THE UNIVERSITY OF CALGARY

The Effects of Cryopreservation on the Frequency and Type of Chromosome Abnormalities in Human Sperm

by

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

MARCH, 1988

ISBN 0-315-42486-9

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "The Effects of Cryopreservation on the Frequency and Type of Chromosome Abnormalities in Human Sperm" submitted by Judy Ellen Chernos in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Cryopreserved semen is used routinely in animal husbandry and for artificial insemination in humans. However, very little attention has been paid to the genetic consequences of freezing and thawing. Utilizing a technique which enables the direct examination of human sperm chromosomes following *in vitro* penetration of hamster oocytes, the effects of cryopreservation on the frequency and type of chromosome abnormalities have been examined.

Semen samples were obtained from 13 normal healthy men. Each ejaculate was split, one-third to be analysed fresh and two-thirds diluted with a cryoprotectant and frozen in liquid nitrogen vapour for later examination. A total of 91 experiments were conducted yielding 454 analysable sperm chromosome complements from fresh sperm and 387 from previously frozen sperm.

An overall abnormality frequency of 17.8% was observed for the prefreeze sperm consisting of 9% structural abnormalities, 7.7% numerical abnormalities and 1.1% numerical with a structural abnormality. The postfreeze sperm had an overall abnormality frequency of 13.4% consisting of 8.8% structural abnormalities, 3.4% numerical abnormalities and 1.3% numerical with a structural abnormality. The overall abnormality frequencies were significantly different between prefreeze and postfreeze data, attributable to differences in the numerical abnormality frequencies alone. Since most of the numerical abnormalities were hypohaploid, suggesting artefactual loss of chromosomes, the data were corrected by doubling

the hyperhaploidy frequency to give a conservative estimate for aneuploidy. The corrected data no longer showed any significant differences between prefreeze and postfreeze data for any category of abnormality.

There was considerable inter-donor variability in sperm chromosome abnormality frequencies, and there was suggestive evidence for donor-dependent responses to cryopreservation. Abnormalities were found to be randomly distributed throughout the sperm haploid chromosome complement and no "hotspots" for chromosome breakage could be identified.

The sex ratios for fresh sperm (52.9% X chromosome-bearing) and previously frozen sperm (53.5% X chromosome-bearing) were not significantly different from each other or from the theoretical 50%.

No correlations could be discerned between individual men's semen parameters and their sperm chromosome abnormality frequencies before or after cryopreservation.

It is concluded, that cryopreservation does not affect the type or frequencies of chromosome abnormalities or alter the sex ratio in human sperm.

Acknowledgements

Many thanks to K. Burns, J. Yamamoto, D. Peterson, L. Long-Simpson, L. Barclay, and E. Ko for their helpful technical assistance and the many hours spent deliberating over karyotypes with me.

Thanks also to Dr. Brian Lowry and the Medical Genetics Unit at the Alberta Children's Hospital for providing me with a stimulating and pleasant environment in which to work.

My sincere thanks to the members of my supervisory committee, Drs. Leon Browder, Bob Church and David Mortimer for constructive input throughout my research and thesis compilation, and thanks to Drs. Fred Rademaker and Gordon Fick for their statistical consultations. Last, but-not-least, I would like to thank my supervisor, Dr. Renee Martin who provided me with encouragement, inspiration and a role model to which I could aspire.

The financial support of the Alberta Heritage Foundation for Medical Research throughout my research is gratefully acknowledged.

Dedication

To Mendy, for his continuous support throughout my academic career and whose constant prodding made me a Ph.D. before a mother.

and

To my parents who always wanted me to become a doctor.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ace	acentric fragment
A.I.	artificial insemination
A.I.D.	artificial insemination by donor sperm
A.I.H.	artificial insemination by husband's sperm
asym.	asymmetrical exchange
B.W.W.	Biggers, Whitten and Whittingham medium
C	celsius/centigrade
cen	centromere
cm	centimetre
comp	complete (rejoining)
csb	chromosome break
csg	chromosome gap
ctb	chromatid break
cte	chromatid exchange
ctg	chromatid gap
del	deletion
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
G	force of gravity
g	gram
hCG	human chorionic gonadotropin
HO	hypohaploidy
HR	hyperhaploidy
HSA	human serum albumin
i	isochromosome
incomp	incomplete (rejoining)
ISCN	International System for Human Cytogenetic Nomenclature
I.U.	International Units
MB	multiple breaks
MB&R	multiple breaks and rearrangements
mg	milligram
min	minute acentric
ml	millilitre

mm	millimetre
N	normal karyotypically
NS	numerically and structurally abnormal
p	short arm of a chromosome
pen-strept	pencillin/stroptomycin antibiotic solution
PMSG	pregnant mare's serum gonadotropin
PVP	polyvinylprollidone
q	long arm of a chromosome
Q-band	quinicrine dihydrochloride banding stain
qr	quadriradial
RPM	revolutions per minute
S+	complex structural rearrangement
ST	simple structural rearrangement
sym	symmetrical exchange
t	translocation
ter	terminal end of a chromosome
tr	triradial
WHO	World Health Organization
ul	microlitre
χ^2	chi squared statistic

I INTRODUCTION

A. Historical Background

Since humans first postulated that sperm cells were the progenitors of life, there has been interest in preserving spermatozoa so as to extend their functional lives. Spallanzani, in 1776, was the first to record his observations that human, stallion and frog spermatozoa could be made temporarily inactive by cooling to the temperature of snow and revived again by warming (Watson, 1979). A century later, Mantegazza, in 1866, had the foresight to recognize the potential social and economic utility of sperm preservation:

> If the human sperm can be preserved for more than four days at the temperature of melting ice without undergoing any change, then it is certain that scientists of the future will be able to improve breeds of horses and cattle without having to spend enormous sums of money in transporting thorough-bred stallions and bulls. It will be possible to carry out artificial insemination with frozen sperm sent rapidly from one locality to another. It should also be feasible for a husband who dies on the battlefield to fertilize his wife and thus to have legitimate sons even after his own death. (In Watson, 1979, p.283)

Today, the predictions of Mantegazza have largely become reality. In the field of animal husbandry, the semen of many economically important species, such as cattle, is routinely cryopreserved in liquid nitrogen resulting in stock improvement. Through banking the semen of genetically superior sires greater widespread access to the sire and more frequent artificial inseminations are possible, even after the donor is no longer available or fertile.

B. Status of Human Sperm Banking

In humans, the advent of successful sperm banking has had important clinical consequences. Artificial inseminations with anonymous donor sperm (A.I.D.) (more recently becoming known as T.D.I., therapeutic donor insemination, due to the unfortunate association of the acronym A.I.D with the disease AIDS) or with a husband's banked sperm (A.I.H.), have become routine practice worldwide as a means of alleviating certain forms of infertility or for avoiding genetically high risk matings. As of 1979, more than 10,000 babies were conceived annually by artificial insemination in the United States (Currie-Cohen, et al., 1979). The 1986 annual review of the Federation CECOS in France (in house publication) reported 2872 couples underwent artificial insemination during 23,000 cycles with cryopreserved semen. In 1986, at the University of Calgary Infertility Clinic, 81 new couples underwent 1428 artificial insemination procedures using cryopreserved semen resulting in 42 successful pregnancies (unpublished statistics,

University of Calgary Infertility Clinic). This constitutes 1/276 live births in the city of Calgary (based on 11,574 births in Calgary) (Alberta Community and Occupational Health, Vital Statistics Annual Review, 1986).

There are many advantages of using banked, cryopreserved semen over fresh semen. The clinician can more easily match the physical characteristics of the sperm donor to the recipient's husband while avoiding incompatible traits such as Rh positive blood type in the case of an Rh negative recipient. Banking ensures that the appropriate donor semen is always available so that ovulation and inseminations coincide, increasing the odds for conception occurring. In addition, since only semen samples which have met the minimal criteria for sperm counts, motility, and other critical semen parameters are banked, the chances for a successful insemination are improved. Men with substandard sperm counts may bank several ejaculates which can later be pooled for A.I.H.. Recently, it has become the usual practice to "quarantine" all sperm to be used in A.I.D. by banking specimens to allow time to test for pathogens including antibodies for acquired immune deficiency syndrome (Guidelines of the Canadian Fertility and Andrology Society 1988). Lastly, an unproven, although potentially important, reason for semen cryopreservation is that it is thought to "freeze out" defective sperm leading to fewer birth defects among the resultant progeny (Glassman and Bennett, 1980; Witherington et al., 1977).

This latter point has never been fully investigated due to the difficulty of follow-up studies, the anonymity of the donor

father and previously, the inability to examine the genetic content of sperm cells directly. A successful pregnancy and live birth have been virtually the only means of monitoring the genetic integrity of the sperm cell after cryopreservation.

C. Clinical Studies on the Effects of Cryopreserving human sperm

A number of studies have been done to compare the outcomes of pregnancies following A.I.D. with fresh sperm and with previously frozen sperm. Witherington et al., (1977) examined the frequencies of abnormalities and spontaneous abortions among 520 births following A.I.D. with previously frozen sperm. They found less than 1% abnormal offspring at birth and less than 8% spontaneous abortions compared to 6% and 10-15% respectively in the general population. Sanger et al., (1979) cited frequencies of 1% for congenital anomalies and 12% for spontaneous abortions in 2,000 live births following A.I.D. with cryopreserved human sperm. These rates were interpreted as lower than those for the general population which they used as a control group. Karow (1979) reported 1% congenital defects and 8% spontaneous abortions among 3,000 pregnancies resulting from A.I.D. with frozen sperm. These frequencies were considerably lower than those compiled by Karow from a literature review of 10,000 births resulting from A.I.D. with fresh sperm: 6% congenital abnormalities and 10-15% spontaneous abortions.

The consensus of such studies was that the traumatic freezing process kills weak or abnormal sperm preferentially.

Unfortunately, the control group was not strictly matched to that of the experimental group for various risk factors such as age and health status and therefore the differing rates of spontaneous abortions and birth defects may be due to some cause other than sperm cryopreservation. An alternative explanation put forth by Sherman (1973), is that cryopreservation minimizes the degree of in vitro aging and thus genetic damage correlated with gamete aging prior to fertilization.

More recently the French collaborative, Federation des Centres d'Etude et de Conservation du Sperme Humain, (Federation CECOS, 1983), analysed results from 2,502 A.I.D. pregnancies (conceived with mainly frozen sperm) in France. They found a 1.8% incidence of malformations, slightly less than for the general population they chose for comparison (Stevenson, et al., 1966). When births with a chromosome anomaly, in particular trisomies, were considered separately and figures corrected for maternal age and mothers over the age of 35 years were excluded, the frequency among A.I.D. births was found to be significantly greater than for the general population (6 observed trisomies versus 3.07 expected based on figures by Hook, 1981a).

Again these findings should not be solely attributed to the cryopreservation process. A similar excess of trisomic births following A.I.D. with fresh semen was reported by Fraser and Forse, (1981). It may be that the A.I.D. treatment of the semen including sperm cryopreservation, contributes to an increased survival rate for hyperhaploid sperm over normal sperm. Or as suggested by Fraser

and Forse (1981), some aspect of semen processing for A.I.D. could be "teratogenic". There may also be a higher risk for chromosomally abnormal offspring among infertile couples due to reproductive dysfunction in the mother as well as the father. Another contributory factor is the use of ovulation inducers prior to artificial insemination which has been found to increase chromosomal abnormalities among spontaneous abortions (Boué and Boué, 1973).

Further evidence that cryopreservation does act differentially on sperm with different genetic content comes from an examination of the sex ratio among births conceived with cryopreserved sperm. In a collaborative study of European and American centres, Mortimer and Richardson (1982) found the proportion of males born after A.I.D. with cryopreserved sperm was 49.7% compared to 57.5% after A.I.D. with fresh sperm. This significant difference in the sex ratio ($p < 0.001$) was interpreted by the authors to reflect the differential cryosurvival of X- and Y-chromosome bearing sperm. However another explanation could be the different timing of insemination regimes employed by clinics using fresh sperm compared to those using banked sperm. It has been suggested by many studies that the sex ratio is influenced by the time of insemination (i.e. Guerrero, 1974; James, 1976; Bartoš, 1980).

D. Animal Studies on the Effects of Sperm Cryopreservation

From the animal systems where artificial insemination with frozen sperm is routinely practiced, there is no reported increase

in the frequency of abnormal progeny. Since birth defects among livestock are of less concern to breeders, less rigorous follow-ups and reporting are more likely than among humans. However, in a study of the effects of sperm cryopreservation on the frequency of chromosome abnormalities in rabbit blastocysts, Robson and Shaver (1979) reported a significant increase in chromosomally abnormal blastocysts when the rabbit was inseminated with previously frozen sperm compared to fresh sperm. Mosaicism was the most frequent abnormality observed suggesting an error of nondisjunction in the embryo rather than in the sperm. However, it is still possible that following cryopreservation, more hyperhaploid sperm fertilized oocytes with subsequent loss of a chromosome in the embryo leading to mosaicism. Alternatively, the cryopreservation process may have a lasting effect on the sperm chromosomes causing them to more frequently undergo nondisjunction.

While there is no direct evidence for haploid gene expression in mature spermatozoa, differential cryosurvival of spermatozoa based on genotype may occur. Evidence that genotype affects the viability of frozen-thawed mouse embryos was reported by Schmidt, et al. (1985).

Considering both human and animal studies together, there is suggestive evidence for the differential survival among cryopreserved sperm based on genetic content.

E. Indirect Studies on the Genetic Effects of Sperm Cryopreservation

While sperm cryopreserved for A.I.D. are under strict

quality control in terms of morphology, counts and motility, before and after freezing, there has been no direct method of assuring that the genetic content of sperm cells is unaffected by freezing. However, as outlined in the preceding sections, there is ample suggestive evidence in the literature that cryopreserving sperm may have genetic consequences.

A number of different approaches have been taken to examine, indirectly, any genetic perturbations caused by cryopreservation of sperm cells.

Very early it was established that the DNA double helix is not disturbed by ultra-cold temperatures. Shikama (1965) examined the effect of freezing and thawing on the stability of the double helix of DNA extracted from calf thymus as measured by hyperchromatic changes. He concluded that the helical structure of DNA is not broken down by freezing and thawing. Soon after, Ackerman and Sod-Moriah (1968) investigated the DNA content of human spermatozoa after storage at low temperatures using Feulgen microspectrophotometry and Webb-Levy chemical determination. Despite the presence of nucleases in human seminal plasma, they found no change in the DNA content of human spermatozoa when stored for up to 75 weeks at ultra-low temperatures. They concluded that the reduced fertilizability of cryopreserved sperm reported by many clinics (Woolley and Richardson, 1978) is associated with altered cellular metabolism rather than "faulty transmission of hereditary material in the spermatozoa" (Ackerman and Sod-Moriah, 1968, p.6).

Two indirect methods have been employed to assess the

genetic effects of cryopreservation on human sperm cells. The first method is based on the intense fluorescence of the distal portion of the Y-chromosome at metaphase after quinicrine staining (Pearson and Bobrow, 1970; Pawlowitzki and Pearson, 1972). The integrity of the Y-chromosome is estimated by staining sperm cells with quinicrine dye and examining for the frequency and intensity of fluourescent F- or Y- bodies in the sperm head before and after freezing. Using this method, Sherman and Char (1974) found no alteration in the F-bodies in freeze-thawed sperm. However the biological basis of this test is unreliable, since there is some question as to whether F-bodies are representative of the presence of the Y-chromosome (Gledhill, 1983). This method would also be unable to detect breaks and rearrangements or aneuploidy in any other chromosome.

A second method has been employed by Huret (1983; 1984) to detect genetic damage to sperm due to freeze-thawing. He compared the decondensing ability of fresh and cryopreserved sperm to assess the stability of the chromatin organization in the condensed nucleus. He found no significant difference between the two treatments using this method and concluded that genetic material is unperturbed by cryopreservation. Again, this method does not preclude limited genetic damage such as aneuploidy, and structural rearrangements since these abnormal sperm are still capable of undergoing all the necessary events of fertilization as evidenced by births with genetic anomalies of paternal origin (Hansson and Mikkelson, 1978, reported that almost 30% of cases of trisomy 21 are of paternal origin).

F. Direct Methods to Assess Genetic Effects of Sperm Cryopreservation

Part of the difficulty in studying directly the cytogenetic content of sperm cells is due to their highly efficient design for transmitting genetic material, intact, to the site of fertilization. Sperm chromatin is highly compact, genetically inactive and biochemically inert and completely insoluble (Pogany, et al., 1981). It is resistant to denaturation by strong acid, protease, or DNase (Miller and Masui, 1982). Despite this incredible nuclear stability, once fusion with and incorporation into an oocyte occurs, the sperm head (nucleus) quickly undergoes decondensation and pronuclear formation (Bedford, 1970; Fraser, 1984).

Successful reactivation of interphase somatic nuclei has been achieved by viral or chemical mediated fusion with rapidly dividing cell types such as HeLa cells (Appels, et al., 1974). A similar approach has been used to induce the functional changes in sperm which occur during fusion with ova (see Yip and Bols, 1982 for a review). In one early study it was possible to achieve cell fusion between rabbit sperm and various somatic cell types, but sperm DNA synthesis was never observed (Sawicki and Koprowski, 1971). Limited success was reported with human sperm/somatic cell fusion as evidenced by nuclear decondensation, a shift from protamine to histone content and measurable amounts of DNA and RNA synthesis (van Meel and Pearson, 1979).

However, it soon became clear that sperm required very specific factors found in the germinal vesicle and ooplasm for

proper pronuclear formation (Usui and Yanagimachi, 1976; Lohka and Masui, 1983b; 1984; Sakai and Shinagawa, 1983). An alternate approach was to simulate the conditions of fertilization and the oocyte milieu *in vitro*. Amphibian oocytes and their ooplasmic extracts have become useful tools for inducing formation of sperm pronuclei. Due to the large size of amphibian oocytes, it is relatively easy to isolate large quantitities of ooplasmic components (Lohka and Masui, 1983a; 1983b; 1984; Lohka and Maller, 1985; Gordon *et al.*, 1985; Ohsumi *et al.*, 1986). While more successful than the somatic cell fusion approach, only rarely have mitotic chromosomes been observed and these were not membrane bound, nor could they be analysed (Lohka and Masui, 1983a).

Other investigators have attempted to obtain sperm pronuclear chromosomes by microsurgically injecting spermatozoa into mammalian oocytes (Uehara and Yanagimachi, 1976; 1977; Thadani, 1979). For human sperm using heterologous oocytes, usually from golden hamsters, has the advantage of being much more readily obtainable material than human oocytes. However the procedure is technically very difficult and data generation is tedious. The first successful formation of analysable human sperm chromosomes using this method has recently been achieved by R. H. Martin's laboratory in Calgary (unpublished results) indicating that there is a future for this approach.

The most important breakthrough in developing a technique for direct examination of human sperm chromosomes was made in R. Yanagimachi's laboratory at the University of Hawaii. Originally

developed as a fertility assay, Yanagimachi et al., (1976) described a technique for *in vitro* fertilization of zona pellucida-free hamster oocytes with human sperm (among others). In 1978, Rudak *et al.* reported the successful culturing of the heterologously fertilized oocytes to obtain human sperm pronuclear chromosomes. Coined the "Humster Assay", this technique provided for the first time, a direct method for assessing chromosomal damage following freezing and thawing (as well as many other environmental factors) while circumventing the uncertainty of indirect methods and difficulties of follow-up clinical studies.

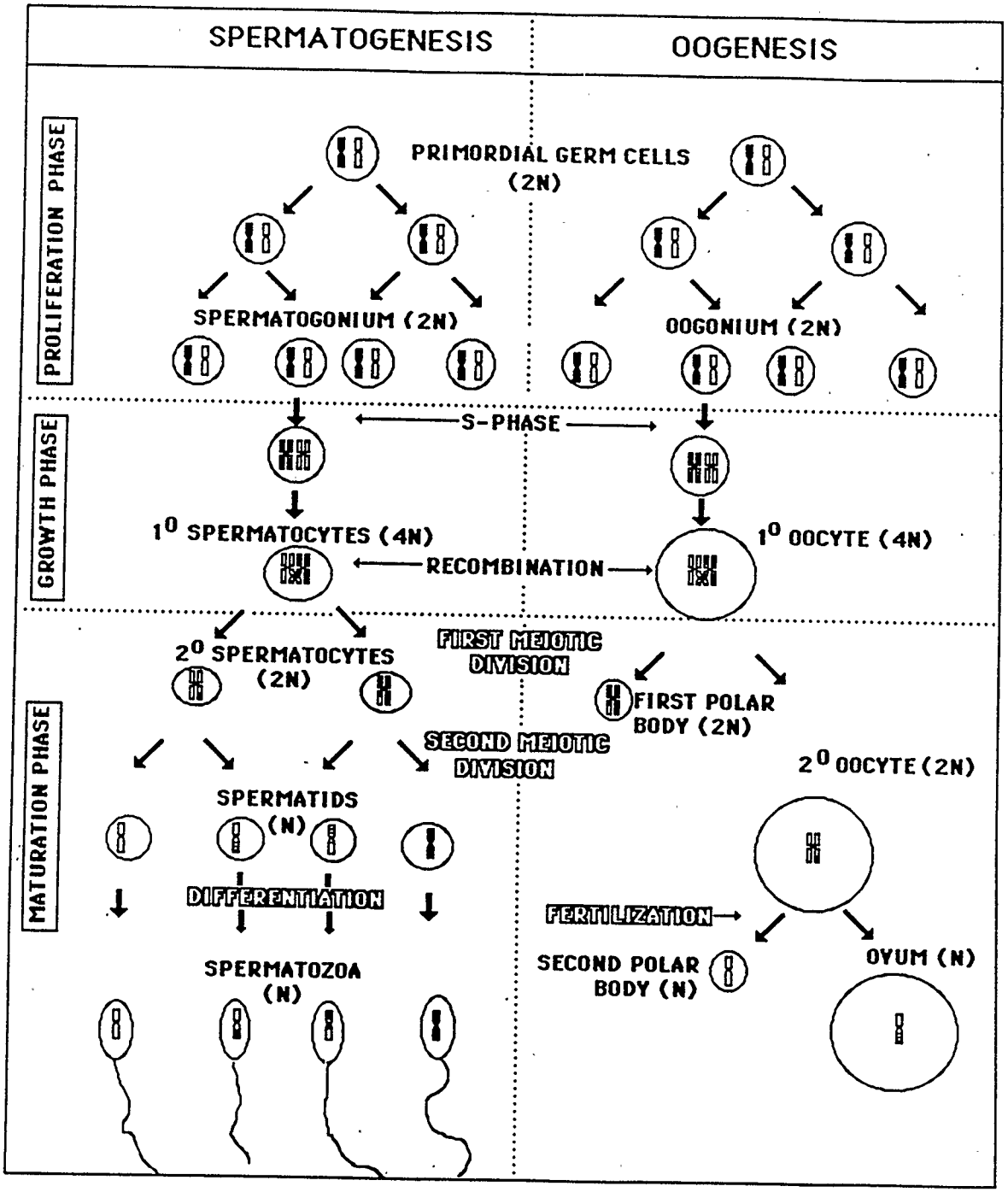
G. Biological Basis of the "Humster Assay"

The successful development of the "humster assay" was made possible by the recent advances made in the field of developmental biology. The normal events of gametogenesis and fertilization are the basis for understanding the "humster assay".

Mammalian sperm and oocytes are formed by parallel genetic events consisting of 1) a proliferation stage by means of mitotic divisions, 2) a growth phase where cells cease mitosis and increase in volume, and 3) a maturation phase consisting of two meiotic divisions reducing to one-half the number of chromosomes and creating diversity through genetic interchange and random chromosome segregation. (See figure 1).

Important differences exist between the male and female versions of gametogenesis. The process of spermatogenesis does not begin until sexual maturity is reached. It then becomes an ongoing

Figure 1: Gametogenesis



process which takes about 65 days to complete (Thompson and Thompson, 1986). Each meiotic product in spermatogenesis is equivalent leading to four functional spermatozoa from each spermatogonium (although there is significant germ cell degeneration during spermatogenesis) (Johnson et al., 1983).

In contrast, oogenesis actually begins well before birth and then arrests at dictyotene of early meiosis I. The continuation of meiosis is stimulated in individual or groups of oocytes (depending on the species) sometime after sexual maturity in response to cyclic hormonal signals. The oocyte, arrested at metaphase II, is then ovulated and only completes the maturation process which may have begun more than forty years before, if it becomes activated by a penetrating spermatozoa. During meiosis in oogenesis, only one functional cell containing a haploid genome and most of the cytoplasm is produced from each oogonium while the rest of the genetic material is discarded in two polar bodies, one at each stage of meiosis.

The mature oocyte, enclosed in a plasma membrane, is also surrounded by a glycoprotein coat called the zona pellucida (Yanagimachi, 1984) and an envelope of cumulus granulosa cells (Scheutz, 1985). Each of these three barriers must be penetrated by a spermatozoa during normal fertilization.

Freshly ejaculated spermatozoa are not immediately capable of fertilizing oocytes. They must undergo a not-well-understood process called capacitation (Bedford, 1970; Soupart and Morgenstern, 1973). In vivo, this occurs within the female genital tract. Upon

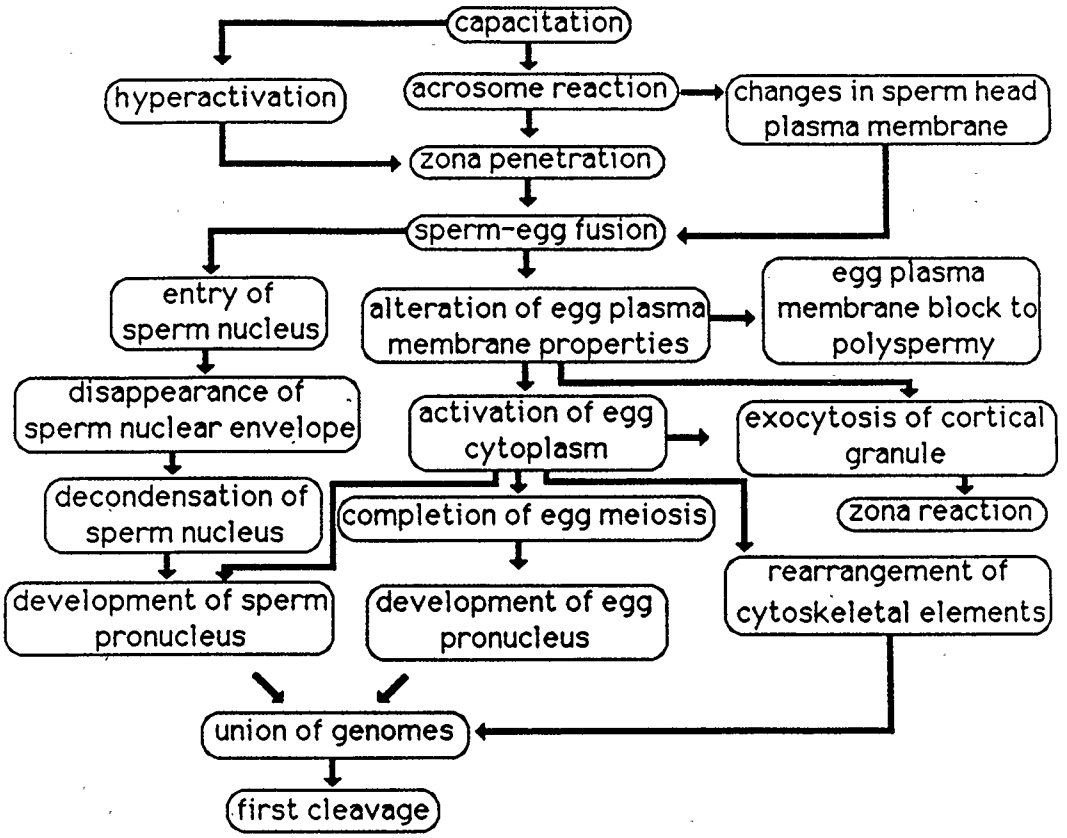
capacitation, the sperm's acrosome can react releasing its enzyme contents essential for the sperm to pass through the outer oocyte investments. The spermatozoon then fuse with the oocyte plasma membrane and the sperm head is incorporated into the ooplasm. Changes in the oocyte membrane and zona pellucida occur to block multiple fertilizations (Yanagimachi, 1984). While the sperm nucleus decondenses and forms a male pronucleus, the oocyte chromosomes complete meiosis and form a female pronucleus. More or less synchronously, DNA synthesis and chromosome duplication occur in both the male and female pronuclei. Cytoskeletal elements move the two pronuclei into close proximity and the nuclear envelopes disintegrate allowing the chromosomes to mingle in preparation for the first cleavage. (See figure 2, based on Yanagimachi, 1984, p 188).

In general, sperm from one species are not capable of fertilizing oocytes from another species. The barrier to heterologous fertilization lies in both the zona pellucida and the plasma membrane of most species' oocytes. The Syrian golden hamster (Mesocricetus auratus) is exceptional. Unlike the oocytes of its close relative the Chinese hamster, when the zona pellucida is removed from golden hamster oocytes, the plasma membrane can be penetrated by sperm from most mammalian species from mice to dolphins to man (Yanagimachi, 1984, table 1). This is perhaps the most critical feature of the "humster assay".

Golden hamsters have well defined estrous cycles and are polyovular. They can be induced to superovulate quite readily

Figure 2:

INTERACTIONS OF MAJOR EVENTS OF MAMMALIAN FERTILIZATION



Based on Yanagimachi, 1984

through injections of exogenous gonadotropic hormones (pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG)) (Scheutz, 1985). The hamster therefore is a useful source for oocytes in systems where the sperm are relatively easy to collect, but the oocytes are not, such as in humans.

Technical advances in the area of in vitro fertilization have made it possible to retrieve, successfully fertilize and culture oocytes in vitro (Yanagimachi, 1984). In addition, an understanding of the necessary events which sperm must undergo to successfully penetrate oocytes has led to the development of techniques for separating spermatozoa from seminal fluids, and suitable culturing conditions to effect capacitation and acrosome reactions.

II RESEARCH OBJECTIVES

The current study was undertaken to examine the cytogenetic consequences of human sperm cryopreservation. The objectives of the study were:

1) to determine whether cryopreservation of human sperm alters the frequency or type of chromosome abnormalities compared to those observed for non-cryopreserved sperm.

2) to examine inter-sperm donor variability to establish if response to sperm cryopreservation is donor-dependent.

3) to examine the effects of cryopreservation on the sex ratio of all sperm successfully penetrating hamster oocytes, and for subpopulations of sperm such as cytogenetically abnormal, to establish if any sex ratio differences exist among these groups.

4) to compare sperm morphological data with sperm chromosome data to establish whether morphological damage due to cryopreservation correlates with rates of chromosome abnormalities overall for individual sperm donors.

III MATERIALS AND METHODS

A. Selection of Donors

Normal healthy males were recruited to provide semen samples for this study. Some of the donors were former donors referred from the University of Calgary Infertility Clinic, some were recruited from staff at the Alberta Children's Hospital and the rest were obtained by word of mouth from acquaintances of the laboratory personnel. Donors ranged in age from 22 to 37 years, mean age 28 years.

The initial criteria for inclusion of a donor in the study followed the guidelines of the University of Calgary A.I.D. programme. These were: 1) sperm concentrations of greater than $60x10^6$/ml; 2) 60% or better sperm survival after cryopreservation; and 3) sperm capable of penetrating hamster oocytes at rates of 40% or more.

The purpose of adopting these criteria was to ensure reproducible results which could be clinically applicable and to maximize the number of sperm chromosome complements obtained per ejaculate.

As data collection began, it became apparent that meeting all of these criteria would not be realistic or necessary for a number of reasons. Firstly, since fertilization occurs *in vitro*, sperm concentrations may be much lower than necessitated by A.I. procedures. In this study sperm cells were washed from the seminal fluids and resuspended to a final concentration of only $10X10^6$/ml,

so low initial sperm count was rarely a problem.

Secondly, recruiting males for regular sperm donations was difficult. As males were not first prescreened on the basis of semen parameters but run on the "humster assay" immediately, a lot of effort had been invested to drop a donor on the basis of low sperm count or poor cryosurvival. If however, a donor had a very low (<5%) hamster oocyte penetration rate in the fresh portion of the ejaculate in conjunction with substandard sperm morphological parameters, no further specimens were obtained from that donor.

Thirdly, individual donor's sperm are known to survive better when frozen in particular cryoprotectants (University of Calgary A.I.D. Clinic, unpublished results). However, in this study only one cryoprotectant was used for all donors to avoid the necessity of running trials with each medium and to ensure homogeneous treatment of all semen samples. Reduced cryosurvival could be compensated for by running two separate experiments on the frozen semen if a sufficient volume had been frozen.

B. Controls

Each semen sample acted as its own internal control. Specimens were split: part of the sample was used fresh in the "humster" assay, and part cryopreserved in liquid nitrogen to be thawed and used at a later date. To ensure that approximately equal data were generated before and after freezing, one-third of the sample was used fresh and two-thirds of the sample was frozen since up to half of the sperm die or lose motility after

cryopreservation. Cohen, et al. (1981), found that fresh and freeze-thawed spermatozoa have similar capacities to bind to the surface of denuded hamster oocytes. Therefore, similar penetration rates could be expected from each part of the split sample.

C. Sample Size Considerations

Donors have been shown to vary considerably with respect to the number of chromosome anomalies naturally present in their sperm cells (Martin, et al., 1983; 1987b; Brandriff, et al., 1985) as well as sperm recovery rates after freezing (Smith and Steinberger, 1973; Cohen, et al. 1981). To minimize this variability, multiple experiments were run on each of several donors to obtain approximately equal data sets.

In some cases, the volume of the initial ejaculate was sufficient to bank semen for two separate postfreeze experiments. Usually, 5 straws (2.5 ml of diluted semen) were used for a single experiment, but as little as 1 straw was found to be sufficient. The advantages of doing two experiments on a single frozen sample were that it generated more chromosome complements since more hamsters were used, compensating for loss of fertility in samples after freezing, and it provided insurance against loss of a specimen due to bacterial contamination.

Data collected by the Federation CECOS (1983) from live births following A.I.D. with previously frozen sperm suggested that there may be a two-fold increase in the number of births with chromosome anomalies over the general population. Using this as a

premise and a baseline of 8.5% chromosome anomalies in fresh human sperm found by Martin, et al., (1983), it was calculated that a minimum of 315 karyotypes from each of the fresh and frozen sperm treatments would be required to show a doubling of chromosome abnormalities (based on statistical functions from Sokal and Rohlf, 1969, pp. 607-610).

Since this study design provided for an internal control in the form of the prefreeze halves of the experiments, as data were generated, a new baseline for chromosome anomalies was established and new projected sample sizes were calculated on this basis. Original projected sample sizes and current estimates for sample sizes required to show at least a doubling or halving of abnormality rates are summarized in Table 1.

TABLE 1: Estimates of Sample Sizes Necessary to Show Two-Fold Differences in Abnormality Rates

	Original sample size (based on Martin et al., 1983) (#karyotypes pre and postfreeze)	Revised sample size (based on prefreeze data, current study) (#karyotypes pre and postfreeze)	
	DOUBLING	DOUBLING	HALVING
Overall Abnormalities	315	120	264
Structural Abnormalities	883	317	679
Numerical Abnormalities	544	285	614

D. Length of Cryopreservation

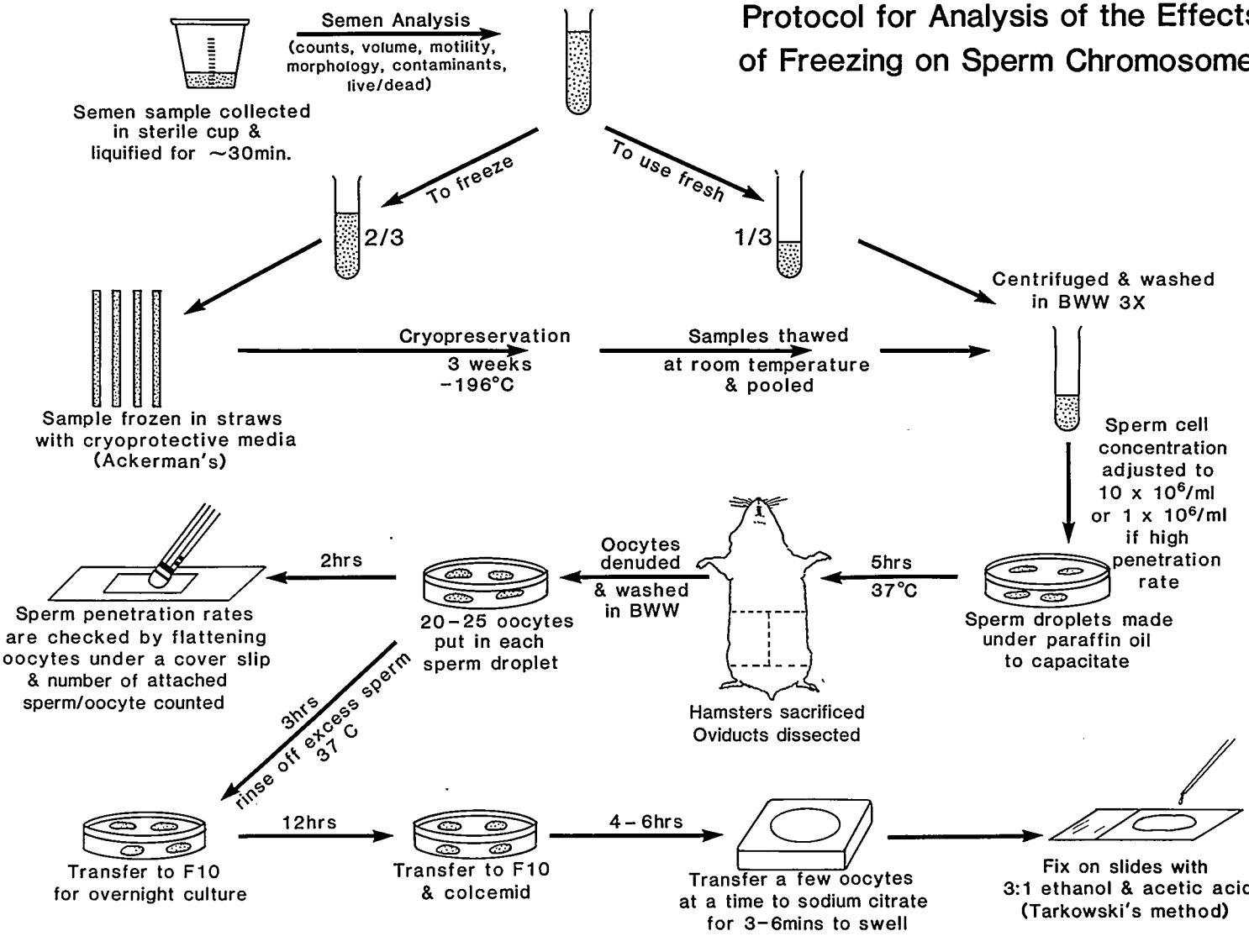
The choice of cryopreservation duration was somewhat arbitrary. Sperm cryopreserved in liquid nitrogen at minus 196 C can have considerable long-term stability (Smith and Steinberger, 1973; Friedman and Broder, 1981). Instances of conception using sperm cryopreserved for more than 10 years have been reported (Witherington, et al., 1977). The harmful effects of cryopreservation occur during the freezing and thawing steps when ice crystals can form. This is evidenced by the lack of correlation between post-thaw survival rates and the duration of sperm storage (Iizuka, 1980). In this study, sperm samples were stored in liquid nitrogen from 4 days, to over 5 months.

E. The "Humster" Assay

The methodology used to obtain human sperm pronuclear chromosomes, facetiously known as the "humster assay", was pioneered in the lab of Ryuzo Yanagimachi at the University of Hawaii, and reported by Rudak et al., in 1978. The technique is fastidious and difficult to master. Even now, almost 10 years after its development, only a handful of laboratories around the world are routinely obtaining human sperm chromosomes (Martin, et al., 1983b; 1987b; Brandriff et al., 1984, 1985; Burns et al., 1986; Sele, et al., 1985; Benet et al., 1986; Kamiguchi and Mikamo, 1986; Jenderny and Rohrborn, 1987; Pellestor et al., 1987.) The technique employed in this study is based on Rudak's original technique as modified by Martin (1983) (see figure 3).

Figure 3: Protocol for the analysis of the effects of freezing on sperm chromosomes.

Protocol for Analysis of the Effects of Freezing on Sperm Chromosomes



F. Sperm Processing and Freezing

i. Fresh Semen

Following at least 24 hours abstinence, semen samples were obtained by masturbation into sterile plastic containers, maintained at room temperature and delivered to the lab within an hour of collection. The semen was liquefied at 37° C with 5% CO_2 in air and 95% humidity for about 20 minutes. All semen processing was carried out in a biological hazards hood (class A) following standard biological containment procedures. Liquefied semen was transferred to a 15 ml sterile centrifuge tube and gently mixed by pipet and agitation. The collection container was rinsed with a small volume of Biggers, Whitten and Whittingham medium at 37°C and set aside.

Semen analysis was carried out on the sample taking note of volume and viscosity, and from a wet mount, motility, forward progression, agglutination, and contaminants. A smear preparation was made for later staining with Giemsa to examine sperm cell morphology (WHO, 1980). Slides were also prepared to estimate proportions of live and dead sperm cells. One drop of whole semen and one drop of either eosin aniline blue (Hackett and MacPherson, 1965) or eosin yellow stain (Eliasson and Treichl, 1971) were mixed on a slide, dried and read at a later time. To determine the initial concentration of sperm cells in the semen sample, a 50µl aliquot of the semen was taken and mixed with 950µl of sperm count diluent. A drop of the suspension was placed on a hemocytometer slide (0.1 mm deep, American Optical) and a cell count made.

One-third of the initial semen volume was diluted with the BWW used to rinse the collection container and fresh BWW added to give a total volume of 10 ml. This suspension was centrifuged at 600 g (1700 RPM) for ten minutes. The supernatant was decanted, and the pellet was resuspended to give a concentration of $10X10^6$ cells/ml. Lower or higher concentrations were used when the donor's sperm penetration rates for the hamster oocyte system were known and dictated the change. The sperm of some donors caused polyspermy at this set concentration while other's sperm did not fertilize well.

Using an Eppendorf pipet with a sterile tip, four 75µl - 100µl droplets were made in a sterile tissue culture dish (Falcon 3002, 60 X 15mm) and covered with prewarmed paraffin oil. Dishes were incubated (at 37°C, 5% CO_2 in air, 95% humidity) for about five hours to allow the sperm to capacitate.

ii. Freezing Semen

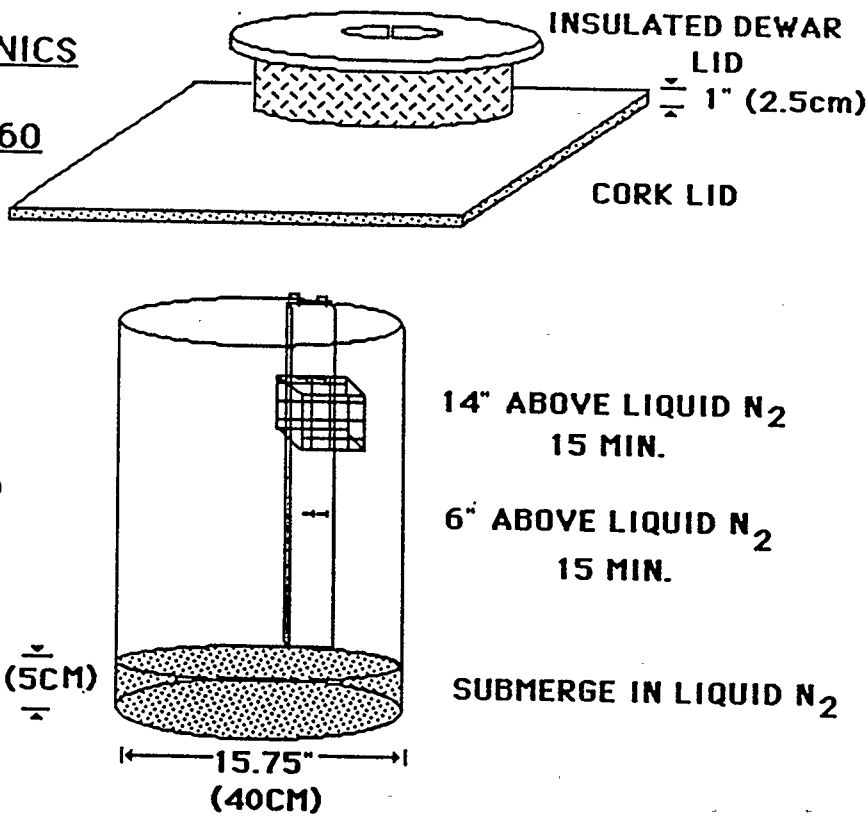
Two-thirds of the initial semen volume was transferred to a plastic mixing cone (Western Breeders, Balzac, Alberta) for freezing preparation. An equal volume of Ackerman's cryoprotectant (Behrman and Ackerman, 1969) (see appendix A for recipe) was added to the semen dropwise with mixing. The mixture was then sucked into 0.5 ml plastic freezing straws (United Breeders, Guelph, Ontario) plugged at one end with wicking and polyvinylpyrollidone (PVP) powder. When the sperm mixture was about 1 cm below the plug, the straw was withdrawn from the cone and sucking continued until the suspension wet the plug and sealed it. The open end was sealed by tamping the straw into PVP powder and placing the straw in water to solidify the

plug. Five straws were bundled together in a labelled plastic tube for freezing. If there were insufficient straws of sperm suspension to fill a tube, then "blank" straws containing only cryoprotectant were inserted to fill gaps. Straws were left at room temperature for 5 - 10 minutes to allow the sperm and cryoprotectant to equilibrate.

Freezing was accomplished at a controlled rate in an MVE Cryogenics tank (Model TA-60) filled to a depth of two inches with liquid nitrogen and left for 30 minutes to establish a stable temperature gradient (see figure 4). Tubes of straws were placed in a metal basket suspended 14 inches above the surface of the liquid nitrogen and cooled for 15 minutes. The basket was then lowered to 6 inches above the liquid nitrogen surface for another 15 minutes. Finally the basket and tubes were submerged in the liquid nitrogen until the bubbling stopped, bringing the temperature of the straws down to -196° C. Tubes were then removed from the freezing tank, placed on pre-cooled metal canes and stored in a large dewar flask filled with liquid nitrogen. Frozen specimens were stored for up to 5 1/2 months prior to use.

To thaw sperm specimens, tubes were removed from liquid nitrogen and the straws separated and left at room temperature for 20 minutes until liquid, then warmed to 37 °C in an incubator. The plugged ends of the straws were cut off and the contents pooled in a sterile centrifuge tube. As with the fresh material, the sperm mixture was diluted with BWW at 37° C to 10 ml and washed three times. Complete sperm analysis was carried out following the final

Figure 4:
MVE CRYOGENICS
TANK
MODEL TA-60



wash. All further processing was identical to that of the fresh material.

G. Media Preparation

i. Biggers, Whitten, and Whittingham (BWW) Media

A modified Krebs-Ringer solution called BWW (Biggers, Whitten, and Whittingham, 1971) was used for human sperm culturing and the in vitro fertilization steps. Composition of the stock solution is described in appendix A. BWW working solution was prepared freshly on the day of the experiment under sterile conditions. To 100 ml of stock solution was added 0.2106 g $NaHCO_3$, 0.37 ml DL lactic acid, and 0.5 g human serum albumin (HSA) and the solution filter-sterilized through a cellulose acetate-nitrate membrane filter, pore size 0.22um into two 50 ml sterile tissue culture flasks. The pH was adjusted to approximately 7.4 using sterile Hepes acid or base as necessary. The BWW working solution was warmed to and maintained at 37°C throughout the sperm preparation and egg processing steps.

ii F10 Working Solution

Ham's F10 with glutamine solution, supplemented with 15% fetal bovine serum, was used as a post-insemination media for overnight culture of the oocytes. Made fresh on the day of the experiment, 7.5 ml of fetal bovine serum (FBS) (inactivated at 56°C for 30 minutes) and 0.5 ml of penicillin-streptomycin antibiotic solution (see appendix A) were added to 42.5 ml of Ham's F10 under

sterile conditions. The pH was adjusted to 7.2 with filter-sterilized 1N HCl or 5% $NaHCO_3$.

H. Procurement and Processing of Oocytes

i. Superovulation of Hamsters

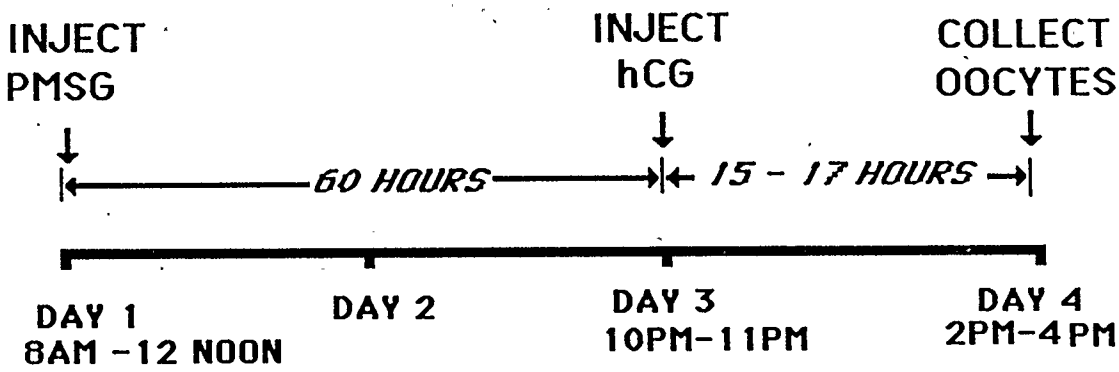
Female Syrian golden hamsters (Mesocricetus auratus) obtained from Charles River Breeding laboratories (Wilmington, Massachussets) at 5-7 weeks of age, were maintained on a constant light cycle of 14 hours light (8 am to 10 pm) and 10 hours dark (10 pm to 8 am) at 22 °C. Hamsters were induced to superovulate by injections of exogenous hormones according to the technique of Yanagimachi (1969). Hamsters were selected at random and injected intraperitoneally with 25 - 30 IU pregnant mare's serum (PMSG) in the morning of Day 1 (see figure 5). Approximately 60 hours later, in the evening of Day 3, a second intraperitoneal injection of 25 - 30 IU of human chorionic gonadotropin (hCG) was administered. 15 - 17 hours post-hCG injection, on Day 4, the cumulus cell mass containing the oocytes could be found in the oviducts. Usually 8 hamsters were sacrificied to provide 300-350 oocytes. However, since oocytes are a limiting factor in obtaining sperm chromosomes, when more data were required, up to 16 hamsters were used.

ii. Collection and Preparation of Oocytes

Hamsters were stunned with ether and killed by cervical dislocation. The oviducts were dissected out and placed in a watchglass (Canlab W2527-3) containing BWW at 37° C. All solutions

Figure 5:

Hormonal Regime for Optimal Oocyte Yield



HAMSTER LIGHT CYCLE : light 8am-10pm
dark 10pm-8am

were maintained at 37°C throughout, and the incandescent room lights were dimmed to prevent premature activation of the oocytes (Hirao and Yanagimachi, 1978). The oviducts were transferred to a second wash of BWW to remove surface blood and debris. One at a time the oviducts were transferred to a watchglass containing 1.5 ml BWW.

With the aid of a dissecting microscope with a red light filter, the oviduct was punctured and the cumulus mass pulled out using fine forceps. The empty oviducts were discarded. When all cumulus cell masses had been collected, 3 mg of hyaluronidase dissolved in 1.5 ml of BWW was added to the watchglass giving a final concentration of 0.1% hyaluronidase. The hyaluronidase dispersed the cumulus cells in 2 - 3 minutes. As the oocytes were freed from the cumulus cell mass and became visible under the dissecting microscope, they were transferred using a micropipet (with rubber tubing and mouthpiece attached) to a fresh wash of BWW in a 9-welled glass plate. Following a second wash of BWW, the oocytes were transferred to a 0.1% trypsin solution which dissolved the zona pellucidae in about one minute. Once the zonae had been removed the oocytes were washed three more times in BWW. Batches of 25 - 30 oocytes were then transferred into the capacitated sperm droplets, distributing them throughout to prevent clumping. Ideally, the processing of oocytes from dissection to coincubation with the sperm took about 30 minutes for 4 hamsters and yielded from 25 - 50 oocytes per hamster.

iii. Fertilization Check

After about 2 - 3 hours of incubation with the sperm, oocytes were examined for evidence of successful fertilization: swollen sperm heads or male pronuclei with tails. A few eggs were removed from the sperm droplets and washed in prewarmed F10 to remove any unattached sperm cells. Four to five oocytes at a time were transferred with a small volume of medium to the centre of a grease-free slide dotted with four drops of paraffin wax or petroleum jelly to support the corners of a coverslip. A glass coverslip was placed over the drop of medium resting on the wax. By pressing down gently on the corners of the coverslip, the eggs could be flattened almost to the point of bursting. Swollen sperm heads and male pronuclei could then be observed using a phase contrast microscope under 160X magnification. If fertilization had not yet occurred to a significant extent, (e.g. 20%) or if there was not appreciable attachment of sperm to the oocyte membrane (< 20), further incubation time was allowed up to a maximum of 4-4.5 hours.

iv. Oocyte Culture

When fertilization had occurred for at least 20% of the oocytes or there was greater than 20 sperm attached to each oocyte, oocytes were removed from the sperm droplets and washed in F10 under paraffin oil at 37° C. The oocytes were then distributed throughout 75ul droplets of F10 solution under paraffin oil in sterile tissue culture dishes for overnight (approximately 12 hours) incubation at 37°C, 5% CO_2 in air, and 95% humidity. The next morning (Day 5),

75µl of 0.8 ug/ml colcemid solution was added to each F10 droplet giving a final concentration of 0.4 µg/ml colcemid. The oocytes were then cultured for a further 4 - 6 hours.

I. Slide Preparation

Oocytes were fixed directly onto glass slides by a modified method of embryo fixation devised by Tarkowski (1966).

Two watchglasses were prepared several hours before slide making by filling them with 1% sodium citrate (hypotonic solution) and adding one to two drops of fetal bovine serum. This prevented the oocytes from sticking to the dish. Just prior to use, this solution was discarded and the dishes filled with fresh hypotonic solution at room temperature.

Oocytes were transferred into the hypotonic solution in batches of about 25 and allowed to swell. Fresh fixative was prepared (3 ml of 95% ethanol and 1 ml of glacial acetic acid) and kept in a closed vial. After three minutes in hypotonic solution, 5 - 10 oocytes, in a small drop of hypotonic solution were placed on an alcohol-precleaned slide. 20 µl of fixative was immediately dropped directly over the oocytes using an automatic pipet with a small bore micropipet tip. As the fixative dispersed and the eggs became visible to the naked eye, a circle was etched around the oocytes on the underside of the slide using a diamond tipped pencil. Before the fixative dried, a second drop was added from a height of 1 - 2 cm. Excess fixative was wiped from the edges of the slide using a tissue. The fixative was allowed to dry slightly,

until the oocytes became visibly flattened, before another drop of fixative was added. Four to five drops of fixative in total were used and gentle blowing after the last drop aided in drying and spreading the chromosomes.

J. Staining and Viewing of Chromosome Preparations

Slides were scanned prior to staining using a phase contrast microscope and chromosome spreads were circled using a diamond etcher objective. After two weeks of aging, slides were stained with 0.5% quinicrine dihydrochloride (at pH 4.4) for 15 - 20 minutes and rinsed 3 times in distilled water (pH 4.4) for a total of 10 minutes. Slides were mounted with distilled water (pH 4.4) under glass coverslips (Corning, No. 1 1/2) immediately prior to examination and photographing with direct fluorescence (D.C. powered HBO W2 mercury lamp with the barrier filter set at = 47 and the excitation filter at BG 3). All spreads containing human chromosomes were photographed using a mounted Nikon C-35 camera with Technical Pan Film (Kodak) and manual exposure.

After viewing and photographing, the slides were frozen to facilitate coverslip removal. The slides were then rinsed in methanol to remove excess wax and oil. Chromosome preparations were subsequently stained with Giemsa to aid and confirm Q-banded analysis. Slides were stained for 5 - 7 minutes in Giemsa (1 part Giemsa solution (Harleco) to 35 parts Gurr buffer), rinsed in distilled water and air dried. Solid stained preparations were examined using a Zeiss Photomicroscope III at 160X to 400X

magnification. Photographs were taken as required using Technical Pan film (35mm) (Kodak) with ASA setting 80, (automatic exposure). All films were developed in D-19 (Kodak) developer and printed on Kodak black and white paper (#1 to 5) by hand as permanent records and for subsequent analysis.

K. Analysis of Chromosome Preparations

Q-banded preparations were karyotyped from photographs according to the International System for Human Cytogenetic Nomenclature (1985) (ISCN 1985).

Abnormal chromosome complements were subclassified as numerically abnormal or aneuploid; structurally abnormal; and numerically and structurally abnormal (possessing both a numerical and structural abnormality in a single spread). If a sperm chromosome complement contained more than one numerical or structural abnormality or a combination of numerical and structural abnormalities it was counted only once in the appropriate class.

The chromosome number was assigned on the basis of the number of centric structures present in the cell regardless of the number of centromeres present in each structure. Thus triradials or other complex chromatid rearrangements were counted as only one structure. In the case of multiply broken and rearranged cells no chromosome number was assigned.

In the event of a double human complement with single or multiple anomalies, then if the two sex chromosomes were alike (i.e. XX or YY), one complement was considered normal while the other was

counted as a single abnormal complement. For the purposes of tallying the sex ratios, such a spread was not included.

In chromosome spreads where human and hamster chromosomes were inter-mixed or in close apposition, both sets of chromosomes were analysed. If there was additional or missing chromosomes which could not be definitively assigned to the human or hamster complement, the spread was excluded from the data. Likewise, if a nonidentifiable acentric fragment or marker was found within a human/hamster hybrid spread, the spread was excluded from the data.

Hypohaploid human spreads were included in the data if: 1) searching the area of the slide around the spread did not reveal extra chromosomes; 2) chromosomes were not excessively spread; and 3) the associated hamster spread was complete. All double human chromosome complements were classified as two normal haploid complements since diploidy could not be confirmed.

Structurally abnormal sperm chromosome complements containing multiple breaks and rearrangements were included in the study if the associated hamster complement was normal. If the hamster complement was similarly affected, then the anomaly was considered to be a technical artefact of the culturing process and the spread was not counted. Gaps of the chromosome (csg) or chromatid (ctg) variety were distinguished from breaks in chromosomes (csb) and chromatids (ctb) by the width of the staining discontinuity. Discontinuities of at least 5 chromatid widths or misalignment of the pieces were considered breaks. The only exception was in the case of centromeric regions which frequently

exhibited extended achromatic "lesions" considered to be normal for this particular chromosome type. Scanning electron microscopy has revealed that these "lesions" or "gaps" are merely regions of chromatin undercondensation (Chernos et al., 1986). Acentric fragments (ace) are non-identifiable pieces of chromosomes without any obvious centromere. Minutes (min) were distinguished from acentric fragments by being smaller than the width of a single chromatid.

L. Sperm Morphological Analysis

Semen smears were flamed to fix the cells and then stained with Giemsa (1 part Giemsa solution (Harleco) to 23 parts Gurr buffer). Preparations were then analysed with regular light microscopy under 400X magnification. 100 - 200 spermatozoa were classified according to the categories set out in the World Health Organization (WHO) publication: "Laboratory Manual for the Examination of Human Semen-Cervical Mucus Interaction", (1980; 1987), for head shape and tail defects. Immature germinal cells and other cell types present in the semen were also classified. Sperm cells were only counted if the tail was still attached. Spermatozoa with "kinked" or "hairpin" tail defects were not counted within the 100-200 classified cells although double tailed spermatozoa were included. Head shape was classified independently from tail defects of the former type. This was because in the previously frozen semen, tail defects were a very common response to osmotic stress brought about by the freezing and thawing processes and thus were not thought to be a legitimate parameter of the sperm profile.

M. Sperm Viability Analysis

Two supravital stains have been employed in this study. A drop of fresh semen was mixed on a slide with a drop of either eosin-blue/aniline or eosin-yellow. The eosin-blue preparations were dried rapidly on a hot plate. The eosin-yellow preparations were air dried at room temperature. Some slides were mounted in Entellan ®. Slides were read under 400X magnification with regular light. Dead cells pick up the stain appearing pink while live cells exclude the stain appearing white.

Early in the study eosin-blue was employed exclusively and the slides were stored and read at a later date. This stain however, originally developed for bull sperm (Hackett and MacPherson, 1965) seemed to yield a very high proportion of dead cells especially on postfreeze specimens. Some eosin-blue preparations were mounted permanently in Entellen ® to rule out leaching of the stain into the sperm (particularly postfreeze cells) prior to reading, contributing to the high dead cell frequency (Mahadevan, 1985). Later in the study, a more reliable stain, eosin-yellow was employed. This stain gave much lower proportions of dead cells and findings were consistent between mounted and unmounted preparations. The eosin-yellow stain also seemed to work well on previously frozen cells. While it was reported by Mahadevan (1985), that supravitally stained postfreeze sperm must be read immediately, it was the finding of this study, that preparations permanently mounted did not develop higher proportions of dead cells with time.

IV RESULTS

A. Summary of Data

The data in this study were generated from 42 fresh ejaculates obtained from 13 normal, healthy males. Five of these donors provided only one specimen because of either poor fertilization in the humster system (donors 3, 4 and 9) or unavailability (donors 5 and 7). A total of 91 separate experiments were conducted yielding 454 analysable sperm chromosome complements from fresh sperm and 387 from previously frozen sperm. A mean of 34.9 karyotypes was analysed on fresh sperm and a mean of 35.2 karyotypes was analysed on cryopreserved sperm from each donor. A summary of the data is provided in table 2.

An overall abnormality frequency of 17.8% (81/454) was observed for the prefreeze sperm compared with 13.4% (52/387) for the postfreeze sperm. The difference between these frequencies was significant by a chi squared test with one degree of freedom (χ^2_{1df}) ($0.01 < P < 0.05$). The breakdown of abnormalities for the prefreeze data was 9% structural abnormalities, 7.7% numerical abnormalities and 1.1% with both a numerical and structural abnormality in a single spread. These compared with the observed postfreeze frequencies of 8.8% structural abnormalities, 3.4% numerical abnormalities and 1.3% numerical with structural abnormalities. Only the frequencies of numerical abnormalities (in particular hypohaploidy) were significantly different before and

Table 2: DATA SUMMARY

	PREFREEZE DATA	POSTFREEZE DATA	$P(\chi^2_{1df})$
23,X	194	188	
23,Y	179	147	
TOTAL NORMAL	373 (82.2%)	335 (86.5%)	
Structural	41 (9%)	34 (8.8%)	P >0.6, n.s.
Numerical/Structural	5 (1.1%)	5 (1.3%)	p >0.6, n.s.
Numerical	35 (7.7%)	13 (3.4%)	P <0.002, **
Hypohaploidy	34 (7.5%)	13 (3.4%)	P <0.005, **
Hyperhaploidy	1 (0.2%)	0 (0%)	P >0.7, n.s.
TOTAL ABNORMAL	81 (17.8%)	52 (13.4%)	P <0.05, *
TOTAL KARYOTYPES	454	387	

*significant, ** highly significant, n.s.=not significant

after freezing ($P < 0.002$) which contributed to the observed difference in the total abnormality frequencies.

On the basis that nondisjunction results in equal proportions of hypohaploidy and hyperhaploidy, it was apparent that hyperhaploid cells were under-represented in both the prefreeze and postfreeze data ($P < 0.001$). Since chromosome loss using Tarkowski's fixation method (Tarkowski, 1966) can occur, the high frequency of hypohaploidy may represent a technical artefact. On this assumption, the data were corrected by doubling the observed hyperhaploidy frequency (including a numerical and structurally abnormal spread) to obtain a conservative estimate of the frequency of aneuploidy. The adjusted data are displayed in table 3.

The differences observed between the prefreeze and postfreeze abnormality frequencies in the full data set were no longer apparent for any category of abnormality in the corrected data set. The adjusted overall abnormality frequencies were 11.8% prefreeze and 10.4% postfreeze.

B. Types of Abnormalities

Table 4 and figure 6 summarize the distribution of abnormal sperm chromosome complements by type of abnormality. The numerical/structural category (NS) included only those spreads which had both an identifiable numerical and structural anomaly. The complex structural category (S+) included all structural abnormalities which involved more than one break (of the chromosome or chromatid type) with or without rearrangements. Simple

Table 3: DATA SUMMARY

ADJUSTED FOR ANEUPLOIDY

	PREFREEZE DATA	POSTFREEZE DATA	$P(X2_{1df})$
23,X	194	188	
23,Y	179	147	
TOTAL NORMAL	373 (88.6%)	335 (89.5%)	
STRUCTURAL	41 (9.7%)	34 (9.1%)	$P > 0.5$, n.s.
NUMERICAL/STRUCTURAL	5 (1.2%)	5 (1.3%)	$P > 0.5$, n.s.
NUMERICAL (based on doubling all spreads with an extra chromosome)	4 (0.9%)	0 (0%)	$P > 0.2$, n.s.
TOTAL ABNORMAL	50 (11.8%)	39 (10.4%)	$P > 0.4$, n.s.
TOTAL KARYOTYPES	423	374	

n.s. = not significant

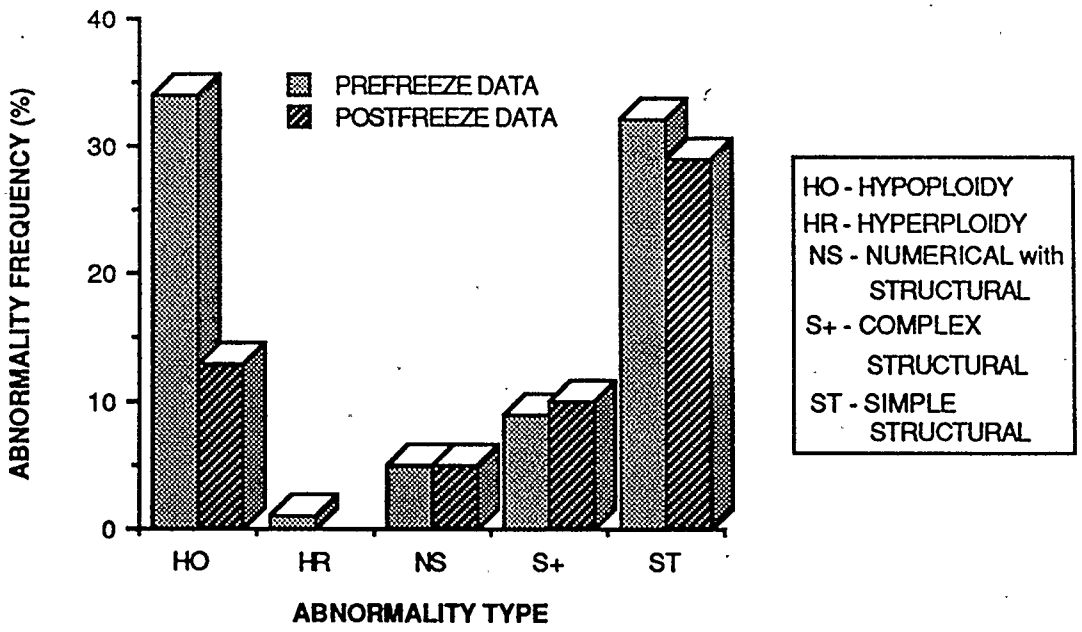
Table 4: SUMMARY OF ABNORMAL SPERM COMPLEMENTS

	PREFREEZE		POSTFREEZE		
ABNORMALITY TYPE	# SPREADS	% ABNORM.[1]	# SPREADS	% ABNORM.[1]	χ^2
Hypohaploidy (HO)	34	42%	13	25%	3.55
Hyperhaploidy (HR)	1	1.2%	0	0%	0.62
Numerical/ Structural (NS)	5	6.2%	5	9.6%	1.01
Complex Structural (S+)	9	11.1%	10	19.2%	3.04
Simple Structural (ST)	32	39.5%	24	46.2%	0.6
TOTALS	81	100%	52	100%	$\chi^2_{4df}=8.8$ $P > 0.05$, n.s.

[1]Proportion of abnormal sperm chromosome complements only.

n.s. = not significant

FIGURE 6
DISTRIBUTION OF ABNORMALITIES (N=133)



structural abnormalities (ST) included isolocus breaks and gaps, chromatid breaks and gaps, acentrics and deletions without rearrangements. There was no significant difference in the distributions of abnormalities between the prefreeze and postfreeze sperm chromosome complements ($\chi^2_{4df}=8.8$, $P> 0.05$).

Ignoring hypohaploidy which has a high degree of uncertainty associated with the observed frequency, simple structural anomalies were the most frequent type of abnormality observed (39.5% of prefreeze and 46.2% of postfreeze abnormal chromosome complements). Of these simple structural anomalies, chromosome breaks with both centric and acentric fragments present were the most common abnormality class observed (see table 5). Chromatid-type abnormalities were less frequent than chromosome-type abnormalities, and this trend was consistent for both prefreeze and postfreeze data sets ($\chi^2_{1df}=1.77$, $P>0.1$).

Details of the abnormal sperm chromosome complements in this study are listed in table 6 by individual donor and fresh versus cryopreserved semen treatment. Examples of normal and abnormal Q-banded sperm complements are shown in figures 7 through 11. The distinctive chromosome morphology of sperm pronuclear chromosomes is evident in these photomicrographs. The centromeric heterochromatin is undercondensed, particularly in the chromosomes with large regions of constitutive heterochromatin (1,9,16,Y). At times this gives the appearance of a gap in the centromere region while at other times the chromatin has a somewhat uncoiled appearance. This is quite striking in the chromosomes of figure 7.

Table 5: BREAKDOWN OF STRUCTURAL ABNORMALITIES

		PREFREEZE	POSTFREEZE	χ^2
Chromatid type				
	simple (ctg)	1	1	
	complex (cte)	3	6	
	MB&R	4	3	
	TOTAL	8 (17%)	10 (25%)	1.5
Chromosome type				
	simple (del, csg, ace, csb, min)	34	29	
	complex (rearrangements)	2	1	
	MB	2	0	
	TOTAL	38 (83%)	30 (75%)	0.273

$\chi^2_{1df}=1.77$

$P > 0.1$, n.s.

Table 6:

ABNORMAL SPERM CHROMOSOME COMPLEMENTS
BY INDIVIDUAL DONOR
(N=133)

	PREFREEZE DATA		POSTFREEZE DATA	
	TYPE	ABNORMALITY	TYPE	ABNORMALITY
Donor 1	ST	23,Y,csb(3)(cen)		
Donor 2	HO	20,Y-10-12-15	HO	22,-X or Y
	HO	21,Y-10-13	HO	22,Y-18
	HO	22,-X or Y	S+	46,XY,csb(11)(cen), del(9)(q22)
	HO	22,-X or Y	S+	MB&R
	HO	22,X-12	S+	MB&R
	HO	22,X-17	ST	23,X,csb(5)(p14)
	HO	22,X-17	ST	23,X,csb(7)(q22)
	HO	22,X-22	ST	23,X,csb(7)(q32)
	HO	22,X-22	ST	23,X,csg(5)(q2)
	HO	22,Y-5	ST	23,X,csg(22)(q13)
	HO	22,Y-16	ST	23,Y+ace
	HO	22,Y-16	ST	23,Y+ace
	HR	24,X+21	ST	23,Y+ace
	NS	21,Y-5-18-22+A	ST	23,Y,csb(6)(q14)
	S+	23,X,Bq-,Cq-, +3ace	ST	23,Y,csb(8)(p12)
	ST	23,X+ace	ST	23,Y,csb(9)(q22)
	ST	23,X+min	ST	23,Y,csb(13)(q21)
	ST	23,X,Cp-	ST	23,Y,csg(9)(p22)

Table 6 - continued

Donor 2	ST	23,X,csb(B)(q)	ST	23,Y,t(9;13)(q34;q14)
	ST	23,X,csg(6)(p21)		
	ST	23,X,csg(14)(q24)		
	ST	23,X,csg(B)(q)		
	ST	23,Y+ace		
	ST	23,Y,csg(1)(q)		
	ST	23,Y,del(1)(q12)		
Donor 6	HO	22,X-5	HO	20,X-1-2-12
	HO	22,X-21	HO	21,Y-8-17
	HO	22,X-E(16/17)	HO	22,X-5-19
	HO	22,X-G	HO	22,X-12
	HO	22,Y-22	HO	22,X-19
	HO	22,Y-D	HO	22,Y-11
	S+	?,Y,MB&R	HO	22,Y-17
	ST	23,X+ace	NS	21,-X or Y-1-16+3ace +cte(complex)
	ST	23,X,csb(5)(q)	NS	21,Y-B-19+2ace
	ST	23,X,csb(16)(p)	NS	22,Y-15,csb(3)(p13)
			S+	22,Y,cte(2;6) (p21;q24)qr,sym,comp.
			S+	22,Y,cte(4;9)(p14;q21) qr,sym,comp,+min
			ST	23,X+ace(2q)
			ST	23,Y+ace

Table 6 - continued

Donor 8	HO	21,X-10,-16	HO	21,Y-18-21
	HO	21,Y-6-16	HO	22,X-17
	HO	22,X-3	NS	22,X-13,del(14)(q13)
	HO	22,X-4	NS	22,Y-14,csg(2)(q13)
	HO	22X,-4	S+	23,Y,csb(11)(p11), cte(acellp11-pter;16) (p;q)tr,1cen,comp.
	HO	22,X-6	ST	23,Y,csb(9)(p13)
	HO	22,X-13		
	HO	22,Y-2		
	HO	22,Y-11		
	HO	22,Y,-12		
	HO	22,Y-14		
	HO	22,Y-15		
	HO	22,Y-20		
	NS	21,X-6,cte(11;19) (pter;pter)tr+ace		
	S+	22,X,cte(13;16)tr		
	S+	MB&R		
	ST	23,X,csb(5)(q12)		
	ST	23,X,csb(12)(cen)		
	ST	23,X,csb)(12)(q13)		
	ST	23,X,del(11)(q14)		
	ST	23,Y,csb(1)(p12)		

Table 6 - continued

Donor 10	NS	21,X-13-22,csg(11)(p15)		
	S+	MB&R		
	ST	23,X,csb(6)(q13)		
Donor 11	HO	22,Y-12	HO	21,Y-10-19
	NS	21,X-1-10+2ace	ST	23,Y,csg(12)(p12)
	S+	?,Y,MB&R	ST	23,Y+min
	ST	23,X,csb(X)(q24)		
	ST	23,X,csg(8)(q22)		
	ST	23,X,ctg(9)(p22)		
	ST	23,X,csb(10(p11)		
	ST	23,Y,csb(3)(q13)		
Donor 12	HO	21,X-21,-22	HO	22,X-22
	S+	22,Y,cte(13;14)(q12;q13)	S+	22,X,cte(8;22)(p12;q13)qr,asym,comp.
	S+	?23,X,MB	S+	23,X,csb(2)(q12),csb(18)(cen),csb(20)(cen)
	S+	?23,Y,MB	S+	23,X,csg(6)(p23),cte(11;15)(p15;q1)tr,incomplete.
	ST	23,X+ace	S+	?X,MB&R
	ST	23,X,csb(1)(p)	ST	23,X,csb(17)(q21)
	ST	23,Y,csb(5)(q14)	ST	23,X,csb(12)(q13)
	ST	23,Ycsb(7)(q34)	ST	23,X,ctg(13)(q21)
			ST	23,Y+2ace(?2csb16)
			ST	23,Y,del(5)(q31)

Table 6 - continued

Donor 13	HO	22,Y-15
	NS	24,X,i(Y)(qter-?cen ?cen-qter)
	ST	23,X,csb(8)(q13)
	ST	23,Y,csb(18)(q12)
	ST	46,XY,csb(5)(q13)

Figure 7: Karyotype of a normal human sperm complement (23,Y) derived from fresh semen. Note the highly extended regions of heterochromatin in 1, 9, 16 & Y and centromeric gaps in other chromosomes (e.g. 4, 11, 12). (X2900)

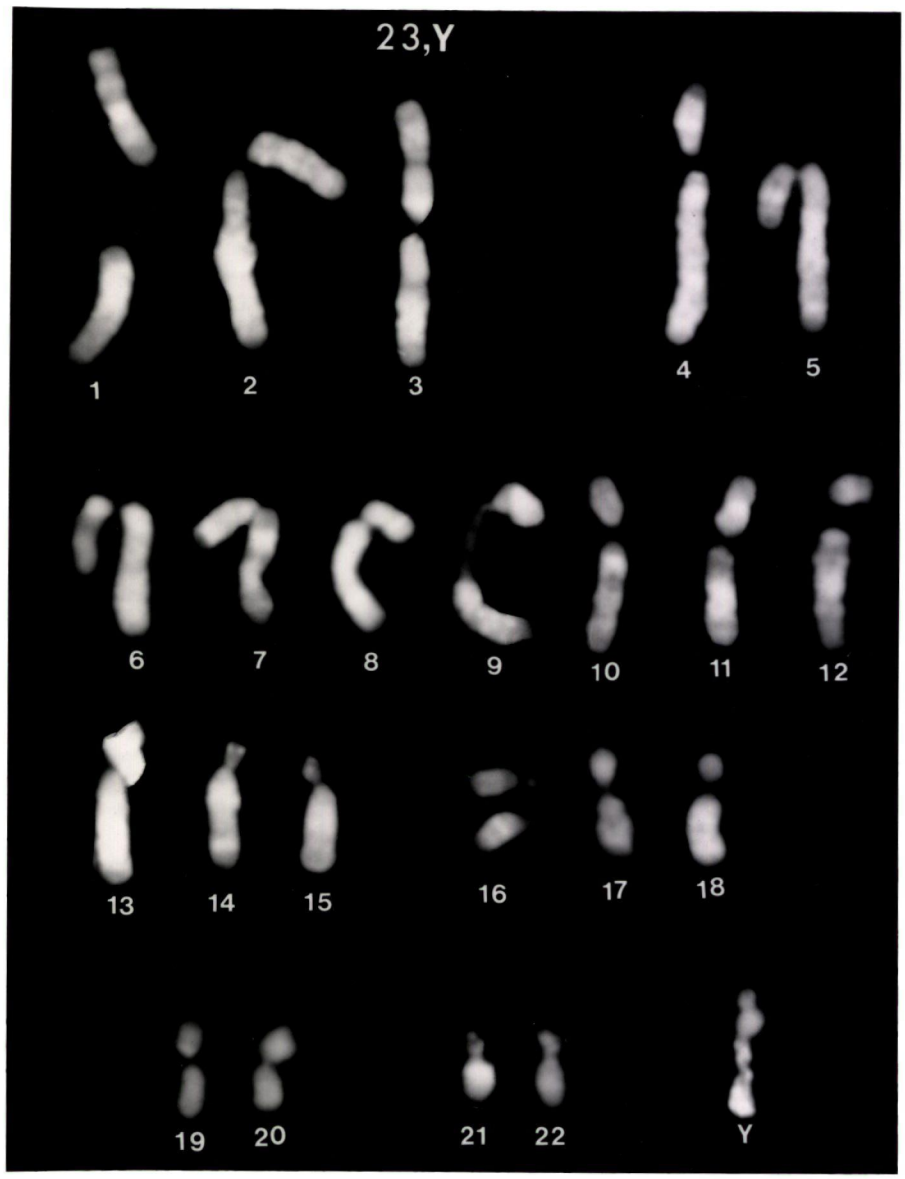

23,Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Y

Figure 8: Karyotype of human sperm complement with both a numerical and structural abnormality (23,X,i(Yq)) derived from fresh semen. (X2800)

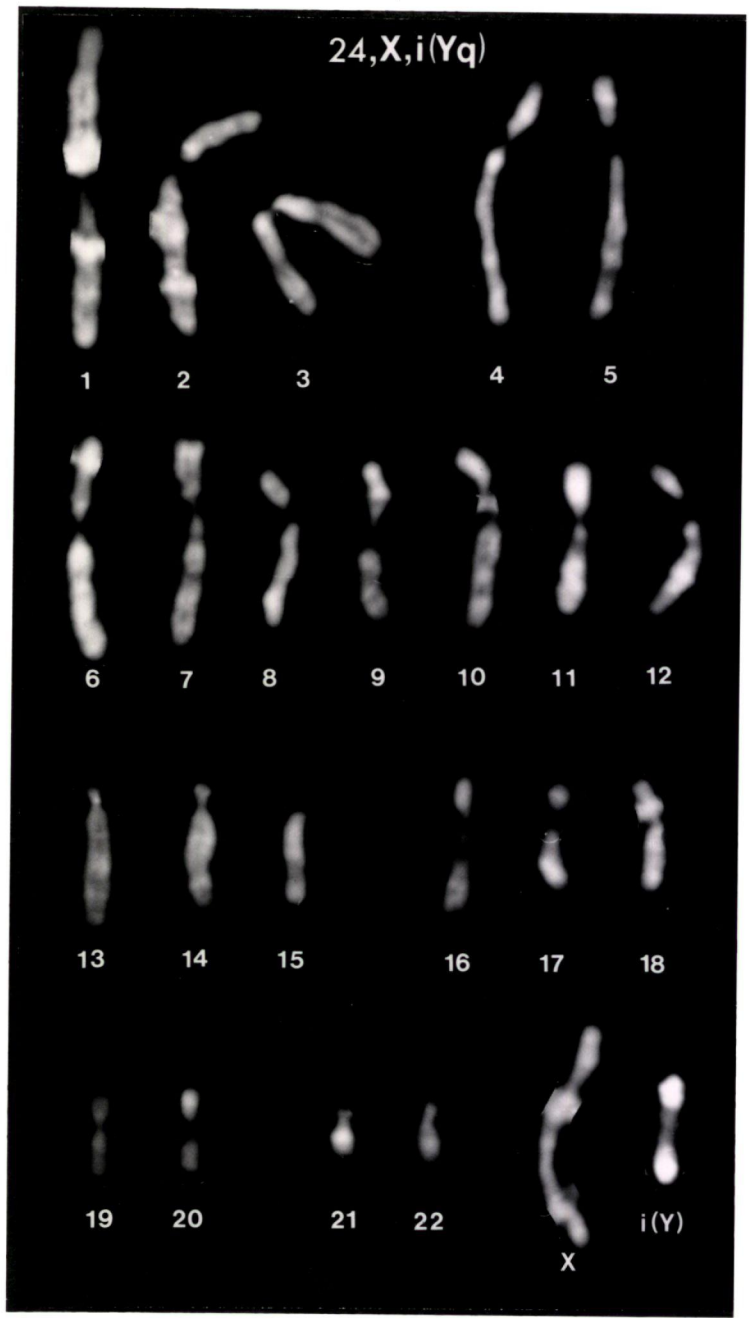

24,X,i(Yq)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
i(Y)

Figure 9: Q-banded and Giemsa stained abnormal sperm complement containing multiple breaks and rearrangements and an X-chromosome derived from fresh semen. (X2900)


MB & R

Figure 10: Karyotype of a normal sperm complement (23,Y) derived from cryopreserved sperm. (X3100)

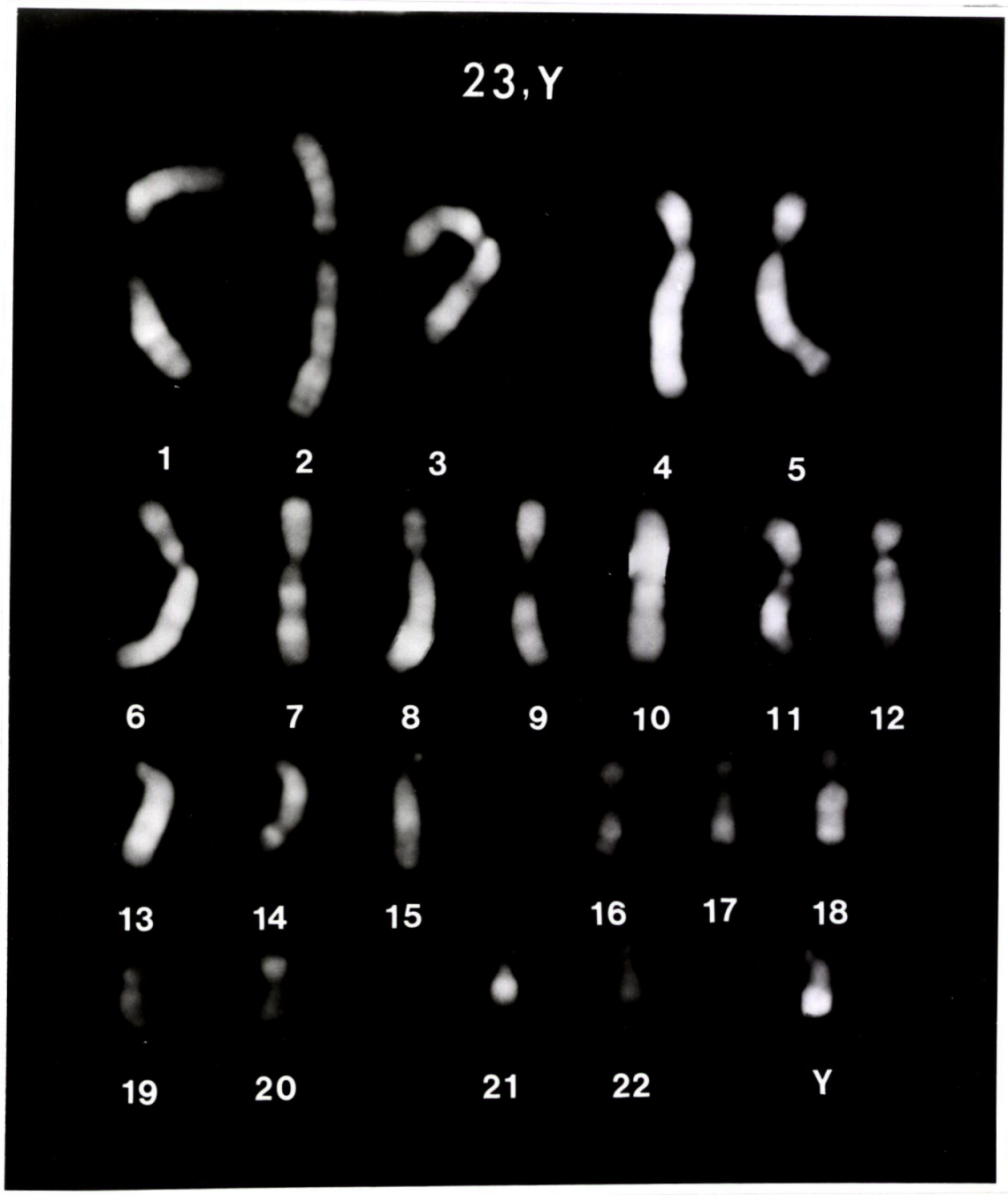
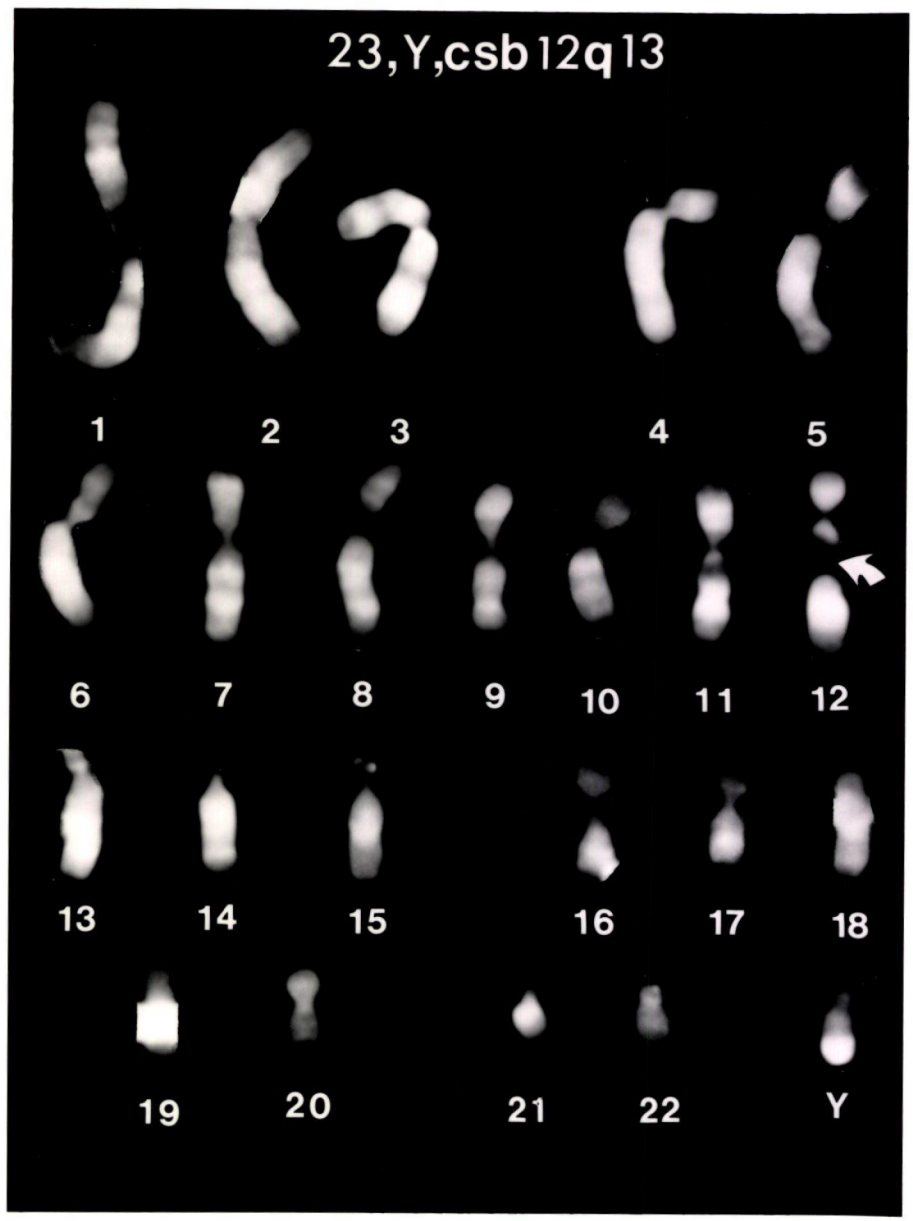

23, Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Y

Figure 11: Karyotype of a structurally abnormal sperm complement (23,Y,csb12q13) derived from cryopreserved sperm. (X3500)


23,Y,csb 12q13
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Y

Banding quality was usually inferior to that obtained with lymphocyte or fibroblast chromosomes. However, as in other cell types, the quality of banding is dependent on the extent of chromosome condensation and how well fixed the preparations are. Spreads with multiple breaks and rearrangements were not usually well banded and in conjunction with the complex rearrangements, they could not be karyotyped in detail.

C. Comparative Abnormality Frequencies Among Donors

Table 7, compares the incidence of abnormalities among 8 donors with one or more abnormal sperm complements. Donors ranged from a low of 10.7% to a high of 33.8% (mean 16.9%) abnormal sperm complements for fresh semen and from 0% to 37.5% (mean 11.45%) abnormal sperm complements for cryopreserved semen. Since some frequencies were based on a small number of analysed sperm complements, they may not accurately reflect a donor's true incidence of sperm abnormalities.

A series of χ^2 tests (1 degree of freedom) were carried out to compare the individual responses to freezing. Donor 2 showed a marked decrease in his sperm chromosome abnormality rate after freezing ($\chi^2_{1df}=25.4$; $P<0.0001$). Donor 11, exhibited a statistically significant increase in his sperm chromosome abnormality frequency after freezing ($\chi^2_{1df}=5.96$; $0.01<P<0.05$). All other donors showed no significant difference in their sperm chromosome abnormality frequencies pre- and postfreeze. A heterogeneity χ^2 test (Sokal and Rohlf, 1969) indicated significant

heterogeneity among the donor's responses to freezing ($\chi^2_{6df}=31.96$, $P<0.001$).

Since it is evident that donor 2 contributes the most to the total χ^2 value and hence the significant heterogeneity among donors observed, the data can be reexamined excluding donor 2. When this is done, the heterogeneity among donors is no longer significant ($\chi^2_{5df}=5.1, P>0.2$).

When the total abnormalities for each donor were broken down by abnormality type, many of the classes were too small for statistically relevant analysis. If only those donors which had more than 30 analysed chromosome complements (in each of prefreeze and postfreeze treatments) were considered, donors 2 and 8 exhibited significantly different distributions of abnormalities before and after freezing (donor 2: $\chi^2_{2df}=24.23, P<0.001$; donor 8: $\chi^2_{2df}=8.25, P<0.05$). However, donors 6 and 12 showed no difference in the distribution of abnormalities (donor 6: $\chi^2_{2df}=0.98$, n.s.; donor 12: $\chi^2_{2df}=0.16$, n.s.). The expected values in many of the cells however, were quite small (< 5) casting some doubt on the validity of these tests in terms of showing true differences in distributions.

D. Distribution of Abnormalities Among Sperm Chromosomes

Given the high abnormality rates observed for both pre- and postfreeze data, it was of interest to discern if the abnormalities were randomly distributed or whether certain chromosomes or loci were more frequently involved.

Table 7: COMPARATIVE ABNORMALITY FREQUENCIES AMONG DONORS WITH ONE OR MORE ABNORMAL SPERM CHROMOSOME COMPLEMENTS (N=8)

DONOR		#NORMAL KARYOTYPES	#ABNORMAL KARYOTYPES	NUMERICAL	STRUCTURAL	NUMERICAL/ STRUCTURAL
1	PRE	7	1(12.5%)	0(0%)	1(12.5%)	0(0%)
	POST	2	0(0%)	0(0%)	0(0%)	0(0%)
2	PRE	49	25(33.8%)	13(17.6%)	11(14.9%)	1(1.4%)
	POST	124	19(13.3%)	2(1.4%)	17(11.9%)	0(0%)
6	PRE	53	10(15.9%)	6(9.5%)	4(6.3%)	0(0%)
	POST	79	14(15.1%)	7(7.5%)	4(4.3%)	3(3.2%)
8	PRE	93	21(18.4%)	13(11.4%)	7(6.1%)	1(0.9%)
	POST	51	6(10.5%)	2(3.5%)	2(3.5%)	2(3.5%)
10	PRE	23	3(11.5%)	0(0%)	2(7.7%)	1(3.8%)
	POST	6	0(0%)	0(0%)	0(0%)	0(0%)
11	PRE	67	8(10.7%)	1(1.3%)	6(8.0%)	1(1.3%)
	POST	5	3(37.5%)	1(12.5%)	2(25.0%)	0(0%)
12	PRE	37	8(17.8%)	1(2.2%)	7(15.6%)	0(0%)
	POST	56	10(15.2%)	1(1.5%)	9(13.6%)	0(0%)
13	PRE	29	5(14.7%)	1(2.9%)	3(8.8%)	1(2.9%)
	POST	11	0(0%)	0(0%)	0(0%)	0(0%)

Abnormal chromosome complements were first classified by the chromosome group(s) involved in the abnormality (summarized in table 8). The expected frequencies were calculated from the number of chromosomes in each ISCN group (ignoring chromosome size differences) as a proportion of the total haploid chromosome number (23) multiplied by the total number of abnormalities classified in each group for each semen treatment. Neither the fresh nor the previously frozen semen exhibited abnormal sperm chromosome distributions which deviated significantly from the expected distributions of abnormalities ($\chi^2_{7df}=7.55$, n.s. for fresh data; $\chi^2_{7df}=2.24$, n.s. for cryopreserved data). In addition, a 2X8 contingency table failed to reveal any difference in the distributions of abnormalities between fresh and previously cryopreserved sperm ($\chi^2_{7df}=4.6$, n.s.).

The high frequency of chromosome loss (hypohaploidy) in this study was somewhat disconcerting. Because of the chance that the fixation technique was contributing to chromosome loss, it was postulated that the smaller chromosomes would be more likely to be lost. Smaller chromosomes might be more easily blown away during the fix dry down and because of their small size, not picked up as strays as readily in the surrounding area. Table 9 summarizes the distribution of hypohaploid sperm complements for prefreeze and postfeeze treatments. There was no significant difference between the observed distributions either prefreeze or postfreeze. Even when the data were pooled into large and small chromosome categories, there was no significant difference from the expected

TABLE 8: FREQUENCIES OF CHROMOSOME GROUPS INVOLVED IN ABNORMALITIES

	PREFREEZE			POSTFREEZE		
ISCN GROUP	OBS.FREQ.	EXP.FREQ.[1]	χ^2	OBS.FREQ.	EXP FREQ.[1]	χ^2
A	10	10.83	0.064	7	8.09	0.147
B	12	7.22	3.16	6	5.39	0.69
C	24	25.26	0.063	21	18.87	0.24
D	11	10.83	0.003	7	8.09	0.147
E	11	10.83	0.003	10	8.09	0.45
F	2	7.22	3.77	5	5.39	0.028
G	9	7.22	0.44	4	5.39	0.358
X/Y	4	3.6	0.046	2	2.69	0.177
TOTALS	83	83	$\chi^2_{7df}=7.55$ $P>0.4$ (n.s.)	62	62	$\chi^2_{7df}=2.24$ $P>0.9$ (n.s.)

[1] based on number of chromosomes per group in haploid complement

n.s. = not significant

distributions prefreeze or postfreeze. A Fisher's Exact Test was also conducted on the pooled data to examine if the pre and postfreeze distributions were different from each other. This gave a value of P=1, indicating that there was no sigificant difference in the distribution of large and small missing chromosomes between semen treatments.

A further hypothesis to be tested regarding the distribution of abnormalities within the haploid chromosome complement was whether structural anomalies were randomly distributed. Several well-documented fragile sites occur in the normal human karyotype (Hecht, 1986) many of which have also been found to be hotspots for chromosome breakage and rearrangements (Kano and Little, 1986; YeWu and Zhou, 1986) and mitotic crossing-over (Therman and Kuhn, 1981).

If the breakpoints observed among the structural anomalies in human sperm cells were randomly distributed, the probability of finding a structural abnormality within a particular chromosome should be proportional to the relative length of the chromosome. On this basis, table 10 was constructed. The observed frequencies of structural abnormalities within the various chromosome groups matched quite closely the frequencies predicted on the basis of chromosome size ($\chi^2_{8df}=4.58$, n.s. for prefreeze anomalies; $\chi^2_{8df}=7.15$, n.s. for postfreeze anomalies). In addition, there was no detectable difference between the prefreeze and postfeeze distributions of structural abnormalities using a 2X8 contingency table ($\chi^2_{7df}=5.93$, n.s.).

Table 9: FREQUENCIES OF CHROMOSOME GROUPS INVOLVED IN HYPOHAPLOIDY

ISCN GROUP	PREFREEZE OBS.FREQ.	PREFREEZE EXP.FREQ.[a]	PREFREEZE χ^2	POSTFREEZE OBS.FREQ.	POSTFREEZE EXP.FREQ.[a]	POSTFREEZE χ^2
A	2	4.96	1.77	1	2.35	0.775
B	4	3.30	0.148	1	1.57	0.207
C	9	11.57	0.57	5	5.48	0.042
D	7	4.96	0.839	0	2.35	2.35
E	7	4.96	0.839	5	2.35	2.99
F	1	3.30	1.603	3	1.57	1.30
G	6	3.30	2.21	2	1.57	0.118
X/Y	2	1.65	0.074	1	0.78	0.062
TOTALS OVERALL	38	38	$\chi^2_{7df}=7.214$ P>0.4,n.s.	18	18	$\chi^2_{7df}=7.84$ P> 0.4, n.s.
Large Chroms.[b] (A.B.C.)	15	19.83	1.176	7	9.4	0.613
Small Chroms.[b] (D,E,F,G)	21	18.17	0.44	11	8.62	0.657
			$\chi^2_{1df}=1.62$ P> 0.1, n.s.			$\chi^2_{1df}=1.27$ P>0.2, n.s.

[a] based on number of chromosomes per group in haploid complement.

[b] not including sex chromosomes aneuploidy

n.s. = not significant.

Table 11 lists the breakpoints involved in the structural abnormalities which could be accurately identified. Three of 26 (11.5%) prefreeze breakpoints and 2/34 (5.9%) postfreeze breakpoints were considered as hotspots for mitotic crossing-over (Therman and Kuhn, 1981). Two of 26 (7.7%) of prefreeze breakpoints and 3/34 (8.8%) of postfreeze breakpoints are common fragile sites in humans. Since more than 80 fragile sites have now been identified, it is not surprising that some of these were at the same loci as breakpoints found in this study and therefore there is likely no biological significance.

Therman and Kuhn (1981), in their study of the incidence and distribution of mitotic crossing-over in Bloom's syndrome patients, hypothesized that Q-dark regions (that is the non-fluorescent bands) are more frequently involved in these exchanges due to the higher gene density and therefore more extended conformation in these regions. Some of the types of structural abnormalities observed in sperm chromosomes can arise post-fertilization and therefore are akin to mitotic cross-over events. The breakpoints identified in this study were classified as Q-bright (brightly fluorescent) or Q-dark (dull) and the frequencies of each type compared. Although there appeared to be more breakpoints located in Q-dark bands for both prefreeze and post-freeze data, these differences were not significant ($\chi^2_{1df}=2.46$ n.s., prefreeze; $\chi^2_{1df}=0.47$, n.s., postfreeze and $\chi^2_{1df}=2.4$, n.s., pooled data).

Table 10: FREQUENCIES OF CHROMOSOME GROUPS INVOLVED IN STRUCTURAL ABNORMALITIES

	PREFREEZE			POSTFREEZE		
ISCN GROUP	OBS.FREQ.	EXP.FREQ.[1]	χ^2	OBS.FREQ.	EXP.FREQ.[1]	χ^2
A	7	8.4	0.23	5	8.4	1.38
B	7	4.3	1.69	4	4.3	0.02
C	14	11.9	0.37	16	11.9	1.41
D	3	3.6	0.1	5	3.6	0.54
E	3	3.1	0.003	4	3.1	0.26
F	1	1.7	0.29	1	1.7	0.29
G	0	1.2	1.2	2	1.2	0.53
X	1	2.05	0.54	0	2.05	2.05
Y	1	0.67	0.16	0	0.67	0.67
TOTALS	37	36.9	$\chi^2_{8df}=4.58$ P>.5,n.s.	37	36.9	$\chi^2_{8df}=7.15$ P>.5,n.s

[1] based on relative length as percentage of total haploid autosome length (ISCN, 1985, appendix 2)

n.s. = not significant.

Table 11: BREAKPOINTS INVOLVED IN STRUCTURAL ABNORMALITIES

BAND	PREFREEZE Q-BRIGHT	Q-DARK	BAND	POSTFREEZE Q-BRIGHT	Q-DARK
1p12	X		2p21		X
1q12		X	2q12	X	
3cen	X		2q13[c]		X
3q13	X		3q13	X	
5q12	X		4p14		X
5q13		X	5p14	X	
5q14	X		5q2	X	
6p21[a]		X	5q13		X
6q13 ?		X	5q31 ?		X
7q34		X	6q14	X	
8q13		X	6q24	X	
8q22[c]		X	7q22 ?		X
9p22		X	7q32[bc]		X
10p11		X	8p13	XX	
11p15 ?		X	9p13		X
11pter		X	9p22		X
11q14 ?	X		9q21	X	
12cen	X		9q22?	XX	
12q13[ac]		X	11cen	X	
13q12		X	11cen	X	
14q13		X	11p11	X	
14q24 ?		X	12p12	X	
18q12 ?[a]	X		12q13[ac]	X	
19pter		X	13q14		X
Xq24		X	13q21 ?		XX
Ycen	X		14q13		X
			17q21[a]		X
			18cen		X
			20cen		X
			22q13 ?		XX
TOTALS	9(34.6%)	17(65.4%)		15(44%)	19(56%)

[a] Hotspots for mitotic crossing-over (Therman and Kuhn, 1981).
[b] One of the most common fragile sites in humans (Hecht, 1986).
[c] Confirmed fragile sites identified by Human gene mapping VII 1983, and HGM VIII, 1985.
? Purported fragile sites (Human gene mapping VIII 1985)

E. Sex Ratios

On theoretical grounds, meiosis in a normal 46,XY male should yield equal proportions of X- and Y-chromosome bearing sperm cells. Table 12 summarizes the sex ratios found in this study for the various categories of sperm chromosome complements. The overall sex ratios were found to be 52.9% prefreeze and 53.5% postfreeze. These ratios are not significantly different from 50%, nor do prefreeze and postfreeze sex ratios differ significantly from each other. The prefreeze sex ratio of normal spreads alone (52%), shows no significant difference from 50% and does not differ from the sex ratio of normal sperm cryopreservation. The postfreeze normal sperm complements do however exhibit a sex ratio which is significantly different from 50% ($0.01 < P < 0.05$).

The sex ratio of abnormal sperm complements alone is significantly different from 50% for prefreeze data, but not for postfreeze data. The prefreeze and postfreeze sex ratios for abnormal sperm complements are skewed in opposite directions resulting in a highly significant P-value when the ratios are compared ($P < 0.005$). This trend is repeated in the structurally abnormal sperm data. The sex ratios of numerically abnormal sperm however, show no significant differences compared with the theoretically expected 50% or between fresh and cryopreserved sperm data. In these subgroups of abnormals, the number of sperm complements is rather small so no firm conclusions can be drawn from these findings.

Table 12: SUMMARY OF SEX RATIOS

X:Y (%X)

	PREFREEZE	$P_{50\%}$ [a]	POSTFREEZE	$P_{50\%}$ [a]	$P_{pre/post}$ [b]
OVERALL	240:208(52.9%)	$p>0.1$ n.s.	207:175(53.5%)	$p>0.1$ n.s.	$P>0.6$ n.s.
NORMAL	194:179(52%)	$P>.04$ n.s.	188:147(56.1%)	$P<0.05$ *	$P>0.1$ n.s.
ABNORMAL	46:29(61%)	$P=0.05$ *	19:28(40.4%)	$P>0.1$ n.s.	$P<0.005$ **
STRUCTURAL[c]	29:13(69%)	$P<0.01$ *	13:22(37%)	$P>0.1$ n.s.	$P<0.001$ **
NUMERICAL[c]	20:17(54%)	$P>0.6$ n.s.	7:9(43.8%)	$P>0.6$ n.s.	7:9(43.8%) n.s.

[a] comparison to theoretical 50% sex ratio, by χ^2_{1df} test

[b] comparison of prefreeze versus postfreeze sex ratios by χ^2_{1df} test

[c] includes karyotypes with both numerical and structural abnormalities

* significant, ** highly significant, n.s. = not significant.

F. Semen Analysis

Table 13 summarizes the major parameters considered in the semen analysis. Semen samples from all donors, including those which left the study after only one donation, underwent complete semen analysis where possible. With the exception of donor 12, all postfreeze fertilization rates were lower than for the fresh counterpart. The mean fertilization rate in the fresh experiments was half of that in the fresh experiments (15% versus 30%). The reduced fertilizing ability is partly attributable to cell death following freezing and thawing as reflected in the percent of live sperm (table 13). Only about half of all sperm survive the freezing and thawing process as indicated by the uptake of supravital stain, and it is evident in these data, that certain donors' sperm survive cryopreservation better than others' (compare donors 6 and 2).

Morphological analysis of spermatozoa yields an average abnormality frequency of 14.4% prefreeze and 13.5% postfreeze. These values are within the normal range reported by the WHO (1980) of 20% with 5% being contributed by tail defects (ignored in this study). Examples of morphologically abnormal sperm are shown in figure 12.

There was no detectable difference in the frequency of abnormal sperm heads following freezing although tail defects of the type induced by osmotic stress were frequently observed in thawed semen. These sperm were still capable of successfully penetrating hamster oocytes *in vitro* as swollen sperm heads with kinked or hairpin tails were observed (see figure 13).

Table 13: SEMEN ANALYSIS DATA

DONOR	AGE	# EJACULATES	PRE/POST	% FERTILIZED OOCYTES	% LIVE SPERM CELLS	% ABNORMAL SPERM HEADS	% IMMATURE GERM CELLS
1	25	2	PRE	30	57	17	0
			POST	10	N/A	N/A	N/A
2	24	6	PRE	53	54	16	1.3
			POST	47	37	22	1.1
3	N/A	1	PRE	N/A	N/A	N/A	N/A
			POST	0	N/A	N/A	N/A
4	24	1	PRE	10	N/A	14	2
			POST	N/A	N/A	N/A	N/A
5	35	1	PRE	0	N/A	12	0
			POST	6	35	18	8
6	28	4	PRE	63	83[a]	15	0
			POST	34	19	16	1
7	27	1	PRE	100	76[a]	27	3
			POST	N/A	32	22	4.3

Table 13 continued.

DONOR	AGE	# EJAC.	PRE/ POST	% FERT.	% LIVE	% ABNORM.	% IMMAT.
8	33	4	PRE	22	76[a]	9	0.7
			POST	14	32	10.1	0.8
9	N/A	1	PRE	0	89	26	1
			POST	N/A	N/A	N/A	N/A
10	27	6	PRE	17	50	14	1.2
			POST	5	41	5.6	3.9
11	37	7	PRE	26	60	7	2.5
			POST	9	29	4.5	0.7
12	27	3	PRE	21	62	10	0.7
			POST	22	30	5.3	0.3
13	22	5	PRE	18	57[b]	5.4	0.6
			POST	3	36	18	1
MEANS	28.1		PRE	30	73.8	14.4	1.2
			POST	15	32.3	13.5	2.2

[a] values provided by U. of Calgary Infertility Clinic

[b] c.f. to 74% when eosin yellow stain exclusively is used

Immature germinal cells were present in low frequencies among the donors in this study (1.2% prefreeze and 2.2% postfreeze). As might be expected, cryopreservation did not seem to influence the incidence of immature sperm cells.

Since there were some obvious effects of cryopreservation on the semen parameters examined in this study, two parameters were investigated for possible correlations to the sperm chromosome abnormality frequencies of individual donors. Table 14 and figures 14 and 15, compare the fertilization rates and frequencies of morphologically abnormal sperm with individual donors' sperm chromosome abnormality rates. By inspection of the scatterplots, no obvious linear trend could be discerned. Calculation of the correlation coefficients confirmed that there were no direct relationships between the pairs of parameters examined. This suggests that the chromosome abnormality rate for each donor was unrelated to that donor's sperm morphological profile and the ability of his sperm to penetrate hamster oocytes.

Figure 12: Examples of abnormal sperm morphology A/DT - amorphous head with double tail; N - normal sperm head; P - pyriform head shape; T - tapered head; LO - large oval head; DH - double head. Double tail defects are noted, but coiled tails as in the sperm with pyriform head are not. (X1400)


A/DT
N
P
N
T
N
LO
DH
N
DT
N

Figure 13: Evidence of successful fertilization: swollen sperm heads (SSH)(arrows) with tails attached within the ooplasm. Normal size sperm are located on the surface of the oocyte. Note the abnormal tail configurations ("corkscrew" and "hairpin") on the successfully penetrated sperm cells. (X1300)

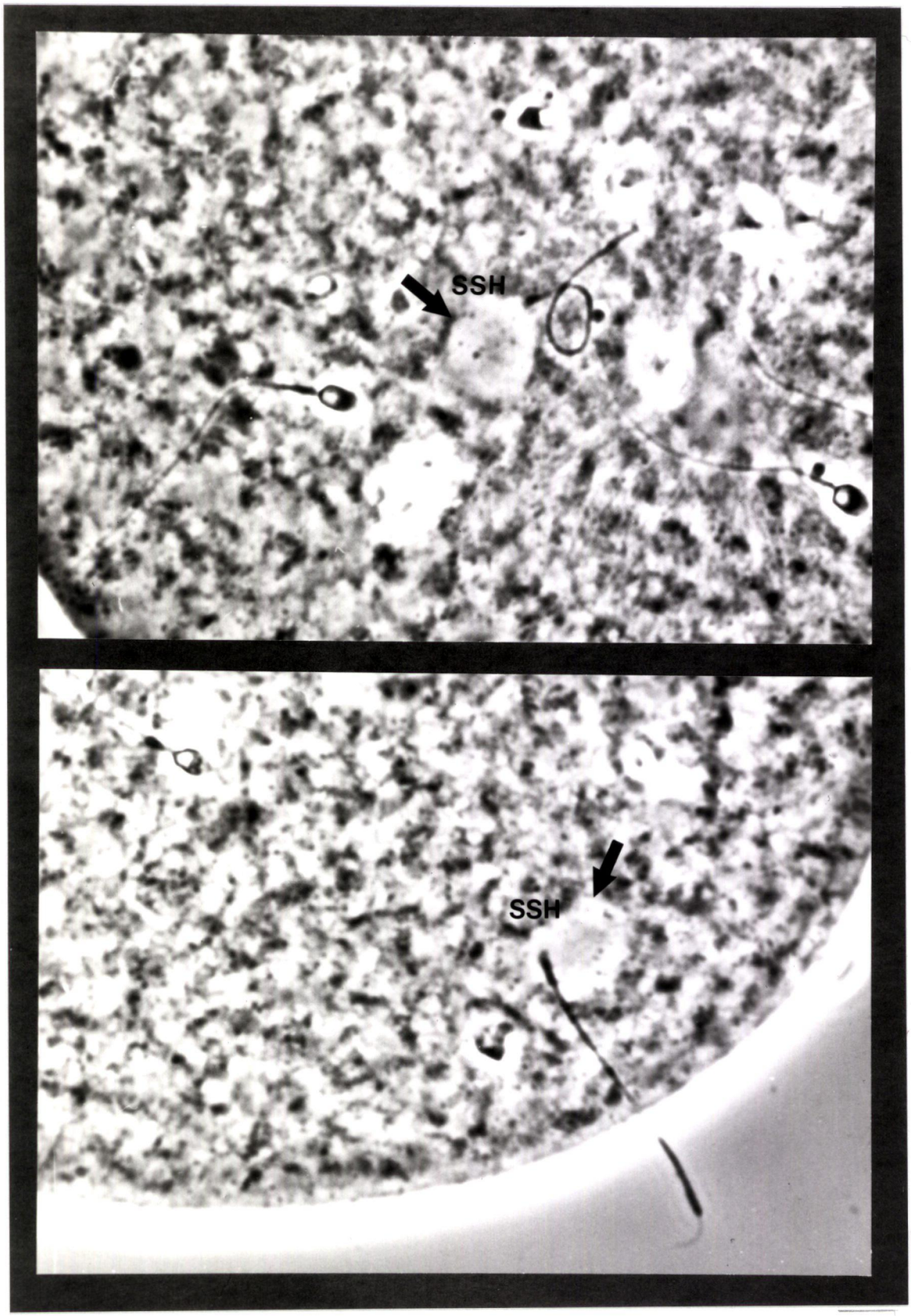

SSH
SSH

Table 14: COMPARISON OF SEMEN PARAMETERS AND CYTOGENETIC ABNORMALITY FREQUENCIES AMONG DONORS WITH >1 ABNORMAL SPERM CHROMOSOME COMPLEMENT

DONOR	PRE/POST	% ABNORMAL KARYOTYPES	FERTILIZATION RATE (%)	% MORPHOLOGICALLY ABNORMAL SPERM
1	PRE	12.5	30	17
	POST	0	10	N/A
2	PRE	33.3	53	16
	POST	13.3	47	22
6	PRE	15.9	63	15
	POST	15.1	34	16
8	PRE	18.4	22	9
	POST	10.5	14	10.1
10	PRE	11.5	17	14
	POST	0	5	5.6
11	PRE	10.7	26	7
	POST	37.5	9	4.5
12	PRE	17.8	21	10
	POST	15.2	22	5.3
13	PRE	14.7	18	5.4
	POST	0	3	18
MEANS	PRE	16.85	31.25	11.68
	POST	11.45	18.0	11.64

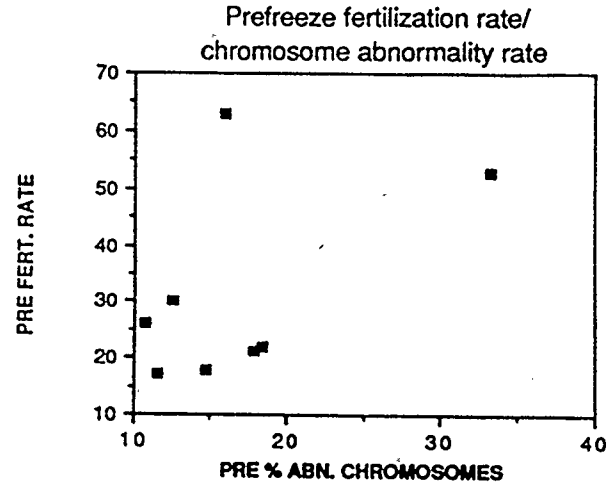
Figure 14: Scatterplots: Semen parameters versus cytogenetic abnormality frequencies-prefreeze data.

a. Fertilization Rate (Pre Fert. Rate) versus Chromosome Abnormality Frequency (Pre % Abn. Chromosomes).

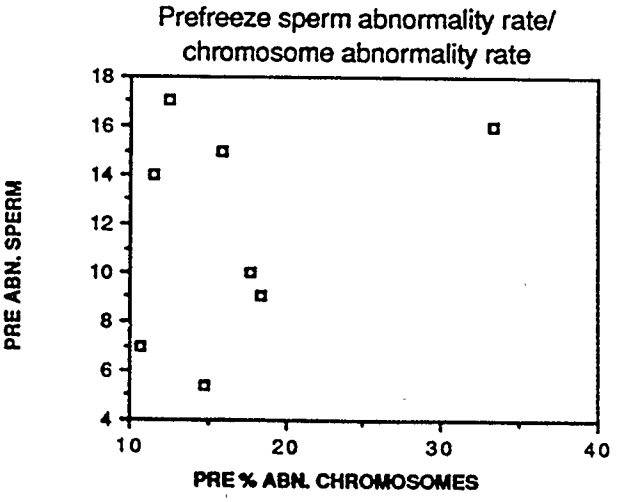
b. Abnormal Sperm Morphology Frequency (Pre Abn. Sperm) versus Chromosome Abnormality Frequency (Pre % Abn. Chromosomes).

c. Fertilization Rate (Pre Fert. Rate) versus Abnormal Sperm Morphology Frequency (Pre Abn. Sperm).

a.


Prefreeze fertilization rate/
chromosome abnormality rate
PRE FERT. RATE
PRE % ABN. CHROMOSOMES
70
60
50
40
30
20
10
10
20
30
40

b.


Prefreeze sperm abnormality rate/
chromosome abnormality rate
PRE ABN. SPERM
PRE % ABN. CHROMOSOMES
18
16
14
12
10
8
6
4
10
20
30
40

c.

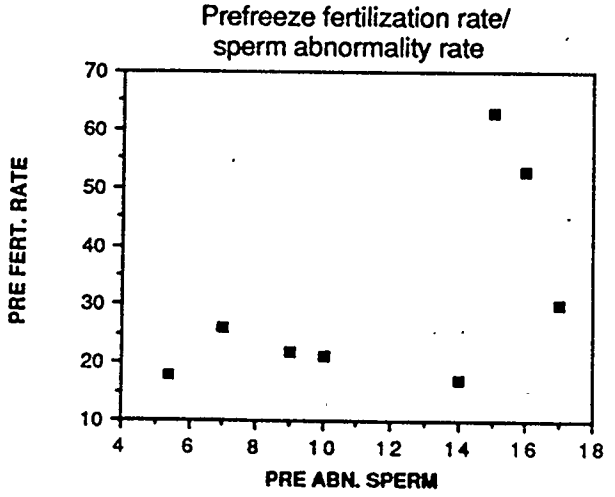

Prefreeze fertilization rate/
sperm abnormality rate
PRE FERT. RATE
PRE ABN. SPERM
70
60
50
40
30
20
10
4
6
8
10
12
14
16
18

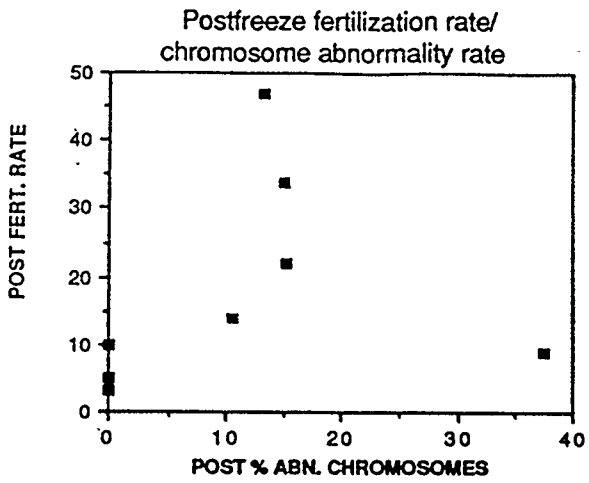
Figure 15: Scatterplots: Semen parameters versus cytogenetic abnormality frequencies - postfreeze data.

a. Fertilization Rate (Post Fert. Rate) versus Chromosome Abnormality Frequency (Post % Abn. Chromosomes).

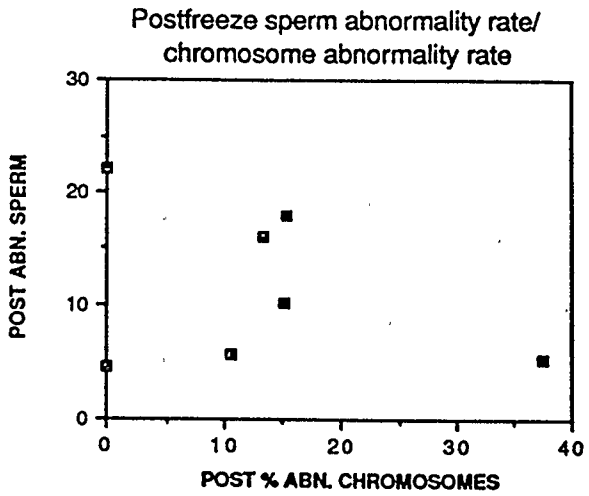
b. Abnormal Sperm Morphology Frequency (Post Abn. Sperm) versus Chromosome Abnormality Frequency (Post % Abn. Chromosomes).

c. Fertilization Rate (Post Fert. Rate) versus Abnormal Sperm Morphology Frequency (Post Abn. Sperm).

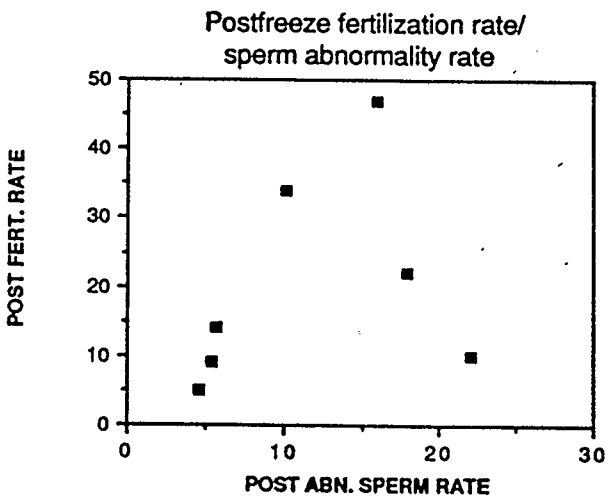
a.


Postfreeze fertilization rate/
chromosome abnormality rate
POST FERT. RATE
POST % ABN. CHROMOSOMES
0
10
20
30
40
50

b.


Postfreeze sperm abnormality rate/
chromosome abnormality rate
POST ABN. SPERM
POST % ABN. CHROMOSOMES
0
10
20
30
40

c.


Postfreeze fertilization rate/
sperm abnormality rate
POST FERT. RATE
POST ABN. SPERM RATE
0
10
20
30
40
50

V DISCUSSION

A. Cryopreservation Results

i. Frequencies of Chromosome Abnormalities

This study has provided the first opportunity for directly examining the genetic effects of cryopreservation on human sperm cells. The results reported here are relatively reassuring.

For the cohort of normal men in this study, no significant difference in sperm chromosome abnormality rates was evident between fresh sperm (17.8%) and previously frozen sperm (13.4%). ($P=0.025$, 95% Confidence interval for difference=$4.4\% \pm 4.9\%$).

Since each semen sample was split, part to be used fresh and part to be cryopreserved, each donor acted as his own control. This ensured that cryopreservation was the only significant variable differing between the two semen treatments.

There were no significant differences in the frequency of hyperhaploid cells (table 2) or in numerically abnormal cells (corrected for excess hypohaploidy) (table 3) before and after freezing. This finding contradicts the evidence from the Federation CECOS, (1983) study which suggested that A.I.D. with cryopreserved sperm may increase the incidence of trisomic births by as much as two fold over the general population. Should this trend be confirmed by other studies on A.I.D. pregnancies, then factors other than sperm cryopreservation must be examined as potential contributory factors.

The data presented here do not support the hypothesis put

forth by many studies on the pregnancy outcomes after A.I.D. with cryopreserved sperm that "defective" sperm are preferentially killed by the freezing process (Witherington et al., 1977; Sanger et al., 1979; Karow, 1979). Only the frequency of hypohaploidy was found to be significantly reduced in this study, but as previously discussed, this group of abnormalities is subject to technical artefact. Corrected values do not show any significant reduction after freezing.

In this study there appeared to be inter-donor variability in response to sperm cryopreservation as revealed by a significant test of heterogeneity (table 7). Donors 2 and 8 exhibited significant differences in sperm chromosome abnormality distributions before and after freezing, while donors 6 and 12 did not. Most of the variability in donors 2 and 8 was due to differences in the frequency of numerically abnormal sperm which has a large component of technical artefact associated with the value because of hypohaploidy. Therefore, while the evidence is not very convincing, the possibility of actual differences in the frequencies of sperm chromosome abnormalities of particular donors due to crypreservation cannot be ruled out.

ii. Distribution of Chromosome Structural Breakpoints

The distribution of chromosome breakpoints has been shown to be non-random among spontaneous structural aberrations in human lymphocytes (Aula and von Koskull, 1976; Mattei, et al., 1979); among induced structural aberrations in human fibroblasts (Kano and

Little, 1986); and among chromosome breaks and sister chromatid reunions in lymphocytes of Bloom's syndrome patients (Kuhn and Therman, 1979). These findings prompted an examination of the breakpoints identified among the structural abnormalities found in this study.

Chi squared tests were conducted to compare the frequency of observed breakpoints from fresh and cryopreserved sperm by ISCN chromosome groups with expected frequencies based on the relative length of chromosomes in each group as a proportion of the total haploid autosome length (ISCN, 1985, appendix 2) (table 10). No significant difference was apparent between the observed and expected distributions suggesting that structural aberrations were randomly distributed throughout the sperm genomes. Cryopreservation also had no effect on the distribution of breakpoints and prefreeze and postfreeze distributions were not significantly different.

Therman and Kuhn (1981) recognized that the preferred sites for mitotic chiasmata were short Q-dark regions. Aula and von Koskull (1976) also reported that the majority of breakpoints they identified were in light G-bands (analogous to Q-dark bands). In this study, the Q-dark bands have greater representation among the breakpoints for both prefreeze and postfreeze data, but the excess was not significant.

Aula and von Koskull (1976) postulated that an underlying structural weakness in the chromatin occurs at boundaries of light and dark bands. Scanning electron micgrographs (SEM) of G-banded chromosomes also indicate that there may be a topological thinning

of the chromosome at the position of G-pale bands (Harrison et al., 1981). Therman and Kuhn (1981) however, suggested that Q-dark regions have higher gene densities and are consequently more active and extended in interphase. This extended conformation may make these regions more susceptible to mutagenic damage such as chromosome breakage and to facilitating pairing and chiasma formation in diploid cells (Therman and Kuhn, 1981).

Juxtacentromeric regions (from q11 to p11) have been identified as "hot" regions for structural rearrangements (Mattei, et al., 1979). In this study, 11.5% (3/26) prefreeze breakpoints and 11.8% (4/34) postfreeze breakpoints were juxtacentromeric. The high incidence of structural aberrations in centromeric regions of sperm pronuclear chromosomes may be related to the phenomenon of centromeric gaps reported by all investigators working in this field. I have shown by SEM that achromatic gaps are regions of disrupted chromatin packing and the chromosome arms are actually continuous (Chernos et al., 1986). As postulated for Q-dark regions, the extended centromeric regions may also be more susceptible to chromatin disruption leading to structural aberrations.

Chromosome fragile sites have been implicated as contributing to the risk of abnormal offspring of carrier individuals (Garcia-Sagredo, et al., 1984; Voullarie, et al., 1987). Martin (1986b) reported an individual with 11.4% of his sperm complements exhibiting a break or gap at fragile site 10q25 as well as a higher than usual incidence of acentric fragments.

Although blood culture could not be done to confirm the fragile site in lymphocytes, the evidence from the sperm data suggests fragile sites predisposed to meiotic chromosome breakage at those loci in this individual.

Some aspect of chromatin structure at the fragile site, or in the chromatin in general, of donors susceptible to expression of multiple fragile sites in their cultured cells (Benet, et al., in press) may predispose to structural abnormalities. An examination of the breakpoints observed in this study was undertaken to identify any accepted fragile sites (Human Gene Mapping VIII, 1985). Two such fragile sites were recognized in each of the prefreeze and postfreeze breakpoints lists (table 11) which is not surprising given that there are over 80 fragile sites recognized (Human Gene Mapping, VIII, 1985).

The data presented here support the conclusion that cryopreservation does not have any influence on the distribution of structural abnormalities and does not cause any "hotspots" for chromosome breakage.

iii. The Sex Ratios

The sex ratios observed in this study (52.9% X chromosome - bearing prefreeze sperm and 53.5% X chromosome - bearing postfreeze sperm) were not significantly different from 50% and were within the range reported by other studies (see table 15). In this study the constancy of the sex ratios before and after cryopreservation (table 12) suggests that there is no differential survival of sperm cells

based on their sex chromosome content.

The apparent reversal of the sex ratios from a higher proportion of X-bearing sperm among chromosomally normal sperm to a higher proportion of Y-bearing sperm among chromosomally abnormal sperm following cryopreservation (table 12) is likely a spurious finding. Only the normal postfreeze ratio and abnormal prefreeze ratio differ significantly from 50% and the sample sizes are small, particularly for the chromosomally abnormal categories. Therefore, more data is required before drawing any biological conclusions from these data.

In studies comparing liveborn offspring conceived by A.I.D. with fresh and cryopreserved sperm, (Mortimer and Richardson, 1982; Alfredsson, 1984) the reported differences in the sex ratios must be attributed to factors other than differential cryosurvival of X- and Y- chromosome bearing sperm. Alfredsson, (1984), found no effect of cryopreservation on the proportion of males born following A.I.. Therefore, he concluded that there was no change in the relative fertility of sperm based on their sex chromosome content after freezing, but suggested some other aspect of A.I.D. such as the use of ovulation inducers, the timing of insemination or maternal age may affect the sex ratio.

iv. Semen Parameters

Cryopreservation of human sperm has been shown to induce morphological changes at the ultrastructural level (Friberg and Nilsson, 1971; Woolley and Richardson, 1978). However, no

significant change in the frequency of abnormal sperm heads following freezing and thawing was observed at the light microscope level of resolution in this study. Even though half of the sperm cells are killed by the freeze-thawing processes, it is evident that morphologically abnormal sperm are not more likely to die during cryopreservation, nor are they being induced.

The combination of increased cell death and ultrastructural changes to the sperm membranes contribute to the reduced fertilizing ability of postfreeze sperm observed in this study. Studies on the use of cryopreserved semen for A.I. are divided on their conclusions about the penetrating ability of postfreeze sperm. Laufer et al., (1985) and Glassman and Bennet, (1980) confirmed the current study's findings of reduced fertilizing rates, while Bordson, et al., (1986) reported no difference in the fertilizing ability of fresh and previously frozen sperm.

Comparisons between individual donor's semen parameters of hamster egg penetration rates and sperm morphology profiles and their sperm chromosome abnormality frequencies failed to reveal any correlations (table 14). Thus it appears that the sperm chromosome abnormality rates of individuals are not directly related to the sperm parameters examined here. Corroborating this finding is the lack of association between chromosome abnormality frequencies and abnormal human sperm morphology among normal men (Martin and Rademaker, in press), and in mice, the lack of correlation between high rates of chromosome imblance and increased abnormal sperm morphology (Redi, et al., 1984).

B. Incidence of Chromosome Abnormalities in Humans

Chromosome abnormalities are a major cause of early fetal loss. The incidence of chromosome abnormalities in first trimester abortions is about 60% (Boué et al., 1975). The overall frequency in spontaneous abortions at all stages is about 50%. Estimates of the frequency of chromosomally abnormal conceptuses vary from 5% (Hook, 1981b) to 50% (Boué et al., 1975). Since so much cytogenetic information is lost through early spontaneous abortions, often in preclinical pregnancies, a truer picture of the spectrum of chromosomal abnormalities at conception can be obtained by examining gametes directly.

While it is possible to examine human oocytes (Jagiello, et al., 1975) and in vitro fertilized embryos (Angell, et al., 1983; 1986; Rudak et al., 1984; 1985), material is scarce and ethically problematic. Conversely, human sperm are simply and readily obtainable, and through in vitro fertilization of hamster oocytes, sperm chromosome complements may be examined directly (Rudak et al., 1978).

Although there is not equal parental contribution of chromosomal abnormalities, significant paternal contribution to the cytogenetic abnormalities of spontaneous abortions and liveborn offspring is becoming apparent. The origin of the extra chromosome 21 in Down Syndrome is paternal in an estimated 30% of cases (Hansson and Mikkelsen, 1978). De novo structural abnormalities were found to be paternal in origin in about 75% of rearrangements examined (excluding Robertsonian translocations) (Chamberlin and

Magenis, 1980; Tomar et al., 1984). Studies on the frequency of chromosome abnormalities in human sperm provide a direct method to determine the paternal contribution of chromosome abnormalities at human conception.

C. Comparative Results from "Humster" Studies

Considerable data have now been accumulated on the incidence of chromosomal anomalies in human spermatozoa. Comparative results from major studies in the world literature are summarized in table 15. All reports indicate that a high proportion of human spermatozoa have an abnormal chromosomal content. The overall abnormality frequencies obtained in this study are quite high compared with the values obtained in other major studies. However, when a correction is made for the high rate of hypohaploidy and a conservative estimate for aneuploidy obtained, the abnormality frequency (11.8%) then falls well within the range reported in the world literature (table 15).

Those investigators employing a modified Tarkowski's technique for fixing eggs have reported an over-representation of hypohaploid cells by about two-fold (Rudak et al., 1978; Sele, et al., 1985; Brandriff et al., 1985; Martin, et al., 1987b; Pellestor, 1988; Benet, et al., in press) Only Jenderny and Rohrborn (1987) are the exception, however their reported overall frequency of aneuploidy is comparatively low and based on a small sample size.

Kamiguchi and Mikamo (1986) have developed an alternative fixation method which has yielded equal frequencies of hyperhaploidy

and hypohaploidy. This finding reinforces the assumption that the observed excess of hypohaploid cells is a technical artefact and justifies the estimates for aneuploidy made in this study and others (Martin, et al., 1987b).

A further consideration is the apparent difference in the frequency of hypohaploidy before and after freezing observed in this study (tables 2 and 4). It is possible that some aspect of cryopreservation influences the likelihood of a chromosome to become displaced from the metaphase plate and lost during fixing. Alternatively, it could be that cryopreservation reduces the chance of a hypohaploid sperm to successfully fertilize hamster oocytes (not a very likely proposition given that there is still a high rate of hypohaploidy in the postfreeze sperm). Most likely, this observation reflects sampling error combined with technical artefact. For example, donor 2 provided 6 semen samples yielding 49 karyotypes from experiments on fresh sperm. However, 12/49 were hypohaploid and of these, 8 (almost 1/4 of all hypohaploids analysed) were generated in a single experiment. This suggests that possibly certain aspects of the conditions for fixing were conducive to chromosome loss. Therefore it is unlikely that any biological significance can be attributed to the observed differences in hypohaploidy before and after freezing. This is corroborated by the disappearance of any significant difference between aneuploidy rates before and after freezing when the data were adjusted for hypohaploidy (table 3).

In the current study the frequencies of structural abnormalites

Table 15: COMPARISON OF "HUMSTER" STUDIES

Study	# Donors	#Karyo-types	%Abnor-mal	%Num-erical	%Struc-ural	N/S	X:Y
Brandriff, et al, 1985	9	2468	9.4%	2.1%	7.3%	-	50.1:49.9
Martin, et al,1987b	30	1582	10.4%	4.7%	6.2%	-	53:47
Kamiguchi, & Mikamo, 1986	4	1091	14%	1%	13%	-	53:47
Sele, et al, 1985;	7	70	14.3%	12.9%	1.4%	-	48.6:51.4
Pellestor, 1988	5	521	6.7%	5.5%	1.5%	-	47.7:52.3
Benet, et al, in press	-	205	12.2%	7.8%	4.4%	-	50.5:49.5
Jenderny & Rohrborn, 1987	6	129	7.8%	1.6%	6.2%	-	51.9:48.1
Rudak, et al, 1978	1	60	5%	3.3%	0	1.6%	-
This study							
Fresh	13	454	17.8%	7.7%	9%	1.1%	52.9:47.1
(corrected)			(11.8%)				
Frozen	13	387	13.4%	3.4%	8.8%	1.3%	53.5:46.5
(corrected)			(10.4%)				

(9% prefreeze; 8.8% postfreeze) were higher than numerical abnormalities in both the observed (7.7% prefreeze; 3.4% postfreeze) and corrected (0.9% prefreeze; 0% postfreeze) data. These results are in good agreement with the findings of Brandriff et al., 1985; Kamiguchi and Mikamo, 1986; and Jenderny and Rohrborn, 1987. The results of the most recent investigation of Martin, et al., (1987) exhibit the same trend of higher frequencies of structural abnormalities than aneuploidy, but the difference is less dramatic. Two European groups however, have observed a reversal of this trend with higher aneuploidy compared to structural abnormality rates (Sele, et al., 1985; Pellestor, 1988; Benet et al., in press) (see table 15).

The most likely explanation for the different trends observed is that of inter-donor variability in rates of sperm cnromosome abnormalities. The frequency of sperm chromosomal abnormalities has been shown to vary considerably among individual men (Martin et al., 1987b). It has also been demonstrated that individual donors' sperm chromosome abnormality rates remain relatively constant through time (Brandriff, et al., 1985). Most cytogenetic studies on human sperm have reported chromosome results on very few donors (only one group has reported on more than 20 normal men: Martin et al., 1987b. Thus the results of the various studies reflect the abnormality frequencies of a particular, small sample of normal men.

Considerable variability exists among the reported frequencies of structural abnormalities in human sperm (table 15). It has been suggested that differences in scoring structural anomalies among the

various investigators contributes to this observed variation (Jenderny and Rohrborn, 1987). For example, in this study sperm complements containing both a numerical and structural anomaly were placed in a separate class (numerical/structural) while other investigators have counted these spreads in each of the numerically and structurally abnormal categories (Martin, et al., 1987b). However, this postulate may contribute to, but is not sufficient to explain, the variability among the studies. During the on-going study conducted in the laboratory of R. H. Martin, the frequency of structural abnormalities initially was 1.7% based on 240 sperm complements (Martin, et al., 1982) compared to their most recent report on 1582 sperm complements with 6.2% structural abnormalities (Martin, et al., 1987b). The same criterion for classification of anomalies and the same technique was used throughout their study so the change in the observed frequencies of structural abnormalities must be attributed to studying more donors and a larger number of sperm chromosome complements. Again this variability among the "humster" studies is likely due to sampling bias. It is reassuring to note that for the same group of donors studied before and after freezing in the present study, the frequencies of structural anomalies are very similar (9% versus 8.8%).

D. Possible Origins of Observed Chromosome Abnormalities in Human Sperm.

i. Gametogenesis versus Post-fertilization

The types of chromosome abnormalities reported in human sperm

cells fall into three main groups based on the likely origins of the abnormalities. The first type consists of those abnormalities which could only have arisen in the primordial germ cells or during spermatogenesis. For this reason they may be of greater genetic significance.

This group includes aneuploidy (excluding hypohaploidy caused by technical artefact) and complex chromosome rearrangements such as translocations, inversions, dicentrics and isochromosomes. Since these structural abnormalities are observed at very low frequencies among human sperm cells (3/133 in this study), aneuploidy, in particular hyperhaploidy, is perhaps the most reliable indicator of the true level of chromosome abnormalities in human sperm. Aneuploidy levels of about 5% found by the "humster" studies are in agreement with the levels of aneuploidy (about 4%) found in unfertilized human oocytes (Jagiello, et al., 1975; Michelmann and Mettler, 1985; Martin, 1986a).

The second group of abnormalities could potentially have arisen during spermatogenesis, but could also be induced in the "humster" system. Less genetic significance can be attributed to this group. Examples of these types of anomalies are chromosome breaks or gaps with both centric and acentric pieces present; deleted chromosomes and spreads with acentric fragments. Chromosome breaks could have arisen relatively late in spermatogenesis (after the spermatogonial mitotic divisions, (see figure 1), or else the acentric fragment would likely have been lost. However, these abnormalities may also be induced within the ooplasm prior to DNA replication (Savage,

1976).

Chromosome gaps may be stable through cell division just as fragile sites are stable and have arisen at any stage of spermatogenesis. Again, however, the origin of chromosome gaps could be a disruption of the chromatin packing at some time after fertilization.

If deleted chromosomes were arising during spermatogenesis, as the result of a chromosome break with subsequent loss of the acentric fragment, the incidence of deleted chromosomes should be much higher than that of acentrics. However, in this study, the reverse was found. Acentric fragments were twice as common as deleted chromosomes in prefreeze sperm, and five times as common in postfreeze sperm (see table 7). This excess of fragments was also reported by Kamiguchi and Mikamo, (1986). This suggests that chromosome breakage is occurring late in spermatogenesis or post-fertilization and many deleted chromosomes are missed due to small, terminal deletions.

The third group of chromosome anomalies are those which almost certainly arose post-fertilization. This group consists mainly of aberrations of the chromatid type, although also included are spreads with multiple breaks with or without rejoining. Chromatid breaks and gaps as well as chromatid exchanges must have arisen during sperm chromatin replication or between replication and chromosome condensation. Although these types of anomalies do not provide much information about meiosis, they are of biological significance as indicators of mutagenic conditions.

ii. Culture Conditions in the "Humster" System

The culturing conditions in the "humster" system are one possible cause of the high frequency of sperm chromosome structural anomalies.

The study reporting the highest incidence of structurally abnormal sperm utilizes culture medium TC 199 (Kamiguchi and Mikamo, 1986). This medium has low levels of folic acid and thymidine which have been implicated in the induction of fragile sites and chromosome breakage (Sutherland, 1979).

The antibiotic Streptomycin, which is added to the culture medium in this study has been shown to be mutagenic in clamydomonas (Sager, 1972). It may have mutagenic effects in mammalian cells as well.

Any agent which affects the overall health of the oocytes, for example bacterial contamination or pH deviations of the media, could lead to structural chromosomal abnormalities. Angell, et al., (1986) have suggested that exogenous hormones, used to induce superovulation, may have some deleterious effect on the oocyte. This in turn may disrupt the egg's ability to properly process the sperm nucleus resulting in chromosome breaks and lesions.

Light is known to spontaneously activate oocytes (Hirao and Yanagimachi, 1978) and it is possible that chromosome abnormalities could be induced if the male and female pronuclei develop asynchronously. A phenomenon referred to as "precocious chromosome condensation" or "pulverization" has been observed when a cell in interphase is fused with a cell in division (Savage 1976).

Similarly, grossly abnormal chromosomes were observed in the "humster" system which were not analysable or recorded although the MB&R spreads may represent a milder form of this same phenomenon.

Although the observed frequencies of structural abnormalities in human sperm are certainly influenced by the technique, these frequencies must also reflect a donor's susceptibility to chromosome damage. Individual donors' structural abnormality frequencies are distinct and stable over considerable time spans (Brandriff et al., 1985). This suggests that there is an interaction between the culture conditions and the donor's physiology which contributes to the donor's observed structural abnormality frequency.

Particular donors may have increased sensitivity to the culture conditions or may have higher innate frequencies of structural abnormalities. One such group of men were cancer patients studied 36 months post-radiotherapy (Martin, et al., 1986). They exhibited almost double the frequency of structural abnormalities of control donors and the frequency of abnormalities was directly dependent on the testicular radiation dose. This provided evidence of the causative relationship between a known mutagen and an increased level of chromosome abnormalities in human gametes (Martin, et al., 1986).

Thus, although the observed frequencies of structural abnormalities in human sperm chromosomes obtained by the "humster" assay may not be the true levels of structural abnormalities in human male gametes, they are biologically relevant and provide a baseline for comparing sperm treatments such as cryopreservation or

donor environmental exposures.

E. Possible Selection of Human Sperm based on Chromosomal Content

The validity of the "humster" technique in the analysis of the products of meiosis hinges on the assumption that there is no selection of sperm by the system. If genetically abnormal sperm are being selected against by virtue of being less successful at fertilizing oocytes than are normal sperm, then the true cytogenetic abnormality rate of human sperm may be much higher than indicated by the "humster" assay. A considerable amount of suggestive data has been accumulated to support the assumption that human sperm are not being selected on the basis of chromosomal content.

Quite high incidences of abnormal conceptuses following *in vitro* fertilization of human oocytes have been reported (Rudak, *et al.*, 1984; 1985; Angell, *et al.*, 1986). Rudak *et al.*, (1985) examined multipronuclear human oocytes fertilized *in vitro* and found that 16.7% of pronuclei were aneuploid suggesting that about 30% of embryos would be chromosomally unbalanced. Angell *et al.*, (1986) reported that 30% of embryos examined by them were chromosomally abnormal, (25% aneuploid and 5% structurally abnormal). Thus it appears that selection is operating in humans mainly at the level of the embryo through high rates of spontaneous abortions and not at the level of preventing conception involving abnormal gametes.

There is however, evidence that selection against genetically abnormal sperm may be occurring prior to sperm formation. Many men carrying reciprocal translocations are detected because of fertility

problems including azoospermia (Micic and Micic, 1984). In sheep, it has also been shown that Robertsonian translocation - carrying rams have high rates of spermatocyte degeneration and a significant dearth of abnormal or carrier offspring suggesting that selection is operating during spermatogenesis (Chapman and Bruère, 1975; Bruère, et al., 1981).

In certain mouse stocks which have high frequencies of spontaneous nondisjunction, aneuploid sperm cells are not eliminated during spermatogenesis or in transit in the female genital tract but can successfully fertilize oocytes (Tates and de Boer, 1984; Redi, et al., 1984). Trisomic and monosomic preimplantation embryos were observed in equal proportions among the offspring of male mice predisposed to aneuploid gametes by virtue of carrying a Robertsonian translocation (Epstein and Travis, 1979). These studies conclude that there is no selection against chromosomally abnormal sperm at fertilization, but monosomic embryos are lost very early in development.

Analogous to the mouse systems where Robertsonian heterozygosity leads to high rates of nondisjunction during meiosis, in humans, carriers of reciprocal translocations and inversions are expected to produce high proportions of abnormal gametes. Sperm chromosome studies on men heterozygous for different reciprocal or Robertsonian translocations showed that normal and balanced gametes were produced in equal proportions as would be theoretically expected since they arise by complementary events (Balkan and Martin, 1983a; Pellestor et al., 1987; Brandriff, et al., 1986b).

In addition, all theoretically - possible types of segregation were observed, not just those which would result in viable offspring (Martin, 1984a; Burns et al., 1986; Brandriff et al., 1986b). The frequency of unbalanced offspring was higher than frequencies observed in studies of fetuses from prenatal diagnosis and liveborns (Balkan and Martin, 1983a; 1983b; Burns et al., 1986; Brandriff, et al., 1986b; Pellestor, et al., 1987). This suggests that all types of sperm cells, normal, balanced and unbalanced, have equal probabilities of fertilizing hamster oocytes.

Another group of men that has been studied by the "humster" assay is former cancer patients who had undergone radiotherapy. These men exhibited sperm chromosome abnormality frequencies far in excess of normal control donors, ranging up to 67% for particular patients (Martin, et al., 1986). It is apparent that chromosomally abnormal sperm are not at a disadvantage in fertilizing hamster oocytes.

Other lines of evidence against sperm selection are derived from the "humster" system. Unlike the limited types of chromosome anomalies found among liveborns and spontaneous abortions (Hook, 1981a), the "humster" technique has yielded trisomies and monosomies for every human chromosome. Also, subpopulations of sperm selected for motility were shown to have chromosome abnormality frequencies that were not significantly different from the unselected sperm (Brandriff et al., 1986a). The present study demonstrates that sperm cells which have survived cyopreservation do not have altered frequencies of chromosome anomalies compared with fresh sperm.

Finally, the morphological data in this study and that of Martin and Rademaker (in press), suggest that there is no correlation between the frequencies of chromosomally and morphologically abnormal sperm.

Although indirect, all these lines of evidence suggest that there is no sperm selection based on chromosomal content operating in the "humster" system. Therefore, it can be concluded, that the sperm chromosome abnormality frequencies obtained by the "humster" assay represent reasonable, biologically significant estimates of the frequency of chromosome abnormalities produced during meiosis.

VI CONCLUSIONS

A group of 13 normal healthy men have been investigated for the effects of semen cryopreservation on their sperm pronuclear chromosomes and semen parameters. The overall frequency of sperm chromosome abnormalities was significantly lower after cryopreservation (17.8% prefreeze versus 13.4% postfreeze). However, when the aneuploidy level was adjusted for the excess of hypohaploid cells, there was no longer any significant difference between abnormality frequencies of fresh (11.8%) and previously frozen sperm (10.4%). Thus the present data suggest that cryopreservation has no effect on the overall incidence of chromosome abnormalities in human sperm.

It has also been demonstrated that the types of sperm chromosome anomalies observed in this study were the same before and after freezing and there was no difference in the distribution of different types of abnormalities caused by freezing.

Sperm chromosome abnormality rates varied considerably among donors both before and after freezing. Only one donor (2) exhibited a statistically significant response to freezing and one donor (11) had a borderline response. While the problem of excess hypohaploidy again arises here, the possibility of true donor differences in response to freezing, as reflected in altered chromosome abnormality rates, cannot be ruled out. Studying a larger number of donors and modifying the technique of egg fixation to reduce the chances of artefactual chromosome loss will help to

resolve this issue.

The abnormalities observed in this study were randomly distributed among the haploid chromosomes. The distributions of abnormalities before and after freezing were also not significantly different. The locations of structural breakpoints could not be shown to be different from expected in this data set and no hotspots for rearrangement could be identified.

The overall sex ratios found in this study were 52.9% X-chromosome bearing for prefreeze sperm and 53.5% X-chromosome bearing for postfreeze sperm. However these ratios are not significantly different from the theoretically expected 50%. The sex ratios before and after cryopreservation are also not significantly different suggesting that there is no differential survival or fertilizing ability of human sperm cells based on sex chromosome content.

The frequency of morphologically abnormal sperm heads was not altered by the freezing and thawing processes although tail defects were induced by osmotic shock. Cell death, as measured by uptake of supravital stain, was the most striking consequence of cryopreservation. This probably contributed most to the reduced fertilizing ability of cryopreserved sperm observed in this study. There was no evidence of any direct relationships between the semen parameters of abnormal sperm morphology and fertilizing ability and sperm chromosome abnormality frequencies. This finding suggests that chromosomally abnormal sperm are not morphologically altered and do not have reduced fertilizing ability.

The findings of this study should alleviate any apprehensions of infertility clinics currently using banked sperm for artificial inseminations. The use of cryopreserved sperm should become the method of choice because of convenience and safety despite the finding that defective sperm are not being preferentially killed off.

The ability to transport frozen sperm of genetically interesting donors to centres for analysis by the "humster" assay should now be possible without fear of confounding results by the cryopreservation process. This will allow analysis of a wider range of donors not currently available for sperm chromosome study.

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VIII APPENDICES

A. Media and Solutions

BWW stock solution (Biggers, Whitten, and Whittingham, 1971)

	grams/litre
NaCl	5.540
KCl	0.356
$CaCl_2$	0.189
KH_2PO_4	0.162
$MgSO_4.7H_2O$	0.294
Pyruvic Acid, Na Salt	1.000
	millitres/litre
Pen-Strept	1.0
0.5% Phenol Red	0.5
Acid Hepes	10.0
Base Hepes	10.0
Distilled H_2O	to 1000.0 ml

Maintain at 4 C for two weeks.

BWW Working Solution

$NaHCO_3$	0.2106 g
DL Lactic Acid	0.37 ml
Human Serum Albumin (HSA) (Fraction V)	0.5 g
BWW Stock Solution	100 ml

Dissolve crystals, mix and filter sterilize through a cellulose acetate/nitrate membrane, pore size 0.22 μm.

Pen-strep for BWW

Streptomycin sulfate	50 mg/ml
Penicillin G, Na Salt	100,000 IU/ml

Freeze in 1 ml aliquots.

Hepes - Acid (2M Hepes in distilled H_2O

Hepes	47.66 g
dH_2O	to 100 ml

Hepes-Base (2M Hepes in 3M NaOH)

NaOH	12 g
Hepes	47.66 g
dH_2O	to 100 ml

F10 Working Solution

Ham's F10	42.5 ml
Fetal Bovine Serum	7.5 ml
(inactivated 56 C, 30 minutes)	
Pen-strept	0.5 ml

Hyaluronidase Solution (0.2%) (Double Strength)

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

Trypsin Solution (0.1%)

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

Colcemid Solution (0.8ug/ml) (Double strength)

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

Pen-Strept For F10 Working Solution

Streptomycin Sulfate	5 mg/ml
Penicillin G, Na Salt	10,000 I.U/ml

Freeze in 0.5 ml aliquots.

Ackerman's Cryopreservative Media

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

Heat inactivate at 56 C for 30 minutes. Adjust pH to 7.2 - 7.4. Store at -20 °C in aliquots of desired volume.

Sperm Count Diluent

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

Giemsa Stain for Chromosomes

1 ML Giemsa Solution (Harleco)
in 35 mls Gurr Buffer

Skim off oxidized surface. Make fresh daily.

Giemsa Stain for Sperm Morphology

1 ML Giemsa Solution (Harleco) in
23 mls Gurr Buffer

Gurr Buffer

1 Gurr ® Buffer tablet (pH 6.8) in

1 L dH_2O

Adjust pH to 4.4

Supravital Stains

Eosin Blue

1 g Eosin bluish

4 g aniline blue

100 ml phosphate buffer working solution *

Dissolve by heating 10 minutes in 85 C water bath. Adjust pH to 6.6.

*Phosphate Buffer: Stock Solution

1.702 g KH_2PO_4 in 100 ml ddH_2O

1.776 g anhydrous Na_2HPO_4 in 100 ml ddH_2O

Working Solution

28.5 ml KH_2PO_4 solution

71.5 Na_2HPO_4 solution

Adjust pH to 7.2

Eosin Yellow

0.5% Eosin Yellow in

0.15 M phosphate buffer.

Adjust pH to 7.4

B. Reagents

$NaHCO_3$	Fisher - S233
DL Lactic acid (6% Syrup)	Sigma L1375
Human Serum Albumin (HSA) Fraction V	Sigma A2386
Pregnant Mares' Serum Gonadotropin (PMSG)	Sigma G4877
Human Chorion Gonadotropin (hCG)	A.P.L. Ayerst
Hepes	Sigma H3375
NaOH	
Hyaluronidase (Type 1-S)	Sigma 3506
Trypsin (Type XII)	Sigma 2884
1X Hams F-10 with Glutamine	Flow 12-403-54
Colcemid (10µg/ml)	Gibco G.D. 1024
Na Citrate	
Ethanol	
Glacial Acetic Acid	
Fetal Bovine Serum (FBS) (Cellect Silver)	Flow 29-161-49
Penicillin G, Na Salt	Sigma P-3032
Streptomycin Sulfate	Sigma S-6501
Bacto Egg Yolk Enrichment - 50%	Difco 3347-72
Glycerol	
Glycine (Reagent Grade)	Fisher G-46
D-Glucose (Dextrose)	Fisher D-16
Sodium Citrate	Fisher S 279
Quinicirne Dihydrochloride	Sigma Q-0250
Giemsa Stain	Harleco 620
GURR ^{R} Buffer tablets (pH6.8)	Hopkins and Williams 065568
Methanol	
Polyvinylpyrollidone (PVP)	
Pyruvic Acid (Na Salt - Type II)	Calbiochem 5510
NaCl	
$CaCl_2$ - pellets	
KH_2PO_4	

$MgSO_4 7H_2O$	
Phenol Red	EM PX0530-3
Entellen® - mounting media	Merck 7961
Eosin Yellowish	
Eosin Bluish	Sigma E-2629
Aniline Blue	Sigma A-9016