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Analysis of Human Sperm Chromatin Integrity

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ANALYSIS OF HUMAN SPERM CHROMATIN INTEGRITY

by

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ABSTRACT
ANALYSIS OF HUMAN SPERM CHROMATIN INTEGRITY

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Determining potential maternal or paternal sources of abnormal chromosomal constitution gives opportunity for preconception genetic counseling. The most direct determination is achieved by analyzing the nuclear constitution of the gametes.

The present study evaluated the integrity of human spermatozoal nuclear material in the two condensation stages of chromatin and chromosomes. Original semen samples (ORI) and their swim-up fractions (SW, selected for motility) from men of known (donors) and unknown (patients) fertility were analyzed. The extent of chromatin condensation was assessed by light microscopy and flow cytometry during the time course of a chemically-induced decondensation reaction.

Motile spermatozoa were used to inseminate hamster oocytes for human sperm chromosomal analysis (original method). A modification of this techniques was introduced in an attempt to overcome the motility barrier required for fertilization. Spermatozoa rendered immotile by cryodamage were directly microinjected into the perivitelline space of hamster oocytes in order to obtain fertilization and possible chromosomal development.

The swim-up-selected spermatozoa showed a higher resistance to the chromatin decondensation assay (10.53% decondensed) than their corresponding nonselected whole semen samples (94.74%). Sperm chromosomal analysis by the traditional technique was restricted to donor samples (86.7% fertilization rate) since all the patients failed to achieve fertilization. Although a high number of chromosomal complements were obtained (2362) only 7.4% provided complete information (range 0-56 complements/donor). The observed X/Y relationship (39/44) was not significantly different than the expected 1/1 ratio. Ten spermatozoa (7.69%) carried structural and 3.08% carried numerical abnormalities. High

rates of fertilization (64-86%) with low rates of polyspermy (< 1%) were achieved by the sperm cells subjected to cryodamage when their acrosomal membranes were completely disrupted. Nuclear material was obtained but karyotyping was not possible due to the poor chromosomal morphology.

Swim-up selected spermatozoa have a higher resistance to the *in-vitro* induced nuclear chromatin decondensation assay (NCDA) than their corresponding ORI samples, which may correlate with their greater nuclear stability. Although SW procedures are invaluable as an aid in infertility treatment due to their selectivity in motility, morphology, fertilizing ability, chromatin resistance, etc. they are not able to discriminate against spermatozoal carriers of genetic defects.

DEDICATION

I dedicate this work to my husband, Mahmood,

my baby, Bijan, and to my family,

for their unconditional love and support.

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Many people have helped me travel this long road, and their cooperation, generosity, and assistance are gratefully acknowledged. I would like to sincerely thank my advisor Dr. R. James Swanson for his contribution of expertise, time, patience and support both in the professional and private life. I would especially like to thank my Dissertation Guidance Committee members Drs. Anibal Acosta, Keith Carson, Susan Lanzendorf, and Patricia Pleban for the time they have spent reviewing this work. I particularly wish to express my gratitute to Anibal A. Acosta, M.D., for making it possible for me to study valuable techniques in Spain. His encouragement has been of immeasurable worth to me.

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CHAPTER I

Introduction

A. Nuclear reorganization of the mammalian spermatozoon

Spermatogenesis is the process of sperm differentiation in the testis, beginning with the mitotic proliferation of spermatogonia (spermatocytogenesis), proceeding through the development of spermatocytes (meiosis), and culminating in morphologic alteration of the haploid round spermatid into the highly elongated, polarized sperm cell (spermiogenesis).

1. Spermatocytogenesis

The replication of stem cells commences in fetal life with the migration of the primordial germ cells into the mesenchyme of the gonadal ridge. After a period of prenatal mitotic division, the gonocytes, which populate the seminiferous cords at birth, remain quiescent until immediately before puberty, when they divide by mitosis to form the spermatogonial population (Muller and Skakkebaek, 1983).

In man, three types of spermatogonia are recognized classically: the A dark, thought to represent the most primitive, the A pale, and the B spermatogonia. They lie adjacent to the basement membrane of the tubule, interspersed with the basal aspects of the Sertoli cells. During cell division, the spermatogonia do not complete cytokinesis and remain linked by intercellular bridges (Dym and Fawcett, 1971).

2. Meiosis

Responding to unknown signals, groups of type B spermatogonia begin meiosis, which involves two cell divisions. The spermatogonia duplicate their DNA (2n,4X), lose

their contact with the basement membrane on the tubule and are then called primary spermatocytes (Clermont, 1963) . After the first division, incomplete cytokinesis causes the formation of a pair of joined secondary spermatocytes (n, 2X) from each primary spermatocyte.

During the second division, the 23 chromosomes, each comprising a pair of chromatids, attach to the spindle, and the chromatids separate. This yields a cell, a spermatid, containing the haploid DNA and chromosomal complement.

3. Spermiogenesis

Spermiogenesis is the process by which a round spermatid transforms morphologically and biochemically into the elongated testicular spermatozoon. This alteration is accomplished without concomitant cell division. The result of spermiogenesis is the morphologically mature spermatozoon. In humans this cell is about 60 μm long and is subdivided structurally into a head and a tail. The head consists of the nucleus with its associated acrosome and is 4.5 μm long, 3.0 μm wide, and about 1 μm deep. Spermatogenesis in humans takes 74 ± 4 days as determined by autoradiographic protocols (Heller and Clermont, 1963; Heller and Clermont, 1964).

The developmental phases are termed Sa_1, Sb_1, Sb_2, Sc_2, and Sd_2 stages according to Clermont (Clermont, 1963; de Kretser, 1969: Holstein and Roosen-Runge, 1981). The changes can be subdivided into nuclear, acrosome formation, flagellar development, redistribution of cytoplasm, and spermiation.

During the Sa_1, and Sb_1 stages, the nucleus, which ultimately forms the head of the sperm, remains centrally placed, but it subsequently is displaced peripherally, coming into apposition with the cell membrane, separated only by the acrosomal cap. There is also a progressive decreasing nuclear volume associated with chromatin condensation,causing the development of a resistance by the DNA to degradation by the enzyme DNA-ase.

Pogany, Corzett, Weston, and Balhorn (1981) measured the amount of DNA

contained within the mouse sperm nucleus and the volume of the nucleus and noted that the mouse sperm DNA could not possibly be packed into the characteristic nucleosome structure of the somatic chromatin. If the DNA of the mouse sperm nucleus were packaged into nucleosomes, it would require 213% of the total nuclear volume.

The DNA of mammalian spermatozoa is greater that sixfold more highly condensed (Wyrobek, Meistrich, Furrer, and Bruce, 1976; Pogany, Corzett, Weston, and Balhorn, 1981; Balhorn, 1982) than the already highly condensed DNA of a mitotic chromosome (Pienta and Coffey, 1984), making it the most highly condensed eukaryotic DNA known. Studies of chromatin condensation during spermiogenesis have shown that it is an ordered process, beginning at the anterior end of the sperm head (at the acrosome) and proceeding towards the tail (Dooher and Bennett, 1973; Zirkin, Soucek, and Chang, 1982). During this condensation the histones are gradually replaced by transition proteins, which are subsequently replaced by protamines, the DNA binding proteins of spermatozoa (Marushige and Marushige, 1975). Mammalian protamines are small, arginine-rich proteins that are present during the late stages of spermiogenesis.

A small portion of the DNA in human sperm nuclei is packaged into histones. Tanphaichitr, Sobhon, Taluppeth, and Chalermisarachai (1978) reported that human sperm chromatin contains about 15 % histones.

Balhorn (1982) proposed a model of protamine-DNA binding where the protamines would lie lengthwise inside the minor grooves of the DNA and would completely neutralize the negative charges of the DNA. The protamine-DNA complex of one strand would fit into the major groove of a neighboring DNA strand, so that the DNA strands of the sperm nucleus would be packed side by side in a linear array, opposite to the negative supercoiled solenoid imposed by the nucleosomal arrangement of the histone-DNA nucleosome complex in the somatic chromatin.

A further degree of organization of the sperm chromatin could be achieved by the

arrangement of the parallel fibers into loop-domains attached to a nuclear matrix similar to the somatic nuclear matrix. The nuclear matrix in hamster sperm (Ward, Partin, Coffey, 1989) seems to organize the DNA into non-supercoiled loops of half the size of the somatic cell loops. Which nuclear structures, if any, are involved in the higher order organization of sperm chromatin is as yet unknown.

Mammalian sperm chromatin is so highly condensed that it is extremely difficult to study with conventional microscopic techniques. Consequently, researchers have applied a variety of techniques (such as circular dichroism: Sipski and Wagner, 1977, and polarizing light: Livolant ,1983) and of extraction buffers to decondense the DNA by solubilizing the protamines and disrupting the nuclear architecture to study chromosomal organization in sperm cells. In this way, Livolant (1983) suggested that the chromatin within the sperm nucleus was composed of sheets of parallel fibers of DNA lying on top of each other. On the basis of freeze-fracture electron microscopy, Koehler and colleagues (Koehler, 1970; Koehler, Wurschmidt, and Larsen, 1983) have proposed a model of sperm chromatin organization in which the DNA is condensed into 20 nm strands that are arranged into several layers of lamellae stacked one on top of the other. Several investigators used high-strength ionic buffers to extract protamines from mouse sperm nuclei and demonstrated that they contain a nonprotamine nuclear matrix, but its role in DNA organization has not been established.

4. Sperm Maturation in the Epididymis

Spermatogenesis itself is not sufficient to produce male gametes capable of successful fertilization. During epididymal transit spermatozoa undergo changes in their morphologic structure, composition of their surface membrane,and capacity for active motility. Morphologic alterations occur with varying severity, depending on the species. Usually, the major changes include acrosomal modifications and the posterior movement and loss of the cytoplasmic droplet, a remaining bit of residual cytoplasm not apparently

required for fertilization.

Biochemical differences arising due to epididymal transit on the other hand, are well-documented and many are clearly related directly to sperm activity. Changes include increases in the disulfide linkages and in resistance to sonication detected in sperm constituents of the nucleus, outer dense fibers, fibrous sheath, and mitochondrial membranes (Henle, Henle and Chambers, 1938; Calvin and Bedford, 1971; Bedford and Calvin, 1974).

B. Genetics and Infertility

Many natural and man-made environmental hazards to which human populations are increasingly exposed, may produce genetic damage. Hazards such as nuclear weapons testing, nuclear energy development and use, medical X-ray technology for diagnostic as well as for therapeutic purposes, and many groups of chemically-active substances used in industry (alkylating agents), medicine (antibiotics and chemotherapeutic agents), agriculture (herbicides and pesticides), and recreational drugs, would all be potential mutagens. Damage at the chromosomal level is detectable by changes in number and structure. These changes are the most important cause of human reproductive failure, and are manifested in sterility, low fertility and a very high rate of mortality among human concepti (Carr, 1971; Jacobs, 1972; Carr and Gedeon, 1977; Boué, Boué, Lazar, and Gueguen, 1973; Bond and Chandley, 1983). That many of these abnormalities are not present or not detected in aborted material or in newborns indicates possible involvement in very early abortions or pre-embryo loss prior to implantation.

Chromosomal abnormalities responsible for reproductive failure can occur *de novo* during gametogenesis or as heritable chromosomal imbalances. These anomalies can cause infertility or sterility in two ways: (1) they can block gametogenesis, or (2) they can

produce unbalanced gametes. In case one, the number of gametes produced will differ depending on whether the blockage is total or partial. In case two, repetitious or subclinical miscarriages associated with embryonic malformations may occur.

The incidence of chromosomal abnormalities in human abortions is extremely high, as much as 61% in the general population (Boué and Boué, 1976) reaching 83% after ovulation induction (Boué and Boué, 1973). In vitro fertilization techniques have contributed to the collection of these data by providing oocytes that remained unfertilized and zygotes that either failed to cleave or that were not transferred because of obvious morphological disorders (degenerated, vacuolated or granulated cytoplasm, polyploidy, delayed cleavage, etc.).

Data on the incidence of chromosomal aberrations in human gametes, both oocytes and spermatozoa, are still scarce. The rate of oocyte abnormalities has ranged from 34% (Martin, Mahadevan, Taylor, Hildebrand, Long-Simpson, Peterson, Yamamoto, and Fleetham, 1986) to nearly 50% (Wramsby, Fredga, and Liedholm, 1987) in freshly recovered non-inseminated oocytes. More data were obtained analyzing unfertilized oocytes after IVF techniques. In these cases, the chromosomal abnormalities ranged from 11% (Spielman, Krüger, Stauber, and Vogerl, 1985) to 65.4% (Wramsby and Fredga, 1988).

In one IVF program (Plachot, de Grouchy, Junca, Mandelbaum, Salat-Baroux, and Cohen, 1988), 38% of the human oocytes recovered and 29% of the pre-implantational embryos showed chromosomal abnormalities.

Until 1978, all information available about the chromosomal constitution of the human gamete was speculative. Facts were inferred indirectly from the chromosomal constitution of concepti surviving long enough to produce clinically recognizable pregnancies. Estimated frequencies of chromosomal abnormalities at conception ranged from 10% (Kajii, Ohama, Niikawa, Ferrier, and Aviracha, 1973) to 20% (Ford and Evans,

1972) to 50% (Boué, Boué, and Lazar, 1975). Relating the true constitution of the male gamete genome to factors influencing production and survival of both chromosomally abnormal sperm as well as chromosomally abnormal concepti requires direct sperm chromosomal analysis.

The importance of determining gamete chromosomal constitution became apparent when specific anomalies related to the meiotic process were described in infertile people with a normal somatic karyotype (McIlree, Price, Brown, Trilloch, Newsam, and Mc Lean, 1966). Reported examples include, (1) presence of quadrivalents in metaphase I (possibly due to a reciprocate translocation, McIlree, Trilloch, and Newsam, 1966), and (2) reduction in the number of chiasmas (Hultén, Eliasson, and Tillinge, 1970; Pearson, Witterland, Khan, De Witt, and Bobrow, 1979).

Since about 20-25% of infertile males show no apparent abnormal semen parameters (Mann and Lutwak-Mann, 1981) and 40-50% have an abnormal basic semen analysis with no identifiable cause, at present, for the poor semen quality (Comhaire, Vermeulen, Ghedira, Mas, Irvine, and Callipolitis, 1983), new factors must be identified to fully explain male infertility problems.

C. Methodologies Used to Study the Sperm Chromatin Structure: Literature Review

1. Chromosomal Studies

Three different evaluative techniques are used to elucidate the existence of chromosomal abnormalities in the progeny of an individual: (1) karyotyping somatic cells (in peripheral blood lymphocytes or bone marrow), (2) studying meiosis in gonadal biopsies (synaptonemal complexes, spermatids, and oocytes), and (3) studying gametal chromosomes. Determining the maternal or paternal source of abnormal chromosomal contribution to the early embryo is paramount. Gamete chromosomal analysis is the only

method that directly reflects meiotic events allowing study of both somatic and meiotic anomalies which are restricted to the germinal line.

Any attempt to analyze male gametal chromosomes, however, immediately elucidates a striking feature of their molecular biology. The DNA of mammalian spermatozoa is the most highly condensed eukaryotic DNA known; more than a six-fold increase in condensation (Wyrobek, Meistrich, Furrer, and Bruce, 1976; Pogany, Corzett, Weston, and Balhorn, 1981; Balhorn, 1982) over the highly condensed DNA of a mitotic chromosome which for human chromosome 4 is in turn 12,400 times more condensed than the naked DNA strand (Pienta and Coffey, 1984). Studies of chromatin condensation during spermiogenesis show an ordered process beginning at the anterior end of the sperm head (the acrosome) and proceeding caudad (Dooher and Bennett, 1973; Zirkin, Soucek, and Chang, 1982). During this condensation the histones are gradually replaced by transitional proteins, which are subsequently replaced by protamines. The DNA binding proteins of spermatozoa are small, arginine-rich protamines present during the late stages of spermiogenesis (Marushige and Marushige, 1975).

Mammalian sperm chromatin is so highly condensed and genetically inactive that it is extremely difficult to study with conventional microscopic techniques. Consequently, researchers have applied a variety of extraction buffers to decondense the DNA by solubilizing the protamines and disrupting the nuclear architecture in order to study the integrity of nuclear content and chromosomal organization in sperm cells.

2. Sperm Nuclear Reactivation.

In the past, five protocols have been developed to reactivate the sperm nucleus. These protocols will be considered in the following order: (a) chemical treatments, (b) fusion with somatic cells, (c) fusion with germ cells, (d) oocyte microinjection, and (e) in vitro culture with amphibian egg extracts.

a. Chemical Treatments

Cells can be incubated with chemical reagents like borate buffer, which give a partial decondensation of the chromatin, or with disulfide bond reducing agents like dithiothreitol (DTT) in Tris(hydroxymethyl)aminomethane (Tris) buffer which gives a high degree of decondensation. Chromosomal reorganization was not observed in either case (Kvist, Afzeliius, and Nilsson, 1980; Huret, 1983).

Early investigations into the denaturability of sperm DNA were based on the hypothesis that misshapen sperm nuclei reflected alterations in chromatin structure (Evenson, Darżynkiewicz, and Melamed, 1980). Because the interactions between DNA and associated proteins affect denaturation of DNA in vitro, it was hypothesized that altered chromatin in vivo might make DNA more susceptible to heat or chemical denaturation. This increased susceptibility to denaturation was confirmed by staining sperm with AO, producing green fluorescence (wavelength 530 nm) in double-stranded (native) DNA (Lerman, 1963), and red fluorescence (wavelength >600 nm) in single-stranded (partially or totally denatured) DNA or RNA (Bradley and Wollf, 1959; Darżynkiewicz, 1979; Darżynkiewicz, Traganos, Carter, and Higgins, 1987). Since there is no significant amount of RNA in mature sperm, red fluorescence reflects the presence of denatured single-stranded DNA. The amount of denaturation is expressed as the amount of red fluorescence divided by total (red plus green) fluorescence, designated α_t (Darżynkiewicz, Traganos, Sharpless, and Melamed, 1975). This value is readily calculated using flow cytometry to measure fluorescence in individual cells. In fertile men, the value for $\alpha_t \pm 1$ S.D. is 0.18 ± 0.01 S.D. for unheated sperm and 0.29 ± 0.03 S.D. for heated (5 minutes at 100°C) sperm (Evenson et al., 1980). Thus, all sperm in a normal ejaculate contain a consistently small proportion of denaturable DNA. In samples from infertile men, α_t values were 0.20 ± 0.02 S.D. for unheated sperm and 0.45 ± 0.11 S.D. for heated sperm, showing a larger proportion of cells with denaturable DNA and a larger amount of denatured-

compared-to-native DNA in each cell.

Flow cytometry measurements have been proposed as a means to identify subfertile semen samples. Since sperm nuclear morphology is related to the chromatin condensation process that occurs during spermatogenesis, it was hypothesized (Gledhill, Darżynkiewicz,, and Ringertz, 1971) that misshapen sperm nuclei at the light microscopic level have an altered chromatin structure. However, it is not clear that molecular abnormalities detectable by flow cytometry are always predictive of abnormal sperm morphology in men. Evenson, Baer, Jost, and Gesch (1986) reported that men recovered from cancer chemotherapeutic treatment may produce normal semen analysis parameters in spite of having abnormal (elevated) denaturability of DNA. Patient fertility status was not given.

One of the most promising features of the DNA denaturability assay is its remarkable consistency in multiple ejaculates from the same man. The values for α_t do not differ by more than 10% unless there has been an intervening alteration in testicular function (Evenson, Jost, Baer, Turner, and Schrader, 1991).

b. Fusion with Somatic Cells

The fusion of spermatozoa with cultures of somatic cells in vitro (Gledhill, Sawicki, Croce, and Koprowski, 1972; Elsevier, and Ruddle, 1976; Phillips and Phillips, 1974) occurs spontaneously and also after treatment with lysolecithin and Sendai virus. However, fusion with cultured cells generally fails to reactivate mature spermatozoa. Reactivation may be due to the lack of some factor or factors necessary for resumption of DNA synthesis that are usually present in the oocyte- inducing decondensation of the tightly packed chromatin necessary for the resumption of DNA synthesis. Even in cases where small amounts of DNA synthesis have been observed in heterokaryons (Bendich, Borenfreund, and Sternberg, 1974; Johnson, Rao, and Hughes, 1970; Sawicki and Koprowski, 1971), there have been no indications of subsequent mitosis.

c. Fusion with Germinal Cells

The presence of specific factors in the germinal vesicle or cytoplasm of the oocyte not found in somatic cells, plays a major role in initiating reactivation (Usui and Yanagimachi, 1976). Therefore, techniques for obtaining sperm chromosomes have been oriented toward fusion of spermatozoa with germinal line cells. The ability to analyze human sperm chromosome complements after penetration of zona pellucida-free hamster eggs (sperm penetration assay, SPA) provided the first opportunity to study the frequency and type of chromosome abnormalities in human gametes. The main drawback of the use of the original SPA to analyze human sperm chromosome complements is the mandatory requirement of sperm motility in order to achieve penetration of the oocyte. This is a major disadvantage because a very interesting group of infertile patients (those with very poor semen parameters, including low or no motility) cannot be evaluated by this methodology. This present work attempted to overcome the motility barrier by using a micromanipulation technique called subzonal sperm injection (SZI) to achieve penetration and possible chromosome development. The most drastic scenario was created in which human spermatozoa were rendered immotile/dead by cryodamage in the absence of a cryoprotective agent. It has been shown by EM studies that freezing protocols (with or without cryoprotectants) disrupt the sperm surrounding membranes to different extent. This work hypothesized that if the inner acrosomal membrane could be exposed by mechanical removal of the plasma membrane and outer acrosomal membrane after cryodamage (as a way of mimicking a physiologic acrosome reaction), then even an immotile/dead sperm should be able to fuse the oolemma. If the proper activation occurred, then the oocyte should be able to phagocyte the attached spermatozoon and induce the normal steps of chromatin decondensation, pronuclei formation and chromosome condensation.

d. Microinjection into Mammalian or Amphibian Oocytes

Male pronuclei are visualized after microinjection of hamster or human sperm into

the perivitelline space (PVS) of hamster zona pellucida (ZP)-intact ova, but sperm pronuclear chromosomes were never observed (Uehara and Yanagimachi, 1976), as a large number of ova were destroyed in the micromanipulation. Identical results were observed when Xenopus ova were used for injection of human spermatozoa.

e. In vitro Culture with Amphibian Egg Extracts

The cytoplasm from mature ova of the Xenopus laevis also supports pronuclear development of human spermatozoa (Lohka and Maller, 1988; Ohsumi, Katagiri, and Yanagimachi, 1986). This animal model has several advantages over the hamster: (1) many more ova can be obtained after superovulation, (2) ova are much larger in size, (3) and there is no need to sacrifice the animals. Cell-free extracts prepared from a large number of ova produce sperm decondensation, pronuclear formation and sometimes visualization of chromosomal threads using either demembranated or capacitated membrane-intact human spermatozoa (Gordon, Brown, and Ruddle, 1985; Goddard and Zenzes, 1986).

Most studies performed so far have used whole semen samples in the analysis of chromatin structure. Because whole semen not only contains the desirable motile cells but may also contain immotile sperm cells, immature germ cells, epithelial cells, immune system cells, microorganisms and debris from various sources, a swim-up fraction of motile sperm will be prepared for the analysis of sperm chromatin as well as for insemination of zona pellucida-free hamster oocytes (Yanagimachi, Yanagimachi, and Rogers, 1976) in order to study the haploid karyotype of human sperm in a modified method (Benet, Genescà, Navarro, Egozcue, and Templado, 1986; Templado, Benet, Genescà, Navarro, Caballin, Miro, and Egozcue, 1988)..

Different separation procedures have been reported to improve the quality of semen samples used in the treatment of infertility, with the wash and swim-up being one of the most widely accepted. This technique allows motile cells to migrate into a nutrient medium

from a washed sperm pellet. Cells recovered in this manner have improved characteristics with respect to the original semen sample. Incremental improvements have been reported in percentage of motility, normal morphological forms, removal of both microorganisms and immature cells (Kuzan, Muller, Zarutskie, Dixon, and Soules, 1987; Wong, Balmaceda, Blanco, Gibbs, and Asch, 1986). Swim-up separation seems to parallel the in vivo selection for morphology and motility that occur in the female reproductive tract.

The percentage of spermatozoa with normal morphology is a good predictor (Kruger et al., 1986; Kruger et al., 1987) of fertilizing ability, and samples with poor morphological characteristics are disadvantaged. Although many morphological changes are obvious, their significance, as well as their relationship to genetic "fitness", are unknown in many cases. Accordingly, sperm cells with misshapen nuclei have been reported to have an altered chromatin structure when induced to decondense by in vitro thermal denaturation and analyzed by flow cytometry (Evenson et al., 1980). The resistance of structurally stabilized mature sperm DNA to denaturation was proposed as an important parameter in evaluating fertility. However, semen samples from patients that were considered to be normal for basic semen parameters including normal morphology but were unable to conceive (Tejada, Mitchell, Norman, Marik, and Friedman, 1984), displayed a higher percentage of cells with unstable chromatin structure after thermal denaturation when compared to fertile men's samples.

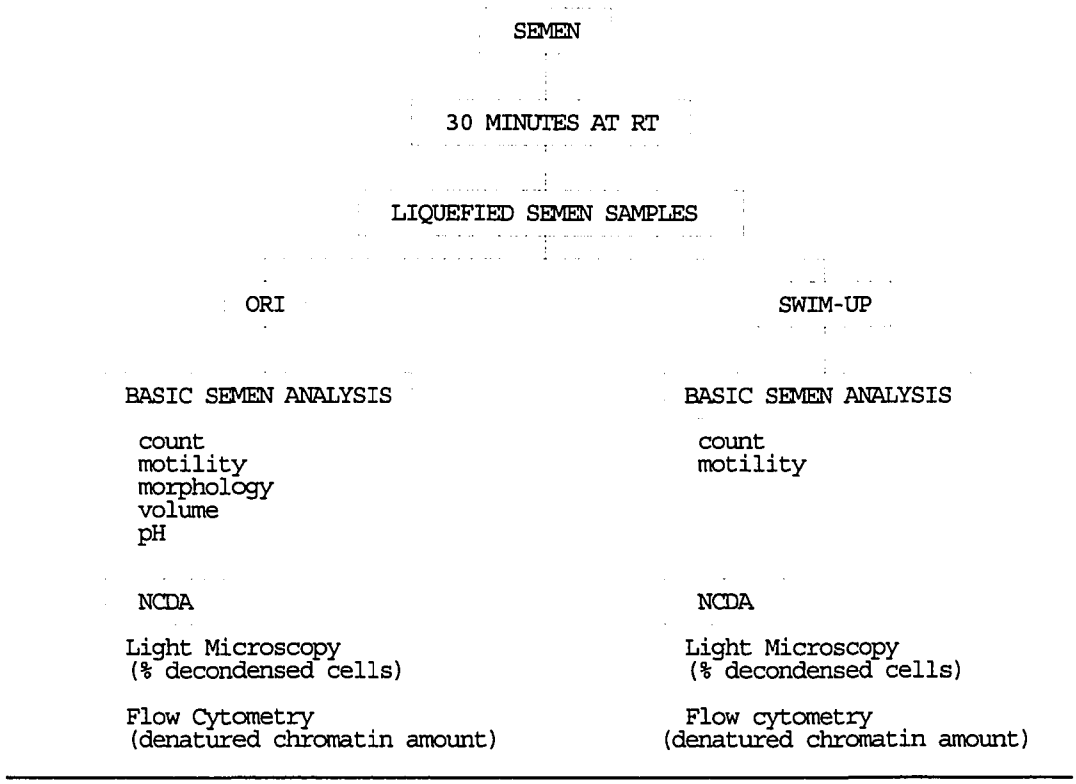
The present study evaluated the integrity of human spermatozoal nuclear material in the 2 condensation stages of chromatin (by the nuclear chromatin decondensation assay) and chromosomes (by the hamster sperm penetration assay).

CHAPTER II

Materials and Methods

A. Human Sperm Chromatin Analysis

1. Outline of the Study Protocol



2. Processing of Semen Samples

Semen samples were obtained from 20 patients attending an infertility clinic and from 3 donors of proven fertility (participants in a sperm donor insemination program). All specimens were collected in special sterile containers by masturbation after a minimum of 3- maximum of 5- days of sexual abstinence. Handling of semen samples was performed wearing powder-free latex gloves. After 30 minutes liquefaction at room temperature (RT), a basic semen analysis provided the following data on each sample: pH, viscosity, viability, morphology (Kruger, Menkveld, Stander, Lombard, VanderMerwe, Van Zyl, and Smith, 1986), sperm count and motility. Sperm count and motility were performed using computer analysis (Cellsoft Semen Analysis System, Labsoft Division of Cryo Resources Ltd., NY). A minimum of 200 cells in no fewer than 4 fields were analyzed on a 5 microliter (µl) droplet of semen (taken from a well-mixed sample) that was loaded into a Makler chamber and placed on the Cellsoft microscope stage warmer (37°C).

The samples were divided in two halves with one half being processed no further and labeled as original (ORI), while the other half being processed for swim-up was labeled as swim-up fraction (SW). The swim-up procedure required the samples to be aliquoted into 4 centrifuge tubes and combined with an equal volume of modified (Martin, Balkan, and Burns, 1983) Biggers-Whitten-Whittingham medium (BWW, Biggers, Whitten, and Whittingham, 1971)-0.3% BSA (bovine serum albumin). Tubes were centrifuged twice for 10 minutes at 270 x g, discarding all supernatants. After gently layering fresh BWW-0.3% BSA over the final pellets, the specimen tubes were incubated for 1 hour at 37°C in 5% CO_2 in air. Finally, the supernatants containing the motile sperm fraction were collected, pooled, and used for the nuclear chromatin decondensation assay (NCDA). Basic semen parameters in ORI and SW fractions were compared between samples for statistical differences by factorial analysis of variance and by the Mann-Whitney test (Siegel, 1956).

3. Nuclear Chromatin Decondensation Assay (NCDA)

The in vivo decondensation of the genetically inactive sperm nucleus triggered after fertilization can be induced in vitro by treatment of sperm with a disulfide reducing agent such as dithiothreitol (DTT) usually in combination with a detergent like sodium dodecylsulfate (SDS), and the chelating agent ethylenediaminetetraacetic acid (EDTA) in the incubation medium (Evenson et al., 1980).

The mechanism of chromatin decondensation has been triggered by 2 necessary, but independent prerequisites: Zn-removal (induced by EDTA-treatment) and demembranation (induced by SDS). In ejaculated human spermatozoa, there is a low incidence of chromatin decondensation in response to SDS alone (Calvin and Bedford, 1971; Ballachey, Evenson, and Saacke, 1988), although the detergent removes the membranous structures.

a. Control samples.

Aliquots of both ORI and SW fractions were diluted 1:1 with buffer I (5 mM borate buffer, pH 8.4, 1.0%w/v SDS, 6 mM EDTA) or 1:9 with buffer II (50 mM Tris buffer, pH 8.0, 3.0% SDS). Six mM EDTA is the lowest concentration that produces a complete removal of Zn (Huacuja, Sosa, Delgado, and Rosado, 1973). These diluents were incubated at room temperature for 0, 5, 10, and 15 minutes in small centrifuge tubes. After each incubation time, samples were immediately assessed for decondensation of the sperm chromatin by both light microscopy and by flow cytometry.

b. Treated samples.

Aliquots of both ORI and SW fractions were diluted 1:9 with buffer II, with the addition of 50 mM DTT (treated); and incubated at room temperature for 0, 5, 10, and 15 minutes in small centrifuge tubes. The degree of chromatin decondensation was also monitored by both light microscopy and flow cytometry.

4. Determination of the Percentage of Decondensed Sperm Heads by Light Microscopy

For the microscopic assessment of the different stages of decondensation, 40 µl droplets of both control and treated samples were carefully smeared on glass slides, air dried, fixed and stained with Diff-Quick stain (Diff-Quik, Baxter, McGaw Park, Il) and a minimum of 400 cells/slide/incubation were scored blindly.

The spermatozoa were classified into 2 major categories: non-decondensed (no fibrillar structures observed in the chromatin) and decondensed (fibrillar structures observed). Further subclassifications were made as indicated by the findings. Classification according to presence or absence of fibrillar structures rather than the traditional "swelling" of the sperm heads was based on the observation of the behavior of sperm chromatin in vivo. Fertilization is not complete until the tightly condensed chromatin fibers of the sperm nucleus unravel into individual chromosomal fibers followed by DNA duplication. DNA then associates with somatic type histones and rearranges into the individual chromosomes of the first mitotic division. The immediate swelling of the heads that occurs as soon as the spermatozoon penetrates an oocyte does not guarantee the further development into chromatin fibers required for normal pronuclear formation. In fact, it has been observed that human and hamster oocytes classified either as "not fertilized" due to lack of pronuclei formation or abnormally fertilized (one-pronuclear embryo), had at least 1 swollen sperm nucleus in their cytoplasm that failed to decondense (Plachot, Mandelbaum, Junca, deGrouchy, Salat-Baroux, and Cohen, 1989). After light microscopy assessment, the percentage of decondensed cells was correlated with incubation time and basic semen characteristics.

5. Flow Cytometric Determination of Sperm Chromatin Decondensation

a. Two-step acridine orange stain for DNA.

At the stated incubation intervals for the NCDA, 0.2 ml of each sample (control and

treated) was mixed with 0.4 ml of a detergent solution consisting of 0.1% Triton X-100 (Sigma Chemical Co., St Louis, MO) in 0.08 N HCl and 0.15 M NaCl to give a concentration of 1-2 x 10^6 spermatozoa/ml, thereby significantly reducing seminal viscosity. Thirty seconds later, 1.2 ml of a solution containing 0.2 M Na_2HPO_4, 0.1 M citric acid buffer (pH 6.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.15 M NaCl, and 6 µg AO/ml will be mixed with the sample (Evenson and Melamed, 1983). Stained samples were immediately introduced into the flow cytometer and analyzed within 1-3 minutes.

Pretreatment of cells with Triton X-100 at low pH rendered the cells permeable to the dye while nucleic acids remain insoluble. Subsequent staining with AO in the presence of chelating agents (EDTA, citrate) resulted in denaturation of all cellular RNA.

b. Flow cytometric measurement of AO-stained cells.

Fluorescence of individual cells was measured at incubation intervals for the chromatin decondensation assay with a Coulter APICX V flow cytometer utilizing an argon-ion laser. The mean green ± coefficient of variation ($X_{F\,530}$ ± CV) and mean red ± coefficient of variation ($X_{F\,600}$ ± CV) fluorescence emission as well as total number of cells read (N) were measured. A minimum of 5,000 cells was recorded for each sample. From these values the total green (T_G) and total red (T_R) fluorescence were calculated as follow:

$$T_G = X_{F\,530} \times N$$

$$T_R = X_{F\,600} \times N$$

If needed, the standard deviation (SD) was calculated as follow:

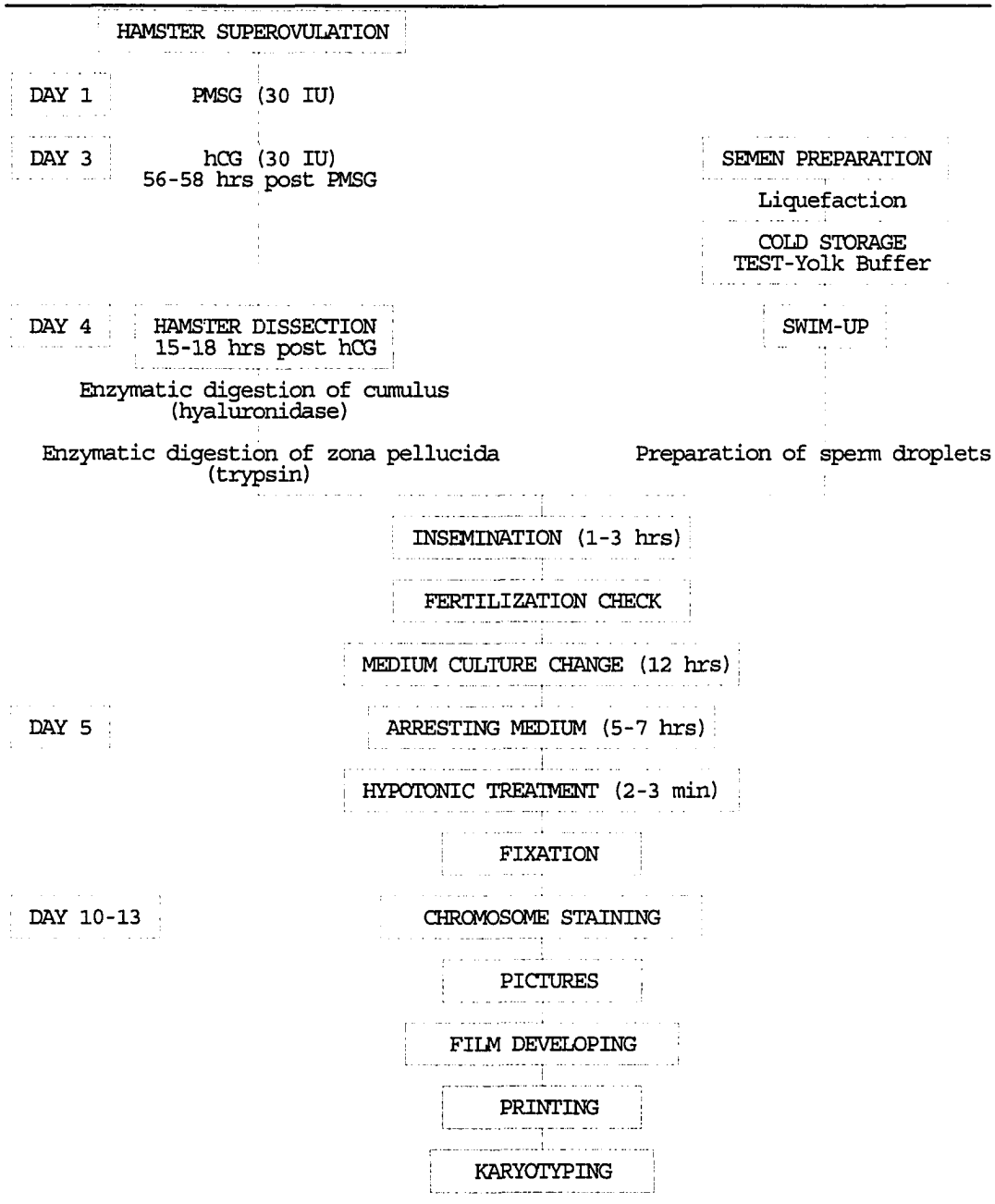
$$SD = X \times CV \times 100$$

Levels of RNA in the sperm nucleus are negligible and do not contribute to the measured fluorescence.

The extent of chromatin decondensation assessed by flow cytometry was correlated with incubation time and with basic semen parameters. Significant differences were analyzed by repeated measures analysis of variance.

B. Human Sperm Chromosomal Analysis

1. Outline of the Study Protocol.



2. Original Sperm Penetration Assay (SPA)

a. Media used.

Modified BWW was used for sperm washing, swim-up and oocyte preparation. Protein-free stock solutions of embryo culture medium were prepared at one to three week intervals. Pyruvate (an essential energy substrate) is labile and undergoes spontaneous decarboxylation in dilute solution. The pH of BWW was adjusted between 7.4 and 7.5 with 1 N NaOH or 1N HCl if necessary. The medium used for oocyte culture was Ham's F10 (Gibco, Grand Island, NY) supplemented with 15% FCS, 100 IU penicillin G/ml, and 50 µg streptomycin sulfate/ml (Ham, 1963). The pH of the Ham's F10 solution was adjusted to 7.2 with 1N HCl.

b. Processing of semen samples

Human semen samples from proven fertile donors were collected on day 3 of the experiment in a sterile container and processed as soon as liquefaction occurred. A basic semen analysis was performed as described in 1b. The liquefied semen samples were mixed with an equal volume of a buffer containing fresh hens egg yolk (TYB, Brandriff, Gordon, and Watchmaker, 1985) and were sealed in a small cryo tube, placed in a jar containing water at RT, and stored for 24 hours in the refrigerator to induce capacitation.

Approximately 3-4 hours before the harvest of the oocytes on day 4, the sperm were washed twice with BWW-0.3% BSA (1:1 dilution) in 15 ml conical centrifuge tubes (600 x g for 10 min). The 1-hour swim-up procedure was performed to separate highly motile spermatozoa, thereby increasing the penetration efficiency. Motile sperm were resuspended in BWW-3.3% BSA to a final concentration of 1-10 x 10^6 motile sperm/ml. One hundred microliter sperm droplets were placed into tissue culture dishes (Falcon, 60x15mm), covered with mineral oil and incubated at 37°C, 5% CO_2 in air and 100% humidity for a few minutes while the oocytes were readied for co-incubation.

Freshly ejaculated mammalian spermatozoa do not have fertilizing ability. This ability is

acquired by a process of maturation during passage of spermatozoa through the female tract or it can be induced by in vitro culture. The process is referred to as capacitation and acrosome reaction which involves biochemical changes in the plasma membrane and inner acrosomal membranes, thereby releasing the acrosomal contents and rendering the inner acrosomal membrane capable of fusing with the oval membrane.

A successful rate of fertilization depends on how well spermatozoa can be capacitated in vitro. Since removal of seminal plasma components initiates the process of capacitation, preincubation of fresh, ejaculated semen samples during prolonged periods of time in simple, chemically-defined media containing glucose, pyruvate, lactate and human serum albumin, renders a proportion of the spermatozoa capable of penetration. In these culture conditions 10-20% of the spermatozoa undergo acrosomal reaction. Multiple testing of several batches of BSA is necessary because capacitation rates of spermatozoa can changed with differing affinities between various lots of BSA and the sperm from an individual semen sample. A strategy to eliminate dependency on BSA is to incubate spermatozoa in TYB at 4°C for a period of 24 to 72 hrs.

c. Hamster care and superovulation.

Adult, 2-5 months old, female hamsters (Mesocricetus aureatus, Charles River Breeding Laboratories) were superovulated by intraperitoneal injection of 30 IU of pregnant mare serum gonadotropin (PMSG, Sigma) in the morning of day 1 of the experiment, followed by 30-40 IU of human chorionic gonadotropin (hCG, Sigma), 16-17 hours prior to sacrifice on day 3 of the experiment. The cumulus cell mass containing eggs arrives in the oviduct 15 to 18 hours after hCG injection with each hamster yielding approximately 40 eggs.

Superovulation provides the experimenter with a large synchronous population of eggs at a predictable ovulation time. Some controversy exists over the supposed inferiority of superovulated compared to spontaneously ovulated eggs. The population of ova has to be large in number and homogeneous in quality (only metaphase II oocytes, MII). Meiotic

maturation is of fundamental importance since it is only after reaching MII that the female gamete is competent to ensure fertilization, cleavage and cellular differentiation. Maturation involves not only nuclear events like the meiotic progression from the dictyotene stage to MII, but also subsequent to breakdown of the germinal vesicle, the appearance of cytoplasmic factors and macromolecules upon which the progress of early embryogenesis depends. The ability of the oval cytoplasm to dissociate the nuclear sperm membrane and totally decondense the penetrated spermatozoon appears to be acquired gradually between germinal vesicle breakdown and MII. The transformation of decondensing sperm into a male pronucleus depends upon the activity of a male pronucleus growth factor in the oval cytoplasm which appears at the time of germinal vesicle breakdown. Its effect disappears or becomes inactive at later stages of development. In polyspermic zona-free hamster ova, multiple decondensing sperm heads fail to develop into male pronuclei, whereas meiotic chromosomes of the ovum form a female pronucleus, suggesting that the factor promoting the female pronucleus development is different from the male pronucleus growth factor.

d. Oocyte retrieval and processing.

After euthanasia, an incision in the linea alba allowed the dissection of the oviducts from the ovary and the uterotubal junction. Remnants of fatty tissue were trimmed off. The dissected oviducts were placed in a sterile petri dish containing BWW-0.3% BSA. The oviducts were flushed using a 3 cc syringe with a 30 gauge needle filled with medium. The dissecting scope used for this purpose was fitted with a fiberoptic light source above and a base light beneath. Masses of cumulus cells were transferred to drops of 0.1 % hyaluronidase until eggs dissociated (3-5 min). Oocytes were washed and put into a 0.1 % trypsin solution until zona pellucida disappeared and the polar body detached. Zona-free oocytes were washed and immediately transferred into sperm droplets and were coincubated in the 5% CO_2 incubator for 1-3 hours, depending on the sperm sample. Handling of eggs must be as quick as possible (30-40 minutes).

e. Fertilization check.

Fertilization checks was performed in order to determine the most appropriate time to transfer the oocytes from sperm droplets to Ham's F10. The timing of these checks depended on sperm motility. A vigorously-swimming sample often required a fertilization check after 1 hour of co-incubation, whereas an asthenospermic sample may have required a longer co-incubation time. The time of co-incubation of zona-free ova with capacitated spermatozoa usually lasted 2-3 hrs.

After incubation, 10-20 eggs were placed on a microscope slide to assess fertilization. Removal of the zona pellucida disturbs the mechanisms that prevent polyspermy so that several spermatozoa may penetrate a zona-free ovum, but heavy polyspermy can be a limiting factor, as oocytes containing more than five decondensing sperm heads leads to poor development of sperm pronuclei. Normally, a maximum of three discrete haploid chromosomal sets from human spermatozoa can be visualized in the ovum cytoplasm. Penetrated ova contained at least one swollen sperm head or one pronucleus with an accompanying tail.

Theoretically, semen samples which produce a high penetration rate with low numbers of penetrating spermatozoa per ovum are best suited for obtaining sperm chromosomes. As the penetration rate increases linearly with increasing concentrations of spermatozoa, manipulations with sperm concentrations may be one effective control and will be used as necessary.

f. Oocyte culture/incubation.

If penetration was equal or higher than 50%, the oocytes were removed from the insemination dish, washed several times and cultured in Ham's F10 medium supplemented with 15% hFCS in the CO_2 incubator for 12.5 hrs. This medium provided all the aminoacids, vitamins and cofactors needed for DNA duplication, chromatin condensation and chromosomal formation. After this first incubation period, oocytes were placed in the

same medium containing 0.4 µg ml of Colcemid (Gibco) and incubated an additional 4-7 hrs in the CO_2 incubator to arrest metaphases.

g. Preparation of slides.

Slides were cleaned with isopropyl alcohol and wiped dry with a lint-free tissue before use.

h. Hypotonic treatment.

Hypotonic medium facilitates the floating of chromosomes in the oocyte's cytoplasm. Hypotonic swelling also frees chromosomes of a ribonucleoprotein complex which usually surrounds them. This swelling improves staining and banding for better observation. Oocytes were therefore kept in hypotonic solution of 1% sodium citrate for 5-10 minutes.

i. Fixation of oocytes.

Fixation was performed at room temperature by dropping 20 µl of an ethanol:acetic acid mixture 3:1 over the oocytes. A very gentle exhalation of breath directly over the slide stopped oocytes from rolling around on the slide while simultaneously added moisture to the slide to stabilize the oocytes. As the drop started to spread out on the slide the oocytes became visible, standing out in relief. A diamond pen was used to scratch a circle on the underside of the slide to mark the area for microscopy. Chromosome grouping was identified by using a phase contrast microscope (Nikon, Diaphot) with 10, 20, 40 and 100x objectives. The type of chromosomal spread and their position on the slide were noted with 10x objective. Chromosomes should be very dark, compact and evenly spaced.

j. Aging of slides.

Many techniques require the fertilized eggs to be aged after slide mounting. One to two weeks at RT, 3 days at 55°C or 29 minutes at 95°C will produce the same results (Holmquist and Motara, 1987). Slides were aged for a minimum of a week at RT before chromosomal staining. The most important effect of aging may be the oxidation of protein sulphydryl groups.

k. Chromosomal staining.

i. Uniform staining. Slides were stained uniformly with Wright stain (Wright Accustain, Sigma) allowing identification of numerical abnormalities, determination of the presence of chromosomal fragments and differentiation between human and hamster chromosomal sets.

ii. G-banding: Wright staining of groups of chromosomes was followed by G-banding which allowed for identification of structural abnormalities in the banded pattern of each chromosome. Wright stain usually varies greatly from batch to batch. The powder is quite stable but the solution starts to precipitate as a surface film at different rates for different components. Individual chromosomes could usually be identified by their banding patterns, but in a proportion of spreads, one or more chromosomes could only be identified according to the Denver group classification (Denver Conference, 1960).

Unstained metaphases with long, well spread chromosomes were G-banded as follows:

1. metaphases were incubated in 2x SSC (salt sodium citrate) solution at 65°C and treated with 0.25% trypsin solution. Time of treatment varied with age and quality of slides.
2. preparations were stained with Wright stain.

l. Photographic techniques.

Stained material were photographed with a Nikon Optiphot microscope with a 60x or 100x lens on a Technical Pan Film 2415 (ESTAR-AH Base Kodak). Films were developed for 8 minutes at 20°C with Kodak HC-110 Developer. Kodak Polycontrast III RC paper was used for printing the photographs.

m. Karyotyping.

Chromosomal aberrations were classified according to the International System for Human Cytogenetic Nomenclature (International System for Human Cytogenetic Nomenclature, 1985). Difficulties to overcome in studying the chromosome banding pattern included the following: (1) obtaining oocytes, (2) penetration rate, and (3)

metaphase stages. Adequate condensation of chromosomes with good spreading and complete blockage of karyogamy will prevent overlapping of human and hamster pronuclear chromosomes. Significant differences of chromosome abnormality frequencies between donors as well as deviations from the expected 1/1 ratio of X:Y chromosomes were analyzed by χ_2.

3. Modified SPA: Subzonal Insertion of Dead Human Sperm

The subzonal insertion of dead human spermatozoa is a completely new approach not only for the study of human sperm chromosomes, but for assisting in fertilization for patients with nonviable sperm cells. To increase the chances of sperm-oocyte interaction, a mechanical disruption of the acrosomal membranes was performed by subjecting the sperm cells to a freezing and thawing procedure in the absence of a cryoprotective agent prior to SZI with hamster oocytes.

a. Semen processing.

Human semen samples were collected by masturbation from 3 healthy fertile donors. After 30 minutes liquefaction at RT, all samples were divided into 3 centrifuge tubes and washed twice with 2 volumes of Ham's F10 with 7.5% hFCS. The pellet in one tube was resuspended in the same medium to a concentration of $10x10^6$ cells/ml and it was named "washed sperm". The 2 remaining pellets were subjected to a 1 hour swim-up procedure in the CO_2 incubator and named "swim-up samples". The swim-up samples were also resuspended to a concentration of $10x10^6$ cells/ml.

One milliliter aliquots of both washed sperm and swim-up samples were subjected to 3 cycles of freezing and thawing. The samples were stored at -20°C for 1 hour and were thawed at RT for 15 minutes (Bocca, Veeck, Swanson, and Morshedi, 1992). A total of 8 fractions were obtained from each original semen sample and were named as follows: washed and swim-up sperm, washed and swim-up sperm frozen-thawed once (Fr-Th x 1), washed and swim-up sperm frozen-thawed twice (Fr-Th x 2), washed and swim-up sperm

frozen-thawed 3 times (Fr-Th x 3). All the sperm fractions were assessed for: viability (by vital Eosin Y staining, Eliasson, and Treichl, 1971), acrosomal status (by FITC-PSA fluorescence staining, Cross, Morales, Overstreet, and Hanston, 1986), and transmission electron microscopy (TEM, Lanzendorf, Maloney, Ackerman, Acosta, and Hodgen, 1988). Untreated washed and swim-up fractions, as well as Fr-Th x 3 washed and swim-up fractions were stored at -20°C for up to one week and before using for SZI with 2 immotile but intact spermatozoa per hamster oocyte.

b. Hamster superovulation/oocyte retrieval.

Obscuring cumulus cells were removed from oocytes with a solution of 0.1% hyaluronidase for 1 minute at RT followed by aspiration and expulsion of the oocytes through a heat-constricted pasteur pipette. Oocytes may be treated with 0.05 M to 0.1 M sucrose in order to shrink the ooplasm to facilitate the successful microneedle insertion under the ZP without damaging the oolemma. Embryos dehydrated in hypertonic sucrose for microinjection were rehydrated before incubation.

c. Subzonal insertion of sperm.

Subzonal sperm microinjection was performed in a 50-100 µl droplet of Ham's F10 medium under mineral oil. Two-to-three dead spermatozoa were injected into the perivitelline space (PVS) of superovulated hamster oocytes. Large diameter egg-holding pipettes (120 µm o.d.) and sharp, bevelled microinjection needles (20-30 degree bevel angle, 10-15 µm o.d.) were manufactured for this procedure.

Micropipettes and microneedles were prepared with a Narishige PB-7 pipette-puller using a double-pull technique on thin-walled glass capillary tubes (0.9 mm o.d., 0.6 mm i.d.; 150 mm length, Drummond Scientific, Broomall, PA). Holding pipettes were made by using four weights and relatively low heater temperatures (heater 1: 20; heater 2: 10.2 on PB-7). This created a pipette with a relatively wide and blunt surface. Holding pipettes were then fire-polished with a Narishige MF-9 microforge to achieve a smooth surface with an i.d. of

20-30 µm and o.d. of approximately 100 µm.

Microneedles were made by using one light weight and relatively high heater settings (heater 1: 30; heater 2: 70.0 on the PB-7). A sharp point was produced by breaking the tip off in the egg-holding pipette or by bevelling on a pipette grinder (Narishige EG-4).

d. Fertilization check.

Micromanipulated and control oocytes were cultured in the CO_2 incubator for 18 hours in Ham's F10-15% hFCS. Oocytes were examined unstained by phase contrast light microscopy for the presence of pronuclei (PN) with extrusion of the second polar body (PB).

CHAPTER III

Results

A. Human Sperm Chromatin Analysis

1. Characteristics of The Semen Samples

A total of 24 semen samples (21 patients of unknown fertility and 3 proven fertile donors) were analyzed in the study (Table 1). Twenty of the 21 patients had normal sperm counts ($\geq 20x10^6$/ml, based on WHO standards); 15/21 had normal motility ($\geq 40\%$, based on WHO standards) and 12/21 had either normal (N) or good (G) prognosis sperm morphology pattern (> 4% normal forms, Kruger et al., 1986). Sample 11 showed bacterial contamination in both the ORI and SW fractions.

All three proven fertile donors had normal count, motility and morphology semen parameters. All the swim-up fractions (Table 2) showed a high percentage of motile cells ($\geq 65\%$) except for patients' samples 4 and 8 which also had an abnormally low percentage of motile cells in their ORI semen.

2. Nuclear Chromatin Decondensation Assay

a. Control samples.

i. Percentage of Decondensed Sperm Heads by Light Microscopy

The first morphological event observed at 5 minutes incubation in buffer control was the loss of the flagellum (Figure 1A, 2), followed by swelling of the sperm nucleus (Figure 1B). No fibrillar structures were observed in the chromatin within the 15 minute incubation time. Swelling of the chromatin was observed in both ORI and SW samples, although no

Table 1: Basic Semen Analysis Results from Original Semen Samples.

Sample number	Concentration (millions/ml)	Percent motility	% N[a]	Volume (ml)	pH
1	171.3	35.4	10.0	1.4	8.3
2	32.2	42.3	4.0	2.7	7.6
3	25.9	67.9	3.7	2.7	7.8
4	280.8	14.4	18.0	1.6	8.1
5	50.1	13.5	12.0	2.3	8.6
6	70.1	65.9	3.7	2.9	8.1
7	92.3	22.0	18.0	1.0	8.4
8	38.6	42.2	14.3	2.5	7.8
9	36.2	68.5	18.0	6.2	7.8
10	63.5	64.7	1.5	4.0	8.5
11[b]	61.8	17.6	2.5	4.3	7.8
12	266.4	79.3	5.0	3.4	7.8
13	14.5	55.1	9.0	2.8	7.8
14	22.5	41.1	15.0	2.2	8.3
15	57.4	41.8	20.5	3.6	7.8
16	174.5	67.8	17.0	1.4	7.8
17	70.7	50.0	4.3	3.3	7.8
18	39.0	63.6	2.0	3.7	8.3
19	32.7	26.7	0.0	3.0	8.2
20	66.0	64.9	3.5	3.3	8.1
21	50.1	13.5	2.3	3.0	8.6
22[c]	279.0	89.8	18.0	1.9	8.3
23[c]	298.1	83.2	16.1	1.5	8.2
24[c]	193.9	80.0	10.0	1.3	8.6

[a]Morphology was assessed by Kruger et al., 1986 strict criteria
[b]Sample showed bacterial contamination in original and swim-up specimens and 20% agglutination.
[c]Donors

Table 2: Swim-up Samples Semen Parameters.

Sample number	Concentration (millions/ml)	Percent motility
1	3.4	84.7
2	2.8	78.6
3	1.5	75.6
4	0.8	47.7
5	2.6	92.3
6	14.4	92.3
7	0.5	29.3
8	1.0	79.6
9	6.4	93.3
10	19.6	87.0
11	1.2	68.3
12	144.8	88.7
13	0.6	73.4
14	1.1	52.6
15	11.9	85.7
16	12.4	85.3
17	43.3	96.9
18	1.1	89.6
19	1.5	94.3
20	15.5	90.3
21	2.6	92.3
22	17.8	64.7
23	40.5	71.6
24	20.1	81.4

Figure 1. **Morphological changes in by cells from DTT-treated semen samples during NCDA assessed by light microscopy.**

A-1: non-decondensed, intact spermatozoa.

A-2: non-decondensed spermatozoa without the flagellum.

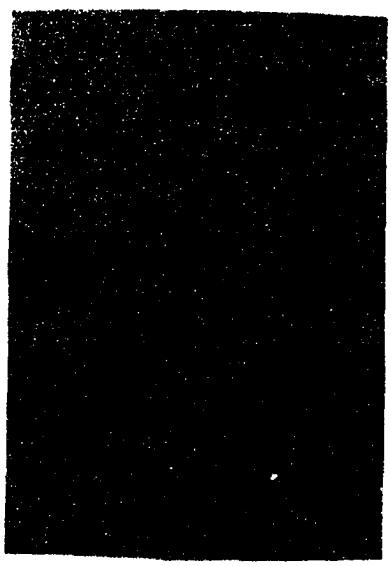
A-3: spermatozoon starting to decondense. Notice the presence of vacuoles in the acrosomal region. Compare the size difference between a starting to decondense cell and a non-decondensed, intacat cell, 400X.

A-4: partially decondensed cells have moderately swollen translucent nucleus showing higher degree of decondensation, 400X.

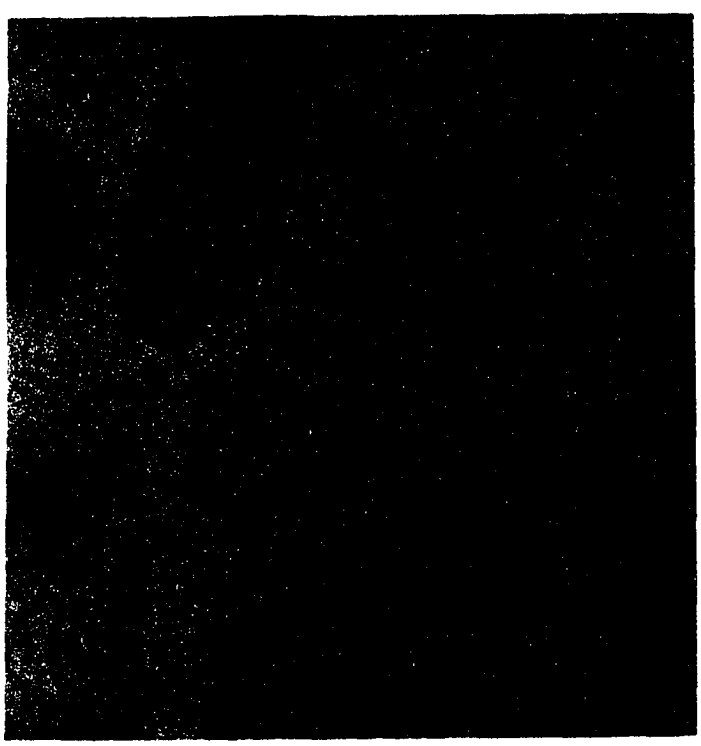
B: non-decondensed, swollen cells. The flagellar loss was followed by swelling of the nucleus beginning from the posterior third portion of the sperm head. The nucleus increased its size, but the chromatin appears dark and homogeneous, 400X.

C: totally decondensed cells with grossly swollen transparent nucleus. Notice the presence of fibrillar structures of different widths in the nuclear chromatin, 1000X.

A



B



C

decondensation into fibrillar structures was achieved at any interval (0/13) when samples were incubated in buffer without a disulfide-bond reducing agent. Neither borate (Bor) nor Tris-control buffers supported decondensation even after 22 hours of incubation at 4°C (samples 11, 13, and 15, data not shown).

ii. Flow Cytometric Determination of Sperm Chromatin Decondensation

The flow cytometer used in the sperm chromatin decondensation assay generated two types of data/graphs: histograms (single-parameter analysis) and scatterplots (dual-parameter analysis) for each sample, at each incubation time. Figures 2 and 3 are computer-drawn histograms of ORI-control sperm cells stained with AO representing fluorescence intensities at 530 nm and at > 600 nm respectively. The coordinates represent fluorescence intensity (abscissa) in arbitrary units and the number of cells (ordinate) scaled arbitrarily. There was a marked increment in fluorescence (both red and green) from 0 to 5 minutes of incubation, in concurrence with the loss of the flagellum observed microscopically, which may have allowed for an easier interaction between the dye (AO) and the chromatin. But mean fluorescence values remained very stable after 5 min incubation for both ORI and SW samples, regardless of the type of control buffer (Bor or Tris) used.

Figure 2. **Pattern of in vitro sperm nuclear decondensation of Control-ORI samples as monitored by flow cytometry (green fluorescence).** Computer-drawn frequency histograms (single-parameter analysis) representing the distribution of cells stained with AO after 0, 5, 10, and 15 minutes of incubation in CONTROL buffer according to their green fluorescence.

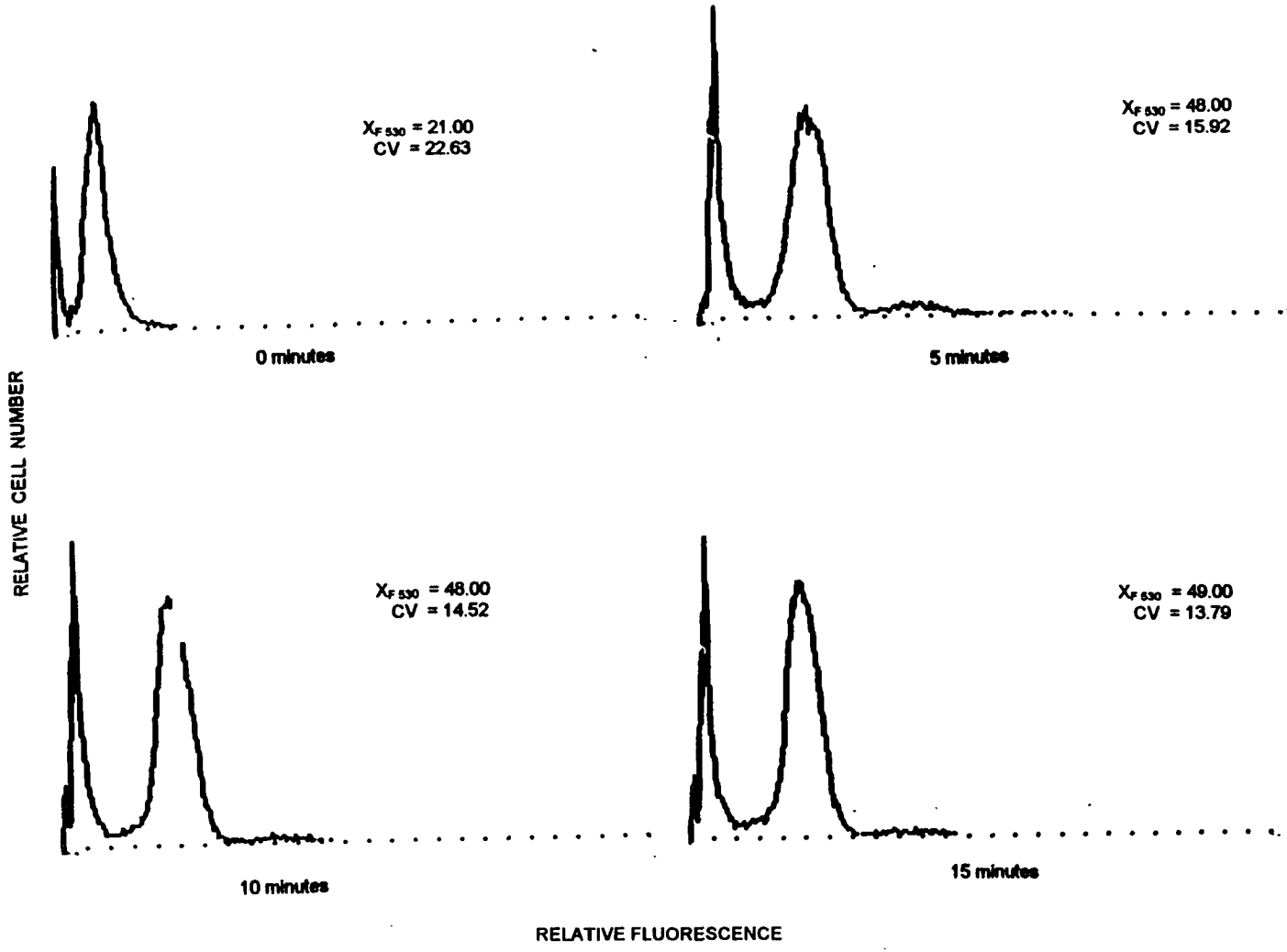
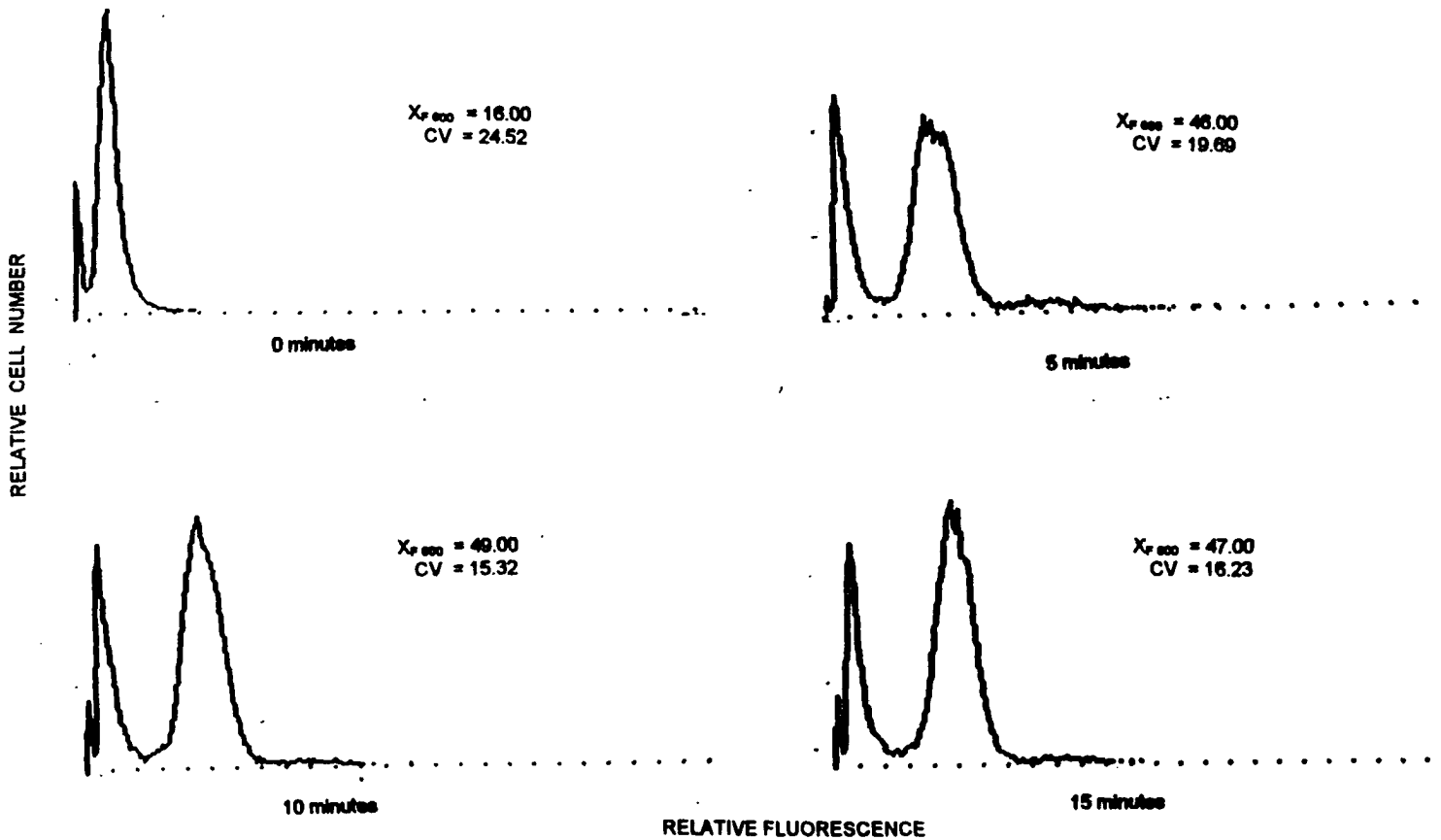


Figure 3. **Pattern of *in vitro* sperm nuclear decondensation of Control-ORI samples as monitored by flow cytometry (red fluorescence).** Computer-drawn frequency histograms (single-parameter analysis) representing the distribution of cells stained with AO after 0, 5, 10, and 15 minutes of incubation in CONTROL buffer according to their red fluorescence.



Figures 4 and 5 are cytograms or scatterplots of green (y axis) vs red (x axis) fluorescence, each dot gives the result for a single cell). Note that the position of the main cluster of nuclei shifted to increased fluorescence values upon incubation time in control buffer; but to a higher extent for ORI (Figure 4) than for SW samples (Figure 5).

The changes in green and red fluorescence, as well as the changes in α_t at each incubation time with control buffer are summarized in Table 3 for ORI samples, and in Table 4 for SW samples. The amount of green and red fluorescence, as well as the α_t values significantly changed upon incubation time for the ORI samples ($F = 9.79$, $p = 0.0466$ for green; $F = 8.87$, $p = 0.0530$ for red fluorescence; $F = 13.14$, $p = 0.0312$ for α_t). These changes were not detected under the LM and probably reflected the higher sensitivity in the detection of slight changes in chromatin structure by the flow cytometer. Conversely, the SW samples had no significant changes in total amount of green fluorescence ($F = 4.35$, $p = 0.1293$), red fluorescence ($F = 3.56$, $p = 0.1622$) or α_t ($F = 1.36$, $p = 0.4030$) upon incubation in the control buffer. These results may reflect the lower stability and higher heterogeneity of cell components of the ORI vs SW samples.

b. Treated samples

i. Percentage of Decondensed Sperm Heads by Light Microscopy

A totally different pattern of chromatin decondensation was observed between ORI and SW samples after incubation in buffer including DTT. Figure 1 shows representative examples of the relative change in sperm nuclear morphology with time in DTT-treated samples, determined by LM. In general, incubation of ORI samples in buffer with DTT allowed the spermatozoa to undergo a series of morphological changes starting from loss of the flagellum, swelling of the sperm nucleus and decondensation of the nuclear chromatin into fibrillar structures (Figure 1, A-C). In all cases, initial size increases occurred while the nuclei remained phase-dark, but as the nuclei continued to swell they became translucent and finally transparent.

Table 3: Total Green (T_G) and Red (T_R) Fluorescence and α_t Values in ORI Control Samples

Sample number	Time (min.)	$T_G/10^4$	$T_R/10^4$	α_t
15 bor	0	37.18	28.33	0.43
	5	69.91	68.42	0.49
	10	70.47	71.15	0.50
	15	71.71	73.48	0.51
16 bor	0	38.15	37.80	0.50
	5	69.72	70.11	0.50
	10	71.71	72.45	0.50
	15	74.14	75.13	0.50
12 bor	0	28.86	25.13	0.47
	5	84.56	84.04	0.50
	10	78.00	79.82	0.51
	15	86.05	85.37	0.50
12 tris	0	29.61	20.49	0.41
	5	96.56	103.99	0.52
	10	93.45	107.62	0.54
	15	91.90	107.42	0.54

$T_G/10^4$: total green fluorescence per 10,000 cells
$T_R/10^4$: total red fluorescence per 10,000 cells

T_G: F = 9.79, p = 0.0466 (S)
T_R: F = 8.87, p = 0.0530 (Marg. S)
α_t: F = 13.14, p = 0.0312 (S)

Table 4: Total Green (T_G) and Red (T_R) Fluorescence and α_t Values in SW Control Samples

Sample number	Time (min.)	$T_G/10^4$	$T_R/10^4$	α_t
15	0	44.42	35.29	0.44
bor	5	69.80	72.87	0.51
	10	69.78	75.60	0.52
	15	68.46	72.51	0.51
16	0	74.69	79.83	0.52
bor	5	80.32	84.28	0.51
	10	90.53	106.41	0.54
	15	82.85	89.35	0.52
12	0	74.79	76.51	0.51
bor	5	76.84	80.01	0.51
	10	79.29	83.38	0.51
	15	81.80	86.08	0.51
12	0	27.17	18.17	0.40
tris	5	73.02	72.67	0.50
	10	89.32	103.41	0.54
	15	84.59	103.57	0.55

$T_G/10^4$; total green fluorescence per 10,000 cells
$T_R/10^4$: total red fluorescence per 10,000 cells

T_G: $F = 4.35$, $p = 0.1293$ (NS)
T_R: $F = 3.56$, $p = 0.1622$ (NS)
α_t: $F = 1.36$, $p = 0.4030$ (NS)

Figure 4. **Pattern of *in vitro* sperm nuclear decondensation of Control-ORI samples as monitored by flow cytometry (scatterplot).** Dual- parameter analysis of the extent of decondensation achieved by sperm cells stained with AO after 0, 5, 10, and 15 minutes of incubation in CONTROL buffer. Each dot in scatterplots represents a single cell whose coordinates are defined by its green (y-axis) and red (x-axis) fluorescence values.

Figure 5. **Pattern of *in vitro* sperm nuclear decondensation of Control-SW samples as monitored by flow cytometry.** Dual-parameter analysis of the extent of decondensation achieved by sperm cells stained with AO after 0, 5, 10, and 15 minutes of incubation in CONTROL buffer. Each dot in scatterplots represents a single cell whose coordinates are defined by its green (y-axis) and red (x-axis) fluorescence values.

The majority (18/19 = 94.74 %) of the ORI samples reached the decondensed level at 15 minutes incubation (Table 5), although the decondensation rates were not uniform among the samples. For example, samples 10, 17, and 20 (with normal morphology), and sample 12 (with poor morphology) showed very low degree of decondensation (< 10 % of nuclei with fibrillar structures) at the end of 15 minutes incubation. In contrast, samples 2, 3, and 23 (with poor morphology), and 12 and 15 (normal morphology) achieved a high degree of decondensation (> 10% of nuclei with fibrillar structures) within 5 minutes of the start of incubation. The percentage of cells in each of the decondensed categories (starting to decondense, partially decondensed and fully decondensed) varied from sample to sample (data not shown). There was an extremely significant ($F = 35.61$, $p < 0.0001$) shift in the distribution of the cells from the non-decondensed state to the decondensed state during the course of the 15 minutes incubation with DTT. Changes were significant for both patients ($F = 17.80$, $p < 0.0001$) and donors ($F = 4.69$, $p = 0.0515$). No significant correlations were observed between the percentage of decondensed cells in the ORI-treated samples and their basic semen parameters at any incubation time (Appendix 1).

The SW-treated samples gave a completely different pattern of chromatin decondensation from the ORI semen: the spermatozoa lost their flagella but they did not undergo the series of morphological changes upon incubation observed for the cells in the ORI-treated samples. Although fibrillar chromatin structures were detected with the light microscope in 15.79 % (3/19) samples (Table 6), these changes were not significant ($F = 1.22$, $p = 0.3129$). No significant correlations were observed between the percentage of decondensed cells in the ORI or SW-treated samples and their semen parameters at any incubation time (Appendix A). Swelling of some nuclei was detected at 15 minutes incubation. Sample 12 deviated from the low percentage of decondensation pattern observed for the SW samples, showing 9.35 % of decondensed nuclei at 15 minutes incubation. However, this particular sample had bacterial contamination as well as more

Table 5: Percentage of Decondensed Cells in ORI Samples Treated With DTT

Sample #	Incubation time			
	0	5	10	15
1	0.00	0.00	14.17	100.00
2	0.00	39.66	39.66	100.00
3	0.00	22.27	76.82	98.21
4	0.00	4.49	2.91	26.18
7	0.00	5.79	18.32	42.16
8	0.00	3.78	14.80	75.64
9	0.00	57.35	93.20	94.37
10	0.00	0.00	0.00	0.00
11	0.30	1.47	15.20	52.30
12	1.22	0.00	1.40	2.48
14	0.00	0.00	31.12	73.39
15	0.00	12.53	60.24	92.72
16	0.00	8.69	35.32	73.49
17	0.00	0.00	1.29	3.14
18	0.00	2.53	18.43	79.96
19	0.00	2.09	15.89	30.22
20	0.00	0.00	0.00	10.29
22	0.00	0.00	64.45	74.58
23	0.00	11.84	49.06	98.11
X ± SD[a]	0.08 ± 0.28	9.08 ± 15.36	29.07 ± 27.99	59.33 ± 36.96

[a] Mean ± SD percentage of decondensed cells for each incubation time
F = 35.61, p < 0.0001, Extr. S.
Patients; F = 17.80, p < 0.0001, Extr. S.
Donors; F = 4.69, p = 0.0515, Marg. S.

Table 6: Percentage of Decondensed Cells in SW Samples Treated with DTT

Sample #	Incubation time (min.)			
	0	5	10	15
1	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
9	0.00	2.50	0.00	0.00
10	0.00	13.10	11.83	1.45
11	5.35	0.00	6.32	0.80
12	0.56	7.30	1.04	9.35
14	0.00	0.00	0.00	0.00
15	0.00	0.00	0.00	0.00
16	0.00	0.00	0.00	0.00
17	0.00	0.00	0.00	0.00
18	0.00	0.00	18.40	0.00
19	0.00	0.00	0.00	0.00
20	0.00	0.00	0.00	0.00
22	0.00	0.00	0.00	0.00
23	0.00	0.00	0.00	0.00
X ± SD[a]	0.31 ± 1.23	1.21 ± 3.36	1.98 ± 4.97	0.61 ± 2.15

[a] Mean ± SD percentage of decondensed cells for each incubation time
F = 1.22, p = 0.3129 (NS)
Patients; F = 1.22, p = 0.3140, NS

than 30 million/ml round cells in ORI specimen smears (Table1). Semen samples with either bacterial infections or exposure to chemicals have been reported to have elevated red fluorescence (decondensed chromatin) (Gopalkrishnan, Hinduja, and Kumar, 1991). These findings showed a high resistance of the selected motile fraction of sperm cells (SW fraction over ORI) to undergo decondensation.

ii. Flow Cytometric Determination of Sperm Chromatin Decondensation

The morphological changes observed in the sperm cells under the light microscope agreed with an increase in AO uptake during the decondensation process. Results of the flow cytometric analysis of patient number 10 are shown in Figures 6 and 7. A pronounced change in the position of the main nuclear cluster occurred over the incubation time for both ORI (Figure 6) and SW-treated nuclei (Figure 7) indicating that nuclear chromatin was sensitive to the chemical decondensation (Figures 5 and 6). However, the positional difference was not as marked for the SW versus ORI nuclei.

The greatest change in ORI-treated samples was seen at 5 minutes of incubation (Table 7), a result also observed for control samples. The addition of DTT gave a typical biphasic pattern in the decondensation process, which has been reported by Jean, Perreault, Auger, Roberts, Chapdelaine, and Bleau (1979). An early increase in fluorescence was detected at 5 minutes that may correlate with changes in chromatin structure (disulfide bond reduction) but with nuclear size almost constant or slightly enlarged (Figure 1B), followed shortly by a decline in the light signal (after 5 minutes) at which time the nucleus becomes extremely enlarged and translucent, thereby scattering less light (Figure 1C). The ORI semen samples showed similar or slightly higher proportions of green than red fluorescence at 0 minutes, but shifted to a strikingly red fluorescence upon incubation, specially for samples 1, 10, 22, and 19 (all with < 10 % normal sperm forms).

The sudden increase of fluorescence from 0 to 5 minutes of incubation in all the ORI

Figure 6. **Pattern of *in vitro* sperm nuclear decondensation of Treated-ORI cells as monitored by flow cytometry.** Computer-drawn scattergrams of the distribution of individual sperm cells stained with AO after 0, 5, 10, and 15 minutes of incubation.

Figure 7. **Pattern of *in vitro* sperm nuclear decondensation of Treated-SW cells as monitored by flow cytometry**. Computer-drawn scattergrams of the distribution of individual sperm cells stained with AO after 0, 5, 10, and 15 minutes of incubation.

Table 7: Total Green (T_G) and Red (T_R) Fluorescence and α_t Values in ORI Samples Treated with DTT

Sample number	Time (min.)	$T_G / 10^4$	$T_R/10^4$	α_t
6	0	34.21	31.40	0.48
	5	86.07	82.82	0.49
	10	85.77	87.29	0.50
	15	80.72	82.93	0.51
5	0	18.02	15.11	0.46
	5	41.35	37.00	0.47
	10	43.61	40.96	0.48
	15	37.66	34.37	0.48
8	0	26.57	27.43	0.51
	5	51.28	55.66	0.52
	10	58.01	79.32	0.58
	15	61.51	82.68	0.57
15	0	42.00	41.30	0.50
	5	91.54	116.93	0.56
	10	83.39	105.86	0.56
	15	89.75	122.33	0.58
16	0	32.83	23.87	0.42
	5	101.64	123.82	0.55
	10	91.22	118.76	0.57
	15	96.25	134.74	0.58

T_G: $F = 28.52$, $p < 0.0001$ (Extr.S)
T_R: $F = 16.15$, $p = 0.0002$ (Extr.S)
α_t: $F = 6.26$, $p = 0.0084$ (V.S)

samples was observed in only half of the SW fractions (Table 8, SW samples 5, 15, and 16), with values decreasing in the remaining three SW fractions during the first incubation period. At 5 minutes the α_t values showed a similar sudden jump in both the control and treated samples. After 5 minutes the α_t values remained constant in the control samples, while the treated sample α_t values continued to increase with incubation time. In general the α_t values (for the same samples) were higher in the treated than in the control samples. This finding was in accordance with the increasing percentage of decondensed cells observed by light microscopy.

The increased level of fluorescence displayed by the ORI ($F = 28.52$, $p < 0.0001$ for green fluorescence, $F = 16.15$, $p = 0.0002$ for red fluorescence, $F = 6.26$, $p = 0.084$ for α_t) over the SW samples ($F = 0.57$, $p = 0.64$ for green fluorescence; $F = 2.24$, $p = 0.13$ for red fluorescence; $F = 4.57$, $p = 0.02$ for α_t) is consistent with the fact that the ORI is an heterogeneous population of mature and immature sperm cells and round cells, as well as live-dead cell mix, with different degrees of sulfhydryl group content and chromatin instability.

B. Human Sperm Chromosomal Analysis

The cytogenetic analysis of human sperm chromosomes was performed from photographic prints obtained at 630X or 1000X. Whenever it was necessary, direct analysis of the slides was used to aid in the karyotyping process. The uniformly stained metaphases only allowed for classification of chromosomes into one of the 7 groups (A through G), except for chromosomes 1, 9, 16 and Y that very often could be easily recognized due to the presence of a very decondensed centromeric chromatin (Figure 8). The more precise identification of each individual chromosome and their cytogenetic abnormalities was achieved after a sequential uniform-G-banding (Benet et al., 1986)

Table 8: Total Green (T_G) and Red (T_R) Fluorescence and α_t Values in SW Samples Treated with DTT

Sample number	Time (min.)	$T_G/10^4$	$T_R/10^4$	α_t
1	0	43.06	62.95	0.59
	5	24.81	44.97	0.64
	10	18.52	36.72	0.66
	15	35.70	71.87	0.67
6	0	15.97	22.10	0.58
	5	9.68	20.10	0.67
	10	11.58	11.69	0.50
	15	53.28	63.82	0.55
5	0	44.10	38.95	0.47
	5	59.97	59.95	0.50
	10	38.74	38.45	0.50
	15	9.70	10.23	0.51
8	0	51.02	48.54	0.49
	5	30.82	42.16	0.58
	10	30.76	46.11	0.60
	1	56.96	84.58	0.60
15	0	45.21	35.84	0.44
	5	83.21	105.49	0.56
	10	91.59	142.26	0.61
	15	88.51	137.57	0.61
16	0	41.87	33.20	0.44
	5	67.30	80.16	0.54
	10	82.15	108.67	0.57
	15	81.99	118.43	0.59

T_G: F = 0.57, p = 0.64 NS)
T_R: F = 2.24, p = 0.13 (NS)
α_t: F = 4.57, p = 0.02 (S)

Figure 8: **A G-banded karyotype of a 23, X, pericentric inversion (2) human sperm.** Notice the pericentric heterocromatic regions of chromosomes 1, 9, and 16 (arrows), 630X.

staining procedure.

The human complements found to be hypohaploid were only considered in the results if: (1) they presented a compact appearance, (2) the associated hamster complement was numerically normal. To minimize artifacts due to the fixation technique, the incidence of aneuploidy could be calculated in a conservative form, as twice the number of hyperhaploid complements (Martin et al., 1987; Templado et al., 1988); as well as in the traditional way (hyperhaploidy + hypohaploidy).

1. Original SPA

A total of 25 donors with normal somatic karyotype 46 XY, and 8 male patients with unknown somatic karyotype were included in this study (Table 9). A total of 6283 hamster oocytes (6036 oocytes for donors and 247 oocytes for patients) were inseminated yielding a mean fertilization rate of 86.7% (13/15) for donors and 0.0% (0/15) for patients (Table 10).

A total of 1746 metaphasic sperm chromosome spreads (1726 from donors and 20 from patients) were obtained. A significantly low number of the chromosomal sets obtained were successfully karyotyped (130/1746). Appendix B gives a detailed analysis of the efficiency of this technique per individual.

The number of analyzable cells ranged from 0 (donors 28, 37, 38, 39, 41, 42, 44, 45 , 46, 47, 48, 49, and all 8 patients) to 56 (donor 27). Some eggs never developed beyond the pronuclear stage (specially if polyspermy, P, was present). Some oocytes were lost (burst) during chromosome fixation. In other oocytes the chromosomes could not be analyzed because of poor morphology (i.e. too thin, T) or inadequate spreading (i.e. clumped, C).

The number of informative spreads obtained in an experiment depends directly on the number of fertilized eggs, which in turn depends on both the number of hamster oocytes obtained and the fertilization rate of a given male. Although all the eight patients included in this study failed to fertilize the hamster oocytes (Appendix B) when assessment of

Table 9 : Basic Semen Analysis Results (Original SPA)

	Concentration (millions/ml)	Percent motile
Donors		
35	330.88	95.48
27	184.60	80.00
25	153.20	91.00
26	106.80	83.97
28	247.57	92.97
29	219.30	97.90
30	248.00	93.00
31	122.61	85.45
32	169.77	82.21
33	134.10	83.00
34	99.80	81.50
37	119.50	86.00
38	218.49	88.69
39	205.13	86.49
40	107.28	79.43
41	105.15	88.70
42	127.00	93.10
43	117.20	94.00
44	247.10	97.00
45	107.00	90.00
46	146.10	91.00
47	193.21	94.00
48	174.30	90.30
49	263.00	97.00
Patients		
a	12.32	9.84
b	22.70	22.90
c	31.70	34.50
d	13.60	3.20
e	24.10	19.50
f	11.38	7.56
g	24.44	19.31
h	0.79	69.40

Table 10. Results of Original Sperm Penetration Assay

Case	No. of inseminated oocytes	% of fertilized oocytes[a]	No. of chromosomal sets obtained	No. of informative sets obtained
Donors (25)	6036	86.7 (13/15)	1726	131
Patients (8)	247	0.0 (0/15)	20	0

[a] % of fertilized oocytes = mean No. of oocytes with swollen sperm heads/ total of 15 inseminated oocytes

swollen heads was performed in the traditional fertilization check, patient' oocytes were processed for chromosomal analysis in the same way as for the donors' oocytes. After fixation and uniform staining, 5 of the 8 patients had hamster eggs displaying more than one nucleus inside the oocyte's cytoplasm. The types of chromatin structures found included presence of: (1) many nuclei (polyspermy) in 2 cases, (2) clumps of chromatin fibers in 2 cases, (3) swollen sperm heads with or without the flagellum in 1 case, and (4) complete lack of fertilization in 3 cases.

Table 11 summarizes the results of the 130 chromosomal complements from which karyotyping was possible: 39 were karyotypes with an X chromosome, 44 were karyotypes carrying a Y chromosome, and 37 had a normal count (23) but their sexual chromosomes were not identifiable. The observed X/Y relationship in this study group (39/44) was not significantly different than the expected 1/1 ratio (X_2= 0.0535, df = 1, p = 0.81708; two-tailed with Yates correction).

Ten spermatozoa (7.69%) carried structural abnormalities, and 4 (3.08%) spermatozoa carried numeric anomalies (0 hyperhaploid and 4 hypohaploid). The observed frequency of aneuploidy (calculated as the sum of hypohaploidy and hyperhaploidy) was 3.08%. Nondisjunction leading to hyperhaploidy of either X or Y chromosomes was not observed in this study.

Structural aberrations observed in this study included chromosome breaks, acentric fragments, deletions, inversions, and dicentric chromosomes (Table 12). Sperm centromeric gaps were not recorded as abnormalities in this study as they appeared to be rather common events in this system.

2. Modified SPA: Subzonal Insertion of Dead Human Sperm

The results of the basic semen parameters of whole semen and their corresponding swim-up fractions are shown in Table 13. All three proven-fertile donors had normal semen count, motility and morphology (data not shown). After three cycles of freezing in Ham's

Table 11: Results of Human Sperm Chromosomal Analysis

Case	No. inf. sets	Counts only	X:Y	He (He+Ho)	Ho	Aneu	No. sets with str. abnormalities
25	3	0	1:2	0	0	0	0
26	3	1	1:1	0	0	0	0
27	56	21	8:17	0	1	1	6
29	36	6	19:11	0	1	1	1
30	15	8	2:5	0	2	2	1
31	3	0	1:2	0	0	0	0
32	1	0	1:0	0	0	0	1
33	1	1	NA	0	0	0	0
34	4	0	2:2	0	0	0	1
35	3	0	3:0	0	0	0	0
36	5	1:4	0	0	0	0	0
Total	130	37	39:44	0	4	4	10

No. inf. sets = Number of informative sets
He = Hyperhaploid sets
Ho = Hypohaploid sets
Aneu = Aneuploid sets
No. sets with str. abnormalities = Number of sets with structural abnormalities

Table 12: Types of Structural Abnormalities

Case	Abnormal complement
27	23,X (per inv 2) 23,X (del 12, inv 2?) 23,X (inv 9) 23,? (dic group A) 21,Y (-3,-7,+ac) 23,Y (inv 2?)
29	23,X (+ac)
30	23,Y,X? (+large submet X?, -15, +ac)
32	23,X (+ac)
34	23,X (+3 ac)

per inv: pericentric inversion
del: deletion
inv: inversion
dic: dicentric chromosome
ac : acentric chromosome
submet: submetacentric chromosome

Table 13: Basic Semen Analysis Results (Modified SPA)

	Donor number 30	27	34
Whole semen			
Concentration (millions/ml)	113.57	69.16	119.86
Percent motility	95.97	83.94	73.06
Swim-up			
Concentration (millions/ml)	31.34	16.66	36.12
Percent motility	98.40	86.41	82.20

F10-7.5% hFCS at -20°C for 1 hour and thawing at RT for 15 minutes, the eight sperm fractions obtained from each whole semen sample were tested for the following parameters: sperm viability (% of dead cells), acrosomal status (% of acrosomal loss), sperm fertilization ability (% of oocytes with two pronuclei after SZI) and sperm ultrastructure (by TEM) (Table 14 for washed sperm and Table 15 for swim-up sperm). Spermatozoa from only 4 of the 8 fractions (ORI, ORI Fr-Th x 3, SW, and SW Fr-Th x 3) were used for SZI. The proposed freezing/thawing protocol rendered similar results in both washed semen and swim-up samples:

1. 100% of the cells lost motility and died after the freeze/thaw x 3 treatment
2. almost 100% of the cells gave a fluorescent staining pattern similar to the one obtained after acrosome reaction has occurred (a complete disruption of the acrosomal membrane was observed under TEM)
3. high rates of formation of two pronuclei (between 64% to 86% for treated samples vs 0% to 17% rate for untreated samples) and low rates of polyspermic (< 1%).

Most of the frozen-thawed spermatozoa had various degrees of acrosomal damage, including the loss of the acrosomal contents (Figure 9). The limiting membranes were wrinkled, irregular and discontinuous. In some cells the membranes were completely detached from the nucleus while in others both the nuclear and inner acrosomal membranes remained attached. In all cases the changes were limited to the apical segment of the sperm while the equatorial segment remained essentially unaffected.

Results of the types and frequencies of nuclear material found in oocytes subjected to SZI are presented in Table 16. After subjecting the sperm to 3 cycles of freezing and thawing analyzable chromosomal complements were only obtained for donor 27. Chromosomal/nuclear material was also obtained for 30 and 34

Table 14: Performance of Washed Sperm after One, Two, and Three Cycles of Freezing/Thawing

	Washed	Fr-Th x 1	Fr-Th x 2	Fr-Th x 3
% dead cells[a]	18.5[d] 23.3 35.7	100.0 99.5 99.0	100.0 100.0 100.0	100.0 100.0 100.0
% acrosomal loss[b]	9.5 ± 0.7 2.0 ± 1.4 5.0 ± 1.4	91.0 ± 2.8 71.0 ± 9.9 46.5 ± 10.6	96.0 ± 1.4 97.5 ± 2.1 94.0 ± 0.0	99.0 ± 1.4 100.0 ± 0.0 96.5 ± 0.0
% fertilized oocytes[c]	0.0 (0/30) 0.0 (0/25) 0.0 (0/25)	NA	NA	80.0(16/20) 81.5(22/27) 64.0(16/25)

[a]n = 200 cells/treatment/donor
[b]mean ± SD
[c]fertilized/inseminated oocytes/donor
[d] results for donors 30, 27, and 34

Table 15: Performance of Swim-up Samples after One, Two, and Three Cycles of Freezing/Thawing

	Swim-up	Fr-Th x 1	Fr-Th x 2	Fr-Th x 3
% dead cells[a]	0.0[d] 0.5 0.0	96.0 99.0 100.0	100.0 100.0 100.0	100.0 100.0 100.0
% acrosomal loss[b]	13.5 ± 2.1 17.0 ± 0.0 12.0 ± 0.0	28.5 ± 3.5 96.5 ± 4.9 89.5 ± 0.7	96.0 ± 2.8 100.0 ± 0.0 96.5 ± 2.1	99.5 ± 0.7 100.0 ± 0.0 99.5 ± 0.7
% fertilized oocytes[c]	17.1(6/35) 5.9(1/17) 0.0(0/15)	NA	NA	78.9(15/19) 86.4(19/22) 6.7(1/15)

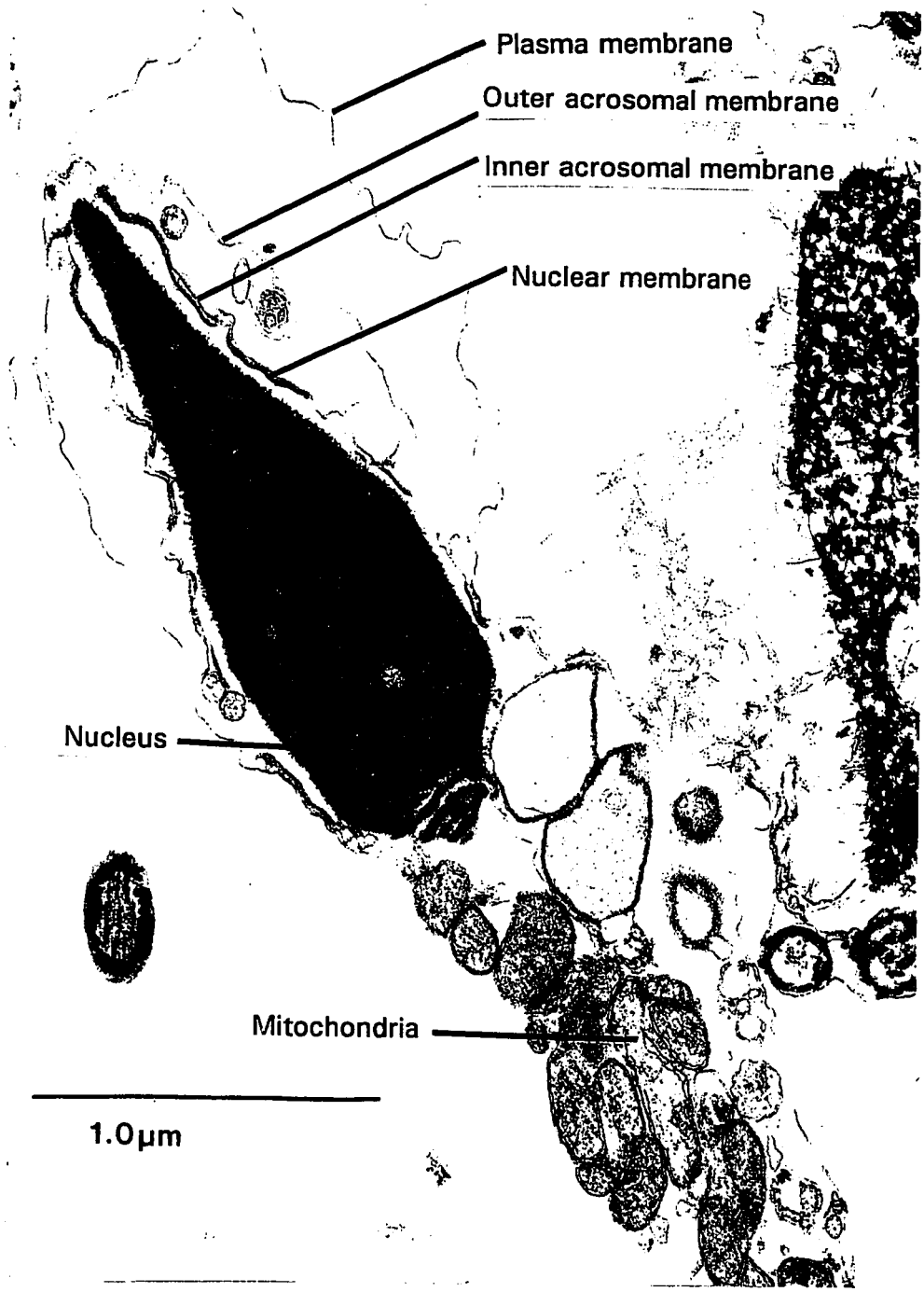
[a]n = 200 cell/treatment/donor
[b]mean ± SD
[c]fertilized/inseminated oocytes/donor
[d] results for donors 30,27, and 34

Table 16: Results of Types and Frequencies of Nuclear Material found in Oocytes Subjected to SZI

Donor	ORI	ORI Fr-Th x 3	SW	SW Fr-Th x 3
30		4 x Ha		5 x Ha
	4 x 1 Nu	2 x 2 CF		
			1 x 2 Nu	3 x 2 C
			8 x Ha + C	5 x 1 Nu + C
				6 x Ha
27	3 x Ha	1 x 1 Nu	2 x 1 C	1 x 2 sets
	2 x 1 Nu	1 x 3 Nu		(22 Ha + 23 Hu)
				1 x 2 C
				7 x Ha
				3 x 2 Nu
				5 x 2 CF
34	5 x Ha	2 x 1 C	2 x 1 Nu	1 x 1 Nu
	1 x 1 Nu	4 x Ha	1 x 2 Nu	3 x 2 Nu
		4 x 1 CF	3 x Ha	1 x 2 CF
		4 x 2 CF		1 x 2 C

Ha: hamster chromosomes with split chromatids
Nu: nuclear material
CF: chromatin fibers
C : clumped chromosomes
Hu: human chromosomes

Figure 9. **Transmission electron micrograph of spermatozoa treated by three cycles of freezing and thawing in buffered medium with no cryoprotective agent.** Most of the cryotreated cells had various degrees of acrosomal damage, including the loss of the acrosomal contents. These changes were limited to the apical segment; the equatorial segment was mainly unaffected. The limiting membranes were wrinkled and less clearly defined. the general structure of the neck region, fiber systems of the tail and the substructure of the flagellar matrix showed a roughly normal picture, 28,000X.



treated samples but karyotyping was not possible due to their poor morphology (clumped or too thin chromosomes). In general, for the untreated samples, nuclear material of probably hamster origin (1-2 nuclei, hamster chromosomes with split chromatids or 1 clump of chromosomes) was obtained, in accordance with the lack of fertilization observed possibly due to the very low rate of acrosomal loss achieved in these cells.

CHAPTER IV

Discussion and Conclusion

A. Human Sperm Chromatin Analysis

Sperm nuclear decondensation is a mandatory step for male pronucleus formation upon fertilization. After separation of the chromatin fibers from the tightly condensed sperm nucleus the duplication of DNA can occur, leading to the association with somatic histones and the recondensation into chromosomes for the first mitotic division. It has been shown that spermatozoa progressively acquire a partial nuclear stability during epididymal transit and that prostatic zinc increases the nuclear stability at ejaculation by protecting the mechanism for chromatin decondensation from oxidative destruction during passage through the female reproductive tract.

Using two experimental approaches, we have found that the stability of human sperm nuclei differs markedly among samples. In the first approach, sperm nuclear decondensation was monitored by flow cytometry after in vitro exposure to a disulfide-bond reducing agent and detergent (DTT/SDS). In the second approach the same sperm cells were analyzed under LM for morphology. We observed changes in the light signal that occurred before changes in nuclear size, as visualized by LM. This early increase in light scatter may correlate with changes in internal structure, possibly related to disulfide bond reduction, that precede chromatin dispersion. The light scatter signal continued to increase as the opaque sperm nuclei began to swell and then declined as the nuclei became translucent and eventually transparent. This biphasic pattern in the light scatter signal suggests that sperm decondensation may be a two-step process, with reduction of at least

some nuclear disulfide bonds occurring early when the sperm nucleus is intact or only slightly enlarged, followed by a dramatic expansion of the nucleus as DTT (or corresponding oocyte factors after fertilization) gains access to the sperm DNA. In vivo changes in sperm nuclear stainability indicative of disulfide bond reduction have been reported to occur during fertilization, shortly after sperm-egg fusion but before the sperm nucleus decondensed (Miller and Masui, 1982) . The pronounced heterogeneous instability of human sperm nuclei from ejaculates observed in the present study agrees with previous reports. A small subpopulation of human sperm was shown to decondense in SDS alone, without supplemental disulfide-bond reducing agent. The addition of EDTA to SDS was reported to increase the proportion of spermatozoa with decondensed chromatin due to zinc-removal (Huret, 1983), but we were not able to observe any decondensation even after 22 hours of incubation in control buffer at 4°C. Bedford, Bent, and Calvin (1973) suggested that decondensation of the sperm nucleus in SDS alone reflected an abnormality of testicular origin and that this might be associated with male infertility. Instead, Blazak and Overstreet (1982) suggested that the heterogeneous response of human sperm nuclei to SDS is more likely to be due to the highly variable transit time of spermatozoa through the human epididymis. Those spermatozoa which readily undergo nuclear decondensation in SDS may be structurally immature and their nuclei may be relatively poor in -S-S- bonds.

The chromatin decondensation mechanism could be reactivated by treating freshly ejaculated spermatozoa with zinc-chelating agents (Kvist, 1980) and it has been suggested that such zinc removal is accomplished during sperm transfer through the female genital tract. This Zn removal would expose free thiol groups in the chromatin and allow the intrinsic mechanism of chromatin decondensation to take place when the spermatozoon is demembranated upon attachment to the oocyte investments and entrance into the ooplasm. After treating 15 fertile men's samples with SDS Blazak and Overstreet (1982) concluded that: (1) the number of spermatozoa with nuclei that are insufficiently stabilized by disulfide

bonds is much higher in the semen of fertile men than was previously thought, (2) significant differences exist among fertile men in the proportions of ejaculated spermatozoa extensively stabilized by disulfide bonds, and (3) removal of seminal plasma and/or cellular contaminants (Zn) is necessary to unveil the instability of the nuclear chromatin in ejaculated spermatozoa of fertile men. Huret (1983) reported that semen samples obtained from men with impaired prostatic secretion showed an appreciably lower resistance to the decondensation treatment. This was considered to be due to a reduction in seminal zinc.

The results reported by Perreault (1985) after microinjection of spermatozoa from different mammals into hamster oocytes also indicated that human sperm nuclei were the most unstable among all the species studied.

In the present study, the treatment of semen samples with buffers containing a detergent and a chelating agent but without a disulfide bond reducing agent supported swelling but not decondensation of the semen samples. The fluorometric analysis followed the same constancy of the response during incubation. However, when DTT was added to the incubation buffer, a high degree of chromatin decondensation and extensive fibrillar structures were observed at 15 minutes for ORI samples (18/19) but not for SW samples (2/19). Although the percentage of cells in each morphological category was variable between samples, decondensation was detected in most of the ORI samples; but decondensation was never observed in SW samples, showing that the population of migrated spermatozoa had a greater nuclear stability than nonselected sperm. When ejaculated human spermatozoa were exposed to a detergent (SDS) alone or in combination with a disulfide bond-cleaving agent (DTT) (Bedford, Bent, and Calvin, 1973) , significant heterogeneity was observed among spermatozoa in the same ejaculate as well as between ejaculates from different semen donors in the degree of nuclear chromatin decondensation (Bedford et al., 1973; Kvist and Eliasson, 1980).

Bedford et al. (1973) suggested that decondensation of the sperm nucleus in SDS

alone reflected an abnormality of testicular origin and that this might be associated with male infertility. Instead, Blazak and Overstreet (1982) suggested that the heterogeneous response of human sperm nuclei to SDS is more likely to be due to the highly variable transit time of spermatozoa through the human epididymis. Those spermatozoa which readily undergo nuclear decondensation in SDS may be structurally immature and their nuclei may be relatively poor in -S-S- bonds. Zirkin et al. (1982) showed that treatment of rabbit sperm with 1% Triton X-100 (or cold shock) and 5 mM DTT resulted in a correlation between the number of sperm with swollen, decondensed nuclei and the ability of the nuclei to serve as templates for DNA synthesis.

The number of cases reported is too low to draw conclusions regarding chromatin characteristics between N, G, and P patterns of morphology; but stable and unstable cells were detected in samples with both P and N morphology patterns. Samples from infertile patients with normal basic semen parameters (including morphology) displayed a low percentage of cells with stable chromatin structure (Tejada et al., 1984). Maybe a more thorough subclassification of sperm head and nuclear defects should be used in the correlation analysis with the fertilizing potential of the same samples.

The relationship between increased levels of F_{530} staining in sperm, and chromatin structure is not clear yet. Current EM studies suggest that the chromatin is not as condensed in sperm with increased F_{530} staining as in normal, fertile controls. The relationship between chromatin condensation and fertility is unclear also, since it is not known whether sperm with less condensed chromatin are on average less likely to successfully fertilize oocytes. In this context, however, other studies have shown a relationship between sperm chromatin structure and fertility. Not only does the in situ DNA of misshaped sperm nuclei have a significantly decreased resistance to thermal denaturation, many morphologically normal nuclei derived from subfertile donors are also abnormally susceptible to in situ thermal denaturation of their DNA (Evenson and Melamed,

1983).

Evenson et al.(1980) have shown a relationship between chromatin structure and fertility for mammalian sperm. Ballachey et al. (1988) found a strong correlation between chromatin structure stability and fertility in cows measured by heterospermic insemination performance.

In conclusion, we have shown that swim-up selected spermatozoa have a higher resistance to in-vitro induced nuclear chromatin decondensation than their corresponding whole semen samples, suggesting an advantage for the use of selected sperm procedures as an aid of infertility treatment. Analysis of the degree of chromatin condensation (by microscopy and/or flow cytometry) should probably be considered as part of the basic semen analysis. Flow cytometric techniques can provide additional pertinent information of sperm maturation and chromatin structure not otherwise available.

A chromatin decondensation test may have important applications in clinical research: (1) as a quality control measure for donor semen in sperm banks and IVF procedures, (2) as a diagnostic tool for use in patients with reduced fertility but apparently normal basic semen analysis, (3) as a part of the assessment of the fertilizing potential of the male partner in a couple experiencing fertility problems, allowing an assessment of the feasibility of artificial insemination by either husband or donor, (4) as a research tool to elucidate classes of male infertility, (5) to investigate the nuclear organization of mammalian spermatozoa, and (6) to facilitate in situ hybridization procedures (e.g., in assaying Y-bearing sperm for use in monitoring sperm separation techniques (Schwerin, Blottner, Thomsen, Roschlau, and Brockmann, 1991).

Additional studies on a larger data set would help to determine which test or combinations of tests will be of greatest use in determining semen quality and evaluating fertility. Further work is necessary to relate chromatin stability to (1) the post-ejaculatory spermatozoal history and (2) abnormal spermatogenesis, so that NCDA can be compared

with other forms of semen evaluation, particularly measures of sperm viability. Not only does the *in situ* DNA of misshaped sperm nuclei have a significantly decreased resistance to thermal denaturation, many morphologically normal nuclei derived from subfertile donors are also abnormally susceptible to *in situ* thermal denaturation of their DNA (Evenson and Melamed, 1983). Flow cytometric techniques can provide additional pertinent information of sperm maturation and chromatin structure not otherwise available.

B. Human Sperm Chromosomal Analysis

The ultimate goal of this technique is to obtain a large number of male pronuclear chromosome spreads suitable for analysis. This requires adequate condensation of chromosomes with good spreading and complete blockage of karyogamy to prevent overlapping of human and hamster pronuclear chromosomes.

Although a high number of chromosomal complements was obtained (2362) in this study a much lower number provided complete and useful information (130, 7.5%). Several factors accounted for this big loss: (1) loss of material during the incubation in 2XSSC solution (due to swelling of the chromatids) that made individual chromosomes superimpose on each other, (2) loss of material during the banding procedure (specially during the trypsinization step), and (3) resistance to, or lack of banding. To secure good banding the treatment has to be adapted to the amount of cytoplasm observed prior to staining, and of course, it is better applied to high quality spreads with prometaphasic chromosomes.

Harrison, Allen, Harris (1983) reported that the changes in chromosome morphology in response to G-banding pretreatment monitored by timed trypsinization were found to be totally reproducible for human lymphocytes but not for sperm chromosomes. With increasing exposure to trypsin (from 5 seconds to 1 minute) a progressive disruption of

chromosomal structure occurred and various orders of chromosomal organization were revealed. In "over-trypsinized" preparations the majority of the integral proteins were removed leaving chromosome "ghosts" in the LM, outlined by aggregated DNA.

It is well known that variations in the degree of G-banding may be observed among metaphases treated at the same time. This may occur as a result of the state of chromosomal condensation at the time of fixation. The heterochromatic region at the centromere in chromosome 1 is a recognized normal human polymorphism (Buckton, O'Riordan, Jacobs, Robinson, Hill, Evans, 1976) . This region may vary in length in different individuals. There is a clear difference in this region between lymphocytic and sperm chromosomes ($p > 0.001$). Thus, the heterochromatic region of chromosome 1 appears to condense differently in the sperm-egg hybrid than in lymphocytes.

The sperm chromosomes are large, with parallel chromatids in which distinctive coils can be seen. Furthermore, in some preparations, the heterochromatic regions of chromosomes 1, 9, 16, and Y seem to be differentially less condensed than the rest of the chromatin. Scanning and transmission electron microscopy techniques have permitted the analysis of gaps on preparations of pronuclear sperm chromosomes (Navarro, Benet, Genescà, Castell, Egozcue, and Templado, 1987) which have revealed that the chromosomes are continuous,linked by fibers of different diameter and length. These gaps are assumed to be regions of undermethylated DNA. Methylation of DNA has a profound effect on its interaction with proteins and it has been shown that methylation of cytosine facilitates the transformation of the B-helix into left-handed Z-DNA. Examination of patterns of methylation of vertebrate DNA, especially the ways in which the patterns differ in different cells, suggests that methylation may have an important role in genome organization. The DNA of most higher eukaryotes is highly methylated, at the 5-position of cytosine in the sequence CpG which confers resistance to digestion with certain restriction enzymes that have this sequence as part of their recognition site and whose activity is

blocked if the C is methylated. But there is a minor fraction of vertebrate DNA (about 1% in mammals) that is non-methylated (Cooper, Taggart, and Bird, 1983) and it is unusually rich in G + C and the normally rare doublet CpG. Several non-methylated regions of this type have been found in the close proximity of genes, at the 5' end (Doerfler, 1983) The non-methylated fraction is not changed in development; it is non-methylated in sperm DNA and in the DNA from all somatic cells that have been analyzed. However, some of the methylated sequences are changed, and there are two quite different types of changes: methylated groups from cytosines in sperm DNA are lost in those genes that become active in many cell types; and undermethylated sperm DNA which gains methylation during development. This is true of the major satellites in calf DNA (Sturm and Taylor, 1981), of the major and minor satellites in the mouse DNA (Sandford, Forrester, Chapman, Chandley, and Hastie, 1984) and of the satellites in human DNA, including one of the Y-chromosome-specific repeated sequences (Cooke, Schmidtke, and Gosdsen, 1982).

The mean number of karyotyped complements per semen sample varied greatly in this study as well as among the different groups of investigators. The means range from 0 (Jenderny and Röhrborn, 1986) to 121 (Kamiguchi and Mikamo, 1986). Some of the differences reside, as noted earlier, in inter-individual variations in penetration rate, which yields variable numbers of chromosome spreads. In addition, the different methods used to capacitate spermatozoa may result in different penetration rates. Past studies also differ in the proportion of numerical versus structural chromosomal abnormalities. An elevated frequency of structural abnormalities, relative to aneuploidy, was observed in three papers (Brandriff, Gordon, Ashworth, Watchmaker, Moore, Wyrobek, and Carrano, 1985; Jenderny and Röhrborn, 1986; Kamiguchi and Mikamo, 1986) , while the opposite was found in at least four articles (Rudak, Jacobs, and Yanagimachi, 1978; Martin and Taylor, 1982; Martin et al., 1983; Benet et al., 1986). Some of the differences in the small studies are clearly not statistically significant. It has been suggested that human oocytes are prone to

humans (Ma, Kalouseck, Zouves, Ho, Gomel, and Moon, 1989) while cytogenetic studies on human sperm have revealed that this cell is prone to *de novo* structural abnormalities (Olson and Magenis, 1988).

Among structural abnormalities, chromosome breaks are the most frequent. These may result from: (1) chromatid breaks that replicate during S-phase after fertilization or (2) chromosome breaks prior to S-phase, which may occur during or after meiosis. Chromatid breaks, acentric fragments, deletions, translocations and exchanges involving complex formations are also found. These types of breaks might be induced in the hamster cytoplasm. As there is a great individual variation in their incidence, this may well be due to individual differences in susceptibility. As more data become available the question of variability in chromosome structural abnormalities will be clarified.

As Martin, 1983 reported "this technique is time-consuming and requires practice and perseverance, but after approximately one year, we found that sperm chromosomal complements could be prepared routinely". The author recognizes the importance of the fertilization check because it is the first opportunity to determine if the technique is working correctly: (1) eggs are living, (2) sperm have attached, and (3) penetration has occurred. Each donor should have a stable fertilization rate.

It is also important to recognize polyspermy. If an egg is fertilized by more than two sperm, further development was arrested. The sperm of some donors consistently fertilize eggs with more than five sperm per egg. If extensive polyspermy is found, the experiment should be abandoned, since no chromosomal spreads will be obtained. The sperm count should be adjusted to 10^6/ml or less in the future with shorter incubation time.

One of the most difficult aspects of Martin's technique is getting well spread chromosomes. Many workers have concluded that clumped chromosomes occur because of poor technique during slide preparation. This is not always the case. In one series of experiments, clumped chromosomes occurred because the pH of the medium was too high

experiments, clumped chromosomes occurred because the pH of the medium was too high during the egg processing stage. It is important to monitor the pH of the medium, to adjust it by bubbling CO_2 through it, and to keep the eggs at RT (out of the incubator) for no longer than 30 minutes.

There are few assays to test the effect of mutagenic agents on gametes. Gametes are of particular concern (compared to somatic cells) because it is important to assess the genetic risk to future generations. Sperm morphology has been suggested as a screen to detect agents which cause genetic damage in sperm (Wyrobek and Bruce, 1978. Martin and Rademaker's (1988) results suggest than an assay of sperm morphology is not a good indication of chromosomal normality since we found no relationship between these two parameters (in a study of 30 healthy men of proven fertility). Past studies by these authors confirm no association between the frequency of morphologically abnormal sperm and the frequency of sperm-chromosomal abnormalities. They have studied two populations of men with high frequencies of chromosomal abnormalities in sperm: patients studied after radiotherapy (Martin, Hildebrand, Yamamoto, Rademaker, Barnes, Douglas, Arthur, Ringrose, and Brown, 1986) and men heterozygous for translocations (Martin, 1987). In the radiotherapy patients, the range in the frequency of chromosomal abnormalities three years after treatment was 6-67% and the frequency was dependent on testicular radiation dose. However, there was no increase in the frequency of morphologically abnormal sperm after radiotherapy and no association between sperm morphology and the testicular radiation dose. Similar results were obtained for men heterozygous for translocations (no correlation between frequency of abnormalities with frequency of morphologically abnormal sperm).

Since chromosomes were obtained only from donors with N pattern correlation between morphology and chromosomal abnormalities was not possible.

It could be argued that the reason they found no association between sperm morphology and chromosomal abnormalities was that the morphologically abnormal sperm

karyotypes. However, in this study, as well as in previous studies (Martin and Taylor, 1982) no association was found between the proportion of hamster eggs penetrated and the proportion of morphologically abnormal sperm.

It must be emphasized that these results provide only indirect evidence since the data are based on correlations in populations of sperm. It is not possible to analyze both the chromosomes and morphology in a given spermatozoon. This might be possible in the future by microinjection of specific sperm into hamster eggs and subsequent analysis of the chromosomes. However, this would require rapid subjective assessment of the morphology of live sperm compared to the more usual assessment of morphology on stained dead sperm.

There are still several problems in the interpretation of the results and in the comparison of the several studies published. A major problem resides in the techniques used to study meiotic chromosomes. All the techniques have disadvantages and it is still not clear whether or not the results obtained by one technique are comparable to those obtained with a second technique. The presentation of results (inclusion of polymorphisms, minor anomalies, and other abnormalities) problems and the process for patient selection for the analysis may also raise problems. Indeed, much confusion still exists between sterility,infertility and subfertility. A standardization in the terminology of male infertility and in the methodology of meiotic chromosome analysis is needed. Altogether, it can be estimated that 20 % of male infertility can be explained by abnormalities in mitotic and/or meiotic chromosomes (DeBraekeleer and Dao, 1991). The evaluation of male infertility should include a somatic chromosomal karyotype and, if normal, a meiotic chromosomal analysis. These analysis should be performed in cases of azoospermia and severe oligozoospermia once other possible causes of male infertility have been excluded. Once established, testing of these parameters will lead to assessing the degree of genomic damage in the male gamete from both natural and man-made hazards. Knowledge gained

will be useful for: (1) genetic counseling of couples in which male carries balanced translocations; (2) analyzing meiotic segregation in the spermatozoa of male carriers; and (3) determining some chromosomal causes of male infertility.

C. Modified SPA: Subzonal Insertion of Dead Human Sperm

A practical consideration arising from the above observations relates to the use of sperm microinjection to assess heterologous sperm chromosomes in hamster oocytes as an alternative to in vitro fertilization of zona-free hamster oocytes. Sperm microinjection bypasses the need for sperm capacitation and AR, making it particularly useful for species/samples that are difficult to capacitate in vitro. Pretreatment of the nuclei with chemical agents or physical treatments or use of spermatid nuclei may resolve this problem. Using the latter approach, it would appear to be important to confirm that the nuclei decondense fully, since partially decondensed sperm nuclei may form pronucleus-like structures with less than the full DNA complement, resulting in abnormal karyotypes.

High rates of normal fertilization were only obtained when dead sperm cells used for SZI had their acrosomal membranes extensively disrupted. Chromosomes complements were also obtained only after 3 cycles of freezing and thawing, although in very few their quality allowed for accurate karyotyping. The reason(s) for this poor chromosomal yield is not known. It could be speculated that the times and conditions of culture after SZI (colchicine treatment, hypotonic treatment, G-banding, etc) should be readjusted in this modified procedure since fertilization steps may proceed at a different sped than in the original SPA. The fact that the conditions/quality of the sperm genetic material may affect the development of good chromosomal morphology can not be discarded, although TEM of the treated spermatozoa did not show gross alterations in their nuclei, and few normal sperm chromosomal complements were obtained from this procedure.

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Appendix A

Correlation between the Percentage of Decondensed Cells in Treated Samples and their Basic Semen Parameters

	Incubation time (min.) 0	5	10	15
ORI Concentration	.3425/.1173[a] .1512[b]	-.2631/.0692 .2764	-.0713/.0051 .7716	-.0889/.0079 .7176
% motility	.2017/.0407 .4076	.1414/.0200 .5636	.3834/.1470 .1051	.1087/.0118 .6577
% N	-.2258/.0510 .3526	.1873/.0351 .4427	.4427/.1959 .0577	.3993/.1594 .0904
SW concentration	-.08/.0064 .7447	.0539/.029 .8265	-.2281/.0520 .3475	.3491/.1218 .1430
% motility	-.3347/.1121 .1621	.3014/.0908 .2099	.0909/.0083 .7112	.2847/.0811 .2375
% N	-.2683/.0720 .2667	-.2925/.0855 .2243	-.4628/.2141 .0460[c]	-.2330/.0543 .3371

[a] = r/r^2
[b] = p
[c] = slope is SD from 0

Appendix B

Per Person Results of Human Sperm Chromosomal Analysis

Case	No. eggs insem.	No. chrom. obtained	No. informative sets
Donors			
25	358	95	3
26	40	273	
27	1031	455	56
28	32	0	0
29	1190	224	36
30	969	356	15
31	63	10	3
32	278	100	1
33	171	32	1
34	369	358	4
35	1255	355	3
36	95	30	5
37	145	0(P)	0
38	44	27(P)	0
39	472	185(P)	0
40	32	3(H)	0
41	70	0(P)	0
42	75	0(P)	0
43	24	19(CF)	0
44	73	0(SH)	0
45	27	0(P)	0
46	43	0(P)	0
47	57	20+(P)	0
48	58	47(CF)	0
49	30	0(P)	0
Patients			
a	15	5(CC)	0
b	11	4(CC)	0
c	20	0(P)	0
d	46	3(CF)	0
e	27	1(NF,Ha)	0
f	78	0(NF,Ha)	0
g	25	7(NF,CC)	0
h	25	0(NF,CC)	0

P = polyspermic
CF = very thin chromatin/chromosomes
CC = clumps of chromatin
SH = sperm swollen heads
NF = no fertilization

Appendix C

Human Subjects

This study used data generated from human semen samples falling under exception number 5 of the regulations for Protection of Human Subjects 45 CFR 46 available from the Office for Protection from Research Risks, National Institutes of Health, Bethesda, MD 20892. Patient semen samples were provided by the Andrology Laboratory of the Jones Institute for Reproductive Medicine, Eastern Virginia Medical School. The laboratory performed the diagnostic tests requested by the referring physicians and the remainder of the sample was used for this research. Patient samples were given codes so that the subjects could not be identified directly or through identifiers linked to the subjects.

Donor semen samples were provided by the Cryolaboratory at the Jones Institute for Reproductive Medicine, Eastern Virginia Medical School. Since the donors were participating in an anonymous insemination program no personal data was available except for the donor codes given by the laboratory.

Appendix D

Vertebrate Animals

Female Syrian outbred golden hamsters (Mesocricetus aureatus) were obtained from Charles River Breeding Laboratories at 8 to 10 weeks of age. Approximately 150 animals were expected to be included in the study (3 animals per 2 sample sets x 50 sets). The techniques for obtaining sperm chromosomes have been oriented toward the utilization of cells from the germinal line (rather than somatic cells) due to the presence of specific factors in the cytoplasm of the oocyte which play a major role in the formation and reactivation of the male pronucleus.

The use of hamster oocytes as "reactivating vehicles" for human sperm was first described by Yanagimachi et al., 1976. As in any cell-cell interaction system, specific and complimentary molecules function to mediate sperm-egg interaction and restrict its specificity. Within mammals, the hamster represents the only easily accessible animal that allows binding and penetration of human spermatozoa to a non-human ovum. Hamsters were housed 2 per cage, at 25°C, with a light cycle of 14 hours light/10 hours dark. Water and food (Purina RMH 3000) were available ad libitum and cages were changed twice weekly. The hamsters were given at least one week post quarantine to acclimate and they were 3 to 6 months of age when used for experiments.

On day 1 of the experiment, hamsters were superovulated with an intraperitoneal (i.p.) injection of 30 IU of pregnant mare serum gonadotropin (PMSG, Sigma), followed 56-58 hours later by an i.p. injection of 30 IU of human chorionic gonadotropin (hCG, Sigma) on day 3 of the experiment.

Ether, a frequently used anesthetic, was used for injections of the animals in order to

minimize discomfort, distress and injury to the animals as well as to the investigators. The advantages of ether are: (1) its simplicity and cost, (2) its rapidly effective producing excellent muscle relaxation, (3) it is easily controlled, and (4) the animals revive rapidly from its effects. The disadvantages are: (1) its highly inflammable and explosive property and (2) it causes excessive salivation and pulmonary irritation which predisposes to subsequent respiratory arrest and/or infection. In spite of this, used under controlled conditions ether provides for accurate and easy handling of for small rodents. To induce anaesthesia the animals were placed in a closed desiccation chamber containing ether soaked cotton or gauze under the perforated platform. Animals should not be exposed directly to the liquid ether because it is a topical irritant.

Hamsters were sacrificed by N_2 asphyxia. N_2 is a colorless, odorless gas, constituting 78% of normal atmospheric air. It is inert, nonflammable, and nonexplosive. The effects of N_2 can be rapid and reliable when properly used and is readily available. N_2 may induce convulsions and vocalization in an animal, and therefore is aesthetically objectionable. In addition, some geographical areas legally prohibit the use of N_2 for euthanasia.

Animals were placed in a covered plastic box and N_2 gas from a gas cylinder was passed via a plastic tube into the box. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (1986) and it presents no particular hazard to the investigators.

AUTOBIOGRAPHICAL STATEMENT

Silvina M. Bocca was born in Rosario, Santa Fe, Argentina on March 30, 1964. She graduated from Universidad Nacional de Buenos Aires, Argentina with a Licenciatura en Ciencias Biologicas degree (or the equivalent to a M.S. degree) in 1987. During those years she obtained teaching assistantships in Cell Biology, Molecular Biology and Genetics as well as a research grant to study the topography of the pseudoacetylcholinesterase in brain tissue.

After obtaining a training fellowship at The Jones Institute for Reproductive Medicine she moved to Virginia and started the Ph. D. program in Biomedical Sciences at ODU/EVMS. Teaching assistantship in Advanced Human Physiology at ODU, as well as research assistantships in the Andrology laboratory/EVMS, Cytogenetics laboratory/EVMS, Embryonics laboratory/ODU allowed her to participate in many research projects, teach courses, supervise students, attend scientific meetings and directly interact with patients seeking infertility treatment.

She was an embryologist at the In Vitro Fertilization (IVF) laboratory in Norfolk, a consultant to the IVF program in Chicago, an ad hoc reviewer for the Journal of Assisted Reproduction and Genetics and the Fertility and Sterility Journal, and was elected to the Honor Society of Phi Kappa Phi, ODU.

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Swanson R. J., Acosta A. A., Austin R., & Bocca S. (1989). Flow cytometry assay. In A. A. Acosta and T. F. Kruger (Eds.). Human spermatozoa in Assisted Reproduction, (pp. 372-377), Baltimore: Williams and Wilkins.

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