 90 did provide semen samples. Volunteers were interviewed early in 1998 using written questionnaires and telephone surveys, about their past occupational exposure to pesticides, and about their intentions for the use of pesticides in the upcoming growing season (1998). The men were classified as exposed or controls based upon their 'current' exposures for the 1998 season (their expectations for pesticide application that year). Actual exposures were later determined from detailed written application records maintained throughout the season, which were mailed to the researchers in Minnesota upon completion. Subjects in the exposed and control groups were matched for age (within 5 years), cigarette smoking (yes or no) and alcohol consumption (yes or no). From the original 90 semen donors, a subset of 40

were chosen based on 'current' fungicide exposure to participate in the aneuploidy study as fungicides were expected to produce aneuploidy events (Lin & Garry, 2000) Twenty men were classified as exposed based on the expectation of fungicide use in the 1998 growing season and another twenty men who anticipated no fungicide use were classified as control subjects. Some of the men in the control group had never been exposed to pesticides (true controls), while others had some historical exposures (1997 and previous years).

c. Semen samples and analysis

Semen samples were collected in Minnesota during October 1998. On the same morning that the men were scheduled to have blood samples drawn, the semen samples were produced at the subjects' homes, collected in a sterile glass jars and placed in insulated containers for transport to the laboratory field site (within 1 hour of collection). Semen analyses were conducted in Minnesota (in part at the home laboratory, and also at the field site) and included: temperature, pH, motility, velocity, concentration, viability, morphologic and morphometric surveys. Motility and velocity measurements were performed using a computer assisted sperm analysis system (HTM-IVOS, Hamilton-Thorn, Beverly, MA). Whole semen was used for the motility analysis, and for velocity determination. The semen samples were diluted 1:1 with Tyrode's buffer, and if necessary, further diluted to lower concentration to <40 million/mL. Motility was measured in MicroCell (Conception Technologies) chambers. Slides were prepared, and morphologic / morphometric analyses were conducted after returning to the main

laboratory. Sperm concentration was measured using IDENT staining (HTM-IVOS, Hamilton-Thorn, Beverly, MA) in MicroCell (Conception Technology) chambers. Sperm viability was determined by hypoosmotic swelling (HOS) assay. Selected semen characteristics are shown for control (see Table 1) and exposed donors (see Table 2).

Table 1: Control donors semen characteristics: sperm concentration (million/mL), abnormal morphology (%), sperm motility (%) and velocity (μm/sec).

Control ID #	Concentration (million/mL)	Morphologically abnormal sperm (%)	Sperm motility (%)	Sperm velocity (μm/sec)
81	74	83	67	112
182	56	68	13	103
256	44	64	53	100
271	246	68	81	107
518	65	72	32	89
532	215	58	81	85
642	76	58	88	105
883	150	97	74	95
1010	47	76	35	77
1011	177	73	62	110
3002	46	85	59	102
3036	23	87	28	90
3071	101	48	83	108
3415	135	76	80	106
3450	26	64	18	109
3487	82	84	58	88
3518	74	82	63	102
4069	104	60	83	116
4071	167	86	80	99
4072	228	80	78	118
Average	107	73	61	101
Reference*	>20	<70	≥50	

*Reference values from WHO, 1999.

Table 2: Exposed donors semen characteristics: sperm concentration (million/mL), abnormal morphology (%), sperm motility (%) and velocity (µm/sec).

Exposed ID#	Concentration (million/mL)	Morphologically abnormal sperm (%)	Sperm motility	Sperm velocity (µm/sec)
43	67	68	92	77
49	217	59	71	83
62	34	79	32	97
83	68	76	20	83
115	63	88	35	72
208	44	64	65	118
254	96	81	14	104
336	29	81	40	85
398	217	67	92	129
430	170	67	92	119
694	72	47	80	90
820	71	74	74	100
941	43	61	55	103
975	126	68	79	93
1071	54	55	46	100
1096	9	55	12	94
3146	87	63	28	92
3149	67	61	39	92
3272	51	74	38	124
3330	31	80	11	70
Average	81	68	51	96
Reference*	>20	<70	≥50	

*Reference values from WHO, 1999.

d. Semen cryopreservation and shipping

Samples were cryopreserved at the field site for transport to the laboratory in Minneapolis. Semen samples were placed in a graduated conical tube and an equal amount of Ackerman's cryoprotectant was added. The mixture was drawn into 0.5 mL plastic freezing straws, and the open end was tapped in polyvinylpyrollidone (PVP) powder and dipped in water to seal the straw. Straws were left at room temperature 5-10 minutes to equilibrate, and then were lowered in two stages (15 minutes each) into a chamber with liquid nitrogen at the bottom. Following this preparatory cooling, the straws were placed in freezing canes and submerged in liquid nitrogen until all bubbling ceased. Canes were removed from liquid nitrogen and placed in a large liquid nitrogen dewar. For shipment to Calgary the specimen straws were removed from the liquid nitrogen dewar, placed in protective sheaths which were sealed in freezer quality zip lock bags, and submerged in dry ice in cardboard encased styrofoam box. Frozen sample straws were received in Calgary in March 1999, and immediately placed into canes and submerged in liquid nitrogen in a storage dewar. Studies by Martin *et al.* (1991a) have shown that the chromosomal constitution of sperm is not altered by cryopreservation.

e. Ethical approval

Ethical approval for this study was granted by the University of Calgary and the University of Minnesota, and subjects gave informed written consent.

f. Researcher blinding

Each of the 40 samples was coded with a random number at the originating laboratory in Minnesota. Only the sample number, marked on each freezing straw, was used to identify donors during FISH analysis and no further information was made available to our laboratory until the data collection phase was concluded. Following completion of slide scoring, donor information including: exposure status grouping (exposed or control), age, occupation, sperm parameters, and specific details of current and historic exposures were sent to our lab. Lacking foreknowledge of the control / exposed status of donors precluded researcher bias toward an expected result during data collection.

g. Sample preparation

i. Sperm washing

Semen straws were thawed and samples were washed three times in 5mL of 0.01 M Tris / 0.9% NaCl under sterile conditions in a fumehood, then were centrifuged at ~600g for six minutes. (The pH and salt concentration of is 0.01 M Tris / 0.9% NaCl is approximate to physiological levels and is used to prevent osmotic damage to the sperm.) Following the final wash, the pellets were resuspended in 0.1 to 0.5mL of 0.01 M Tris / 0.9% NaCl to bring the sperm concentration to approximately 60 x 10^6 sperm / mL (Ko & Martin, 1999), and stored at 4°C until slides were prepared. Slide preparation generally took place immediately following sperm washing, but occasionally more slides were

required at a later date, and in these cases the suspensions were used to prepare slides several months later.

ii. Slide preparation

One to two µL of the sperm suspension was placed on the centre of a slide (precleaned, superfrost, 25 X 75 X 1 mm slides from VWR, Baie d'Urfe, Quebec). Using the side of a pipette tip, the liquid was spread over a 1 cm^2 area. The slide was allowed to air dry and the location of the sample was indicated by etching the back of the slide with a diamond pen. Slides were stored in slide boxes at room temperature in the dark until they underwent decondensation. Following slide preparation, a minimum of one day wait was necessary before decondensation to allow sperm to adhere to the slides and limit loss of sperm in the decondensation solutions. Prepared slides can be stored at room temperature for long periods of time before they are decondensed, in this study slides were decondensed up to 5 months after preparation.

iii. Sperm decondensation

In general, slides were placed in a 10mM DTT / 0.1M Tris solution for 25 minutes, followed by immersion for 2.5 hours in a solution containing 10mM LIS, 1mM DTT / 0.1M Tris (Pinkel *et al.*, 1986). Sperm heads swell during decondensation, and should enlarge to 1.5 - 2 times the size of an

undecondensed sperm head. It was empirically observed that sperm cells from some donors were more resistant to the decondensation process, and the methodology was modified appropriately. Slides of these donors' sperm were therefore decondensed for 30 min / 3.0 hours or 35 min / 3.5 hours, as necessary. Following decondensation, slides were rinsed briefly in 2XSSC, air dried, and stored in the dark at room temperature until hybridization. Slides could be hybridized immediately after decondensation, and were generally used within a month of being decondensed. In a few rare cases, slides were used up to 8 months after decondensation with good hybridization results.

h. Fluorescence *in situ* hybridization

i. Probes

Five DNA probes were utilized in this study (see Table 3). The autosomal probes for chromosomes 13 and 21 were commercially prepared locus specific probes from Vysis (Downers Grove, IL):

- LSI 13, SpectrumGreen™ - 13q14
  (RB-1 locus)
- LSI 21, SpectrumOrange™ - 21q22.13 - q22.2
  (loci: D21S259, D21S341, D21S342)

Three probes were utilized for sex chromosome analysis: a commercially prepared chromosome enumeration probe (CEP) specific to a sequence near the centromere of the Y chromosome Vysis (Downers Grove, IL), and two probes

prepared in our laboratory from satellite DNA near the centromeres of chromosome 1 (which was used as an internal control) and the X chromosome.

- CEP Y, SpectrumOrange™ -Yq12
  (satellite III, DYZ1 locus)
- X_c, FluoroGreen™
  (∝-satellite)
- pUC1.77, FluoroBlue™
  (satellite III)

Chromosome 1 and X chromosome probes were prepared and available in the laboratory prior to the beginning of this study. The protocol for the preparation of probes for chromosome 1 (pUC177) and X (X_c) is as follows: Probes were prepared from chromosome specific sequences kindly provided by HJ Cooke, Edinburgh; and E Jabs, John Hopkins, respectively. Transformed bacteria containing the plasmid of interest were grown in Luria Broth with ampicillin at 37°C overnight. Following centrifugation, the pellet of bacteria was resuspended and the bacteria lysed to release the plamid DNA. Plasmid DNA was separated using a cesium chloride gradient, and collected from the appropriate fraction with a 21 gauge needle. Extractions with water-saturated n-butanol and dialysis against low TE, followed by ethanol precipitation were used to clean the DNA which was re-dissolved in low TE (Sambrook, 1989). Spectrophotometry (at 260nm and 280nm) was used to measure DNA concentrations. Plasmid DNA was digested with the appropriate restriction enzyme to separate the vector from the chromosome specific sequence, and then run on an agarose gel overnight

with ethidium bromide. The band representing the insert DNA was electroeluted (Maniatis, 1982), and further purified by repeating the above extractions and precipitation. The X probe was labelled with FluoroGreen™ (fluorescein-11-dUTP), and the chromosome 1 probe was labelled with FluoroBlue™ (coumarin-4-dUTP), using the manufacturer's recommended nick translation protocol. (Amersham Pharmacia Biotech, Bais d'Urfé, Quebec, Cat# RPN 2121 and Cat# RPN 2123 respectively).

Table 3: DNA probes used and their sources.

Probe	Source
13q14 (RB-1 locus)	Vysis (Downers Grove, IL)
21q22.13-q22.2 (loci: D21S259, D21S341, D21S342)	Vysis (Downers Grove, IL)
X_c (∝-satellite)	Jabs, et al. 1989
Y (satellite III) (DYZ1 locus)	Vysis (Downers Grove, IL)
pUC1.77 (satellite III)	Cooke, et al. 1979

ii. Hybridization

A mixture of the desired probes (see Table 4) was prepared, heated in a water bath at 70 - 75°C for 5 minutes, then snap-cooled on ice. Sperm slides were denatured in 70% formamide / 2 x SSC (pH 7.5) at 70 - 75°C for 5 minutes, then snap-cooled and dehydrated through a series of two minute immersions in 70%, 85%, and 95% ethanol at -20°C. Slides were then air dried and pre-warmed to

37°C on a slide warmer. Probe mixture was pipetted onto each dried slide and a glass coverslip was applied. The edges of the coverslip were sealed with rubber cement, and slides were incubated in a dark, humid chamber at 37°C for 16 -120 hours. Slides were generally incubated for 16 - 24 hours (Ko & Martin, 1999), but slides for donors that were observed to have poor quality hybridizations (ex. small, faint signals) were incubated for longer periods of time to allow more of the labelled probe to bind to the target sequences.

Table 4: Components and combinations for the probe mixtures used for autosomal and sex chromosome hybridizations.

Probe Mix Components (for a single slide)	Chromosome 13 & 21 Hybridizations	Sex Chromosome Hybridizations
LSI buffer (Vysis)	8 µL	
SpectrumGreen™ 13	1 µL	
SpectrumOrange™ 21	1 µL	

MM 2.1		8 µL
Fluorogreen™ X		1 µL
SpectrumOrange™ Yq		0.3 µL
FluoroBlue™ 1		1 µL

Total Volume (µL)	10 µL	10.3 µL

iii. Post - hybridization

Coverslips were removed and slides underwent one post-hybridization wash in 2 x SSC at 68 - 72°C for 2 minutes. Slides were rinsed in 2XSSC / 0.1% NP - 40 at room temperature, and slides with XY1 probes were counterstained with 0.05µg / mL propidium iodide for 5 seconds at room temperature. Slides were

air dried in the dark, mounted in antifade (XY1 slides) or 50:50 DAPI II / antifade (13/21 slides) and were stored in the dark until scored.

i. Scoring

Slides were scored using either a Zeiss Axiophot or Zeiss Axioplan microscope equipped with four filter sets: an FITC single bandpass filter set, an FITC / rhodamine dual bandpass filter set, a DAPI single bandpass filter set and an FITC / rhodamine / DAPI triple bandpass filter set (Carl Zeiss Canada, North York). The following scoring criteria were used: sperm were scored only if they were intact with a clearly defined border, did not overlap other sperm and were not decondensed to more than twice the size of a non-decondensed sperm. (Over-decondensed sperm can result in signal-splitting due to the diffuse nature of sperm chromatin (Wyrobek *et al.*, 1990). In order to score two same coloured signals in a sperm head as two distinct signals, they were required to be separated by at least 1 signal diameter (domain), and be of the same general size, shape and intensity. Due to the disproportionately large size of the CEP Yq probe signal, an exception to the one domain separation criterion was made, and only one half domain distance was required to count two Yq signals as separate if they met the other scoring criteria.

i. Scoring with the use of multi-colour FISH

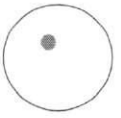
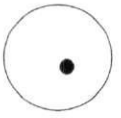
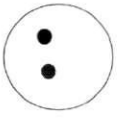
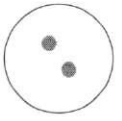
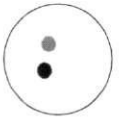
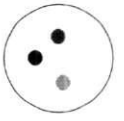
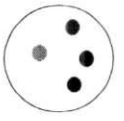
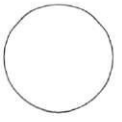
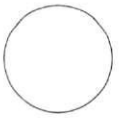
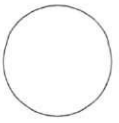
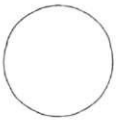
Multi-colour FISH is used to distinguish between disomy and diploidy, nullisomy

and hybridization failure, which single colour FISH cannot do (see Table 5). When scoring autosomes with a single fluorescent probe, a normal cell displays a single signal. Disomy and diploidy cannot be distinguished because both exhibit two signals, and similarly, nullisomy for the chromosome in question and hybridization failure both present with no signals. Scoring of the sex chromosomes with only two different coloured probes (one for the X and a second colour for the Y) leads to similar problems. Disomy and diploidy both show two signals (either two signals of the same colour in cases of XX or YY, or one signal of each colour for XY), while nullisomy and hybridization failure for the sex chromosomes both display no signals in the cell. To distinguish between nullisomy and hybridization failure, and disomy and diploidy, an additional autosomal probe is added to the hybridization mixture for both autosomal and sex chromosome analysis. Studying chromosomes in this manner allows clear determination of numerical abnormalities present.

As can be seen in Table 5 (below), when multi-colour FISH is applied to autosomes: a normal cell will contain one signal of each colour, a disomic cell will contain two signals of one colour and a single signal of the second colour (indicating that only one chromosome is present in two copies). Diploidy is distinct as it exhibits two signals of each colour. Nullisomy will present one signal (either colour) and hybridization failure will present no signals as both probes have failed to bind to the DNA. Using multi-colour FISH to study the sex chromosomes involves three different coloured probes: one for the X

chromosome, another for the Y chromosome, and the third for an autosome. For our study, chromosome 1 was included in the sex chromosome hybridizations as an internal autosomal control. The presence of the third probe allows determination of the numerical abnormality: a normal cell contains one autosomal probe and either a single X or Y signal, a disomic cell displays a single autosomal signal and two sex chromosome signals (either XX, YY, or XY), a diploid cell exhibits two sex chromosomes (XX, YY, or XY) and two autosomal signals, nullisomy for the sex chromosomes shows only an autosomal signal and cells where the hybridization has failed have no signals at all.

Table 5: Demonstration of the necessity of using multi-colour FISH for discerning disomy from diploidy, and nullisomy from hybridization failure.

	Autosomes		Sex Chromosomes	
	One colour FISH	Two colour FISH	2 colour FISH	3 colour FISH
Normal				
Disomy				
Diploidy				
Nullisomy				
Hybridization Failure				

j. Data analysis

Upon completion of scoring, donor status (control or exposed) for each of the samples was received from the originating laboratory. This information was used to combine the data into groups: (1) control donors / autosomes, (2) control donors / sex chromosomes, (3) exposed donors / autosomes, and (4) exposed donors / sex chromosomes. Percentage of sperm carrying X chromosomes was used to determine the sex ratio of the sperm, with an expected ratio of

approximately 50:50. As missing chromosomes can be technical artefacts (the assumption that absence of a signal in the cell indicates the chromosome is not present may not be definitive, as it could be due to poor hybridization or the absence of the probe sequence on the chromosome), the only numerical abnormalities reported in our data were cases of hyperploidy (disomy and diploidy).

i. Statistical analysis

Statistical work was completed primarily by Alfred W. Rademaker, Ph.D. of Northwestern University, Chicago, IL. Donors in the exposed and control groups were compared for age using a two sample t-test, smoking and alcohol consumption using a two-tailed Fisher's exact test, and abnormality frequencies were compared using two-tailed z-tests, adjusting for within person clustering of the data (Donner & Klar, 1994).

3. Results

a. Donors

Donors were originally classified as either control or exposed, based upon their intended exposure to agricultural fungicides during the 1998 growing season. Exposed donors (n=20) were men who reported anticipating exposure to fungicides during the 1998 growing season, and the twenty control donors reported no intended fungicide exposure. Four subjects within the control group had never been occupationally exposed to pesticides, but the remainder did have historical exposures to various pesticides. Age (within 5 years), smoking (yes or no) and consumption of alcoholic beverages (yes or no) were intended to be matched between the two groups. The mean age of donors in exposed (38.75 years) and control (39.10 years) groups was not statistically different ($p>0.05$) and there was no significant difference between the donor groups for cigarette smoking ($p=0.51$), however alcohol consumption was significantly different between the two groups ($p=0.03$), with more drinkers among the exposed subjects. All donors were healthy with no chronic medication use and no chronic disease. The age, occupation, smoking and alcohol consumption (drinking) status for each of the subjects are provided in Table 6 (control donors) and Table 7 (exposed donors).

Table 6: Control donors characteristics: age, occupation, smoking and alcohol consumption (drinking) status.

Control ID #	Age	Occupation	Smoke	Drink
81	42	Machinist	Y	N
182	45	Beet Pulp	Y	Y
256	45	Pesticide Applicator	N	Y
271	50	Pesticide Applicator	N	Y
518	44	Truck Driver / Farmer	N	N
532	45	Food Processing	Y	N
642	30	Burial Vault Installation	Y	Y
883	55	Tractor Driver	Y	N
1010	51	Maintenance	N	N
1011	46	Sunflower Plot Maintenance	Y	N
3002	22	Photographer	N	N
3036	43	Dairy Farmer	N	N
3071	24	Sales	N	Y
3415	20	Law Enforcement	N	N
3450	49	Machinist	N	N
3487	26	Law Enforcement	Y	N
3518	32	Farmer / Beet Processor	N	Y
4069	35	Music Teacher	N	N
4071	54	Clinic / Hospital Maintenance	Y	N
4072	24	Laboratory Technician	Y	Y
Average	39.10			

Table 7: Exposed donors characteristics: age, occupation, smoking and alcohol consumption (drinking) status.

Exposed ID#	Age	Occupation	Smoke	Drink
43	32	Pesticide Applicator	Y	Y
49	**41**	Pesticide Applicator	N	Y
62	53	Pesticide Applicator	N	N
83	38	Pesticide Applicator	N	N
115	45	Pesticide Applicator	N	Y
208	43	Pesticide Applicator	N	Y
254	45	Pesticide Applicator	N	Y
336	29	Pesticide Applicator	N	Y
398	38	Pesticide Applicator	Y	N
430	45	Pesticide Applicator	N	Y
694	27	Pesticide Applicator	N	Y
820	39	Pesticide Applicator	N	Y
941	36	Insurance Sales	N	Y
975	51	Pesticide Applicator	N	Y
1071	43	Student	Y	N
1096	40	Pesticide Applicator	Y	Y
3146	24	Pesticide Applicator	N	Y
3149	26	Pesticide Applicator	Y	Y
3272	25	Pesticide Applicator	N	Y
3330	55	Pesticide Applicator	Y	N
Average	38.75			

b. Exposures

Pesticide exposures were classified as current (for the 1998 growing season) or as historical (1997 and previous years). The application season in 1998 included the use of herbicides from late March to late June, insecticides between the end of May and the beginning of September, and fungicide application in July through to late October. Semen samples were collected from both control and exposed donor groups in October 1998. Chemicals used by our donors were classified as herbicides (H), insecticides (I), and fungicides(G), and noted below in Table 8 (control donors) and Table 9 (exposed donors).

Table 8: Current and historic exposures of control donors. Abbreviations are: herbicides (H), insecticides (I), and fungicides(F).

Control ID #	Current Exposures	Historic Exposures
81		H
182	H	H
256	H	H I F
271	I	H I
518		H I F
532		H I
642		H
883		H
1010		H
1011		H I
3002		H* I*
3036	H	H I
3071		
3415		
3450		H I
3487		H* I* F*
3518		H I* F
4069		
4071		
4072		

*Indicates a exposure reporting discrepancy between written and phone questionnaires. If men indicated exposure to a chemical on either survey, we have included it in their exposure profile.

Table 9: Current and historic exposures of exposed donors. Abbreviations are: herbicides (H), insecticides (I), and fungicides(F).

Exposed ID#	Current Exposures	Historic Exposures
43	F	H F
49	H F	H I F
62	H I F	H I F
83	H I	H I F
115	H F	H I F
208	H I	H I
254	H I F	H I F
336	H F	H I F
398	H	H I
430	H F	H I F
694	H I F	H I F
820	H I F	H I F
941		H I F*
975	H I	H I F
1071		H I F
1096	H	H I F
3146	H	H I F
3149	H F	H I F
3272	H F	H I F
3330	H	H I F

*Indicates a exposure reporting discrepancy between written and phone questionnaires. If men indicated exposure to a chemical on either survey, we have included it in their exposure profile.

c. FISH analysis

Multi-colour FISH analysis was used to determine disomy and diploidy frequencies for chromosomes 13, 21, X and Y, and examples are presented in Figures 2,3 and 4. Two colour FISH for chromosomes 13 and 21 and three colour FISH for the sex chromosomes were utilized to permit distinction between disomy and diploidy, and between nullisomy and lack of hybridization. A minimum of 10,000 sperm were scored for each donor hybridization (ex. 10,000 sperm scored for chromosomes 13 & 21, and another 10,000 scored for the sex chromosomes). A total of 809,935 sperm were scored. Hybridization efficiency was 99% throughout the study. This was determined by randomly selecting slides which were scored normally and in addition were scored for the presence of sperm that had no signals at all. Slides with the greatest number of sperm cells that had failed to hybridize exhibited less than 1% hybridization failure, as fewer than 100 sperm were without signals within the 10,000 counted. FISH data was collected over an eighteen month period, and all scored by the same individual. To determine scoring consistency over time, slides scored early in the project were re-scored at the end to compare the aneuploidy frequencies noted, and no major variances were noted (see Tables 10 & 11).

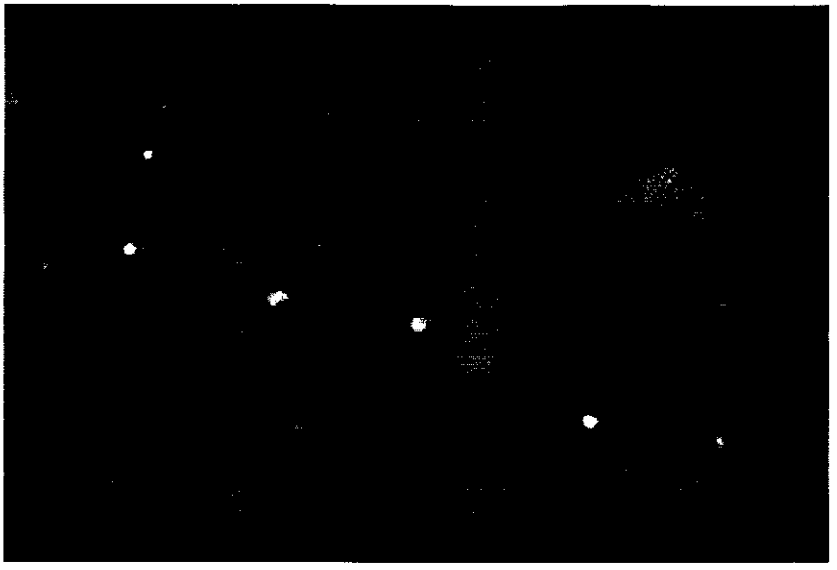


Figure 2: Fluorescence *in situ* hybridization in human sperm for chromosomes X (green), Y (red), and 1 (blue). Two normal X-bearing, and one normal Y bearing sperm are shown.

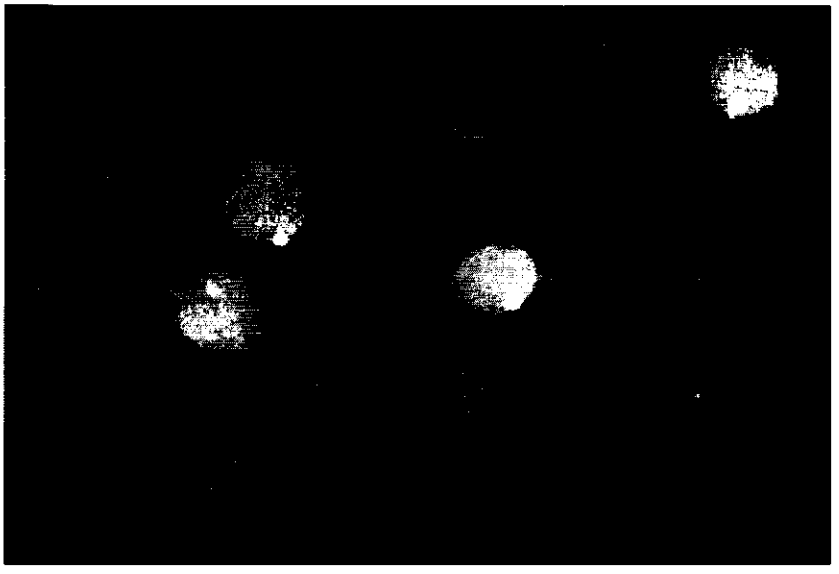


Figure 3: Fluorescence *in situ* hybridization in human sperm for chromosomes 13 (green) and 21 (red). Four normal sperm are shown, each with one chromosome 13 and one chromosome 21.

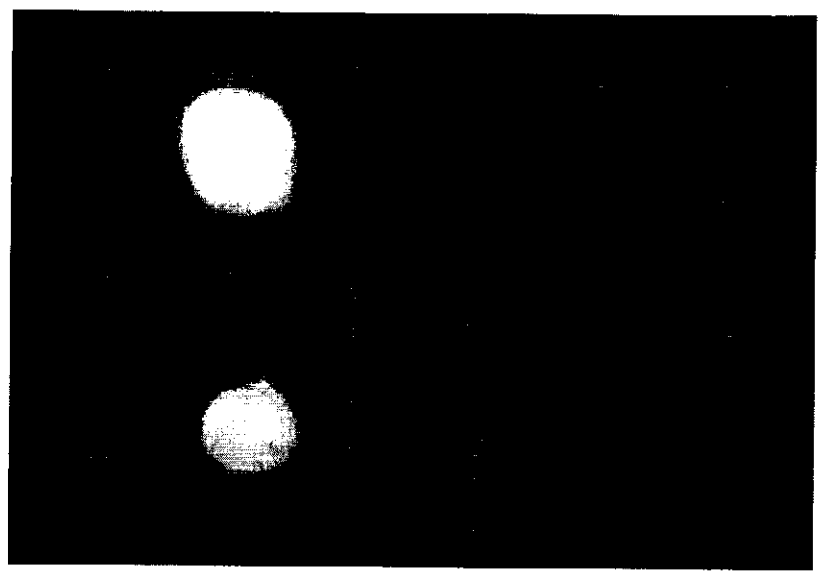


Figure 4: Fluorescence *in situ* hybridization in human sperm for chromosomes 13 (green) and 21 (red). Two normal sperm, and one disomic for chromosome 21 are shown.

Table 10: Comparison of autosomal disomy and diploidy frequencies scored during the experiment with frequencies recorded upon scoring the same slides after completion of the research.

	Slide #398		Slide #975	
	Original	Re-score	Original	Re-score
Disomy 13	0.13%	0.10%	0.17%	0.22%
Disomy 21	0.23%	0.24%	0.28%	0.23%
Diploidy	0.27%	0.32%	0.56%	0.52%

Table 11 : Comparison of sex chromosomal disomy and diploidy frequencies scored during the experiment with frequencies recorded upon scoring the same slides after completion of the research.

	Slide #43		Slide #208	
	Original	Re-score	Original	Re-score
XX	0.07%	0.11%	0.07%	0.08%
YY	0.02%	0.05%	0%	0.03%
XY	0.42%	0.51%	0.72%	0.69%
Dipl	0.17%	0.13%	0.09%	0.09%

d. Assumptions

FISH is an indirect technique because it utilizes the signals of fluorescent probes to indicate the presence of chromosomes, rather than examining the chromosomes directly as is done with karyotyping. This type of analysis involves some basic assumptions, including: a single fluorescent signal represents a single complete chromosome, and the combination of two signals for

chromosome 13 and one for chromosome 21 represents disomy 13. (This signal combination could represent a diploid cell with a lack of hybridization for one chromosome 21, but disomy 13 is more likely.)

e. Data

Individual results for autosomal disomy frequencies in control donors and exposed men are presented in Table 12. There were no significant differences in autosomal aneuploidy frequencies between the exposed and control groups. The mean frequency of disomy for chromosome 13 was 0.43% for control donors and 0.38% for exposed donors (p=0.64). The mean frequencies for chromosome 21 in the two groups were 0.41% and 0.55% respectively (p=0.14). While there are no significant differences between the two populations, chromosome 13 and 21 disomy frequencies in this study are elevated above human sperm aneuploidy frequencies observed in normal men previously studied in our lab. In a study of 18 normal men, disomy frequencies for chromosome 13 (0.13 ± 0.11%) and chromosome 21 (0.37 ± 0.39%) were lower than the percentages of disomy noted in both our donor groups (McInnes *et al.*, 1998a).

Sex chromosome frequencies (see Table 13) showed no statistically significant differences between groups. Control and exposed donors both displayed a mean frequency of 0.05% XX disomy (p=0.78), and while control donors had a slightly higher mean frequency of (0.04%) YY disomy compared to exposed men

(0.03% YY), the difference was not meaningful (p=0.46). The greatest difference between control and exposed donors was the mean frequency of XY disomy with 0.51% and 0.40% respectively, however, this variation between the groups was not significant (p=0.24). Statistical analysis determined no significant differences between disomy frequencies of the control and exposed groups. Sex chromosome disomy frequencies in this study were within the range of values observed in normal men, with the exception of XY disomy, which was elevated compared to previous research in our lab (Kinakin *et al.*, 1997).

Diploidy frequencies (see Table 14) in control and exposed men were not significantly different, with mean frequencies of 1.14% and 0.77% respectively based on autosomal probes (p=0.18), and mean diploidy frequencies were 0.27% for controls, and 0.16% for exposed donors (p=0.18), when estimated by sex chromosome scoring.

i. Significant Findings

The data show no real trend towards increased frequencies of aneuploidy with pesticide exposure. However, a few significant findings were revealed through statistical analysis. Men exposed to pesticides showed an altered sex ratio in their sperm, with a significantly higher percentage of X bearing sperm (see Table 15) compared to the control donors (p=0.04). When the exposed group of men was narrowed down to those exposed specifically to fungicides (n=11), and compared to the controls (n=20), the fungicide exposed men showed a lower

frequency of XY disomy, with borderline significance (p=0.04). No other significant differences were noted.

Control			Exposed		
Donor #	Disomy 13 (%)	Disomy 21(%)	Donor #	Disomy 13 (%)	Disomy 21 (%)
81	57 (0.57)	41 (0.41)	43	62 (0.44)	100 (0.72)
182	21 (0.21)	61 (0.61)	49	9 (0.09)	20 (0.20)
256	31 (0.31)	20 (0.20)	62	43 (0.43)	24 (0.24)
271	71 (0.71)	20 (0.20)	83	17 (0.17)	89 (0.89)
518	104 (1.04)	121 (1.21)	115	12 (0.12)	51 (0.51)
532	11 (0.11)	21 (0.21)	208	7 (0.07)	45 (0.45)
642	50 (0.50)	58 (0.58)	254	18 (0.18)	76 (0.76)
883	20 (0.20)	30 (0.30)	336	32 (0.32)	39 (0.39)
1010	31 (0.31)	65 (0.65)	398	13 (0.13)	23 (0.23)
1011	15 (0.15)	33 (0.33)	430	4 (0.04)	16 (0.16)
3002	34 (0.34)	52 (0.52)	694	88 (0.88)	71 (0.71)
3036	69 (0.69)	39 (0.39)	820	54 (0.54)	142 (1.42)
3071	23 (0.23)	31 (0.31)	941	55 (0.55)	35 (0.35)
3415	69 (0.69)	35 (0.35)	975	17 (0.17)	28 (0.28)
3450	31 (0.31)	28 (0.28)	1071	19 (0.19)	63 (0.63)
3487	53 (0.53)	53 (0.53)	1096	86 (0.86)	40 (0.40)
3518	56 (0.56)	21 (0.21)	3146	18 (0.18)	49 (0.49)
4069	11 (0.11)	24 (0.24)	3149	165 (1.65)	21 (0.21)
4071	81 (0.81)	46 (0.46)	3272	9 (0.09)	58 (0.58)
4072	25 (0.25)	15 (0.15)	3330	55 (0.55)	129 (1.29)
MEAN	43.15 (0.43 ± 0.06)	40.7 (0.41 ± 0.05)	MEAN	38.38 (0.38 ± 0.09)	54.85 (0.55 ± 0.08)

Table 12: Number and percentage (in parentheses) of sperm disomic for chromosomes 13 and 21 in exposed and control populations. Row of mean values includes percentage ± standard error (in parentheses.)

Control				Exposed			
Donor #	XX (%)	YY (%)	XY (%)	Donor #	XX (%)	YY (%)	XY (%)
81	3 (0.03)	4 (0.04)	27 (0.27)	43	7 (0.07)	2 (0.02)	42 0.42
182	6 (0.06)	2 (0.02)	5 (0.05)	49	0 (0.00)	1 (0.01)	14 0.14
256	1 (0.01)	0 (0.00)	47 (0.47)	62	4 (0.03)	4 (0.03)	27 0.23
271	10 (0.10)	5 (0.05)	26 (0.26)	83	1 (0.01)	0 (0.00)	124 1.24
518	5 (0.05)	6 (0.06)	83 (0.83)	115	5 (0.05)	4 (0.04)	14 0.14
532	0 (0.00)	3 (0.03)	43 (0.43)	208	7 (0.07)	0 (0.00)	72 0.72
642	6 (0.06)	2 (0.02)	50 (0.50)	254	5 (0.05)	1 (0.01)	23 0.23
883	0 (0.00)	1 (0.01)	42 (0.42)	336	7 (0.07)	3 (0.03)	53 0.53
1010	21 (0.21)	22 (0.22)	130 (1.30)	398	0 (0.00)	0 (0.00)	17 0.17
1011	0 (0.00)	0 (0.00)	22 (0.22)	430	7 (0.07)	10 (0.10)	24 0.24
3002	4 (0.04)	1 (0.01)	49 (0.49)	694	5 (0.05)	2 (0.02)	19 0.19
3036	5 (0.05)	6 (0.06)	41 (0.41)	820	9 (0.09)	8 (0.08)	35 0.35
3071	5 (0.05)	12 (0.12)	72 (0.72)	941	5 (0.05)	3 (0.03)	33 0.33
3415	0 (0.00)	1 (0.01)	38 (0.38)	975	5 (0.05)	0 (0.00)	23 0.23
3450	19 (0.19)	1 (0.01)	57 (0.57)	1071	7 (0.07)	4 (0.04)	50 0.50
3487	2 (0.02)	2 (0.02)	116 (1.16)	1096	4 (0.04)	1 (0.01)	30 0.30
3518	1 (0.01)	4 (0.04)	58 (0.58)	3146	2 (0.02)	2 (0.02)	32 0.32
4069	8 (0.08)	4 (0.04)	13 (0.13)	3149	10 (0.10)	12 (0.12)	50 0.50
4071	10 (0.10)	4 (0.04)	92 (0.92)	3272	6 (0.06)	6 (0.06)	28 0.28
4072	0 (0.00)	1 (0.01)	15 (0.15)	3330	2 (0.02)	0 (0.00)	96 0.96
MEAN	5.3 (0.05 ± 0.01)	4.05 (0.04 ± 0.01)	51.3 (0.51 ± 0.07)	MEAN	4.85 (0.05 ± 0.01)	3.12 (0.03 ± 0.01)	39.90 (0.40 ± 0.06)

Table 13: Number and percentage (in parentheses) of sperm disomic for sex chromosomes in exposed and control populations. Row of mean values includes percentage ± standard error (in parentheses).

Control			Exposed		
Donor #	Autosomal (%)	Sex Chromosome (%)	Donor #	Autosomal (%)	Sex Chromosome (%)
81	27 (0.37)	4 (0.04)	43	98 (0.70)	17 (0.17)
182	52 (0.52)	7 (0.07)	49	8 (0.08)	5 (0.05)
256	0.71 (71)	19 (0.19)	62	35 (0.35)	7 (0.06)
271	57 (0.57)	5 (0.05)	83	108 (1.07)	12 (0.12)
518	475 (4.54)	20 (0.20)	115	30 (0.30)	7 (0.07)
532	47 (0.47)	15 (0.15)	208	44 (0.44)	9 (0.09)
642	117 (1.16)	46 (0.46)	254	84 (0.83)	28 (0.28)
883	54 (0.54)	19 (0.19)	336	143 (1.41)	28 (0.28)
1010	279 (2.72)	158 (1.56)	398	27 (0.27)	5 (0.05)
1011	80 (0.79)	21 (0.21)	430	39 (0.39)	21 (0.21)
3002	45 (0.45)	5 (0.05)	694	47 (0.47)	4 (0.04)
3036	74 (0.74)	13 (0.13)	820	273 (2. 66)	39 (0.39)
3071	146 (1.44)	45 (0.45)	941	54 (0.54)	6 (0.06)
3415	125 (1.24)	24 (0.24)	975	56 (0.56)	3 (0.03)
3450	53 (0.53)	16 (0.16)	1071	98 (0.97)	33 (0.33)
3487	178 (1.75)	23 (0.23)	1096	32 (0.32)	17 (0.17)
3518	236 (2.31)	90 (0.89)	3146	67 (0.67)	9 (0.09)
4069	30 (0.30)	9 (0.09)	3149	76 (0.76)	28 (0.28)
4071	114 (1.13)	3 (0.03)	3272	64 (0.64)	9 (0.09)
4072	34 (0.34)	3 (0.03)	3330	191 (1.88)	31 (0.31)
MEAN	115.2 (1.14 ± 0.24)	27.25 (0.27 ± 0.08)	MEAN	77.16 (0.77 ± 0.14)	15.74 (0.16 ± 0.03)

Table 14: Number and percentage (in parentheses) of diploid sperm for autosomes (13 & 21) and the sex chromosomes in exposed and control populations. Row of mean values includes percentage ± standard error (in parentheses).

Table 15: Number and percentage (in parentheses) of X bearing sperm.

Control		Exposed	
Donor #	X - bearing (%)	Donor #	X - bearing (%)
81	4750 (47.54)	43	5049 (50.54)
182	4685 (46.90)	49	4954 (49.59)
256	4774 (47.79)	62	5729 (47.79)
271	4794 (47.99)	83	4866 (48.71)
518	4878 (48.82)	115	4854 (48.59)
532	5029 (50.34)	208	4874 (48.79)
642	4897 (49.01)	254	4881 (48.86)
883	4934 (49.39)	336	4778 (47.83)
1010	4667 (46.72)	398	5131 (51.36)
1011	4823 (48.28)	430	4789 (47.94)
3002	4615 (46.20)	694	4902 (49.07)
3036	4578 (45.83)	820	4893 (48.98)
3071	4959 (49.64)	941	4660 (46.65)
3415	4919 (49.24)	975	5010 (50.15)
3450	4521 (45.26)	1071	4978 (49.83)
3487	4557 (45.52)	1096	4936 (49.41)
3518	4865 (48.70)	3146	4810 (48.15)
4069	4878 (48.83)	3149	4731 (47.36)
4071	4828 (48.33)	3272	5054 (50.59)
4072	4971 (49.76)	3330	4910 (49.15)
MEAN	4796 (48.01)	MEAN	4891 (48.95)

4. Discussion

The purpose of this study was to determine if pesticide exposure was associated with increased aneuploidy and diploidy frequencies in human sperm, in an attempt to elucidate the causes of reproductive difficulty observed in men with occupational exposures to pesticides (Garry *et al.*, 1996). When the frequency of birth anomalies among the offspring of pesticide appliers in Minnesota was compared to that of the general population, a highly significant difference was noted ($p<0.001$). Regions of the state which have frequent use of chlorophenoxy herbicides / fungicides show significantly increased frequencies ($p<0.01$) of abnormal births in the winter (corresponding to spring conceptions). Based on these observations, this collaborative study was initiated to determine if aneuploidy plays a role in the increased abnormalities observed.

A number of factors must be taken into consideration when studying sperm aneuploidy and other sources of male reproductive difficulty induced by environmental exposures. The time frame between exposure and semen analysis is very important, because spermatogenesis has several distinct stages, and at each stage the cells have different susceptibilities to chemical damage. Male gametogenesis lasts approximately 65 days and includes mitotic divisions of stem cells, that produce spermatogonia which progress through two rounds of mitosis lasting approximately 16 days in humans. Next, spermatocytes undergo meiosis (~25 days), followed by differentiation of spermatids during which an acrosome develops and cells elongate (~16 days). Testicular sperm continue to

mature (~7days) as they move to the epididymis where they spend an estimated 8-17 days before they are ejaculated or resorbed (Adler, 1996). Therefore, depending upon the type of damage expected, semen samples must be taken at an appropriate interval subsequent to the exposure. Another factor to consider when analysing the results of environmental exposures is the effect of confounding factors such as age, smoking, and alcohol consumption. Significant relationships between paternal age and germ cell aneuploidy have been noted in a number of studies (Lorda-Sanchez *et al.*, 1992; Martin *et al.*, 1995; Robbins *et al.*, 1995; and Griffin *et al.*, 1995) which all demonstrate increasing sex chromosome disomy with increasing paternal age. Cigarette smoking and alcohol consumption are also significantly associated with sperm aneuploidy (Robbins *et al.*, 1997b; Rubes *et al.*, 1998; Harkonen *et al.*, 1999) which is discussed later in more detail. Maternal exposures are also a confounding factor for aneuploidy studies if fertility or affected births are used as a measure of damage caused by paternal exposure, as chemical exposures before pregnancy can influence aneuploidy in oocytes (Yin *et al.*, 1998a; Yin *et al.*, 1998b; Marchetti *et al.*, 1996; Eichenlaub-Ritter *et al.*, 1996 and 1995), and maternal exposures during pregnancy can affect birth anomalies (Cziezel *et al.*, 1993) independent of paternal contributions.

A small number of studies have been done specifically on the effects of pesticide exposure on human male reproduction, and even fewer have looked particularly

at germ cell aneuploidy. Related research includes: occupational exposures and lifestyle factors such as smoking and alcohol consumption, as well as the many chemicals used in medical therapy and their effects on semen quality, fertility and germ cell aneuploidy. Human studies have inherent limitations as researchers cannot expose people to dangerous substances for obvious ethical reasons, so studies are necessarily limited to the effects of accidental exposures, and the observation of groups of people for the affects of exposures to potentially dangerous substances in high concentrations or for long periods of time (ex. workplace exposures). These studies do not necessarily have clearly defined exposed and control populations, and therefore many confounding factors can be present. Animal studies of a specific exposure are a useful supplement to human studies since exposure can be properly controlled to be the only variable parameter. In addition, animals studies provide the opportunity to delve deeper into the mechanisms of how a specific chemical acts within the tissue or cell type of interest. While these studies can be very informative, care must be taken in drawing parallels between human and animal exposure outcomes as they are comparable in some instances, but cannot always be assumed equivalent.

a. Occupational exposures

i. Human research

(1) Semen quality

In a study of human semen quality, De Celis *et al.* (2000) noted significantly increased frequencies of abnormalities in the semen of workers occupationally exposed to hydrocarbons in a rubber factory. In a comparison of 48 exposed workers and 42 controls, significant differences were noted in a number of semen parameters, with exposed workers displaying increased viscosity ($p=0.01$), poor or absent liquefaction ($p=0.002$), oligospermia ($p<0.001$), asthenozoospermia ($p<0.001$), and teratozoospermia ($p<0.001$). While it was not possible to quantify the specifics of the men's exposures, a number of hydrocarbons were observed in air quality tests and the men's altered sperm parameters were attributed to their exposures to these chemicals (De Celis *et al.*, 2000).

Similarly, male workers in lead acid battery factory in South Africa were studied by Robins *et al.* (1997) for effects of lead exposure on semen quality. Ninety-seven men took part in the semen analysis, which demonstrated a significant relationship ($p=0.01$) between abnormal sperm morphology (<5% normal morphology) and cumulative blood lead levels (years of exposure × µg/dL). The concentration of lead present in the semen (µg/dL) was significantly associated with decreased sperm density (<20 million/mL). Although no significant associations were observed between the measured lead exposure parameters and motility or total sperm count, the results of this study indicate a detrimental effect on sperm morphology and semen concentration with occupational exposure to lead (Robins *et al.*, 1997).

(2) Fertility

In other research on occupational exposures, lead and other heavy metals was linked to male infertility by a study conducted in Finland (Sallmen *et al.*, 2000). In this retrospective study, 4146 male subjects employed in a number of industries where they are subjected to heavy metals (ex. lead smelting, car radiator repair, metal scraping, painting, glass manufacturing, etc.) were assessed for occupation exposure and categorized by blood lead levels. Blood samples had been taken during employment by the Finnish Institute of Occupational Health for the purpose of biological monitoring for exposure to inorganic lead: 681 subjects had low level or no exposure (0.0 - 0.4 μmol/L), while the remaining 3465 men had exposure levels ≥0.5 μmol/L and were divided into five groups based on their blood lead levels (0.5-0.9, 1.0-1.4, 1.5-1.9, 2.0-2.4, ≥2.5 μmol/L). First marital pregnancies were utilized as a measure of fertility, and in couples where the men were not occupationally exposed to lead 21.3% of couples had no pregnancies, while in men with various levels of lead exposure the percentage of couples with no pregnancies varied from 27 - 35% in the five exposed groups (26.8, 27.8, 29.8, 29.5, 35.4%) which corresponded to relative risks of not bearing children of 1.27 (confidence interval 1.08-1.51), 1.35 (1.12-1.63), 1.37 (1.08-1.72), 1.50 (1.08-2.02) and 1.90 (1.30-2.59) (Sallmen *et al.*, 2000). The authors did not mention the statistical significance of this data, but did point to the relationship of increasing infertility risks to increasing levels of lead in the men's blood samples.

Employees in the Italian mint are subjected to a variety of metal fumes and solvents day to day, and the effect of these exposures on their reproductive health was evaluated by Figa-Talamanca *et al.* (2000), who compared reproductive histories and time to pregnancy (TTP) of the mint's workers with the administrative staff (with no occupational exposures). Of the 302 workers employed at the mint, 167 took part in the study and were employed in either administrative, technical, stamper or founder positions. Air concentrations of metals and solvents were determined from reports of environmental monitoring conducted at the factory since 1980, and reproductive history was collected by interviews with the male employees. The results of this study demonstrated increased TTP among production workers when compared to administration staff. Relative risk estimates of delayed TTP (> 6 months) of production workers compared to administration were: technical 2.58 (95% CI 0.51-13.09), stampers 3.03 (95% CI 0.40-23.04), founders 2.19 (95% CI 0.34-14.20), after controlling for maternal and paternal age, smoking, alcohol consumption, pregnancy planning and father's education level. While no outcomes were significant (as the small number of participants in the study limited its statistical power), it should be noted that all exposed men reported seeking medical advice for infertility more frequently and had longer TTP than unexposed workers. Figa-Talamanca *et al.* (2000), believe that their findings are not incompatible with the idea that heavy metal fumes and solvents cause fertility problems for exposed men.

(3) Spontaneous abortions / Live births

Figa-Talamanca *et al.* (2000) also reported an increase in frequency of spontaneous abortions among exposed workers at the Italian mine compared with administrative controls. The percentage of spontaneous abortions (spontaneous abortions / total pregnancies) for each job set was: administrative 13.3%, technical 16.5%, stampers 19.6% and founders 13.8%. Again, comparisons between the employment groups did not yield any statistically significant differences due to the small sample size, but the frequencies appear to be elevated in all production workers, with larger increases in technical and stamper positions. This data agrees with results of research on workers in a pharmaceutical factory who showed significantly increased frequencies of spontaneous abortions and significantly decreased live birth frequencies.

Prasad *et al.* (1996), examined the reproductive performance of 300 men who worked manufacturing sulfonamide medications, and compared them to 360 men from the same socioeconomic group not exposed to radiation, toxic chemicals or drugs. To control for the potentially confounding effects of cigarette smoking, the control and exposed groups were analysed in two parts: smokers and non-smokers. In exposed men, the frequencies of both live births and spontaneous abortions were significantly different ($p<0.05$) from the controls, with decreased live births and increased spontaneous abortions observed in both smoking and non-smoking comparisons (Prasad *et al.*, 1996).

(4) Birth abnormalities

Blatter *et al.* (1997) conducted a study of the relationship between paternal occupational exposures around the time of conception and the occurrence of spina bifida in offspring. Information was collected via postal questionnaires and telephone interviews, and focussed on the time period ranging from three months before until one month after conception. Exposures to the fathers of children born with spina bifida between 1980 and 1992 (n=222) were compared with the fathers of 764 children born in the same regions and in municipalities of a comparable size, who were born without spina bifida. Statistically significant associations were noted for men with low exposure to welding fumes (odds ratio (OR) = 1.6; 95% confidence intervals (CI) = 1.0-2.6), and low exposure to UV radiation during welding (OR = 2.6; 95% CI = 1.2-5.6). However, it was noted that this was primarily due to other professions using welding equipment (ex. painters, printers, plastic and paper workers), as less of these men used protective equipment (46%) than professional welders (86%).

Birth defects brought about by exposures during military service were a source of great debate following the Vietnam war when veterans suffered a number of aliments that they felt were associated with their service time in Vietnam. "Agent Orange" (a herbicide mixture used to kill vegetation and expose enemy armies) was an exposure that many veterans felt had lead to birth anomalies in their children. Erickson *et al.* (1984) published research that did not support or deny the veterans' claims. Results of the study noted no increased risk of Vietnam

veterans fathering children with birth anomalies ($p>0.05$). Men who were analysed independently based upon their greater opportunities for exposure to Agent Orange also showed no increased risk of having babies with all types of birth defects combined ($p>0.05$), however there were a few specific defects (spina bifida, coloboma, cleft lip without cleft palate, and unspecified neoplasms) that had elevated risks ($p<0.05$). Erickson *et al.* (1984) were careful to explain that while these elevated risks may be a result of exposures during the Vietnam war, they could also be due to some other unidentified risk or merely a chance event. This type of indeterminate finding is a drawback associated with epidemiological studies. In order to determine more about the specifics of exposures to occupational chemicals, we turn to animal studies to add to our knowledge.

ii. Animal research

(1) Sperm aneuploidy

Acrylamide (AA) is an industrial chemical widely used in sewage and waste water treatment, the paper industry, and in research labs, among others (Gutierrez-Espeleta *et al.*, 1992). When male mice are exposed to this chemical, various types of reproductive damage have been observed. Acrylamide was tested by Schmid *et al.* (1999) using multi-colour FISH to determine induction of aneuploidy in mouse sperm cells. Male mice were treated with a singe intraperitoneal injection of acrylamide (60 or 120 mg/kg) and sacrificed 22 days

later. Multi-colour FISH analysis of epididymal sperm revealed that male exposure, to either concentration, of acrylamide did not cause significant increases in aneuploid or diploid mouse germ cells.

(2) Structural chromosomal aberrations

In a study of physical DNA damage in mouse embryos following paternal exposure, Marchetti *et al.* (1997) treated male mice with five consecutive daily doses of AA (50 mg/kg body weight), and mated them with untreated females at eight time periods following treatment to cause fertilization with sperm that were affected by the chemical at various specific stages of development. The greatest chromosomal damage with AA treatment appeared in zygotes fertilized with spermatozoa that were immature when the AA treatment occurred (mated 6.5 days after the AA doses), where 76% of zygotes had damage ($p<0.01$). Chromosomal aberrations were analyzed using four DNA painting probes, and included both balanced and unbalanced exchanges which were induced in equivalent frequencies with the exception of the mice mated 9.5 days after treatment (corresponding to elongated spermatids) where significantly more ($p<0.02$) unbalanced exchanges were noted.

(3) Embryonic abnormalities

Titenko-Holland *et al.* (1998) treated male mice with AA (50 mg/kg for 5 days) and mated them with untreated females 5-17 days following treatment. Analysis

of the embryos produced following paternal AA treatment demonstrated significantly increased abnormalities compared to control embryos. The abnormalities observed included: single-cell embryos ($p<0.001$), embryos with <10 cells ($p<0.001$) and embryos with at least one lysed blastomere ($p<0.001$). Analysis of micronuclei in all embryos showed more micronuclei present in embryos from the treated group than the control ($p<0.05$), and in morphologically normal embryos this association was stronger ($p<0.01$). Nuclei of the embryonic cells were also effected by AA treatment as morphologically normal embryos had smaller nuclei in the treated group than in the controls ($p<0.05$), and in abnormal embryos the treated cases had fewer cells which had considerably larger nuclei than in the controls ($p<0.001$). From these studies it is obvious that paternal acrylamide exposure has seriously deleterious effect on reproductive function.

Occupational exposures including those outlined above are of great concern as people can be exposed to very high levels of dangerous chemicals in their workplace which can have a detrimental effect on their reproduction. Another area of concern involves chemicals that people voluntarily expose themselves to, such as cigarette smoking and consumption of alcohol and caffeine. While there are recognized risks associated with these lifestyle choices, many people still choose to continue these activities despite our growing knowledge of their potential harm. Recent studies on human reproduction are adding to our knowledge about these lifestyle exposures, allowing all of us to make more informed choices.

b. Lifestyle factors

i. Human research

(1) Sperm parameters

In research looking at smoking, caffeine and alcohol consumption, Vine *et al.* (1997) studied semen parameters including: sperm density, semen volume, motility, morphology, sperm nuclear size, shape and chromatin structure, in 88 male volunteers. All study participants were healthy Caucasian males between 18 and 35 years of age. Using a multivariate analysis of covariance, Vine *et al.* (1997) found a weak association between altered semen parameters and caffeine intake, but there were no other significant associations between the tested lifestyle factors and sperm morphometry found in this study.

In contrast, Rubes *et al.* (1998) noted significantly altered semen characteristics associated with cigarette smoking. Semen analysis was conducted in part by technicians, and in part by computer aided analysis. There is no comment about the number of technicians who scored the samples, or of observers being blinded to exposure status. Significant changes in semen quality observed were: decreased sperm motility in smokers ($p<0.02$), and an increased frequency of abnormal forms in the semen of smokers ($p=0.01$) when compared to non-smokers, suggesting that exogenous chemicals can alter human sperm parameters.

(2) Sperm aneuploidy

Robbins *et al.* (1997b) noted a significant relationship between smoking, the consumption of caffeine and alcohol with increased frequencies of aneuploidy in male gametes. Coffee drinkers showed a distinctly linear relationship of caffeine intake with increasing aneuploidy frequencies: XX18 ($p=0.04$), XY18 ($p=0.0002$), YY18-18 ($p=0.03$), and XY18-18 ($p=0.001$). Alcohol consumption also displayed a significant linear relationship with chromosomal abnormalities: XX18 ($p=0.04$), XX18-18 ($p=0.014$), XY18-18 ($p=0.0002$). Cigarette smoking was evaluated by the presence of a nicotine metabolite (cotinine) in the urine, which was significantly associated ($p=0.05$) with XX18 sperm when age and alcohol consumption were controlled for. The results of this research by Robbins *et al.* (1997b), show a very strong relationship between these specific environmental exposures and altered chromosome complements in human sperm, suggesting potential harm for the offspring of men exposed to these chemicals.

The results of Rubes *et al.* (1998) support this connection between cigarette smoking and sperm aneuploidy as they too found a statistically significant relationship. The semen of 25 men (10 heavy smokers and 15 non-smokers) of the same age (18 years) was studied for sperm aneuploidy. Significantly increased frequencies of Y disomy ($p<0.001$) were found in the sperm of smokers, and overall aneuploidy (including chromosomes 8, X and Y) also demonstrated statistically significant increases ($p<0.01$).

In a study of pesticide exposure and sperm aneuploidy, Harkonen *et al.* (1999) inadvertently observed a relationship between smoking and sperm aneuploidy. Semen from 32 Danish farmers was analysed for aneuploidy of chromosomes 1 and 7 before and after exposure to fungicides. The men were divided into smoking and non-smoking groups and aneuploidy frequencies before pesticide exposure displayed a significant increase in chromosome 1 disomy ($p=0.02$).

Smoking was also significantly related to increased frequencies of sperm disomy in the results of a study by Shi *et al.* (2001a) comparing 21 smokers and 10 non-smokers in China. All subjects were healthy, did not consume alcohol, and there were no statistically significant differences in age or semen parameters between the two groups. Chromosomes 13, 21 X and Y were analysed by multi-colour FISH. Results showed a significant increase in chromosome 13 aneuploidy in the sperm of both light smokers ($p=0.01$), and heavy smokers ($p=0.001$) when compared to non-smokers, but no significant differences were noted in the aneuploidy frequencies of chromosome 21, or the sex chromosomes (Shi *et al.*, 2001a).

(3) Spontaneous abortions / Live births

In a study looking at spontaneous abortion and live birth frequencies in men occupationally exposed to pharmaceuticals, Prasad *et al.* (1996) also noted a significant difference between smokers and non-smokers in their control group.

Reproductive performance of the wives of 360 men not exposed to chemicals in their workplace, were divided into smoking and non-smoking groups (each n=180). Spontaneous abortions were significantly more frequent ($p<0.05$) among smokers, as was a significant decrease in live birth frequencies ($p<0.05$) when compared to the non-smokers in the control group (Prasad *et al.*, 1996).

However, two other studies (Windham *et al.*, 1992; Chatenoud *et al.*, 1998), both looking at maternal and paternal smoking noted that a mother's smoking was associated with increased risk of pregnancy loss, while a father's smoking had no significant relationship to spontaneous abortion frequencies in either study.

ii. Animal studies

(1) Live births

Bielawski and Abel (1997) orally dosed male rats with alcohol (6, 4, 2 and 0 g/kg), and after eight hours mated them with untreated females for up to 7 days after intubation. They repeated the experiment on two separate occasions, and noted two distinct effects of paternal alcohol consumption on the live born offspring in both trials. With single doses of alcohol, there were significant dosage related increases in the number of runts in the litters ($p<0.003$; $p<0.01$), and a significant linear association between increasing alcohol dose and malformation frequencies in the offspring ($p<0.02$; $p<0.001$). Decreased litter size ($p<0.03$) was also significantly connected to paternal alcohol treatment in

the first run of the experiment, but not the second, and as the result was not repeated they did not consider it a strong association.

Pollard & Smallshaw (1988), noted significant effects on two generations from ingestion of large amounts of caffeine. Male rats were treated daily with 30 mg/kg of caffeine (the equivalent of 10-12 cups of brewed coffee) by oral gavage for a minimum of 15 days before mating with naive females. In the F1 generation, both sexes of offspring sired by the treated rats had significantly decreased birth weight ($p<0.001$), and significantly increased mortality during the second week of life ($p<0.01$). A second generation was produced by randomly choosing male and female F1 progeny from control and treated males, and mating them with controls from the F0 generation (not their own parents). The outcomes of the F2 generation included: significantly increased birth weights for both sexes when F1 females sired by treated males were backcrossed with F0 control males, and for the male breeding line (F1 males backcrossed with F0 control females) the caffeine group displayed significant percentages of F2 litters that were aborted (Pollard & Smallshaw, 1988).

c. Medical exposures

i. Human studies

(1) Sperm aneuploidy

Results of karyotype and FISH analysis on four testicular cancer patients whose

sperm were analysed for aneuploidy before and 2-13 years after BEP (bleomycin, etoposide and cisplatin) chemotherapy (CT), were published by Martin (1998). Using the human sperm / hamster oocyte technique, 788 karyotypes were analysed (236 before and 552 following CT treatment). No significant differences in chromosomal abnormalities or numerical abnormalities were noted in the comparison. Previous analysis of the same men using multi-colour FISH for chromosomes 1, 12, X and Y yielded comparable results, and none of the frequencies were significantly different from 10 normal control donors (Martin, 1998).

Another study from the same research group with analysis conducted at different times relative to CT treatment yielded different results. In this research, semen samples were analysed by multicolour FISH before, during, and after CT treatment. The study initially included four testicular cancer patients, but due to the treatment regimen three of the four men had no sperm available for analysis during the treatment. Sperm were analysed for the remaining subject before, during (56 days after beginning CT), and after (366 days after beginning CT) treatment. A significant increase in sex chromosome aneuploidy was noted during and post-CT compared to pre-CT aneuploidy frequencies, and a significantly increased frequency of disomy was noted post-CT when compared with pre-CT and during CT values (Martin *et al.*, 1999). The authors suggest that the effects of this particular CT treatment may cause only short term effects on developing sperm and not influence the spermatogonial stem cells, so

aneuploidy frequencies are only increased during and for a limited period following the chemotherapy. This would not have been noted by the first study as only pre-CT and post-CT analyses were performed, and the post-CT time frame was between 2-13 years. As spermatogenesis only lasts ~65 days in humans (Adler, 1996) so no affected sperm would be present 2 years after treatment had ceased.

Robbins *et al.* (1997a) noted a highly significant association between chemotherapy (CT) and sperm aneuploidy in eight male Hodgkin's Disease patients. The patients received NOVP treatment (Novanthrone, Oncovin, Vinblastine, Prednisone), and their sperm was evaluated before, during and after CT treatment. Robbins *et al.* (1997a) reported a 5 fold increase in disomy and diploidy with errors occurring at MI and MII. Significant differences were noted between pre-CT and during-CT frequencies of XX8 ($p<0.05$), XX88 ($p=0.02$). And while the abnormality frequency was very high during treatment, the levels slowly tailed off with the passing of time after the CT was complete (Robbins *et al.*, 1997a).

ii. Animal studies

(1) Sperm aneuploidy

Marchetti *et al.* (2001) tested a drug used routinely in cancer therapy for induction of aneuploidy in mouse spermatozoa. Etoposide was given by

intraperitoneal injection to male mice at doses comparable for the range received in humans for CT. Control mice received identical injections without the etoposide. Sperm from treated and control mice was harvested 6, 16, 40, 64 hours and 10 days after injection. Numerical abnormalities were increased in sperm of the treated mice compared to controls, with diploidy significantly increased ($p<0.01$) in 16, 40 and 64 hour harvest times. Treated mice were mated with untreated females at various times post treatment to promote fertilization by sperm affected at different stages of development: 6.5 days (early spermatozoa), 24.5 days (pachytene spermatocytes), 34.5 days (preleptotene spermatocytes), and 41.5 days after etoposide treatment (differentiating spermatogonia). To determine if these abnormalities were transmitted, zygotes were analysed for hyperploidy and a significant increase was observed for 24.5 and 34.5 day mating times ($p<0.05$). These results indicated that etoposide induces aneuploidy in germ cells undergoing meiosis, and these numerical abnormalities can be transmitted to the progeny (Marchetti *et al.*, 2001).

In another study, Shi *et al.* (1999) orally treated male mice with griseofulvin (an oral antibiotic fungicide) at various doses (500, 1000 and 2000 mg/kg). Samples were taken from the testes 22 hours following treatment and FISH analysis was conducted on the sperm using probes for chromosomes 8, X and Y. Hyperhaploidy frequencies were significantly increased in the sperm of treated mice at all dosages, but aneuploidy frequencies were not dosage related: 500 mg/kg ($p<0.01$), 1000 mg/kg ($p<0.05$), 2000mg/kg ($p<0.01$). Frequencies of

diploidy sperm were significantly increased at all doses, and demonstrated a dosage response with a p-value of $p<0.01$ for the two higher dosages (Shi *et al.*, 1999).

Two compounds used for medical treatment (diazepam - active ingredient in Valium; thiabendazole - anthelminthic) were tested by multi-colour FISH to determine induction of aneuploidy in mouse sperm cells. Schmid *et al.* (1999) orally treated male mice for 11 consecutive days with diazepam (75, 150, or 300 mg/kg), or thiabendazole (100 or 300 mg/kg). Males were sacrificed 22 days following treatment, and sperm was harvested from the epididymis. Multi-colour FISH analysis revealed that male exposure to diazepam (300 mg/kg) induced significant elevations in aneuploidy ($p<0.05$), and diploidy ($p<0.05$) frequencies in mouse germ cells. While, thiabendazole treatment did not induce an increase in aneuploidy, at the 300 mg/kg dose it did produce a significant rise in the frequency of diploid sperm ($p<0.05$).

(2) Sperm DNA damage

Intraperitoneal injection of etoposide in male mice (previously discussed in more detail) and subsequent analysis of sperm DNA by Marchetti *et al.* (2001) showed significantly increased structural chromosomal aberrations($p<0.01$) in MI and MII metaphases from treated mice compared to controls. Mating of treated male mice with untreated females produced zygotes with significantly increased

frequencies of structural abnormalities corresponding to: pachytene spermatocytes ($p<0.001$), and preleptotene spermatocytes ($p<0.001$). Chromosome aberration frequencies were not significantly increased in early spermatozoa or differentiating spermatogonia. Results of this study indicate that etoposide induces damage primarily in germ cells undergoing meiosis, causing structural abnormalities that can be transmitted to the progeny.

Another study by Harrouk *et al.* (2000) measured DNA damage in sperm following treatment with the anti-cancer agent, cyclophosphamide. Male rats were treated daily with saline or 6 mg/kg cyclophosphamide for 4-6 weeks. Following treatment, the exposed males were mated with untreated females. The females were sacrificed on day 0 of pregnancy to permit collection of a single-celled zygote. A significant increase in DNA damage was noted with strand breaks observed in much higher frequencies ($p<0.001$) in zygotes produced by treated males, compared to controls. Specifics of the DNA damage were determined by utilising antisense RNA to determine levels of transcription of specific genes in 1-8 cell zygotes. Four DNA repair gene families were analysed: NER (nucleotide excision repair), MMR (mismatch repair), RCR (recombination repair) and BER (base excision repair). Expression profiles of all four families were altered in cyclophosphamide exposed subjects, with members of the NER, MMR, and RCR families showing increased transcripts compared to controls, while BER and other RCR family members displayed decreased transcription in exposed subjects vs. controls. From this research, Harrouk *et al.*

(2000) demonstrate the presence of DNA damage, and suggest its location within the genome by measuring transcription levels in early rat zygotes.

(3) Sperm apoptosis

We have looked at various types of sperm abnormalities and genetic damage caused by environmental exposures. In cases of extreme exposure, severely damaged sperm are eliminated by apoptosis to limit the chances of fertilization by damaged germ cells. Sjoblom *et al.* (1998), injected male rats intraperitoneally with one of two anti-cancer agents - etoposide (10 mg/kg) and adriamycin (5mg/kg), and observed tissue sections of the rats' testes harvested 2, 4, 6, 18, 24 and 48 hours after their treatment. They noted DNA damage by analysing the frequency of apoptotic cells at various points in germ cell differentiation. In male rats treated with etoposide, significant increases in the frequency of apoptotic cells were observed 18, 24 and 48 hours after injection in several developmental stages including: A_4 spermatogonia, intermediate spermatogonia, B spermatogonia, preleptotene spermatocytes, pachytene spermatocytes and meiotic spermatocytes, ($p<0.01$ for all types). Adriamycin treated rats did not display the same degree of apoptotic response, but did display increased apoptosis 18 hours after treatment in leptotene spermatocytes ($p<0.01$), 24 hours after treatment in intermediate spermatogonia and pachytene spermatocytes ($p<0.05$), and in A_4 spermatogonia and pachytene spermatocytes 48 hours after treatment ($p<0.01$) (Sjoblom *et al.,* 1998).

Another anti-cancer treatment, cisplatin, was tested by Zhang *et al.* (2001) to determine if it increased apoptosis in mouse sperm. Mice in the treatment group were injected intraperitoneally with 1, 5 or 10 mg/kg of cisplatin and controls were injected in the same manner with saline alone. At 1, 3 and 7 days post treatment, four mice from each group were sacrificed and both testes removed. Histological examination of paraffin-embedded sections was used to detect apoptotic cells and statistically significant differences were observed both between the control and treated groups, and within the treated group itself. Apoptotic indices (AI) were significantly different between the control mice and all cisplatin doses for all three time periods ($p<0.0001$). Within the exposed mice, there were statistically significant differences between AIs in each of the dosage groups analysed on day 7 ($p<0.005$) and at the highest treatment dosage (10 mg/kg) there were significant differences between AI scores for testes analysed after each of the three time periods ($p<0.0001$).

(4) Birth anomalies

Jenkinson & Anderson (1990) analysed the fetuses sired by male rats treated with cyclophosphamide to determine if paternal exposure to this anti-cancer compound effects offspring. Daily doses of 3.5 mg/kg were given for the first four weeks, and then raised to 5.1 mg/kg, with doses 7 days per week up to week 12 and 5 days per week afterward. Males were caged with virgin females on weeks 1-11, 21 and 30, and following mating, pregnant females were sacrificed on day 20 of pregnancy. Cyclophosphamide treatment resulted in a

significant increase in pre-implantation loss ($p<0.01$), a highly significant increase in the percentage of runts and gross abnormalities per litter ($p<0.001$). Abnormal fetuses were primarily noted for mating times corresponding to sperm first exposed to cyclophosphamide in meiotic or pre-meiotic stages of development (Jenkinson & Anderson, 1990).

d. Pesticide exposures

i. Humans

(1) Fertility

In 1977, Whorton *et al.* proposed a connection between exposure to pesticides and male factor infertility. Over half of the 25 non-vasectomized employees of a California pesticide factory demonstrated infertility due to oligo- or azoospermia, and the men's sperm counts were significantly associated inversely with their years of employment at the factory ($p<0.001$). Quantification of the men's chemical exposures was not possible, but the authors speculated that exposure to a nematocide produced at the factory. 1,2-dibromo-3-chloropropane (DBCP) was known to cause sterility in animals (Torkelson *et al.*, 1961), and was thought to be responsible for the severely reduced sperm counts observed in these men (Whorton *et al.*, 1977).

Thonneau *et al.* (1999) studied male agricultural workers from France and Denmark who were exposed to pesticides in the calendar year before the birth of

their youngest child (n=589), and compared time to pregnancy (TTP) with men from the same regions who were not exposed to pesticides (n=343). Adjusted fecundability ratios were not significantly different from one for any of the groups analysed, therefore Thonneau *et al.* (1999) concluded that there was no effect of pesticide exposure on the fertility of the men studied.

(2) Birth anomalies

A few years later, the relationship between paternal pesticide exposure and birth defects was analysed by a study in New Zealand. Smith *et al.* (1982), considered rates of birth defects and pregnancy loss over an 11 year period among men who were professional pesticide sprayers and used 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and compared to a control group not exposed to the pesticide. Results from the study showed no statistically significant increases in congenital defects or miscarriages in 1172 births following paternal exposure to pesticides (relative risk estimates were 1.19 and 0.89 respectively).

Increased frequencies of birth defects were noted by a study of a number of populations in rural Minnesota . In an attempt to determine if there was a relationship between male pesticide exposure and birth anomalies, Garry *et al.* (1996) examined all births in the state registry for the years 1989-1992 (n=210,723 live births) and compared births to men who were state-licensed

private pesticide appliers (n=4935) with the rest of the population. Birth anomalies in the offspring of pesticide appliers were significantly elevated ($p<0.001$) over those of the general population. Specific classes of birth anomalies were also noted to have significantly higher frequencies among pesticide appliers' offspring: circulatory/respiratory ($p=0.05$), musculoskeletal/integumental ($p=0.02$), and urogenital anomalies ($p=0.02$). To further examine this relationship, a large study has been undertaken to investigate how the paternal exposure results in detrimental effects in the offspring. Blood chemistry, chromosome damage in somatic and germ cells, semen parameters and sperm aneuploidy (detailed in our study) are all being studied to determine if the mechanism of the chemical(s) can be elucidated.

(3) Sperm aneuploidy

Our study focussed on men with occupational exposure to pesticides, and the frequencies of aneuploidy in their sperm. Recently, a small number of other studies have been done on human germ cell aneuploidy following pesticide exposures. Padungtod *et al.* (1999) studied the frequency of sperm aneuploidy in men occupationally exposed to pesticides. Seventy-five men participated in the study, which assessed semen parameters and aneuploidy. Twenty-nine randomly chosen subjects had their semen samples analysed by FISH to determine sperm aneuploidy frequencies: 13 exposed men from a pesticide manufacturing plant, and 16 control subjects who worked in a textile factory and had no pesticide exposures. All the men ranged from 20 - 40 years of age, were

generally healthy, and were required to have been employed at their particular factory for a minimum of 3 months prior to the study. FISH analysis utilized centromeric probes for chromosomes 18 and X, and a satellite III DNA probe for distal Yq. A total of at least 2500 sperm were scored for each man by two or three technicians blinded to the subject's exposure status. Scoring criteria for disomy included: the two signals must be equal in intensity, separated by a minimum of one signal domain, regular in appearance, not diffuse, and both clearly within the sperm head. Results of this study showed a significant increase in YY disomy (p=0.009) among the men exposed to pesticides compared to the unexposed men (Padungtod *et al.*, 1999). Following an adjustment for inter-technician effect, regression coefficients were statistically significant with a rate ratio of 1.51 (95% CI, 1.04-2.20).

In another study of male pesticide exposure, Harkonen *et al.* (1999) analysed the sperm of 30 healthy farmers (aged 29 - 49 years) before and after exposure to fungicides using FISH. Two autosomal probes were used: one for the pericentric heterochromatin of chromosome 1 (pUC1.77), and a second specific to alpha satellite DNA on chromosome 7. Ten thousand (10,000) sperm were scored for each individual by three different scorers, two scoring 2500 and the third scoring 5000. Scorers were blinded to the subjects' identities and exposure standings, and their scoring criteria were that for a sperm with two signals to be disomic: signals must be compact and separate with a distance of at least one signal diameter between them. Sperm with a single red or a single green signal (or no

signals at all) were considered unsuccessful hybridizations. The results of this study showed no significant change in aneuploidy frequencies (Harkonen *et al.*, 1999).

ii. Animals studies

(1) Sperm aneuploidy

Originally an agricultural insecticide, trichlorfon later also became utilized as an experimental therapy for Alzheimer disease (Tariot *et al.*, 1997). Reports of the deleterious effects of trichlorfon exposure have included cell cycle delay, nondisjunction, and aneuploidy in lymphocytes. Sun *et al.* (2000), observed a dose-dependent increase of aneuploidy in mouse germ cells at three different dosages. Male mice were given a single intraperitoneal injection of either 200, 300, or 405 mg/kg of body weight, sacrificed after 22 days and epididymal sperm were collected and analysed by multi-colour FISH. The aneuploidy frequencies of chromosomes 8, X, and Y were compared with sperm of control groups produced concurrently with each treatment group to avoid scoring biases. Overall aneuploidy frequencies were calculated for each group, and a significant difference was observed between exposed and control mice at each of the dosages: 200 mg/kg ($p<0.05$), 300 mg/kg ($p<0.01$) and 405 mg/kg ($p<0.01$) (Sun *et al.*, 2000). In addition, this study conducted an *in vitro* assay of trichlorfon exposure to try to determine the mechanism of action, mitotic indices were measured for V79 hamster cells in culture at a number of levels of trichlorfon

treatment. Experimental findings showed an association between increasing trichlorfon concentration and increasing numbers of mitoses with spindle disturbances, as well as a reduction in the mitotic index at intermediate trichlorfon concentrations (Sun *et al.*, 2000). The results are consistent with the affects of a spindle poison. Nondisjunction or chromosome loss could ensue from damage or destruction of the meiotic spindle, suggesting that the mechanism of action of trichlorfon could lead to aneuploidy.

(2) Sperm morphology

Abnormal sperm morphology was utilized to measure the effect of the organophosphate pesticide methamidophos on spermatogenesis. Burruel *et al.* (2000), gave a single intraperitoneal injection of 0.0, 0.5, 3.75, 5.0 or 7.5 mg/kg of methamidophos to 8 week old male mice, and four weeks later, the males were sacrificed and epididymal sperm harvested. Sperm were assessed and significant increases in morphologically abnormal sperm were correlated with increasing pesticide dosage ($p<0.005$).

In another study of an organophosphate pesticide, an acute dosage of curacon (20, 40 or 60 mg/kg) was given to male mice. Thirty-five days following treatment, the mice were euthanized and sperm retrieved from the epididymis for analysis. El Nahas *et al.* (1989), noted significantly increased abnormalities in all dosage groups: 20 mg/kg ($p<0.05$), 40 mg/kg ($p<0.01$) and 60 mg/kg ($p<0.01$).

(3) Structural chromosomal aberrations

El Nahas *et al.* (1989) also analysed chromosomal aberrations (ex. breaks, deletions, fragments, translocations, etc.) induced in spermatozoa of male mice by the pesticide, curacon. Dosage corresponding to 1/24 (12 mg/kg), 1/8 (36 mg/kg) and 1/4 (72 mg/kg) LD_{50} were administered by oral gavage in a single acute dose, or daily for 5 consecutive days. Mice given the single treatment were killed 24 or 48 hours after treatment, subacute treatment ended with euthanisation 24 hours after the last dose was administered. Approximately 50 metaphases per mouse were scored to determine chromosomal aberration frequencies. Among mice given a single dose of curacon, all dosages and times before sacrifice yielded statistically significant increases in chromosomal aberrations observed in epididymal sperm: 12 mg/kg after 24 and 48 hours ($p<0.05$), 36 mg/kg after 24 hours ($p<0.01$), 36 mg/kg after 48 hours ($p<0.001$), and 72 mg/kg after 24 and 48 hours ($p<0.001$). Mice treated daily for five consecutive days showed highly significant increases in chromosomal aberrations at both 12 and 36 mg/kg. All subjects treated at 1/4 LD_{50} (72 mg/kg) died before completion of their treatment and sperm could not be analysed.

Fahmy *et al.* (1998), also noted increased chromosomal aberrations in the spermatozoa of mice treated with organophosphate pesticides. Three insecticides, profenofos, buprofezin or super royal, were given orally to male mice in doses of 1/16, 1/8, and1/4 LD_{50}. Samples were taken from the testes 24 hours after treatment and chromosomal aberrations in primary spermatocytes

were analysed. Profenofos treatment showed significantly increased frequencies of chromosomal aberrations at the two higher doses (p=0.01), while the other two chemicals did not produce significant increases at any of the tested dosages.

iii. Our research

We noted no trends in our data suggesting an increase in aneuploidy frequencies in the sperm of men exposed to pesticides. To control for confounding variables which have been shown to have an influence on sperm aneuploidy frequencies such as: paternal age (Griffin *et al.* 1995; Robbins *et al.*, 1997b), cigarette smoking (Robbins *et al.*, 1997b; Rubes *et al.*, 1998; Shi *et al.*,2001a), and alcohol consumption (Robbins *et al.*,1997b), the subjects for the overall study were matched for age (within 5 years), smoking (yes or no) and alcohol consumption (yes or no). The subset of 40 men who participated in our aneuploidy research were age matched (see Tables 4 & 5), with mean ages of 39.10 (control donors) and 38.75 years (exposed donors), and were also matched for cigarette smoking, with 9/20 control donors and 6/20 exposed donors being smokers (p=0.51, Fisher's exact test). However, the difference in alcohol consumption between exposed and control donors was significant (p=0.03), with 7/20 controls vs. 15/20 exposed subjects drinking alcohol. (Alcohol consumption was classified as ingesting one or more alcohol-containing drinks an average of more than once per month.) Studies on paternal alcohol consumption have reported various results: Vine *et al.* (1997) described no

association between alcohol intake and abnormal male germ cell morphology, but Robbins *et al.* (1997b) noted a significant association with sperm disomy and diploidy. Effects on offspring were investigated by Bielawski & Abel (1997) who noted significantly increased frequencies of malformations in offspring of mice with acute paternal alcohol ingestion. The increased frequency of drinkers in our exposed subjects does not seem to have influenced our results as no significant elevation in their sperm aneuploidy frequencies were noted compared to the controls (who were significantly less likely to consume alcohol).

(1) Comparisons to other studies in humans

Results of our research tend to agree with the findings of the work by Harkonen *et al.* (1999), who found no significant increases in sperm aneuploidy for chromosomes 1 and 7 in Danish farmers after they were exposed to fungicides and other pesticides (compared to their aneuploidy frequencies before exposure). Concerns about matching men for age, smoking and alcohol consumption between exposed and control groups was not an issue as the same men were sampled before and after pesticide exposure. Statistical analysis showed no significant associations of age or alcohol consumption with sperm aneuploidy frequencies (within samples taken either before or after exposure), yet smoking did have a significant association with chromosome 1 disomy ($p=0.02$) in the before exposure samples. The men were divided into smoking and non-smoking groups for analysis to control for this effect (Harkonen *et al.*,

1999). A total of 10,000 sperm were scored for each subject by three individuals (two scoring 2500 and one scoring 5000) who were blinded to the identity and exposure status of the subjects' samples. There was no discussion of consistency between scorers, or total numbers of sperm scored by each individual. Harkonen *et al.* (1999) deliberately collected their semen samples 35-50 days after the farmers had sprayed agricultural chemicals to allow retrieval of sperm that would have been exposed as spermatocytes, to maximize the possibility of observing aneugenic effects of the pesticides.

Our results did not agree with Padungtod et al. (1999), who noted a significant increase in Y chromosome disomy frequencies ($p=0.009$) in the sperm of men employed in a factory producing organophosphate pesticides. Thirteen exposed and 16 unexposed men took part in the sperm aneuploidy analysis. The mean age (control 32 years, exposed 32 years) and smoking status (69% of subjects smoked in both groups) were reported to be equivalent between the exposed and control groups, but alcohol consumption was not, with only 13% of unexposed subjects being drinkers compared to 23% of the exposed population (significance of this difference was not discussed). FISH analysis of chromosome 18 and the sex chromosomes was completed by multiple individuals, with two or three scorers (blinded to the donor's exposure status) analysing each subject's sample. A minimum of 2500 sperm were scored for each subject. Statistical analysis of the technicians' scoring revealed that each individual scored an approximately equivalent number of sperm within the

exposed and control groups, and that there was one scorer who had a tendency to tally lower abnormality frequencies than the other two. The time frame of sample collection was not critical for this study as men were required to have been employed at the pesticide factory for a period of at least three months, to be considered for participation. As human spermatogenesis lasts approximately 65 days (Adler, 1996) the three month criterion would ensure chemical exposure at all stages of spermatogenesis, allowing the observation of aneuploidy induced during the meiotic stages of sperm production.

Neither our research nor the study by Harkonen *et al.* (1999) had attempted to quantify the type of exposure the men received, as various chemicals were used in a number of different concentrations, and different modes of application were employed, with no consistencies between exposed individuals. All of these factors make it difficult to make an accurate of determination the men's exposures. However, Padungtod *et al.* (1999) were able to make a reasonable estimate of their subjects' exposures by randomly measuring pesticide residues on their skin, and completing metabolite analysis of the men's urine after a day at the factory. In this more controlled environment, it can be estimated that men in the factory all have similar exposure levels, and mean measurements of air chemical levels showed: ethyl parathion (0.02 mg/m^3) and methamidophos (0.03 mg/m^3). It is logical to think that overall exposure levels would be far greater for men constantly in contact with pesticides in a production factory setting, than for men who use them outside on a seasonal basis for crop applications. As well,

one would expect that the chemicals are present in much higher concentrations during production than they are in commercial products. For these reasons, it is possible that results from our study (and that by Harkonen *et al.*, 1999) did not display increased aneuploidy frequencies in sperm, because the exposure levels were not consistently high enough to induce this affect. And the results from Padungtod et al. suggest that organophosphate pesticides can generate aneuploidy at higher concentrations.

(2) Significant results

One significant difference was noted in our study: the sperm of exposed donors had an increased percentage of X bearing sperm compared to controls ($p=0.04$). Based on the chromosomal segregation in meiosis, the ratio of X-bearing to Y-bearing sperm is expected to be 1:1, however the subject groups displayed 48.48% (controls) and 49.37% X-bearing sperm (exposed). Increased numbers of female births in populations with paternal environmental exposures have been noted by a number of studies (Whorton *et al.*, 1983; Potashnik & Yanai-Inbar, 1987; Mocarelli *et al.*, 2000; Figa-Talamanca & Petrelli, 2000). Offspring of men exposed specifically to fungicides in Minnesota have given preliminary indications of increased proportions of female births: in >300 children born to fungicide exposed men, the secondary sex ratio was 0.8, which is significantly different($p=0.02$) from the expected 1.06 (Garry, personal communication).

(3) Difficulties encountered

Our expectation at the outset of this study was that we would observe increased frequencies of aneuploidy in the sperm of our exposed subjects vs. controls due to pesticide exposures, and perhaps due specifically to fungicide exposures. Possible explanations for the lack of significant increases in aneuploidy include: poorly defined control and exposed groups, difficulties quantifying exposures, and the possibility of spermatogonial stem cell damage from previous contact with pesticides or other harmful environmental agents.

The overall study consisted of 90 men in three groups: (1) exposed, who used fungicides a minimum of 10 days per year; (2) regional controls, who lived in the same region and used pesticides five or fewer days per year; and (3) urban controls, who were employed in occupations without significant environmental chemical exposure (ex. postal workers, bus drivers, computer programmers). The subgroup of forty men selected for this study were intended to have been 20 exposed subjects (with the highest levels of fungicide exposure), and 20 urban controls. However, semen samples were not available from urban controls, and 20 subjects from the regional control group had to be used. Of the 20 men selected, 4 were 'true' controls with no current or historical exposure, and the remaining 16 were men who had historical exposures to pesticides but no 'current' exposures (pesticide exposures during the 1998 growing season). All subjects were evaluated before the 1998 season commenced, and placed in exposed or control groups based on their intent of using agricultural pesticides in

the upcoming growing season. Cryopreserved semen samples were sent to our laboratory in March 1999, and FISH analysis began shortly thereafter. The lists of donors for the aneuploidy study (this research) were generated prior to the end of the 1998 season based on the control and exposed groups determined earlier that year. It was later realised from completed exposure records received from the subjects (after semen collection had taken place in October) that the exposure status of some of the men changed, as some men who had planned to use pesticides did not and others who had not intended to use pesticides did so. Therefore we were left with groups of donors that were not exactly as proposed: our control group consisted of 16 men with no current exposures, and 4 men with current exposures (3 herbicide exposures, and 1 insecticide exposure); and our exposed group consisted of 18 men with current exposures and two men with no current exposures (see Tables 6 & 7 in Results section). To further complicate the analysis, the time frame of chemical exposure relative to spermatogenesis from stem cell to mature spermatozoa in the ejaculate (approximately 80days - Adler, 1996) had to be taken into consideration. As semen samples were collected in October, any exposure that occurred more than 80 days before that would not be relevant in our investigation, therefore, herbicide applications (which extended from March to late June) would not affect sperm collected in our samples. Thus only insecticide and particularly fungicide exposure would be affecting the spermatogenesis assayed in October. Based on the altered exposure within our study groups and the consideration of exposure and germ cell development time frames, we opted to do an additional analysis limited to

men with current fungicide exposures. When we compared aneuploidy frequencies of men exposed to fungicides in the 1998 growing season (n=11) with our control group, we noted no statistically significant differences.

A concern for all studies of occupational exposure to pesticides is the difficulty of quantifying the type and levels of exposure the participants are subjected to. Each man in our study used different chemicals, in various concentrations, for differing durations and with different application methods. All of these things combine to make an accurate determination of exposures very difficult. As well, details of potency of active chemicals in commercial preparations, and their mechanisms of action and interactions between chemical compounds in the human body should ideally be taken into account, but this information was not available for our study. Another factor that should be considered is what personal protection measures are utilized by the appliers. Due to previous experience with questions about validity of reporting vs. the actual use of protective measures (Garry *et al.*, 2001) the use of protective gear was not taken into account in this study.

The overall study, of which our research is a part, began based on significantly increased frequencies of birth anomalies being noted in the offspring of pesticide appliers compared to the general population of Minnesota (Garry *et al.*, 1996). It is possible that we have not noted increased aneuploidy frequencies in male germ cells due to the fact that the chemicals these men were exposed to cause

damage of a different type (ex. chromosomal breakage, aberrant exchange, point mutations or other structural chromosomal damage that would not be observed in our aneuploidy analysis. The birth anomalies present in significantly increased frequencies in pesticide appliers vs. the general population included: circulatory / respiratory, urogenital, and musculoskeletal / integumental (Garry *et al.*, 1996). As no increase in the frequency of chromosomal abnormalities was noted in live births between appliers and the general population, it is possible that aneuploidy is not one of the effects of the chemicals to which our subjects were exposed.

Another possible reason for failing to notice differences in the aneuploidy frequencies between exposed and control donors is that all but four of our 20 donors have historical exposure to various types of pesticides. If these pesticides damage spermatogonial stem cells, then resulting spermatozoa will be damaged, regardless of current exposures. Data from Kinakin *et al.* (1997) comparing aneuploidy frequencies of chromosomes 13, 21, X and Y from 18 normal men are comparable to our results, therefore we can assume that damage to spermatogonial stem cells did not play a factor in our results.

5. Conclusions

The results of this study indicate no significant relationship between male pesticide exposure and increased sperm aneuploidy frequencies. Other human research in this field has provided mixed results (Harkonen *et al.*, 1999; Padungtod *et al.*, 1999), and more work is necessary to make any strong conclusions. A statistically significant association was noted between pesticide exposure and increased proportions of X-bearing sperm, which is seemingly in agreement with secondary sex ratios noted following paternal exposure (Potashnik & Yanai-Inbar, 1987; Mocarelli *et al.*, 2000; Figa-Talamanca & Petrelli, 2000). Research in this area using animal models might further elucidate the reasons for the apparent selection against Y-bearing sperm (and male births) in the sperm (and offspring) of men with environmental chemical exposures.

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Appendix A - Media and Solutions

0.01M Tris -- 0.9% NaCl
9g NaCl
100mL 0.1M Tris
~900mL distilled water
Dissolve NaCl in 0.1M Tris solution. Bring volume to 1 litre with distilled water. Autoclave and store at room temperature.

0.1M Tris
100mL 1M Tris, pH 8.0
900mL distilled water
Mix Tris solution with distilled water and adjust pH to 8.0. Aliquot desired volumes, autoclave and store at room temperature.

1M Tris
121.1g Tris base
800mL distilled water
~42mL concentrated HCl
Combine Tris with distilled water, then add HCl. After the solution has returned to room temperature, adjust the pH to 8.0 and add distilled water to a volume of 1 litre. Aliquot desired volumes, autoclave and store at room temperature.

10mM DTT in Tris
400µL 1M DTT
40mL 0.1M Tris
Add DTT to Tris solution, stir and use only on the day prepared.

1M Dithiothrietol (DTT)
3.08g DTT
20mL sterile distilled water
Disolve DTT in water, make 500µL aliquots and store at -20°C.

10mM LIS, 1mM DTT in Tris
20 mL 20 mM LIS
20 mL 0.1 M Tris
40 µL 1 M DTT
Add LIS and DTT to Tris solution, stir and use only on the day prepared.

20mM lithium diiodosalicylate (LIS)
0.792 g LIS
100 mL sterile distilled water
Dissolve LIS in sterile distilled water and store at room temperature.

2 × standard saline citrate SSC

200 mL 20 × SSC
480 mL distilled water
Combine 20 X SSC with distilled water, autoclave and store at room temperature.

20 × (SSC)
175.2g NaCl
88.2g sodium citrate
concentrated NaOH
distilled water
Mix NaCl and sodium citrate in distilled water. Use concentrated NaOH to adjust pH to 7.4, and bring volume up to 1 litre with distilled water. Aliquot desired volumes, autoclave and store at room temperature.

70% formamide / 2 × SSC
35 mL ultrapure formamide
5 mL 20 × SSC
sterile distilled water
Combine formamide and SSC. Adjust pH to 7.5 with 1 M HCl. Bring volume to 50 mL with sterile distilled water. Store in a Coplin jar at 4°C. Use for up to 20 slides or one month.

Formamide purification
Remove products of degradation before using formamide in any solutions: Add 1 g Amberlite™ MB--1 resin for each 100 mL formamide, mix the beads well with the formamide, and filter through a paper filter. Store ultrapure formamide at 4°C.

MM2.1 High stringency hybridization buffer
5.5 mL ultrapure formamide (use purification above)
0.5 mL 20 × SSC
1 g dextran sulfate
Combine formamide and SSC in a test tube. Add dextran sulfate and heat at 70°C to dissolve. Allow mixture to cool to room temperature. Adjust pH to 7.0 and bring volume to 7 mL with sterile distilled water. Store at -20°C in 1 mL aliquots.

2 x SSC / 0.1% NP-40
500 mL 2 x SSC
0.5 mL NP-40
Mix 2 x SSC and NP-40, allow bubbles to dissipate and store at room temperature.

Antifade
5 mg p-phenylenediamine

10 mL PN buffer
10 mL glycerol
Dissolve p-phenylenediamine in PN buffer. Combine this mixture with an equal volume of glycerol. Store at -20°C in 1 mL aliquots.

PN Buffer
3.8 L sodium phosphate, dibasic solution
~200 mL sodium phosphate, monobasic solution
~ 4 mL Nonidet™ P-40
While stirring sodium phosphate, dibasic solution, monitor pH and slowly add monobasic solution. When mixture reaches pH 8.0, measure the volume and add 1 mL Nonidet™ P-40 per litre of solution.

Sodium phosphate, dibasic
107.2 g $Na_2HPO_4 \cdot 7H_2O$
distilled water
Dissolve sodium phosphate, dibasic in distilled water and bring volume up to 4 L.

Sodium phosphate, monobasic
3.9 g $NaH_2PO_4 \cdot 2H_2O$
distilled water
Dissolve sodium phosphate, monobasic in distilled water and bring volume up to 250 mL.

Appendix B - Reagents and Commercially available products

	Company	Catalogue #
amberlite™ resin	ICN Biomedical	150330
DAPI II (4',6-diamidino-2-phenylindole)	Sigma	D9542
dextran sulfate	BDH	3710
DTT (dithiothreitol)	Sigma	D9779
Fluoroblue™	Amersham	RPN 2123
Fluorogreen™	Amersham	RPN 2121
glycerol	BDH	B10118
hydrogen chloride	BDH	B1025-74
LIS (lithium diiodosalicylate)	Sigma	D3635
microscope slides	VWR	48393 048
Nonidet™ p-40	Sigma	N6507
p-phenylenediamine	Sigma	P6001
paper filter	Whatman	1004 150
propidium iodide	Sigma	
sodium chloride	BDH	B10241
sodium phosphate, monobasic	Fisher	S381
sodium citrate	Fisher	S279
sodium phosphate, dibasic	BDH	ACS810
sodium hydroxide	BDH	B10252
Spectrumgreen™ 13 (LSI)	Vysis	32-192018
Spectrumorange™ Y (CEP)	Vysis	32-130024
Spectrumorange™ 21 (LSI)	Vysis	32-190002
Tris base (Trizma® base)	Sigma	T8524
Tyrode's buffer	Sigma	T2145
ultrapure formamide	ICN Biomedical	71937