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Numerical Chromosome Abnormalities in Sperm From Oligoasthenoteratozoospermic Patients and Fertile Males

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Abstract

NUMERICAL CHROMOSOME ABNORMALITIES IN SPERM FROM OLIGOASTHENOTERATOZOOSPERMIC (OAT) PATIENTS AND FERTILE MALES

Myung-Geol Pang

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Artificial fertilization protocols have been developed which bypass natural barriers for reproduction. One aspect of infertility which has received little attention is the potential importance of aneuploidy in sperm. To more clearly understand the cytogenetic make-up of sperm from OAT patients, multi-probe, multi-color FISH was performed to determine aneuploidy.

The introduction of intracytoplasmic sperm injection (ICSI) has revolutionized protocols used in in vitro fertilization centers. The pregnancies resulting from ICSI suggest an increased frequency of sex chromosome aneuploidy in livebirths. Preliminary data by others' suggest that sperm tail swelling patterns following hypo-osmotic swelling (HOST) can be predictive of fertilizing ability. To clarify the relationship between sperm tail swelling patterns, aneuploidy, and fertilizing ability, we developed a technique

combining HOST and FISH.

Using multi-probe, multi color FISH and developed formulae, we determined total aneuploidy in sperm from proven fertile donors and OAT patients. In controls, the total aneuploidy ranged between 4.1 and 7.7%. For OAT patients, the total aneuploidy was between 43 and 69%. Diploidy was found in 0.04% of sperm in controls and ranged between 0.4 and 9.6% in OAT patients.

To determine if sperm selection by swim-up can separate haploid from aneuploid sperm, we used three-probe, three-color FISH on sperm in the swim-up and pellet fractions. Between 35 and 52% of sperm from OAT patients in the swim-up fraction and 36 to 49% from the pellet were aneuploid. No significant difference was observed. However, the percent of diploid sperm predominantly resided within the pellet for all OAT and proven fertile donors.

We performed HOST and FISH on sperm from OAT patients undergoing ICSI. Data suggests that sperm tail swelling patterns A, B, D, E, F and G may be aneuploid in OAT patients. No aneuploidy was found in sperm with a C type tail swelling pattern in either OAT or normal controls. Diploid sperm in controls and OAT patients were only found in types A and G tail swelling patterns. Preliminary data suggests that there may be a correlation between HOST and aneuploidy for OAT patients.

Of the 30 OAT patients studied, 26 series of ICSI were performed. Sixty-eight percent of oocytes fertilized (165 embryos / 242 oocytes). Following fresh embryo transfer, 1 preclinical abortion, 1 first trimester loss, and 2 term

deliveries (2/26; 7.7%) occurred.

Our data show significant increases in the frequencies of diploidy, autosomal disomy, autosomal nullisomy, sex chromosome number, and total cytogenetic abnormalities in sperm from OAT patients versus controls. The data suggest that meiotic errors occur at highly elevated frequencies in the germ cells of severely affected OAT patients.

Dedication

This thesis is dedicated to my

Parents: Kee-Bong Pang and Dong-Soon Shin

Wife: Yung-Jou Kim

Sons: Won-Kee and Jun-Kee

whose love, encouragement and support have enabled me to pursue my education and accomplish this goal.

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Chapter 1. Introduction, Hypothesis and Specific Aims

Artificial fertilization protocols have been developed which bypass natural barriers for reproduction. Genetic based barriers are poorly understood and require research initiatives to clarify the genetic component of infertility.

Until recently, successful therapy for the treatment of infertile males with a low sperm count with or without a high percentage of sperm with abnormal forms and poor motility was very difficult. This dramatically changed with the advent of micromanipulative technologies such that a single spermatozoan can now be directly injected into the cytoplasm of an ovum. The introduction of intracytoplasmic sperm injection (ICSI) in 1992 now permits oligo-, astheno-, and azoospermic males' sperm to be used in in vitro fertilization protocols.

One aspect of infertility which has received little attention is the potential importance of aneuploidy in sperm. What is the cytogenetic make-up of sperm from infertile males? Is there an association between a sperms' fertilizing ability and chromosome abnormalities? Do techniques that bypass barriers to normal fertilization increase the parents' risk of having a fetus or liveborn with numerical or structural chromosome abnormalities?

Review of the Literature (1995)

In the past, it has been difficult to predict fertility from in vitro measures of sperm quality (Bavister, 1990). The development of experimental techniques to predict sperm function is complicated by the fact that many interacting biological processes are required for normal sperm function in vivo. For example, sperm with damaged acrosomes but normal motility probably lack fertilizing capacity in vivo (Saacke and White, 1972). Additional parameters such as surface charge, chromosome complement, surface adhesiveness, or the expression of specific membrane receptors might also affect sperm transport and in vivo fertilization. The elucidation of the biological, including the genetic, processes required for normal sperm function in vivo is a prerequisite for the full understanding of the ontogeny of male infertility.

Meiotic Chromosome Studies in Males

In an extensive and early study, Skakkebaek et al. (1973) reported on the meiotic chromosomes of 18 control and 74 infertile men. They reported on the frequency of numerical chromosome aberrations, chiasma frequency, the presence of unpaired homologous chromosomes, and the occurrence of polyploidy. All subjects had normal somatic karyotypes. Normal controls ranging in age from 19 to 39 had sperm counts greater than $60x10^6$/ml and sperm of good motility with less than 50% morphologically abnormal cells. The infertile men ranged in age from 23 to 51 years and were classified as infertile

by the authors on the basis of aspermia, severely impaired fertility, poor sperm motility, and / or abnormal sperm morphology. Testicular material was obtained and orcein stained meiotic chromosome preparations were analyzed from each individual. Meiotic cells were scored for the relative frequency of cells in the first meiotic division (MI) and in the second meiotic division (MII), chiasma frequency at MI, non-pairing of both autosomes and sex chromosomes at MI, and polyploidy. Structural chromosome abnormalities were not evaluated.

Skakkebaek et al. (1973) questioned whether decreased sperm production in the infertile men was due to inhibition before MI, between MI and MII, or following MII. Their results showed that the mean ratio of MII to MI cells was 0.9 in controls and 0.5 in the infertile males. The lower value in the latter group indicates that, in these men, many cells failed to progress from MI to MII. Therefore, the patients' infertility was, at least in part, a consequence of the failure of primary spermatocytes to divide to form secondary spermatocytes. Twelve of the eighteen infertile males whose spermatogenesis progressed to MII had MII / MI ratios below that of the lowest control. An additional three infertile males showed complete inhibition of spermatogenesis between MI and MII. These data were supportive of previous work (McIlree et al., 1966; Hult'en et al., 1970; Pearson et al., 1970) showing six out of fifty-three infertile males with an arrest of spermatogenesis between these stages.

Their data also showed that the relative frequency of spermatogonial metaphases was higher in infertile men (19.7%) versus normal controls (9.4%). Skakkebaek et al. (1973) suggested that the absolute number of spermatogonia may be the same in both groups. The elevated frequency seen in the infertile subjects may have resulted from the reduction in their number of spermatocytes.

Skakkebaek et al. (1973) found an average of 51.2 and 48.7 chiasmata per cell for normal controls and infertile males respectively. The difference was not significant. However, significantly greater variability in chiasma frequency did occur within the infertile male population with 10 of 17 infertile patients having chiasma frequencies lower than the lowest of the 6 normal controls.

Skakkebaek et al. (1973) evaluated pairing of sex chromosomes and autosomes at MI. The failure of the X and Y chromosomes to be paired, seen in 15% of 1159 cells, was the most common finding noted. Their data showed only a few small autosomes separated in a rare cell. In these regards, no significant differences existed between infertile patients and normal control donors. Unpaired homologous chromosomes at metaphase I could lead to nondisjunction and the production of aneuploid sperm. Such abnormalities could give rise to zygotes showing for example, 47,XXY (Klinefelter syndrome), 45,X (Turner syndrome), and 47,XY,+21 (Down syndrome).

Polyploidy was observed in 4.8% of spermatogonial (mitotic) metaphases and in 1.3% of MI metaphases in both infertile and control subjects. There was a significant difference in the frequency of polyploidy at MII in the two populations. At MII, polyploidy was seen in 3.6% of control and 10.6% of patient metaphases. Polyploidy seen at any of these stages results from either the abnormal occurrence of two rounds of DNA synthesis (endoreduplication) or from an abortive nuclear division. These abnormalities, along with failure of secondary spermatocytes to complete the second meiotic division (an abnormality which could not be scored by the technique used by Skakkebaek et al) will potentially produce diploid sperm.

By this time, polyploid cells had been found in mice and other mammalian species (Hult'en et al., 1970) and were believed to result from cell fusion or endoreduplication (Beatty, 1961). In addition, diploid spermatozoa had been found in animals and man (Beatty, 1961; Gledhill, 1964; Salisbury and Baker, 1966; Sumner, 1971; Sumner et al., 1971). The finding of increased polyploidy at MII by Skakkebaek et al demonstrated that failure of a meiotic division may also generate polyploidy and that polyploidy generated in this way occurs at elevated frequencies in infertile males.

Several studies published from the mid-1970's to the mid-1980's demonstrated that the incidence of meiotic chromosome abnormalities in

infertile males was much higher than in fertile males (Zuffardi and Tiepolo, 1982). The collective results of Chandley et al. (1976), Ferguson-Smith (1976), Hendry et al. (1976), Mi'ci'c et al. (1981), and Koulischer et al. (1982) indicated that an average frequency of 8.9% (98/1101) of infertile males had elevated frequencies of meiotic abnormalities, especially meiotic arrest and desynapsis (the failure of homologous chromosomes that have synapsed normally during pachynema to remain paired during diplonema; desynapsis is usually the result of a failure of chiasma formation). Individual studies showed frequencies that ranged from 1.44% to 17%. Koulischer et al. (1982) suggested that this wide range was primarily due to different interpretations of what should be scored as a meiotic abnormality. They held that one should not include anomalies such as separation of the sex chromosomes at metaphase I as a meiotic abnormality.

In 1983, Egozcue et al. (1983) performed meiotic studies on spermatocytes from testicular biopsies or semen samples from 1100 infertile males attending fertility clinics. "Meiotic arrest", probably corresponding to the decreased MII / MI ratio reported by Skakkebaek et al (1973), was the most common anomaly noted. This was followed in frequency by the absence of germ cells and then by desynapsis. Somatic abnormalities, which included Robertsonian translocations, reciprocal translocations, inversions, a deletion, a mosaic 45,X/46,XY karyotype, a mosaic 46,XY/47,XXY karyotype, and a 47,XYY karyotype, were seen in 1.9% of the subjects. The authors presented the results

of semen analysis in 55 patients with meiotic and somatic abnormalities. Only 2 of the 55 were normozoospermic. Oligoasthenoteratozoospermia and oligoasthenozoospermia were found in 14 cases each. Asthenozoospermia was found in 7 cases, followed by oligozoospermia in 5, azoospermia in 4, cryptozoospermia in 4, asthenoteratozoospermia in 2, oligoteratozoospermia in 2, and polyasthenoteratozoospermia in 1 patient.

Chromosome Analysis of Spermatozoa and the Male Pronucleus

In order to assess the value of estimating the ploidy of sperm cells by the size of the sperm head, Carothers and Beatty (1975) first classified sperm heads as being small, medium, or large based upon subjective size evaluation of Feulgen stained preparations and then correlated these subjective determinations with photometric determinations of ploidy based on light absorption by the Feulgen stained DNA. Their data on rabbits showed that haploid or diploid sperm, as determined by the Feulgen-DNA absorption assay, were always classified as being small or large in size, respectively. In humans however, no consistent relationship was noted between sperm head size and ploidy. While all small sperm heads were determined to be haploid by the Feulgen-DNA assay, haploidy was also the most common finding for both the medium and large nuclei. Based on the Feulgen-DNA absorption assay of 5554 spermatozoa from two normal controls, 99.3% were haploid, 0.56% were diploid, and 0.07% had higher ploidy levels. These data closely resemble those of

Sumner et al. (1971), who evaluated 1670 spermatozoa from four patients of an infertility clinic by fluorescence and DNA content analysis. Sumner et al reported 98.98% of sperm as haploid, 0.96% diploid, and 0.06% tetraploid. Considering the small number of subjects in both of these studies, it remained unclear as to whether infertile males, relative to normal fertile donors, show a significant increase in either their absolute number or percent of diploid sperm.

Numerical and structural chromosome abnormalities play an important role in the etiology of prenatal wastage. Approximately eight per cent of all human conceptuses surviving long enough to produce clinically recognized pregnancies have a chromosome aberration in either structure or number. It is estimated that approximately 50% of all abortions are chromosomally abnormal (Hassold et al., 1978). Most numerical chromosome abnormalities arise *de novo* due to an error in chromosome disjunction during meiosis in a parent or as a result of a disjunctional error in mitotic divisions in the zygote. In contrast, most major structural abnormalities (e.g. translocations and inversions) are transmitted to offspring from a carrier parent; the remainder arise *de novo*.

The frequency of *de novo* chromosome abnormalities in sperm is only partially known. Prior to the mid-1970's , the inspection of sperm chromosomes proved impossible because, in the course of spermatogenesis, sperm chromatin becomes highly compacted and does not become visible again until the first

cleavage division of the newly formed zygote. In 1976, Yanagamachi et al. developed a technique for the *in vitro* fertilization of zona-free golden hamster eggs with capacitated human sperm which then decondensed within the ooplasm. Using this technique, Rudak et al. (1978) established a system in which human sperm were fused with Syrian hamster eggs which then proceeded through the initial stages of development. The human sperm heads decondensed and the human chromatin formed metaphase chromosomes in preparation for first cleavage. This allowed the first extended cytogenetic observation of countable human sperm chromosomes in which numerical as well as structural abnormalities could be identified. From 1978 until 1992, many investigators used the human-hamster fusion technique to analyze the chromosome constitution of approximately twenty thousand sperm. The results of Rudak et al. (1978), Sele et al. (1985), Brandiff et al. (1985), Jerderny and Rohrborn (1987), Kamiguchi and Mikamo (1986), Martin et al. (1982, 1983, 1991), Estop et al. (1991), Rosenbush and Sterzik (1991), Benet et al. (1992), and Tateno et al. (1992) are summarized in Table 1.

The data summarize chromosome analyses for hypoploidy, hyperploidy, percent aneuploidy, conservative estimate of aneuploidy, and number of structural abnormalities. Hypoploidy refers to cells containing one or more fewer chromosomes than the normal haploid number; hyperploidy refers to cells containing more chromosomes than the normal haploid number, percent

aneuploidy is the sum of percent hypoploid plus the percent hyperploid, and a conservative estimate of aneuploidy is defined as twice the percent of hyperploidy.

Twelve investigators reported on % hypohaploidy, % hyperhaploidy, % aneuploidy, and % structural abnormalities; ten of twelve also reported on the X:Y sex chromosome ratio. One group reported on % aneuploidy and % structural abnormalities. Overall, the investigators reported a mean % hypohaploidy of 3.19%, a mean % hyperhaploidy of 1.95%, a mean % aneuploidy of 4.28%, a mean % of structural abnormalities of 6.54%, and an X:Y sex chromosome ratio of 52.5%:46.6%.

The findings of Martin et al. (1982, 1983, 1991) and Brandriff et al. (1985) will be reviewed in detail to illustrate the values of such studies. In 1983, Martin et al reported on the karyotypes of one thousand human male pronuclei from thirty three normal donors using the human-hamster fusion technique. Donors ranged in age from 22 to 50 and had no history of chemotherapy, radiation treatments, or infertility. Twenty-one were of proven fertility while twelve had no confirmed children. Sixty-five percent of the chromosome spreads were successfully karyotyped using Q-banding; in the remaining cells, chromosomes were placed in groups using routine Giemsa staining. The total frequency of abnormalities was 8.5%. Fifty-two were aneuploid (24 were hyperhaploid, 27

were hypohaploid, and one had double aneuploidy) and 33 showed structural chromosome abnormalities. The 24 hyperhaploid sperm complements included 17 with one extra chromosome and 7 with more than one extra chromosome. Eighteen of the 27 hypohaploid complements were missing one chromosome and nine were missing more than one chromosome. The percent of X-bearing sperm was 53.9%, whereas the percentage of sperm with one Y chromosome was 46.1%. Structural chromosome abnormalities included 2.2% chromosome breaks, 0.2% chromosome gaps, 0.2% fragments, 0.1% deletions, 0.4% dicentrics or quadriradials, and 0.2% reciprocal translocations.

Aneuploidy was seen in all chromosome groups and Martin et al. suggested that all chromosomes may be equally susceptible to nondisjunction. Numerical chromosome abnormalities were more common than structural aberrations. The frequency of abnormalities was not uniform across all donors; two males of unproven fertility had 33% of the total structural aberrations identified. The frequency of aneuploidy was higher in the human chromosome complement than in the hamster chromosome complement. The numbers of hyperhaploid to hypohaploid complements (24 and 27 respectively) were close to the predicted 1:1 ratio expected if all aneuploidy results from meiotic nondisjunction and there is no differential selection against the resulting hypo- and hyperhaploid gametes. Martin et al. did not select donors based on their ability to fertilize hamster eggs. Consequently, the number of sperm

complements analyzed per donor varied between 4 and 134. Martin et al stated that, with this range, they could not adequately assess the variation in the frequency of chromosome aberrations between donors.

In 1984, Brandriff et al. compared the frequencies of structural and numerical chromosome aberrations in 909 karyotypes from four healthy donors. They reported frequencies of structural abnormalities of 1.3, 4.8, 9, and 10.4%. The frequencies of aneuploidy were 1.3, 1.4, 1.4, and 1.9% in these four donors. For three donors, the frequencies of structural abnormalities were significantly higher in their sperm than in their peripheral blood lymphocytes. Sex chromosome frequencies did not significantly differ from the expected 1:1 ratio.

Brandriff et al. in 1985 analyzed the chromosomal constitution of an additional 2468 male pronuclei. Healthy donors (n=11) ranging in age from 21 to 49 participated in the study and, as in previous studies, donor to donor variation in the ability of their sperm to fuse with hamster eggs was noted. Variations in fusion rate necessitated repeat samples from donors. Numerical as well as structural abnormalities were scored. Of the 2468 karyotypes analyzed, 7.7% had structural abnormalities and 1.7% were aneuploid. Aneuploidy included 0.9% hypohaploid and 0.7% hyperhaploid. The sex chromosome frequencies were 50.1% and 49.9%.

The human-hamster technique is difficult, time consuming, and expensive. Sperm from some males produce a high mitotic index of metaphase chromosomes whereas other males' sperm yield few metaphase chromosome spreads, possibly indicating genetic variability in the ability of human sperm to fertilize hamster eggs *in vitro*. Large numbers of suitably-primed golden hamsters are needed to provide the eggs and the ability to produce metaphase chromosome preparations of suitable quality is problematic. Chromosomes may be clumped or too widely spread. The chromosomes are somewhat resistant to banding with results usually inferior to those of high resolution banding techniques applied to typical somatic cells. Furthermore, it is possible that structural or numerical chromosome abnormalities may be induced by the culture system or harvest procedure (Genesca et al., 1990), or that some sperm may be selected for or against by the in vitro fertilization system (Brandriff et al., 1984). Fewer than twenty thousand sperm have been analyzed to date using this technique. As aneuploidy in human sperm is rare, precise quantification of aneuploidy rates is not feasible (Martin and Rademaker, 1990).

Fluorescent in situ hybridization (FISH) is a powerful tool which permits the direct visualization of specific chromosomes or chromosome regions (Kearns and Pearson, 1994a,b). Using DNA probes specific for a particular chromosome, FISH provides a rapid, alternative technique for the detection of aneuploidy and polyploidy in human sperm. Two types of nonradioactive hybridization methods

exist, direct and indirect. In the direct method, the detectable reporter molecule (fluorochrome) is bound directly to the nucleic acid probe so that formed hybrids can immediately be visualized using a fluorescent microscope. The indirect method uses hapten labeled probes and subsequent signal detection by affinity cytochemistry. Recently, the availability of microscopes equipped with double or triple band pass filter sets has permitted the simultaneous detection of two or more differentially labelled probes.

The successful application of the FISH technique to mammalian spermatozoa depends primarily upon the efficiency of probe penetration following decondensation of the sperm nucleus. The chromatin of mammalian sperm is packaged in a condensed and inactive state. The histones found in somatic nuclei are replaced by sperm-specific protamines during late spermatogenesis. Intermolecular disulfide bonds form between adjacent protamines during epididymal maturation and maintain the tightly packaged state of chromatin. This tight packaging of the DNA-protamine complex in the human sperm nucleus makes it difficult for DNA probes to access target chromatin. Thus, sperm nucleus decondensation must precede the successful application of FISH.

Since 1984, at least fourteen studies using FISH on sperm chromosomes from normal men have been reported. The results from Joseph et al. (1984),

Guttenbach and Schmidt (1990, 1991), Pieters et al. (1990), Coonen et al. (1991), Han et al. (1992, 1993), Holmes and Martin (1993), Robbins et al. (1993) Bischoff et al. (1994), Guttenbach et al. (1994a,b), Miharu et al (1994), and Pang et al. (1995) are summarized in Table 2.

Disomy frequencies for chromosomes 1, 3, 7, 10, 11, 12, 15, 16, 17, 18, X and Y have been reported in sperm from normal donors. Seven investigators have reported on the disomy frequency for chromosome 1; mean = 0.37% (range 0.06 - 0.8%). In comparison, only one group has reported on the disomy frequency for chromosome 15; mean = 0.2%. Seven investigators each have reported on the disomy frequency for the X chromosome; mean = 0.24% (range 0.03 - 0.38%), whereas eight investigators have reported on the disomy frequency for the Y chromosome; mean = 0.13% (range 0 - 0.27%). Representative studies are now discussed in detail.

In an early attempt to analyze the chromosome complements in human sperm, Joseph et al. (1984) used ^{3}H-labelled satellite DNA probes to detect aneuploidy for chromosomes 1 and Y but noted that the sperm heads were too condensed for precise signal detection. The disomy frequencies for chromosomes 1 and Y were 0.35% and 0.18% respectively.

Guttenbach and Schmid (1990) performed nonradioactive in situ

hybridization on sperm nuclei from eight normal donors using a biotin-labeled Y chromosome-specific satellite DNA probe. The sperm nuclei were not chemically pretreated to disrupt disulfide bonds but were prepared for FISH by incubation in Hanks' balanced salt solution. Guttenbach and Schmid noted that they did not hypotonically treat the sperm and stated that it was not necessary to chemically decondense the sperm heads because they felt the sperm naturally decondensed during the DNA denaturation process. They reported 50.3% X (no Y signal) and 49.4% Y-bearing sperm. The frequency of disomy for the Y chromosome was 0.27%, which exceeds the observed frequency of XYY livebirths of 0.1%. They speculated that either 24,YY sperm have a reduced fertilizing capacity or that chance led to the discrepancy. An alternative explanation is that some of the YY bearing sperm were diploid as a consequence of nonreduction of chromosome number, specifically failure of MII. Hybridization with only one probe cannot distinguish between disomy due to nondisjunction or diploidy due to nonreduction. The ability of Guttenbach and Schmid to successfully identify the presence of a Y chromosome without chemical decondensation, and the observation of the expected 1:1 ratio, suggests that some sperm nucleus decondensation did indeed occur, possibly during denaturation.

Studies have shown that partially decondensed human sperm nuclei contain a complex network of chromatin fibers (Evenson et al., 1978) and

Wyrobek et al. (1990) analyzed and discussed the importance of sperm nuclei decondensation in order for successful probe hybridization to take place. Sperm nuclei from three normal donors were decondensed with lithium diiodosalicylate (LIS) and then analyzed by FISH for the presence of a Y chromosome. The decondensed sperm nuclei showed the presence of one Y chromosome in 50.1% of the nuclei examined. Their results of 50% of human sperm carrying a Y chromosome is consistent with data reported by others using the human-hamster fusion technique and supports the hypothesis that X and Y-bearing human gametes are produced in equal numbers.

Coonen et al. (1991) decondensed sperm nuclei using dithiotreitol (DTT) treatments of varaible duration and analyzed sperm from 32 normal healthy men by FISH for the presence of chromosome 1. They reported that chromosome 1 specific signals were observed in 40-90% of the analyzed sperm and hypothesized that this variation was due to maturational heterogeneity of the sperm present in the ejaculate. Alternatively, variability in signal detection may have resulted from the variable duration of DTT treatment. They reported that 0.67% (range 0.2-1.3%) of successfully hybridized sperm had two copies of chromosome 1. This frequency represents the sum of cells disomic for chromosome 1 and diploid cells.

Han et al. (1992) separately probed decondensed sperm nuclei by FISH

using probes for chromosomes 17 and X from 13 healthy donors. Sperm nuclei decondensation was by a modification of the method of West et al. (1989) using 6mM EDTA and 2mM DTT. Han et al distinguished between diploid and disomic sperm based upon the size of the sperm head; sperm nuclei of normal size with two hybridization signals were scored as disomic, whereas large nuclei with two hybridization signals were classified as diploid. Hybridization signals for chromosome 17 were detected in 96.1% of sperm nuclei analyzed; 95.4% were haploid (range 89.8-99.3%), 0.33% disomic (range 0.16-0.54%), and 0.37% diploid (range 0.00-.89%). The hybridization results for the X chromosome showed 48.2% of sperm nuclei with positive signals; 47.7% haploid (range 43.4-50.4%), 0.29% disomic (range 0.18-0.4%), and 0.2% diploid (range 0-0.55%).

Holmes and Martin (1993) also evaluated FISH for its ability to successfully detect aneuploidy in human sperm. Repetitive DNA sequences specific for chromosomes 1, 12, and X were used separately as probes and decondensed sperm nuclei were used as target DNA. Decondensation protocols used cetyltrimethylammonium bromide and DTT. A total of 10524 sperm was analyzed for chromosome 1, 10263 sperm nuclei were scored for chromosome 12, and 7003 sperm were analyzed for the X chromosome. Hybridization efficiency was greater than 98%. Reported disomy frequencies were 0.06% for chromosome 1, 0.04% for chromosome 12, and 0.03% for the X chromosome. Holmes and Martin compared their frequencies using the FISH technique to

published data using the human-hamster fusion protocol and found no significant differences.

Using simultaneous two-probe, two-color FISH, Han et al. (1993) studied the sex chromosome constitution of sperm nuclei obtained from 12 normal donors ranging in age from 20 to 45 years. Diploid and disomic sperm were distinguished as described above. Ninety-six percent of 12,636 decondensed sperm nuclei showed fluorescent labeling. Forty-seven percent were X and 46.8% were Y. 0.28% were disomic XX and 0.18% were diploid XX. 0.21% were disomic YY and 0.17% were diploid YY. The frequency of XY bearing sperm was 0.83%. When compared to sperm karyotyping data, these FISH sex chromosome aneuploidy rates are much higher.

Robbins et al. (1993) used FISH to evaluate aneuploidy for chromosomes 1 and Y in sperm from three healthy men whose sperm had been previously studied using the human-hamster fusion technique. Frequencies of sperm disomic for chromosome 1 were 14.2 / 10,000 and for chromosome Y were 5.6 / 10,000. These frequencies were not significantly different from the frequencies of hyperhaploidy seen using the human-hamster technique. However, the frequencies of disomy for chromosomes 1 and Y in one donor were approximately 2.5X higher than in the other two donors. Such donor to donor variation has been observed with the human-hamster sperm karyotyping

technique (Martin et al., 1991).

Oligoasthenoteratozoospermic patients (n = 9) of an in vitro fertilization program as well as normal controls (n = 4) were studied by Pang et al. (1995). Four of the nine patients previously had their sperm utilized for intracytoplasmic sperm injection (ICSI). Sperm nuclei decondensation was accomplished using a modification of the method by West et al. (1989) using 6mM EDTA and 2mM DTT. Simultaneous two-probe two color FISH was performed using probe sets for either chromosomes 7 and 18, 11 and 12, or X and Y. One thousand sperm were scored for each probe set. Two-probe, two-color FISH enables one to successfully differentiate disomy due to nondisjunction or diploidy due to nonreduction (except for the sex chromosomes).

A sperm cell was scored as disomic for a particular autosome if it showed two signals for that chromosome but not for the other simultaneously probed autosome. The absence of signal for a single chromosome was scored as nulliosomy for that chromosome. Cells were scored as diploid if there were two signals for both probed chromosomes. Sperm showing signal for neither chromosome of a probe set were not scored, as this outcome may have been an artifact resulting from inadequate decondensation of the cell's nucleus. The mean sum of the frequencies of sperm with disomy for any of the four tested autosomes was 0.53% (range 0 - 1.1%) for the controls and 5.5% (range 1.5 -

9.5%) for the patients. The corresponding data for autosomal nullisomy were 0.45% (range 0 - 0.7%) for fertile controls and 9.2% (range 1 - 18.1%) for the oligoasthenoteratozoospermic patients. From these data, it was calculated that the average frequency of aneuploidy, expressed on a per chromosome basis, was 0.25% for controls and 3.7% for patients. Under the assumption that all 22 autosomes disjoin at the same frequency as the 4 tested autosomes, and that nondisjunctional events occur independently, Pang et el calculated that, in non-polyploid sperm, the total frequencies of autosomal aneuploidy would have been approximately 5.3% for controls and 56.1% for the oligoasthenoteratozoospermic patients.

The frequencies of nondisjunctional gain or loss of sex chromosomes could not be established when using an X and Y pooled probe set. Cells with two sex chromosomes could be diploid due to failure of a meiotic division or disomic due to nondisjunction.

As determined from analyses using the autosomal probes, proven fertile donors showed a frequency of diploidy of less than 0.1% (range 0 - 0.1%), while patients' frequencies ranged between 0.3 to 9.8% with a mean of 2.1%. For the oligoasthenoteratozoospermic patient with 9.8% diploid sperm, Pang et al identified the type of error which primarily occurred. Of the 1000 sperm analyzed for the X and Y chromosomes, 14.6% had two signals; of these, 11.1%

were XY, 2.4% were XX, and 1.1% were YY. Based upon the high frequency of diploidy found using autosomal probes, it was concluded that most of the cells with 2 sex chromosomes must be diploid. The distribution of sex chromosome types indicates that there was a failure of the first meiotic division in the majority of diploid sperm cells. This was deduced from the meiotic behavior of the sex bivalent. If the first division is abortive, the sex bivalent and all autosomal bivalents will not disjoin and the resulting sperm will have both an X and a Y chromosome, as well as two of every autosome. On the other hand, if a normal first meiotic division is followed by an abortive second division, the two chromatids of all chromosomes, including the single X or Y chromosome, do not separate and the sperm will have either two X's or two Y's.

The data of Pang et al. (1995) show a significant increase ($p<0.05$) in the frequencies of diploidy, autosomal disomy, autosomal nullisomy, sex chromosome number, and total abnormalities from oligoasthenoteratozoospermic patients versus proven fertile donors. The data suggests that meiotic errors occur at highly elevated frequencies in oligoasthenoteratozoospermic patients. Such errors may be causally related to the patients' decreased fertility.

It is clear that FISH can be a rapid, reliable method for numerical chromosome evaluation of decondensed human sperm nuclei. The procedure is

easily learned, shows a high efficiency of hybridization, permits the rapid scoring of thousands of sperm nuclei, and is very sensitive. Sperm nuclei decondensation, probe labelling, hybridization conditions, and immunocytochemistry are parameters which require optimization for enhanced signal detection by FISH. Successful FISH requires high efficiency rates of hybridization while not destroying sub-populations of sperm nuclei. Overall comparisons for disomy frequencies between FISH and sperm karyotyping shows fair consistency between the two techniques. However, investigator to investigator variability exists for FISH results on disomy frequencies studying fertile donors. This variability may have resulted from different degrees of sperm nuclei decondensation, yielding variable probe access for FISH. Additional factors, such as variation in signal scoring criteria, fluorescence microscope configuration, as well as DNA cross-hybridization and donor to donor variation may have contributed to this chromosome frequency variability.

Tremendous progress has been made over the past quarter century in studying the cytogenetics of male gametogenesis and the resulting gamete. Cytogeneticists have gone from studying spermatogenesis itself, which sheds light on the nature of meiotic errors, to the study of the chromosomes of successful sperm cells; that is, those cells which succeed in fertilizing hamster ova. Studies merging molecular techniques and conventional cytogenetics are now beginning to bridge the gap between what we have learned about the

meiotic process in males and what we know of the mitotic chromosomes of zygotes.

The results of meiotic studies of spermatogenesis and FISH studies of sperm are consistent with one another. Taken collectively, the meiotic studies have shown that failure of primary spermatocytes to progress from MI to MII is common in infertile males. Although there is currently sparse FISH data from infertile males, the data of Pang et al. (1995) suggest that, in at least some cases, frequencies of diploidy will be found to be elevated. It is probable that most diploid sperm have an XY sex chromosome constitution. Diploid XY sperm results from a failed first meiotic division (as frequently observed in the meiotic studies), followed by an essentially normal second meiotic division.

Meiotic studies also revealed that infertile males may have a small decline in their average chiasma frequency. Some infertile men show a marked reduction in chiasma frequency and desynapsis. Decreased chiasma frequency could result in premature disjunction of bivalents (desynapsis). In turn, this would increase aneuploidy as the resulting univalents may migrate randomly to one pole or the other during anaphase of MI. The limited FISH data of Pang et al. (1995) on sperm from oligoasthenoteratozoospermic patients suggest increased frequencies of aneuploidy in such males.

It is difficult to relate the results of sperm studies utilizing FISH to the studies of the male pronucleus chromosomes. The human sperm-hamster egg fusion technique data summarized was on sperm from normal men and can only assess the cytogenetic status of sperm capable of successful fertilization. FISH, on the other hand, assesses all sperm. If aneuploidy has no effect on the fertilizing capacity of sperm and if the calculation of the mean aneuploidy rate for controls given by Pang et al. (1995) is correct, then there is good agreement between these studies. Using unweighted means, the average frequencies of nullisomy plus disomy in the studies of male pronuclei given in Table 2 was 4.7%. Pang et al. (1995) estimated the overall frequency of autosomal nullisomy plus disomy in sperm from normal donors to be 5.3%.

In order to assess the role that chromosome complement plays in normal and abnormal fertility, additional molecular cytogenetic studies must be done on sperm samples from men with normal fertility but also from men with abnormal semen. Care should be taken to fully characterize each patient's clinical picture so that data on subjects with identical presentations can later be pooled. Correlations of meiotic cytology and FISH studies on sperm from the same individuals may also prove highly informative.

To more clearly understand the cytogenetic make-up of sperm from OAT patients undergoing ICSI, FISH will be used to determine numerical

chromosome abnormalities. Total aneuploidy frequencies, fertilization rates, and pregnancy results will be discussed.

Hypothesis and Specific Aims Hypothesis - Sperm from oligoasthenoteratozoospermic (OAT) males will exhibit a significantly higher percentage of numerical chromosome abnormalities when compared to fertile donors.

To more clearly understand the relationship between aneuploid sperm and fertilization or pregnancy rates, we will use multi-probe, multi-color FISH on sperm from OAT patients undergoing ICSI to determine the frequency of numerical chromosome abnormalities. We will examine the effect that sperm selection by swim-up may have on separating haploid from aneuploid sperm fractions. We will present an analysis of the outcome of ICSI using sperm from these men who may be at risk for producing chromosomally abnormal concepti. We propose the following specific aims:

1) To develop formulae for the estimation of the frequencies of aneuploidy and diploidy in sperm studied by FISH. Aneuploidy observed in the chromosomes of male pronuclei, as seen after fertilization of hamster eggs by human sperm, will be reviewed with data on sperm chromosomes visualized by FISH.

2) To determine aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y and diploidy in sperm from fertile men using FISH.

3) To detect aneuploidy by FISH for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13,

17, 18, 21, X and Y and diploidy in sperm from oligoasthenoteratozoospermic (OAT) patients undergoing ICSI. Aneuploidy frequencies, fertilization rates and pregnancy results will be discussed.

4) To detect aneuploidy by FISH for chromosomes 1, 13, 18, 21, X and Y and diploidy in sperm from OAT patients undergoing ICSI after isolation of the motile sperm fraction Aneuploidy frequencies, fertilization rates and pregnancy results will be discussed.

5) To develop a technique to correlate aneuploidy and sperm tail swelling patterns following hypo-osmotic swelling and fluorescence in situ hybridization (FISH) in sperm from oligoasthenoteratozoospermic (OAT) patients undergoing ICSI Aneuploidy frequencies, fertilization rates and pregnancy results will be discussed.

Chapter 2. Theoretical Considerations and a Comparison of FISH Data with the Results of Studies of the Chromosomes of Male Pronuclei

Introduction

Our understanding of the male's chromosomal contribution to the zygote has come a long way in the last 20 years. In 1976, Yanagamachi et al. developed the technique for the in vitro fertilization of golden hamster eggs by human sperm. Their pioneering work led to many studies of the male-derived chromosomes that can be seen at the first mitotic metaphase in the zygote and which are referred to here as pronuclear chromosomes to distinguish them from chromosomes visualized in sperm cells using fluorescence in situ hybridization (FISH).

The frequencies of numerical chromosome aberrations observed in 14 studies of pronuclear chromosomes from normal men are summarized in Table I (derived, in part, from data presented by Jacobs, 1992, and Kearns et al., 1996). In summary, the mean frequency of aneuploidy, if calculated as the sum of nullisomy and disomy, was 2.9%. A more conservative view (Jacobs, 1992) is that, because inadvertent scoring of broken cells may lead to inflated nullisomy frequencies, aneuploidy is best estimated as twice the disomy frequency. This assumption leads to a mean estimate of 1.9% aneuploidy.

The study of male pronuclear chromosomes is expensive, time consuming,

requires considerable technical skill, and samples only sperm with fertilizing capability. Therefore, such studies are being supplemented or supplanted by FISH studies on sperm. The use of FISH has many advantages. The technique is easily learned, is much less expensive per unit of information, and large sample sizes are easily obtained. Many studies using FISH (Holmes and Martin, 1993; Martin et al., 1993; Robbins et al., 1993; Williams et al., 1993; Bischoff et al., 1994; Lu et al., 1994; Miharu et al., 1994; Wyrobek et al., 1994; Chevret et al., 1995; Martin and Rademaker, 1995; Martin et al., 1995a and 1995b; Moosani et al., 1995; Blanco et al., 1996; Martin et al., 1996; Pellestor et al., 1996a and 1996b; Spriggs et al., 1996; Pang et al., 1996) have sample sizes which exceed the total number of studied pronuclear karyotypes from normal men. In addition, sperm are ontogenetically closer to spermatocytes than are male pronuclei. Therefore, aneuploidy rates seen by FISH may more closely approximate the frequency of the non-disjunctional events which occurred during spermatogenesis.

On the other hand, studies of male pronuclear chromosomes have their advantages. On a per cell basis, FISH provides data on only the few chromosomes which are simultaneously probed. Each pronuclear karyotype, however, provides numerical and structural information on all 23 chromosomes in the haploid set making the total frequency of aneuploid gametes easy to calculate. The results of studies of pronuclear chromosomes may also more closely reflect the actual risks to human embryos from aneuploid sperm as only sperm cells with demonstrable fertilizing ability

are sampled.

Accurate determination of the aneuploidy frequencies of sperm will significantly improve our understanding of the cytogenetics of spermatogenesis. This report presents formulae for the calculation of the frequency of diploidy and the frequencies of disomy, nullisomy, and total aneuploidy for individual chromosomes from FISH data. Procedures for using these frequencies to estimate total aneuploidy in sperm are developed and are then used to compare published FISH data on aneuploidy in sperm with the data on pronuclear chromosomes.

Estimation of the Frequency of Diploid Sperm

Most recent studies of aneuploidy and diploidy in human sperm used two-probe, two-color FISH to simultaneously score sperm cells for the copy number of two different chromosomes. In some studies, three-probe, three-color FISH was used to study the gonosomes plus one autosome. Sperm samples were usually obtained from men with normal semen parameters, and low frequencies of cytogenetically abnormal cells were found. Under these circumstances, it is reasonable to estimate the frequency of diploidy as equal to the frequency of sperm showing two signals for each of two probed autosomes. However, this approximation leads to a slight overestimation of the true frequency of diploidy as the calculation erroneously includes those rare cells which are disomic for both chromosomes as a consequence of non-disjunctional events which affected both probed chromosome pairs. This assumption, as well as the assumption

that all cells showing hybridization signals for neither probed chromosome are artifacts resulting from hybridization failure rather than double nullisomics, may be inappropriate in studies of populations with markedly higher aneuploidy rates. The following more precise procedure is therefore proposed.

Our argument assumes 1) that the directions of anaphase I and/or II movement of two simultaneously non-disjoining chromosomes are random, or independent events (i.e., that the directionality of one bivalent or chromosome's non-disjunctional anaphase migration has no effect on the directionality of the other bivalent or chromosome's non-disjunctional anaphase migration) and 2) that in gametes resulting from spermatocytes with non-disjoining chromosomes, there is no additional aneuploidy resulting from anaphase lag (i.e., that non-disjunction alone is responsible for the production of gametes which are aneuploid for more than one chromosome). Under this assumption, nullisomy and disomy rates for specific chromosomes will be equal in gametes which are aneuploid for two autosomes probed using two-color, two-probe FISH.

Let "(wA,zB)" designate the category of sperm which has "w" copies of autosome "A" and "z" copies of autosome "B", where A and B are the two probed autosomes. Both w and z can have values of 0, 1, or 2. For the (2A,2B) category, we must distinguish three types: (obs.2A,2B) are observed double disomic sperm, (red.2A,2B) are reduced sperm (i.e., sperm produced by two meiotic divisions; non-polyploid sperm) which are aneuploid double disomics, and (dip.2A,2B) are diploid

sperm resulting from failure of either the first or second meiotic division. Somewhat similarly, there are three types of (0A,0B) sperm: (obs.0A,0B) are observed cells with no signal, (art.0A,0B) gametes are artifacts resulting from a technical failure of probe to anneal with target sequence and (nul.0A,0B) are sperm which are nullisomic for both chromosomes. Let "N(wA,zB)" designate the number of scored sperm with the partial karyotype of (wA,zB). Then, it is clear that

(eq. 1) $N(obs.2A,2B) = N(dip.2A,2B) + N(red.2A,2B)$

and

(eq. 2) $N(obs.0A,0B) = N(nul.0A,0B) + N(art.0A,0B)$.

Then, under our assumptions that the directionality of non-disjunctional events is random and independent and that loss of laggards can be ignored in spermatocytes in which non-disjunction is occurring,

(eq. 3) $N(red.2A,2B) = N(nul.0A,0B) = N(2A,0B) = N(0A,2B)$

or

(eq. 4) $N(red.2A,2B) + N(nul.0A,0B) = N(2A,0B) + N(0A,2B)$.

Together, these imply that

(eq. 5) $N(red.2A,2B) = N(nul.0A,0B) = (1/2)(N(2A,0B) + N(0A,2B))$.

Since rearranging the terms in eq. 1 indicates that

(eq. 6) $N(dip.2A,2B) = N(obs.2A,2B) - N(red.2A,2B)$,

it follows that:

(eq. 7) $$N(dip.2A,2B) = N(obs.2A,2B) - (1/2)(N(2A,0B) + N(0A,2B)),$$

or, in other words, that the number of diploid sperm can be estimated as the number of sperm with two signals for both probed chromosomes minus one-half the sum of the number of cells disomic for one chromosome and nullisomic for the other. The frequency of diploidy should therefore be calculated as

(eq. 8) $$\text{frequency of diploid sperm} = \frac{N(dip.2A,2B)}{\text{No. of sperm expressing one or both probes} + N(nul.0A,0B)}$$

$$= \frac{N(obs.2A,2B) - (1/2)(N(2A,0B) + N(0A,2B))}{\text{No. of sperm expressing one or both probes} + (1/2)(N(2A,0B) + N(0A,2B)).}$$

The numerator of the final working equation is from eq. 7. The second term in the denominator adds to the sample those cells that are nullisomic for both chromosomes and is derived from eq. 5.

To illustrate the potential value of this correction, we will consider data presented by Pang et al. (1995) on four males with oligoasthenoteratozoospermia. In a sample of 3,688 signal-expressing cells probed for chromosomes 11 and 12, 39 expressed two signals for both probed chromosomes and five expressed two signals for

one chromosome and none for the other. The frequency of diploidy would be estimated as 1.06% if it were calculated as 39/3,688. Using eq. 8, the frequency drops to 0.99%, a decrease of 6.5%. An atypical fifth patient, who had approximately 10% diploid cells in his semen, was a statistical outlier and was therefore omitted from this calculation.

Calculations of Autosomal Disomy, Nullisomy, and Aneuploidy Frequencies

We will consider the calculation of the frequencies of numerically abnormal gametes in the population of reduced (i.e., non-polyploid) sperm. We feel that, when calculating nullisomy, disomy, or total aneuploidy frequencies, the diploid gametes should not be included in the population considered as, from the study of the chromosomes of male pronuclei, there is no evidence that diploid sperm have fertilizing ability. Also, if diploid gametes were included in the calculation, individual-to-individual or population-to-population variation in diploidy frequencies would generate misleading variability in the calculated aneuploidy frequencies.

For the calculation of the frequency of disomy of autosome "A", an error occurs if the frequency is estimated without taking into account the presence of (red.2A,2B) gametes in the total population of reduced gametes. With these corrections,

$$\text{(eq. 9)}\quad \text{frequency of disomy of autosome "A"} = \frac{N(2A,1B) + N(2A,0B) + N(\text{red.}2A,2B)}{\text{No. sperm expressing one or both probes} - N(\text{dip.}2A,2B) + N(\text{nul.}0A,0B)}$$

$$= \frac{N(2A,1B) + N(2A,0B) + N(red.2A,2B)}{\text{No. sperm expressing one - N(obs.2A,2B) + N(red.2A,2B) + nul.0A,0B). or both probes}}$$

The third term in the numerator will be estimated from eq. 5. Collectively, the second and third terms in the denominator remove diploid gametes from the sample. The fourth term in the denominator adds those gametes that are nullisomic for both chromosomes. The gamete types represented by the last two terms are expected to collectively occur at a frequency equal to that of cells with disomy for one probed chromosome and nullisomy for the other (see eq. 4). The working formula for the calculation of disomy frequencies for autosomes studied with two-probe two-color FISH therefore should be

(eq. 10) $$\text{frequency of disomy of autosome "A"} = \frac{N(2A,1B)+N(2A,0B)+(1/2)(N(2A,0B)+N(0A,2B))}{\text{No. of sperm expressing one-N(obs.2A,2B)+N(2A,0B)+N(0A,2B). or both probes}}$$

Similarly, the working formulas for the frequencies of nullisomy and aneuploidy of autosome "A" are

(eq. 11) $$\text{frequency of nullisomy of autosome "A"} = \frac{N(0A,1B) + N(0A,2B) + (1/2)(N(2A,0B) + N(0A,2B))}{\text{No. of sperm expressing one-N(obs.2A,2B)+N(2A,0B)+N(0A,2B) or both probes}}$$

and

$$\text{(eq. 12)}\quad \text{frequency of aneuploidy of autosome "A"} = \frac{\text{N(2A,1B)+N(0A,1B)+2(N(2A,0B)+N(0A,2B))}}{\text{No. of sperm expressing one-N(obs.2A,2B)+N(2A,0B)+N(0A,2B). or both probes}}$$

Calculations Using Data on Gonosomes and One Autosome

The notation used in the following discussion is similar to that used above. Here, "XY", "XX", and "YY" denote disomy for the indicated gonosome(s) and "0G" denotes gonosomal nullisomy.

For three probe, three color FISH of the gonosomes and one autosome, the possibility that a reduced sperm will have two copies of either the same or two different gonosomes as well as two copies of the autosome should be considered. For example, the number of diploid sperm resulting from failure of the first meiotic division, i.e., (dip.XY,2A) sperm, should be estimated as

$$\text{(eq. 13)}\quad N(\text{dip.XY,2A}) = N(\text{obs.XY,2A}) - N(\text{red.XY,2A}) = N(\text{obs.XY,2A}) - N(\text{XY,0A}).$$

Here, the most suitable estimate of N(red.XY,2A) is N(XY,0A) and not 1/2[N(XY,2A) + N(0G,2A)], i.e., a term analogous to the last term in eq. 7, because (0G,2A) gametes, i.e., gametes with gonosomic nullisomy and autosomal disomy, result from first or second division non-disjunction.

As an example of the calculation of the frequency of a gonosomal aneuploidy, we will consider disomy XX:

$$\text{eq. 14)}\quad \text{frequency of XX disomy} = \frac{N(XX,1A)+N(XX,0A)+N(\text{red.}XX,2A)}{\text{No. of sperm expressing at least one probe}\ -N(\text{obs.}XX,2A)+N(\text{red.}XX,2A)+N(\text{nul.}0G,0A).}$$

The working formula derived from eq. 14 is

$$\text{(eq. 15)}\quad \text{frequency XX disomy} = \frac{N(XX,1A) + 2\,N(XX,0A)}{\text{No. of sperm expressing at least one probe}\ -N(\text{obs.}XX,2A)+N(\text{red.}XX,0A)+N(0G,2A).}$$

The effects of these corrections on calculated disomy, nullisomy, and aneuploidy frequencies in sperm from normal subjects are likely to be negligible as the values of all correction terms are very low. However, their effect may not be trivial in subjects with abnormal spermatogenesis.

Approximation of Overall Aneuploidy Frequencies

If data on the aneuploidy frequencies of all 23 pairs were available, we could calculate an overall aneuploidy rate after making the assumption that, within a given primary or secondary spermatocyte, the probability of one chromosome's non-disjunctions independent of the probability of any other chromosome's non-disjunction.

Where a_1 = the probability of aneuploidy for the first studied autosome,

a_2 = the probability of aneuploidy for the second studied autosome, ...

a_{22} = the probability of aneuploidy for the 22nd studied autosome, and

a_g = the probability of aneuploidy for the gonosomes,

we can calculate that, among reduced gametes,

(eq. 16) frequency of normal haploid sperm = $(1-a_1)(1-a_2)...(1-a_{22})(1-a_g)$.

Since, among reduced gametes, the sum of haploid and aneuploid gametes equals unity, we can estimate that

(eq. 17) frequency of aneuploid sperm = $1 - (1-a_1)(1-a_2)...(1-a_{22})(1-a_g)$.

This equation presupposes an ideal condition which is unlikely to be met in the near future, i.e., that all 23 aneuploidy frequencies are known. Nonetheless, in the absence of complete data (i.e., where "n" of the 22 autosomes have been studied and the n th chromosome has a frequency of aneuploidy of "an"), we can approximate the total aneuploidy frequency by assuming that the unknown frequencies are each equal to the average of the known autosomal frequencies. We can then estimate total aneuploidy as

(eq. 18) frequency of aneuploid sperm = $\{1-a_1\}\{1-a_2\}...\{1-a_n\}\{1-[(a_1+a_2+...+a_n)/n]\}_{22-n}\ \{1-a_g\}$.

From a strict mathematical viewpoint, as an equality, the validity of this relationship is contingent on homogeneity of autosomal aneuploidy frequencies, a

condition which may not be the case. Spriggs et al. (1996) presented data indicating that disomy frequencies of chromosome 21 and the gonosomes were higher than those of other chromosomes and hypothesized that the relatively short length available for crossingover in these chromosomes puts them at increased risk of first division non-disjunction. However, from a practical viewpoint, the probable absence of homogeneity is less likely to lead to significant error than are sampling error, investigator-to-investigator variation in the application of scoring criteria, and, perhaps, the failure of non-disjunctional events to be independent of one-another.

Testing for the Independence of Non-Disjunctional Events

It will be important to learn if meiotic non-disjunctional events are randomly distributed among spermatocytes or, alternatively, if these events co-occur within spermatocytes at lower or higher than expected frequencies. The above formulae for estimating total aneuploidy assume randomness. If, on the other hand, non-disjunctional events co-occur at higher than expected frequencies, these equations are invalid and, at best, will only be useful for establishing an upper limit on the estimated total frequency of aneuploidy.

In addition, knowing the nature of the distribution of non-disjunctional events among spermatocytes may provide insight into the causality of these meiotic errors. A random distribution of meiotic errors within cells would suggest that defects within individual chromosomes (e.g., defective centromeres) are causal. Co-occurrence at

higher than expected frequencies would suggest that physiological imbalances which may vary in intensity over time or space (e.g., nutritional or energetic inadequacies) may be involved.

In order to test for the independence of non-disjunctional events, the observed numbers of (1A,1B), (aneuploid A,1B), (1A,aneuploid B), and (aneuploid A,aneuploid B) gametes can be compared with the corresponding expected numbers by Chi-square analysis. While the first three categories can be directly observed, we must use an estimate of the total number of gametes aneuploid for both of two probed autosomes. Since (2A,2B) sperm may be either aneuploid double disomic or diploid, and (0A,0B) sperm may either be double nullisomic or represent hybridization failure, the only double aneuploids which can be reliably scored as such will be the (2A,0B) and (0A,2B) sperm. Since all four types are expected with equal frequency (eq. 3), the number of double aneuploids should be taken as twice the sum of N(2A,0B) and N(0A,2B). The best estimate of the actual frequency of double aneuploids is therefore

$$\text{(eq. 19)} \qquad \text{frequency of double aneuploidy} = \frac{2(N(2A,0B) + N(0A,2B))}{\text{No. of sperm expressing one -(obs.2A,2B)+N(2A,0B)+N(0A,2B). or both probes}}$$

Under the assumption of independence, the frequency of gametes aneuploid for both A and B is expected to be the product of the individual aneuploidy frequencies. Similarly, the frequency of monosomy for both is expected to be the product of the two

monosomy frequencies. The frequency of each single aneuploid class is expected to be the product of the frequency of monosomy of one chromosome times the frequency of aneuploidy of the other.

Analysis of published FISH Data

Most two-color and three-color FISH studies on sperm have not included data on the frequency of nullisomy or on the total frequency of aneuploidy. Instead, emphasis has been placed on disomy frequencies. Among exceptions, Lu et al. (1994) published aneuploidy frequencies but did not separate these values into their two components. Bischoff et al. (1994) and Martin et al.(1995b) are the only large studies which include both nullisomy and disomy frequencies. Bischoff et al., using probes for 11 autosomes and the gonosomes, observed 252 nullisomic sperm (= 0.35%) and 203 disomic sperm (= 0.28%) in a total of 72,001 cells. Somewhat similarly, in a study of 225,846 sperm, Martin et al., using probes for two autosomes and the gonosomes, observed average nullisomy and disomy frequencies of 0.43% and 0.23%, respectively. With the exception of Chevret et al. (1995), none of the two-color and three-color FISH studies included primary, i.e., "raw", data. Therefore, there is currently an inadequate amount of data for the direct calculation of the total frequency of aneuploidy in human sperm or for calculating the frequency of gametes with double aneuploidy.

In contrast to the paucity of data on nullisomy, twelve large studies using two-probe or three-probe FISH have reported disomy frequencies. Their data are

summarized in Table 2. The tabulated mean disomy frequencies for individual chromosomes, which ranged from 0.06 to 0.26% for autosomes and averaged 0.40% for the gonosomes, can be used to estimate total frequency of aneuploidy if we assume that the frequencies of nullisomy and disomy are equal, i.e., that twice the disomy frequency equals the aneuploidy frequency. Substituting twice the mean per chromosome disomy frequencies into eq. 18, we estimate that 7.5% of sperm are aneuploid. If we limit our consideration to the seven studies which used the conservative scoring criteria of Martin and Rademaker (1995), i.e., the last seven studies in Table II, our estimate of total aneuploidy decreases to 6.6%. If nullisomy is more common than disomy, as the data of Bischoff et al. (1994) and Martin et al. (1995b) suggest, these estimates may be underestimates.

Recently, Pellestor et al. (1996a,b) demonstrated that the primed in situ technique (PRINS), in which biotinylated and/or digoxigeninated DNA is synthesized on the microscope slide using primers specific to the chromosome(s) of interest, can be used to detect disomy in sperm. Using PRINS, autosomal disomy frequencies, expressed on a per chromosome basis, averaged approximately 0.3%, which is twice the average autosomal disomy frequency seen in recent FISH studies. As Pellester et al. used the scoring criteria developed by Martin and Rademaker (1995), the factors which lead to the higher frequency estimates seen with PRINS are obscure.

Our analysis of data on the chromosomes of the male pronuclei seen after the

fertilization of hamster ova by human sperm showed that the frequency of aneuploid pronuclei probably lies within the range of from 1.9% (when calculated as twice the disomy frequency) to 2.9% (when calculated as the sum of the nullisomy and disomy frequencies). The markedly higher frequency of aneuploidy estimated from the FISH studies tabulated in Table 2 suggests either that aneuploidy is not randomly distributed among sperm cells, that aneuploid sperm have diminished fertilizing capacity in the hamster ovum assay, or that current FISH techniques and scoring criteria result in an overestimation of aneuploidy frequencies.

Table 1. Frequencies of numerical chromosome abnormalities and X:Y ratio in male pronuclei from normal subjects.

Study	Number of Karyotypes	% Hyppohaploid	% Hyperhaploid	% Aneuploid	Conservative Estimate of Aneuploidy (%)	X-Y Ratio
Rudak et al. (1978)	60	3.3	1.7	5.0	3.3	57.0:43.0
Martin et al. (1982)	240	2.1	5.4	7.5	10.8	59.6:40.4
Martin et al. (1983)	1000	2.7	2.4	5.2	4.8	53.9:46.1
Brandriff et al. (1984)	909	1.0	0.6	1.5	1.2	- - -
Brandriff et al. (1985)	2468	0.9	0.7	1.7	1.5	50.1:49.9
Sele et al. (1985)	70	7.1	5.7	12.9	11.4	- - -
Kamiguchi & Mikamo (1986)	1091	0.5	0.5	1.0	1.0	53.0:47.0
Jenderny & Rohrborn (1987)	129	0.8	0.8	1.6	1.6	51.9:48.1
Mikamo et al. (1990)	9280	0.6	0.7	1.3	1.4	- - -
Martin et al. (1991)	5629	3.5	0.6	4.2	1.2	- - -
Estop et al. (1991)	555	6.3	2.0	8.3	4.0	54.5:45.5
Rosenbush & Sterzik (1991)	413	1.0	1.0	2.0	2.0	49.2:50.8
Benet et al. (1992)	505	9.1	2.0	11.1	4.0	50.4:49.6
Tateno et al. (1992)	519	- -	- -	0.8	- -	- - -
Jenderny et al. (1992)	450	0.9	0.9	1.8	1.8	47.4 : 52.5
Marquez et al. (1996)	771	7.4	2.5	9.9	4.9	50.8 : 49.2
Weighted Means		1.9	0.9	2.7	1.8	52.0:48.0

Table 2. Disomy frequencies, in percent, observed in reduced, i.e., non-polyploid, sperm.

	Chromosome																					
Study	1	2	3	4	6	7	8	9	10	11	12	14	15	16	17	18	20	21	XX	YY	XY	X&Y Total
Goldman et al. (1993)	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.40 [1]
Williams et al. (1993)	--	--	--	--	--	--	--	--	--	--	--	--	--	0.13	--	0.08	--	--	0.04	0.05	0.09	0.18
Bischoff et al. (1994)	--	--	0.38	0.28	0.11	0.06	0.09	--	0.22	0.09	0.30	--	0.20	0.39	0.13	0.18	--	--	0.38	0.08	0.12	0.59
Chevret et al. (1995)	0.17	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.03	0.01	0.40	0.44
Rousseaux & Chevret (1995)	--	0.20	--	0.08	--	--	--	--	--	0.09	--	0.17	--	--	--	--	--	0.10	--	--	--	--
Martin & Rademaker (1995)	0.08	--	--	--	--	--	--	--	--	--	0.17	--	--	--	--	--	--	--	0.07	0.16	0.12	0.35
Martin et al. (1995a)	0.08	--	--	--	--	--	--	--	--	--	0.17	--	--	--	--	--	--	--	0.12	0.16	0.12	0.40
Martin et al. (1995b) [2]	0.11	--	--	--	--	--	--	--	--	--	0.16	--	--	--	--	--	--	--	0.07	0.18	0.16	0.41
Moosani et al. (1995) [3]	0.09	--	--	--	--	--	--	--	--	--	0.15	--	--	--	--	--	--	--	0.08	0.18	0.16	0.42
Martin et al. (1996)	0.09	--	--	--	--	--	--	--	--	--	0.16	--	0.11	--	--	0.11	--	--	0.07	0.21	0.15	0.43
Spriggs et al. (1996)	--	0.08	--	0.11	--	--	--	0.14	--	--	--	--	--	0.11	--	--	0.12	0.29	--	--	--	--
Blanko et al. (1996)	--	--	--	--	0.14	--	--	--	--	--	--	--	--	--	--	--	--	0.38	--	--	--	--
Unweighted Means	0.10	0.14	0.38	0.16	0.13	0.06	0.09	0.14	0.22	0.09	0.19	0.17	0.16	0.21	0.13	0.12	0.12	0.26	0.11	0.13	0.17	0.40

[1] calulated by substracting the frequency of diploid sperm from the total frequency of sperm with gonosomal disomy
[2] includes the data of Spriggs et al. (1995)
[3] excludes data for chromosomes 1, 12, 15, 18, X and Y previously reported by Spriggs et al. (1995)

Chapter 3. Detection of Aneuploidy for Chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X , Y and Diploidy in Sperm from Fertile Men

Introduction

Fetal wastage frequently results from the presence of numerical and structural chromosome abnormalities. Hassold et al. (1978) estimated that approximately 50% of first trimester spontaneous abortions have such abnormalities and aneuploidy is present in approximately 1 in 300 newborns (Jacobs, 1992). Most numerical chromosome abnormalities arise de novo due to meiotic nondisjunction (NDJ) in a parent or from mitotic NDJ in the zygote or embryo. It is likely that most aneuploid zygotes result from aneuploid ova. A review by Pellester (1991) indicated that 22.9% of studied metaphase II oocytes were aneuploid. Since second division NDJ would generate additional aneuploidy, the frequency of aneuploidy in the female pronucleus is probably markedly higher.

Much of what we know about the frequency of aneuploidy in sperm comes from the study of the chromosomes seen at the first mitotic metaphase following the fertilization of hamster eggs by human sperm. Rudak et al. (1978) established a system in which human sperm were fused with Syrian hamster eggs and made the first extended cytogenetic observations of countable human sperm chromosomes in which numerical as well as structural abnormalities could

be identified. However, such studies sample only those sperm which have fertilizing capability. From 1978 to 1992, many investigators used this technique to determine aneuploidy frequencies in a total sample of approximately 22,000 sperm from normal subjects. Summarizing these studies, Hoegerman et al. (1996) estimated the frequency of aneuploidy in male pronuclei to be between 1.8 and 2.7%.

Since 1984, at least 21 studies using FISH on sperm chromosomes from normal men have been reported. FISH is a powerful tool for the detection of specific chromosomes or chromosome regions (Kearns and Pearson, 1994a,b). Using chromosome-specific DNA probes, FISH provides a rapid, alternative technique for the detection of aneuploidy and polyploidy in human sperm without selection for fertilizing ability. Many early studies used one-probe, one-color FISH. With one-probe, one-color FISH, a sperm cell displaying two signals (and therefore assumed to be disomic) for the probed chromosome could be either an aneuploid sperm which resulted from meiotic NDJ or a diploid sperm which resulted from nonreduction (i.e., the failure of one of the meiotic divisions). Therefore, the results of these early studies cannot be used for calculating the frequencies of either aneuploidy or diploidy. In these studies, and in more recent reports using two and three-color FISH, there has been striking investigator-to-investigator variability in reported aneuploidy frequencies.

Wyrobek et al. (1990) showed that incomplete sperm decondensation may result in a spurious increase in nullisomy frequencies. Further studies by Wyrobek et al. (1993), using confocal microscopy on decondensed human sperm nuclei, suggested that signals generated using satellite DNA probes may appear split into two to eight domains. Martin and Rademaker (1995) showed that variability in apparent disomy frequency may result from variability in scoring criteria. They compared two criteria for scoring disomy of chromosomes 1, 12, X, and Y in sperm from five normal men. One scoring criterion used one-half the diameter of a fluorescent signal as the minimum distance between two dots to be counted as two signals. The other criterion required that a full signal domain diameter be the minimum distance between two signals for the cell to be scored as disomic. The disomy frequency decreased significantly when a full diameter was used as the scoring criteria for chromosomes 1, X, and Y. However, the disomy frequency for chromosome 12 did not change between the two scoring methods. Martin and Rademaker suggested that the fluorescent signals for chromosomes 1, X, and Y were frequently split into more than one domain in decondensed human sperm interphase nuclei and that using the one-half domain scoring criteria could lead to an over estimation of disomy for some chromosomes. The wide range in published disomy frequencies for some chromosomes may therefore be a consequence of variability in scoring criteria with some workers overestimating disomy frequencies as a consequence of scoring split signals as two chromosomes.

Hoegerman et al. (1996) reviewed 13 studies on normal donors which used two or three color FISH. Using the mean disomy frequencies of the 19 studied chromosome pairs, it was estimated that approximately 6.6 to 7.5% of sperm are aneuploid. These values are roughly three fold higher than what had been found in studies of pronuclear chromosomes.

In this study, we performed simultaneous two-probe, two-color FISH using direct labeled satellite or contig DNA specific for chromosomes 4, 6-13, 17, 18, and 21 and three-probe, three-color FISH for chromosomes 18, X and Y on decondensed sperm nuclei from four proven fertile donors to determine frequencies of aneuploidy and diploidy. Our results suggest that approximately 3.7% of sperm are aneuploid and 0.036% are diploid.

Materials and Methods

Evaluation of Protocols for Sperm Decondensation and Sperm Head Swelling

Preliminary studies were conducted to determine the efficiency of decondensation protocols. Sperm obtained from seven normal donors by masturbation was immediately washed after liquefaction and then decondensed using modifications of the method of West et al. (1989). Our original protocol entailed mixing 1ml aliquots of semen with 4 ml phosphate buffered saline (PBS). Following centrifugation, pellets were resuspended in 1 ml PBS

containing 6mM EDTA, pH 7 at room temperature (RT). After centrifugation, pellets were resuspended in 1ml of PBS containing 2mM dithiothreitol (DTT), pH 7 at RT for 45 min. Following mixing with 2 ml PBS and centrifugation, pellets were resuspended by vortexing. While vortexing, 5 ml of fixative (3 parts methanol :1 part glacial acetic acid) were gently added. All centrifugations were at 300g for 5 min. Slide preparation was accomplished using the smear method and short term slide storage was at 4 °C.

To attempt to reduce the frequency of sperm DNA which failed to hybridize to probes, we changed the temperature for decondensation in DTT to 37 °C. When comparing these protocols, the degree of sperm decondensation and head swelling was monitored by measuring nuclear length, area, perimeter, and degree of roundness using FISH analysis software (Biological Detection System, Gaithersburg, MD). Apparent copy number (see scoring criteria) for chromosome 1 and, separately, for the gonosomes was determined by FISH using satellite DNA probes for loci D1Z1 , DXZ1 and DYZ3.

Semen donors for aneuploidy studies

The four normal subjects used to determine aneuploidy frequencies ranged in age from 29 to 33 years. Their sperm counts varied from 79 X 10^6/ml to 160 X 10^6/ml (mean = 123 ± 36.6 X 10^6/ml). Between 63 and 80% (mean = 69.5 ± 7.6 %) of sperm were motile and normal morphology, by strict criteria,

was seen in 17 to 30% (mean = 23.5 ± 6.0%). All have at least one child. All donors abstained from sexual activities and alcohol consumption for three days prior to donation. Semen was obtained by masturbation and, after liquefaction, sperm were immediately decondensed using the modification of the method of West et al. (1989) described above.

FISH

Two-probe two-color FISH was performed using probe sets for either chromosomes 4 and 6, 7 and 18, 8 and 13, 9 and 17, 10 and 21, or 11 and 12. Three-probe, three-color FISH for chromosomes X,Y, and 18 was also performed. Approximately 1000 sperm per subject were scored with each autosome probe set and approximately 2000 sperm per subject were scored using the X, Y, 18 probe set. Two-probe or three-probe FISH was used to differentiate disomy due to nondisjunction from diploidy due to nonreduction. Simultaneous scoring of two autosomes also provided an internal control to differentiate nullisomy from lack of hybridization.

Alpha satellite and contig probes (Table 3) were obtained from Vysis (Downers Grove, IL). For chromosome 1, the probe (puc 1.77) was laboratory-prepared by biotin labeling with subsequent avidin-fluorochrome detection. When two or three chromosomes were simultaneously probed with alpha satellite sequences, hybridizations were performed using 20 ng of each labeled

probe in a hybridization mix of 60% formamide, 2XSSC, pH 7.0 (TV = 10 μl). For probe sets consisting of one satellite sequence and one contig, a mixture of 20ng of the satellite sequence and 60-100 ng of preannealed contig DNA in a hybridization mix of 60% formamide, 2XSSC, pH 7.0 (TV = 10 μl) was prepared.

Hybridization mixes were added to prewarmed slides (42 °C), covered with 22 x 22 mm coverslips and sealed with rubber cement. Slides were denatured at 80 °C for 5 min. All slides were hybridized in a moist chamber for 2 - 20 hr at 42 °C.

Stringency washes of 3 X 10 min in 50% formamide, 2XSSC, pH 7.0, followed by 10 min 2XSSC, pH 7.0 and by 10 min 2XSSC, 0.1% NP-40, pH 7.0, all at 37 °C, were performed. Transition to antifade was accomplished by a 5 min PBS, pH 7.0 wash at RT. Coverslips were added over 13 μl antifade either with (for two-color FISH) or without (for three-color FISH) 0.6 μg/ml 4,6-diamidino-2-phenyl-lindole (DAPI) counterstain. Microscopy was performed using a Nikon epi-fluorescent microscope equipped with a 20x PlanApo objective, a 40x 1.3 na fluorite objective, a 60x 1.3 na fluorite objective, and a 100X 1.4 na plan apochromatic objective, one beam splitter, and one emission filter. Multiple fluorescent signal detection was accomplished using a Ludl filter wheel with six different excitation filters. Images were captured using a Photometrics (Tucson,

AZ) Series 200 cooled CCD camera (grade 2 chip) controlled by a MacIntosh Quadra 800 computer using BDS Image and FISH analysis software.

Scoring criteria

Nuclei were scored only if they were not over-decondensed, did not overlap and were intact with clearly defined borders. A sperm was scored as disomic for a particular chromosome if it showed two signals for that chromosome and one each of the simultaneously probed chromosome(s). Consistent with the scoring criteria of Martin and Rademaker (1995), two spots separated by less than the diameter of one domain were scored as a single signal. The absence of signal for a single chromosome was scored as nullisomy for that chromosome. Sperm showing signal for none of the chromosomes of a probe set were scored as such but were not included in the calculation of nullisomy frequencies as this outcome may be an artifact resulting from unsuccessful hybridization due to inadequate nuclear decondensation. A cell was scored as diploid if there were two signals for both probed chromosome pairs.

Statistical Analyses

Data were analyzed using SAS for Chi-square and Fisher's exact tests where appropriate.

Results

Comparison of Protocols for Sperm Decondensation and Head Swelling

Comparisons of the physical parameters of sperm and of the apparent copy number of chromosome 1 and the gonosomes in sperm decondensed at RT or at 37 °C are shown in Tables 4 and 5 respectively. Sperm incubated at 37 °C showed significant increases ($p<0.05$) in length, area, perimeter, and degree of roundness (p2a). There was a significant decrease ($p<0.05$) in the frequency of nuclei displaying no signal but no statistically significant change in the frequency of nuclei with two signals in samples decondensed at 37 °C. This suggested that incubation at 37 °C may lower the frequency of cells with spurious "nullisomy" due to hybridization failure without inducing spurious "disomy" resulting from increased distances between split signals. We therefore decondensed all test samples using DTT incubation at 37 °C.

Hybridization efficiency and X : Y ratio

For the 12 autosomes studied in the 12 normal donors, the mean hybridization efficiency, defined as the mean frequency of nuclei showing signal for at least one probed sequence, was 0.996. The frequencies of X and Y bearing sperm did not differ from the expected one to one ratio $\chi^2 = 0.1$, $p > 0.99$).

Determination of the frequencies of aneuploidy and diploidy

Complete data from two-probe, two-color studies of autosomes are shown in Table 6. Summary data on autosomal nullisomy, disomy and total aneuploidy (here expressed as the sum of nullisomy and disomy) are presented in Table 6. Table 5 lists results of the three-probe, three-color study of the X and Y chromosomes and chromosome 18.

On a per chromosome basis, the mean frequency of disomy for autosomes was 0.17% (range 0.05 to 0.28%). This frequency was almost twice the mean frequency of nullisomy (0.09%, range 0 to 0.18%). For the gonosomes, nullisomy (0.20%) was approximately as common as the sum of disomy and trisomy (0.18%). First division gonosomal NDJ was observed more frequently than second division NDJ (seven XY sperm versus four XX or YY) but the difference is not significant.

With the autosome probe sets, 10 of the 23,735 cells (Table 6) which expressed probe had two copies of both probed chromosomes. Similarly, with the X/Y/18 probe set, 12/8,026 cells (Table 7) had two gonosomes and two copies of chromosome 18. We can therefore crudely estimate the frequency of diploid sperm as 22/31,761 = 0.07%, or approximately 1/1400 sperm. Only three of the 12 diploid cells found using the X/Y/18 probe set were XY. While the deviation from a one to one ratio of XY to (XX + YY) cells is not statistically

significant ($\chi^2 = 3.0$, $0.10 > p > 0.05$), the lower frequency of XY diploid sperm suggests that failure of the second meiotic division may be more common than failure of the first.

Since, in most samples, the number of aneuploid sperm counted was low (<5 / 1000), Fisher's exact test was used to test for variability among donors. At the $p < 0.001$ level, there were no significant differences in aneuploidy levels among donors. However, at the $p < 0.05$ level, significant differences were found in the two-probe, two-color FISH data for nullisomy 7, nullisomy 18, and disomy 18.

Discussion

This study reports on the detection of aneuploidy in human sperm from four proven fertile donors for chromosomes 4, 6-13, 17, 18, and 21 using two-probe, two-color FISH and for chromosomes X, Y and 18 using three-probe, three-color FISH. Decondensation of sperm nuclei was accomplished using a modification of the method of West et al. (1989). In a preliminary study, a modified protocol, which includes a 37 °C incubation in 2mM DTT, produced larger sperm heads and a significant reduction in the number of sperm with no signal for chromosome 1. This revised protocol was therefore used to study aneuploidy frequencies in the fertile donors.

For the 12 studied autosomes, approximately 1000 nuclei per subject were scored using each probe set. For the gonosomes and chromosome 18, approximately 2000 nuclei per subject were scored. For the autosomes, nullisomy frequencies ranged from 0 to 0.18% (mean = 0.09%). Disomy, with frequencies which ranged from 0.05 to 0.28% (mean = 0.17%), was significantly more common. Gonosomal hyperhaploidy and hypohaploidy frequencies were 0.20% and 0.18% respectively.

Most FISH studies have not reported nullisomy frequencies (Hoegerman et al., 1996), presumably because the frequency of sperm with no signal for a particular probe was much higher than both the measured disomy frequency and the nullisomy frequency anticipated from studies of male pronuclei. However, if the decondensation reaction progresses to the point where all targets are accessible to the probes, and if hybridization is 100% successful, the most accurate frequencies obtained would be those for nullisomy as these frequencies would not be influenced by variability in the frequency of split signals. In this study, the modified decondensation protocol yielded nullisomy frequencies which were generally lower than disomy frequencies and a very low frequency of cells with no signal for all simultaneously utilized probes. We therefore consider our nullisomy data to be at least as reliable as, if not more reliable than, our disomy data.

Under the assumptions that the 10 autosomes which we did not study undergo NDJ at a frequency equal to the mean frequency of NDJ of the 12 studied autosomes and that nondisjunctional events occur independently, we used the aneuploidy frequencies in Table 8, where aneuploidy frequencies are given as the sum of nullisomy and disomy frequencies, and equation 18 of Hoegerman et al. (1996), to estimate that 5.9% of sperm from these four proven fertile donors are aneuploid. In a manner analogous to that used for the analysis of data from pronuclear chromosomes where a conservative estimate of total aneuploidy was often estimated by doubling disomy frequencies (which were low relative to nullisomy frequencies), we can double our per chromosome nullisomy frequencies (which here are low relative to disomy frequencies) and the substitute these values into equation 18 of Hoegerman et al. (1996). This yields a conservative estimate of 3.7% total aneuploidy in our normal donors.

A third estimate of total aneuploidy can be derived by doubling our per chromosome disomy frequencies and then substituting these values into the same equation. That calculation leads to an estimate of 7.7% aneuploid sperm. The magnitude of this value suggests that our decondensation protocol may have increased the distance between split signals more than it increased the diameter of such signals and that our disomy data are therefore overestimates of the true aneuploidy frequencies.

The first two estimates (5.9% and 3.7%) are lower than the lowest estimate of total aneuploidy (6.6%) calculated by Hoegerman et al. (1996) using data from previously published FISH studies. That analysis, like the third analysis above, estimated per chromosome aneuploidy by doubling per chromosome disomy frequencies. The disparity between the results of the current calculations which include nullisomy data and the calculation based on previously published data suggests that the previously published disomy frequencies may have also been, on average, overestimates. If so, the scoring criteria for determining chromosome copy number in sperm may require refinement.

It is difficult to reconcile the results of sperm studies using FISH to cytogenetic data on male pronuclear chromosomes obtained following the fertilization of hamster ova by human sperm. All estimates of total aneuploidy in sperm (3.7% to 7.7%) are markedly greater than the observed aneuploidy frequency (1.9 to 2.9%, as estimated by Hoegerman et al. (1996) seen in male pronuclei studied by the human sperm - hamster egg fusion technique. Factors, in addition to scoring criteria, which may account for the discrepancy include non-independence of nondisjunction events and aneuploidy-associated diminution of fertilizing capacity.

If multiple nondisjunctional events occur in primary and/or secondary

spermatocytes at higher frequencies than would be expected under the assumption that NDJ is randomly distributed among these cells, the equation used to calculate total aneuploidy rates would yield overestimates of the total frequency of aneuploid gametes. Efficient testing of this hypothesis will require the use of multicolor FISH to simultaneously score aneuploidy for three or more chromosomes.

There is evidence that some specific aneuploidies do diminish fertilizing capacity. Zackowski and Martin-DeLeon (1989) showed that, in male mice doubly heterozygous for two Robertsonian translocations which had one original chromosome in common, some unbalanced karyotypes produced in equal frequencies by meiosis had, depending on the age of the sperm, unequal frequencies in zygotes studied at metaphase of the first cleavage division.

In humans, it would appear that not all aneuploid states diminish fertilizing capacity. Ferguson-Smith (1983) found equal transmission of unbalanced karyotypes by female (44/378 = 11.6%) and male (27/231 = 11.7%) reciprocal translocation carriers.

In contrast to the transmission data on reciprocal translocation carriers, males carrying a 13/21 or 14/21 Robertsonian translocation are significantly less likely to produce Down's syndrome fetuses (0/53 = 0%) than are women carrying

these translocations (24/157 = 15.3%) (Ferguson-Smith, 1983). It is unlikely that imprinted loci are responsible for the lower frequency of functionally trisomy 21 fetuses which result from unbalanced paternal gametes. A normal female and a normal male with t(21q:21q) chromosomes of paternal origin were described, respectively, by Robinson et al. (1994) and Blouin et al. (1993) and a normal female with a similar chromosome of maternal origin was reported by Creau-Goldberg et al. (1987). Thus, since individuals with either paternal or maternal uniparental disomy have normal phenotypes, the existence of clinically significant imprinted alleles on chromosome 21 is highly unlikely. In addition, Stoll et al. (1995) found that the phenotypic variability of Down's syndrome individuals did not correlate with the parental origin of the extra chromosome as might occur if there were imprinted loci on chromosome 21.

As imprinting is an unlikely cause of the low frequency of Down's syndrome among the progeny of male carriers of Robertsonian translocations, diminished fertilizing capacity of sperm with disomy 21 is likely. If several additional human chromosomes have similar effects when disomic in sperm, aneuploidy-associated diminution of fertilizing capacity could account for much of the difference between the aneuploidy frequencies reported from studies on male pronuclei and FISH studies on sperm. Therefore, if scoring criteria are valid, FISH will yield the most accurate estimates of aneuploidy in sperm.

Table 3. Probes used for two- and three-color FISH.

Chromosome	Sequence Type	Locus or Loci
4	alpha satellite	D4Z1
6	alpha satellite	D6Z1
7	alpha satellite	D7Z1
8	alpha satellite	D8Z1
9	alpha satellite	D9Z1
10	alpha satellite	D10Z1
11	alpha satellite	D11Z1
12	alpha satellite	D12Z1
13	contig	RB1
17	alpha satellite	D17Z1
18	alpha satellite	D18Z1
21	contig	D21S259-D21S341-D21S342
X	alpha satellite	DXZ1
Y	alpha satellite	DYZ3

Table 4. Effects of decondensation procedure on sperm head parameters.

	Decondensation Procedure		
	None	DTT at Room Temp.	DTT at 37°C
Relative Area	1 ± 0.20[1]	1.67 ± 0.27	2.06 ± 0.32
Relative Perimeter	1 ± 0.11	1.28 ± 0.14	1.42 ± 0.15
p2a [2]	1.15 ± 0.08	1.12 ± 0.05	1.09 ± 0.03
Relative Length	1 ± 0.16	1.33 ± 0.14	1.47 ± 0.16

[1] Mean ± 1 S.D.

[2] Measurement of relative roundness where 1.00 is a perfect circle

Table 5. Comparison of hybridization efficiencies for chromosome 1 and the gonosomes after decondensation at 37°C or room temperature (RT).

	Chromosome 1					
	No Signal		One Signal		Two Signals	
Donor	37°C	RT	37°C	RT	37°C	RT
1	3	12	997	985	4	3
2	2	15	996	981	2	4
3	1	13	995	983	4	4
4	3	9	994	987	3	4
5	1	8	993	987	6	5
6	5	13	991	984	4	3
7	6	18	989	978	5	4
Total	21	88	6955	6885	28	27
Percent	0.3	1.26	99.3	98.36	0.4	0.39

	Gonosomes							
	No Signal		One Signal		Two X's		Two Y's	
Donor	37°C	RT	37°C	RT	37°C	RT	37°C	RT
1	5	14	991	983	2	0	2	3
2	6	13	992	986	1	1	2	0
3	2	16	993	979	3	3	2	2
4	4	18	994	981	0	1	2	0
5	3	16	994	980	2	2	1	2
6	7	13	986	979	4	5	3	3
7	9	19	985	976	3	3	3	2
Total	36	109	6935	6864	15	15	15	12
Percent	0.51	1.56	99.06	98.06	0.21	0.21	0.21	0.17

Table 6. Autosomal nullisomy, monosomy and disomy observed in 1000 cell samples of decondensed sperm from four normal subjects. Note that "1A,1B" denotes sperm monosomic for chromosomes "A" and"B", "2A,0B"denotes sperm disomic for "A" and nullisomic for "B", etc.

Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
4 (=A)	A	989	2	0	1	1	0	0	0	7
and	B	992	2	1	0	0	0	0	0	5
6 (=B)	C	992	1	2	2	1	0	0	0	2
	D	987	1	2	1	1	0	0	1	7
	Sum	3960	6	5	4	3	0	0	1	21
Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
7 (=A)	A	980	2	3	0	0	0	3	0	12
and	B	989	4	0	0	0	0	0	0	7
18 (=B)	C	996	2	0	0	0	0	0	0	2
	D	989	0	0	3	0	0	0	0	8
	Sum	3954	8	3	3	0	0	3	0	29
Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
8 (=A)	A	990	1	1	2	0	0	0	1	5
and	B	987	5	2	0	0	0	0	0	6
13 (=B)	C	991	1	3	0	0	0	0	0	5
	D	984	2	2	0	1	0	0	1	10
	Sum	3952	9	8	2	1	0	0	2	26
Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
9 (=A)	A	991	1	2	1	0	0	0	0	5
and	B	989	3	3	1	0	0	0	0	4
17 (=B)	C	994	2	1	1	0	0	0	0	2
	D	984	2	2	1	0	0	0	2	9
	Sum	3958	8	8	4	0	0	0	2	20
Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
10 (=A)	A	982	2	2	2	3	0	0	2	7
and	B	993	3	0	0	0	0	0	0	4
21 (=B)	C	992	2	2	0	0	0	0	0	4
	D	985	1	3	0	1	0	0	1	9
	Sum	3952	8	7	2	4	0	0	3	24
Chromosomes	Subject	1A,1B	2A,1B	1A,2B	0A,1B	1A,0B	0A,2B	A,0B	A,2B	No Signal
11 (=A)	A	987	3	0	0	4	0	0	0	6
and	B	991	0	0	3	2	0	0	0	4
12 (=B)	C	993	2	2	0	0	0	0	0	3
	D	988	0	0	2	1	0	0	2	7
	Sum	3959	5	2	5	7	0	0	2	20

Table 7. Chromosome constitution of sperm scored using three-color FISH for chromosomes 18, X and Y.

	Signals Present	Subject A	B	C	D	Sum
Normal	X,18	994	989	1000	1001	3984
	Y,18	994	996	988	999	3977
Gonosomal Nullisomy	18	5	2	6	3	16
	Percent	0.25	0.1	0.3	0.15	0.2
Gonosomal Disomy	XX,18	0	1	1	1	3
	YY,18	0	0	0	0	0
	XY,18	3	0	3	1	7
	XX	1	0	0	0	1
	Sum	4	1	4	2	11
	Percent	0.2	0.05	0.2	0.1	0.14
Gonosomal Trisomy	XYY	0	1	1	0	2
	XXY,18	0	1	0	0	1
	XXY,2(18)	0	0	1	0	1 [1]
	Sum	0	2	1	0	3
	Percent	0	0.1	0.05	0	0.04
Autosomal Nullisomy	X	0	0	0	0	0
	Y	5	3	2	1	11
	XX	1	0	0	0	1
	Sum	6	3	2	1	12
	Percent	0.3	0.15	0.1	0.05	0.15
Autosomal Disomy	X,2(18)	1	2	1	2	6
	Y,2(18)	0	1	0	1	2
	XXY,2(18)	0	0	1	0	1 [1]
	Sum	1	3	1	3	8
	Percent	0.05	0.15	0.05	0.15	0.1
Diploidy	XX,2(18)	2	1	1	3	7
	YY,2(18)	1	1	0	0	2
	XY,2(18)	1	0	1	1	3
	Sum	4	2	2	4	12
	Percent	0.2	0.1	0.1	0.2	0.15
	No Signal	0	1	1	2	4
	Percent	0	0.05	0.05	0.1	0.05
	Sum	2007	1999	2005	2015	8026

[1] Not included in calculations. If not artifactual, this sperm cell could have resulted either from three nodisjunctional events or from nonreduction preceeded or followed by nondisjunction.

Table 8. Summary data on the frequency, in percent, of autosomal aneuploidy.

Chromosome	Disomy	Nullisomy	Trisomy	Aneuploidy
4	0.15	0.10	0.00	0.25
6	0.13	0.08	0.00	0.20
7	0.28	0.11	0.00	0.43
8	0.23	0.05	0.00	0.28
9	0.20	0.10	0.00	0.30
10	0.20	0.05	0.00	0.25
11	0.13	0.13	0.00	0.25
12	0.05	0.18	0.00	0.23
13	0.20	0.03	0.00	0.23
17	0.20	0.00	0.00	0.20
18 [1]	0.09	0.14	0.00	0.22
21	0.18	0.10	0.00	0.28
Mean, Autosomes	0.17	0.09	0.00	0.26
X & Y	0.14	0.20	0.04	0.38

[1] Weighted mean of two-probe and three-probe data.

Chapter 4. Detection of Aneuploidy by Fluorescence In Situ Hybridization In Sperm From Oligoasthenoteratozoospermic Patients Undergoing ICSI

Introduction

Worldwide, infertility affects approximately 15% of couples (Baker et al., 1986). Male factor infertility is the primary problem in about half of these couples. Predicting the extent of their infertility from *in vitro* measures of sperm quality is complicated by the many interacting biological processes required for normal sperm function (Bavister, 1990). For example, sperm with damaged acrosomes but normal motility probably lack fertilizing capacity in vivo (Saacke and White, 1972). Additional parameters such as surface charge and adhesiveness, chromosome complement, and the expression of specific membrane receptors might also affect sperm transport and *in vivo* fertilization rates. Elucidation of the biological, including genetic, factors required for normal sperm function in vivo is a prerequisite for the full understanding of the ontogeny of male infertility. One aspect of infertility which has received little attention is the potential role of aneuploid sperm. What is the cytogenetic make-up of sperm from infertile males? What is the incidence of chromosomal abnormalities in somatic tissue (e.g. white blood cells) in infertile males? Is there an association between a sperm's fertilizing ability and chromosome abnormalities? Do techniques that bypass barriers to normal fertilization increase the parents' risk of having a fetus or liveborn with numerical or structural chromosome

abnormalities?

Fetal wastage frequently results from the presence of numerical or structural chromosome abnormalities. Hassold et al. (1978) estimated that approximately 50% of first trimester spontaneous abortions are chromosomally abnormal; of these abnormalities, approximately 96% have numerical abnormalities; structural aberrations are found in the remainder (Thompson et al., 1991). Jacobs et al. (1992) estimated that aneuploidy is present in approximately 1 in 300 newborns. Numerical chromosome abnormalities arise de novo due to meiotic nondisjunction (NDJ) in a parent or from mitotic NDJ in the embryo. Most aneuploid embryos result from aneuploid oocytes. Pellester reviewed the results of cytogenetic studies of over 1500 oocytes and showed that approximately 30% of metaphase II oocytes are aneuploid. Since second division NDJ would produce additional aneuploid gametes, the frequency of aneuploidy in the female pronucleus is likely much higher.

Molecular studies on the origin of trisomy for chromosomes 2-12, 13-15, 16, 18, 21, 22, XXY and XXX in 1207 informative concepti have shown 89.6% to be of maternal origin, with MI errors predominating (Koehler et al., 1996). However, studies of 133 informative XXY concepti showed 44% were of paternal origin; the XY gamete resulting from meiosis I (MI) NDJ. The available evidence suggests that maternal errors predominate for most trisomies.

However, paternal errors of NDJ may be preferentially associated with live offspring having sex chromosome aneuploidy.

Cytogenetic studies of somatic cells from infertile men have shown an increase in the incidence of constitutional chromosome anomalies as compared to normal fertile controls (Baschat et al.,1996; Chandley, 1979; Bourrouillou et al., 1985). Baschat et al. (1996) performed cytogenetic analyses on 32 males with impaired semen parameters. Two of 32 (6.3%) were found to have chromosomal abnormalities; one 45,XY,t(21;22)(p11;q11) and the second with 46,X,t(Y;22)(q12;p11). Both males had oligoasthenoteratozoospermia. Eighteen males with oligoasthenoteratozoospermia had karyotypes performed and 2 were abnormal. Of the 18 OAT males studied, 36.9% successfully fertilized eggs. In the two cases with paternal translocations, the fertilization rate was 57.6%. ICSI failed for the couple with the paternal t(21;22)(p11;q11). However, ICSI was successful for the couple with the paternal t(Y;22)(q12;p11); resulting in a twin pregnancy. Cytogenetic analysis on amniocytes at 14 weeks gestation showed one normal 46,XX fetus and one abnormal male karyotype with an unbalanced Y,22 translocation. De Braekeleer and Dao (1991) reviewed the literature on mitotic studies in males with azoospermia and severe oligozoospermia (count < 10 X 10^6 / ml) and showed that 12% had a cytogenetic abnormality. Overall, in 2105 somatic cell karyotypes from males with oligozoospermia, 12 were 47,XXY, 8 were mosaic Klinefelter syndrome, 14 had other numerical sex chromosome

anomalies, 9 had Y structural abnormalities, 32 had Robertsonian translocations, 20 had reciprocal translocations, 10 had inversions, and marker chromosomes were identified in eight patients. Among 1450 azoospermic men, somatic cell karyotypes showed 159 with Klinefelter syndrome, 13 mosaic XXY, 12 with other sex chromosome aneuploidy, 15 had Y structural abnormalities, 11 were XX/XY mosaics, 2 had Robertsonian translocations, 11 had reciprocal translocations, 1 had an inversion, and 1 had a marker chromosome. However, these studies did not determine cytogenetic aberrations in sperm. Structural aberrations in gametes can also result in chromosomally abnormal embryos. Robertsonian translocations, the result of fusion of the long arms of acrocentric chromosomes, generate a risk to carriers of having liveborn offspring with unbalanced karyotypes. Segregation of the paired chromosomes produces normal gametes, balanced translocation carriers, and chromosomally unbalanced progeny.

The cytogenetic detection of chromosome rearrangements in band q11 of the Y chromosome in sterile males suggested an association between such structural abnormalities and infertility (Tiepolo and Zuffardi,1976; Sandberg,1985; Chandley et al.,1989). Subsequent molecular analysis identified seven intervals within the Y chromosome (Vergnaud et al., 1986). The azoospermia factor (AZF) locus was localized to interval 6 at band Yq11.23 (Anderson et al.,1988; Bardoni et al., 1991). Southern blot analyses detected

microdeletions within this interval in 2 of 19 (10.5%) oligo- or azoospermic patients (Ma et al.,1992; Vogt et al.,1992). Nagafuchi et al. (1993) identified deletions within interval 6 in 6 of 50 (12%) infertile patients analyzed. Ma et al. (1993) isolated and characterized a gene family, called the Y chromosome RNA recognition motif (RBM1), that localized within this interval. They identified deletions within RBM1 in two infertile males studied. Stuppia et al. (1996) used STS-PCR to identify deletions within interval 6 of Yq11.23. Microdeletions were identified in six of 33 (18.1%) idiopathic oligo- or azoospermic patients studied. Vogt et al. (1996) evaluated 370 males with azoospermia or severe oligospermia for deletions of 76 loci within Yq11. Twelve males showed *de novo* microdeletions of several DNA loci, while one patient had a Mendelian inherited deletion. The deleted loci were mapped to three subregions of Yq11; one to the AZF locus and the other two proximal to AZF. The observed deletions were not overlapping but the size of deleted DNA was similar. Histological studies of testis tissue showed a correlation between spermatogenesis disruption and deleted loci. Vogt et al. (1996) proposed the presence of three spermatogenesis loci within Yq11 that are expressed during different phases of male germ cell development. Up to this point in time, microdeletion analyses on somatic cell DNA from oligo- or azoospermic males have shown between 3 and 18.1% of patients studied with deletions in interval 6 of Yq11. Corroborative data exists which suggests the presence of genes associated with spermatogenesis at additional loci within Yq11.

It is clear that somatic cells from infertile males show elevated frequencies of chromosome abnormalities and between 3 and 18% of those with azoospermia or oligospermia show microdeletions within interval 6 of Yq11.23.

However, what is the cytogenetic make-up of sperm from infertile males? The meiotic abnormalities described by Skakkebaek et al. (1973) and others (McIlree et al., 1966; Hult'en et al., 1970; Pearson et al., 1970; Zuffardi and Tiepolo, 1982; Chandley et al., 1976; Ferguson-Smith, 1976; Hendry et al., 1976; Mi'ci's et al., 1981; Koulischer et al., 1982) provided circumstantial evidence that some proportion of gametes from infertile males may be either aneuploid or diploid. Are there associations between aneuploidy in sperm, fertilizing ability, and frequency of livebirths? Is there a preferential contribution from male gametes to specific chromosome aneuploidies in progeny? Do techniques that bypass barriers for normal fertilization increase one's risk of having a fetus or liveborn with numerical or structural chromosome abnormalities?

Therapy for the treatment of infertile males has changed dramatically over the past several years. In vitro fertilization (IVF) protocols incorporating intracytoplasmic sperm injection (ICSI) are being performed in several centers and ICSI is now the treatment of choice for severe male infertility. Using ICSI, fertilization and pregnancy rates approach those of natural conception, even in

the presence of compromised semen parameters (Van Steirteghem, et al., 1993; Nagy et al., 1994). Even immature spermatozoa, aspirated from the epididymis or testes, are successfully used to fertilize eggs.

The possibility that aneuploid offspring may be relatively common following ICSI is suggested by the results of Tournaye et al. (1995) and cumulative data reported by Van Steirteghem (IX World Congress on In Vitro Fertilization and Assisted Reproduction, April 3-7, 1995, Vienna Austria). They reported on 1275 consecutive treatment cycles using ICSI performed in 919 couples. All men had male factor infertility and all couples had at least one failed conventional IVF treatment cycle. In some couples, the male had semen parameters incompatible with standard IVF or was afflicted with excretory azoospermia which required microsurgical epididymal sperm aspiration or testicular sperm retrieval. Results showed a diploid (2PN) fertilization rate of 47.7% per retrieved oocyte-cumulus complex and 66.4% per successfully injected metaphase II oocyte. The clinical pregnancy rate was 28.4% per started cycle or 31.3% per transfer. The mean maternal age was 32 years. Five fetuses with sex chromosome aneuploidy (47,XXX, 47,XYY, two 47,XXY, 46,XX / 47,XYY) and one fetus with trisomy 20 were diagnosed prenatally. The outcome of those pregnancies was not reported, although it can be safely assumed that the trisomy 20 fetus was electively or spontaneously aborted. An additional pregnancy yielded a Down syndrome newborn. Sex chromosome

aneuploidy was 1.0% (5/491). The expected frequencies of 47,XXX, 47,XYY and 47,XXY are approximately 3 per 1000 (0.3%) livebirths. The frequency of sex chromosome aneuploidy observed in this study is therefore 3-4 times higher than the frequency seen in newborns from the general population (Thompson et al., 1991).

In't Veld et al. (1995) performed prenatal diagnosis for advanced maternal age on 12 patients whose pregnancies (9 singletons and 3 sets of twins) were established by ICSI in IVF clinics in Belgium and the Netherlands. Four of 15 fetuses (27%) were diagnosed with chromosomal abnormalities. Cytogenetic results identified two XXY's, one 45,X/46,X,dic(Y)(q11)/46,X,del(Y)(q11), and two with Turner syndrome. Hoegerman et al. (1995) using data on sperm from nine OAT patients using two-probe, two color FISH, estimated the total frequency of sex chromosome aneuploidy to be approximately 5%. They hypothesized that the markedly higher frequency of sex chromosome aneuploidy observed by In't Veld et al. (1995) probably resulted from studying a non-random sample or from sampling error.

The use of ICSI has revolutionized assisted reproduction technologies, but the genetic risks associated with ICSI remain unclear. To evaluate the cytogenetic make-up of sperm from oligoteratozoospermic males, Pang et al. (1994) performed a preliminary study on 15 OAT males using FISH to determine

aneuploidy for chromosomes 1, X and Y. OAT was defined by the criteria of both Kruger et al. (1986) for morphology and the WHO (1992) for concentration and motility. Semen parameters were considered abnormal if the sperm concentration was less than $20x10^6$ /mL, less than 50% showed progressive motility, and less than 14% had normal morphology. The data showed significant increases in aneuploidy for all chromosomes analyzed in OAT patients versus controls. Moosani et al. (1995) reported both sperm karyotypes and FISH analyses for chromosomes 1, 12, X and Yin sperm from five infertile men with normal somatic karyotypes. Two were teratozoospermic, two were oligospermic, and one was asthenozoospermic. FISH analyses showed a significant increase versus normal controls in the frequency of disomy for chromosome 1 in three of five patients (teratozoospermic and oligozoospermic) analyzed. One oligozoospermic patient showed a significant increase in the frequency of disomy for chromosome 12. The frequency of XY disomy was significantly increased in four of five patients studied. Sperm karyotypes showed a significant increase in the frequency of numerical abnormalities relative to controls. Miharu et al. (1994) analyzed sperm from nine fertile and 21 infertile males whose semen parameters were not given for numerical abnormalities for chromosomes 1,16, X and Y by FISH. They found no significant differences between fertile and infertile males in the frequency of numerical abnormalities as determined by one-color FISH. XY aneuploidy could not be classified because they did not perform two-probe, two-color FISH for the sex chromosomes.

To more clearly understand the cytogenetic makeup of sperm from OAT males undergoing ICSI, we evaluated sperm from fresh ejaculate of nine severe oligoasthenoteratozoospermic patients and four proven fertile donors for aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y. Aneuploidy frequencies, fertilization rates, and pregnancy results will be discussed.

Materials and Methods

Sperm decondensation and sperm head swelling

Sperm obtained from four normal subjects and nine OAT patients by masturbation were immediately washed after liquefaction and decondensed as previously described (Pang et al., 1996). Briefly, fresh ejaculate was immediately rinsed in phosphate buffered saline (PBS), pH 7 and incubated for 5 min at room temperature (RT). Following centrifugation, the pellet was mixed with 1 mL PBS containing 6mM EDTA, pH 7.0, and incubated for 5 min at RT. Sperm head decondensation was accomplished using 2mM dithiotheitol (DTT), pH 7.0 for 45 min at 37 °C. Following centrifugation, the pellet was washed in PBS and the pellet was isolated by centrifugation.. The supernatant was discarded and the pellet was resuspended in fresh fixative (3 parts methanol: 1 part glacial acetic acid). Slide preparation was by the smear method and short term slide storage

was at 4 °C .

Normal semen donors

The four normal subjects used to determine control aneuploidy frequencies ranged in age from 29 to 33 years (mean = 31.3 years). Their sperm counts varied from 79 X 10^6/mL to 160 X 10^6/mL (mean = 123 x 10^6/ml). Between 63 and 80% (mean = 69.5 %) of sperm were motile and normal morphology, by strict criteria, was seen in 17 to 30% (mean = 23.5%). The mean percentage of dead sperm from controls was 10.3% (Table 9). All donors abstained from sexual activities and alcohol consumption for 3 days. All have at least one child. Semen was obtained by masturbation and sperm were immediately decondensed as described above. Aneuploidy frequencies in these donors were described in detail by Pang et al. (1996).

OAT Patients

The nine OAT patients' ranged in age from 25 to 39 years. Semen was obtained by masturbation and sperm were decondensed as described above. Sperm counts were between 2 and 15 x 10^6/mL. Motility ranged between 17.9 and 41.1% and between 1 and 4.4% sperm showed normal forms. The percent of dead sperm ranged between 7.6 and 40.7% (Table 9).

FISH

Two-probe two-color FISH was performed using probe sets for either chromosomes 4 and 6, 7 and 18, 8 and 13, 9 and 17, 10 and 21, or 11 and 12. Three-probe, three-color FISH for chromosomes X,Y, and 18 was also performed. Probes used for FISH were from loci D4Z1, D6Z1, D7Z1, D8Z2, D9Z4, D11Z1, D12Z3, 13q22 contig (RB1), D17Z1, D18Z1, 21q22.1 contig (D21S259-D21S341-D21S342), DXZ1, and DYZ3. Alpha satellite and contig DNA probes were obtained from Vysis, Inc. (Downers Grove, IL). All hybridizations for patients' and control sperm were performed at the same time. Approximately 1000 sperm per subject were scored with each autosome probe set and approximately 2000 sperm per subject were scored using the X, Y, 18 probe set. Two-probe or three-probe FISH was used to differentiate disomy due to nondisjunction from diploidy due to nonreduction. Simultaneous scoring of two autosomes also provided an internal control to differentiate nullisomy from lack of hybridization.

When two or three chromosomes were simultaneously probed with alpha satellite sequences, hybridizations were performed using 20 ng of each labeled probe in a hybridization mix of 60% formamide, 2XSSC, pH 7.0 (TV = 10 μl). For probe sets consisting of one satellite sequence and one contig, a mixture of 20ng of the satellite sequence and 60-100 ng of preannealed contig DNA in a hybridization mix of 60% formamide, 2X SSC, pH 7.0 (TV = 10 μl) was prepared. Hybridization mixes were added to prewarmed slides (42 °C) and covered with

22 x 22 mm coverslips which were sealed with rubber cement. Slides were denatured at 80 °C for 5 min. All slides were hybridized in a moist chamber for 2 - 20 hr at 42 °C.

Stringency washes of 3 X 10 min in 50% formamide, 2XSSC, pH 7.0, followed by 10 min 2XSSC, pH 7.0 and by 10 min 2XSSC, 0.1% NP-40, pH 7.0, all at 37 °C, were performed. Transition to antifade was accomplished by a 5 min PBS, pH 7.0 wash at RT. Coverslips were added over 13 μl antifade either with (for two-color FISH) or without (for three-color FISH) 0.6 μg/ml 4,6-diamidino-2-phenyl-lindole (DAPI) counterstain.

Microscopy was performed using a Nikon epi-fluorescent microscope equipped with a 20x PlanApo objective, a 40x 1.3 na fluorite objective, a 60x 1.3 na fluorite objective, and a 100x 1.4 na plan apochromatic objective, one beam splitter, and one emission filter. Multiple fluorescent signal detection was accomplished using a Ludl filter wheel with six different excitation filters. Images were captured using a Photometrics (Tucson, AZ) Series 200 cooled CCD camera (grade 2 chip) controlled by a MacIntosh Quadra 800 computer using BDS Image and FISH analysis software.

Scoring criteria

Nuclei were scored only if they were not over-decondensed, did not

overlap and were intact with clearly defined borders. A sperm was scored as disomic for a particular chromosome if it showed two signals for that chromosome and one each of the simultaneously probed chromosome(s). Consistent with the scoring criteria of Martin and Rademaker, two spots separated by less than the diameter of one domain were scored as a single signal (Martin and Rademaker, 1995). The absence of signal for a single chromosome was scored as nullisomy for that chromosome. Sperm showing signal for none of the chromosomes of a probe set were scored as such but were not included in the calculation of nullisomy frequencies as this outcome may be an artifact resulting from unsuccessful hybridization due to inadequate nuclear decondensation. A cell was scored as diploid if there were two signals for both probed chromosome pairs.

Statistical Analyses

Data were analyzed using SAS for Chi-square and Fisher's exact tests where appropriate.

Results

Two-Probe, Two-Color FISH

Summary data on autosomal and gonosomal (sex chromosome) nullisomy, disomy and total aneuploidy (expressed as the sum of disomy and nullisomy) are presented in Table 10. Disomy frequencies for autosomes and

gonosomes in OAT males ranged between 0 and 5.4%. In contrast, the disomy frequencies in controls ranged between 0.05 and 0.20%. Nullisomy frequencies for autosomes and the sex chromosomes in OAT males showed a range between 0 and 7%. Nullisomy frequencies in controls ranged between 0 and 0.75%. Aneuploidy, defined as the sum of disomy and nullisomy, for autosomes and gonosomes in OAT males ranged between 1.1 and 7.5%. In contrast, aneuploidy in controls ranged between 0.20 to 0.43%. The range of diploid sperm in OAT patients ranged between 0.4 and 9.6%. In contrast, controls showed a mean of 0.04% diploid sperm.

Three-Probe, Three-Color FISH

Table 11 shows results of three-probe, three-color studies of the X and Y chromosomes and chromosome 18. Gonosomal nullisomy in patients ranged between 0.39 and 2.54%. Controls showed a mean of 0.2%. Gonosomal disomy ranged between 1.36 and 4.87%. In contrast, 0.14% of control sperm was disomic. Gonosomal trisomy ranged from 0 to 0.18% in OAT patients and was found at a mean of 0.05% in controls. Three-probe, three color FISH of OAT patients showed a chromosome 18 nullisomy range between 1.37 and 3.85%. In contrast, controls showed a mean of 0.15%. Disomy for chromosome 18 ranged between 0.29 and 2.62% for OAT patients, whereas controls showed a mean of 0.01%. The range of diploid sperm in OAT patients ranged between 0.49 and 9.59%, while controls showed a mean of 0.15%.

For two, or three-probe FISH, the percent of sperm showing no signal from OAT patients ranged between 2.7 and 7.5%, whereas no signal for controls showed a mean of 0.04%. The frequencies of X and Y bearing sperm did not differ from the expected one to one ratio in normal donors.

Determination of total aneuploidy and diploidy in OAT patients and controls

Frequencies of total aneuploidy were estimated by calculating, using formulae developed by Hoegerman et al. (1996), per chromosome aneuploidy frequencies as either the sum of disomy and nullisomy, twice the disomy frequency, or twice the nullisomy frequency. These calculations assume independence of non-disjunctional events within the same cell. The total aneuploidy in sperm from nine OAT patients ranged between 33 and 74%. In contrast, the total aneuploidy in sperm from controls showed a mean range between 4.1 and 7.7%. The percent of diploid sperm in OAT males ranged between 0.4 and 9.6%, whereas controls showed a mean of 0.04%.

Statistical Analyses

Using Chi-square analysis and Fisher's exact test, we compared the frequencies of aneuploidy for individual chromosomes both within patients and among patients. Due to the heterogeneity of aneuploidy frequencies both within and between OAT patients, data could not be pooled. In contrast, data from

controls were homogenous and therefore were pooled.

Comparison of numerical chromosome abnormalities in OAT patients and controls

For 108 individual measurements of disomy frequencies (12 chromosomes X 9 subjects) in OAT patients, 90 comparisons to control means showed a significant increase ($p<0.01$), 13 showed no difference, and 5 were significantly lower ($p<0.01$). Similarly, for the 108 measurements of nullisomy, 100 showed a significant increase ($p<0.01$), 6 showed no difference, and 2 were significantly lower ($p<0.01$) than controls.

Comparisons of nullisomy versus disomy within individuals

Control FB showed a significant ($p<0.01$) excess of disomy over nullisomy. Patients OH and OI showed a significant ($p<0.01$) excess of disomy, while patients OB, OC, OD, OE, OF, and OG showed a significant ($p<0.01$) excess of nullisomy. Overall, in the OAT patients', nullisomy predominated.

Comparisons of nullisomy versus disomy for all chromosomes

In donors, chromosome 7 showed a significant ($p<0.01$) preponderance for disomy, whereas in OAT patients, chromosomes 4, 7, 8, 11, 18, and 21 showed a significant ($p<0.01$) excess of nullisomy.

Comparisons of the frequency of diploid sperm in OAT patients versus controls

The frequencies of diploid sperm was significantly increased ($p<0.01$) in OAT patients versus controls.

Sex chromosomes

No significant variation was noted across control donors in the frequencies of XX disomy, YY disomy, XY disomy, and gonosomal nullisomy. There was a significant increase ($p<0.01$) in all observed types of sex chromosome aneuploidy in all OAT patients, except OB (XX), OF (sex chromosome nullisomy), and OH (gonosomal nullisomy), compared to normal donor means. In OAT patients, XX disomy, XY disomy, YY disomy and gonosomal nullisomy occurred at equal frequencies. Sex chromosome aneuploidy was significantly increased ($p<0.01$) in OAT patients versus controls.

Comparisons of the frequency of no signal cells in OAT patients versus controls

There was a significant increase ($p<0.01$) in sperm from OAT patients with no signal versus normal controls.

Results of assisted reproductive technologies using sperm from OAT patients

Six series of ICSI were performed using ova fertilized by sperm from 5 of the OAT patients. Patient OC had no embryo transfer due to fertilization failure. Patient OD had two embryo transfers and OE one embryo transfer with no resultant pregnancies. OF had a preclinical spontaneous abortion and OH had a first trimester spontaneous abortion. No successful livebirths resulted.

Discussion

Over the past twenty years several studies have provided valuable information on the cytogenetic make-up of sperm from normal men. Using the human-hamster fusion technique, the frequencies of numerical chromosome aberrations observed in 16 studies of male pronuclear chromosomes were summarized and weighted means calculated by Hoegerman et al. (1996). The frequency of aneuploidy ranged between 1.9 and 2.9%. An alternative technique to determine aneuploidy in sperm is FISH (Kearns and Pearson, 1994a,b). FISH can directly identify aneuploidy and polyploidy in sperm . The FISH technique obviates the need to fertilize hamster eggs and permits the characterization of sperm without a selection bias based upon fertilizing ability. Hoegerman et al. (1996) reviewed 13 studies on normal donors using two or three color FISH. Calculations based upon the mean disomy frequencies of the 18 studied autosomes and sex chromosomes, estimated that between 6.6 and 7.5% of sperm from normal men are aneuploid.

In the current study, the total aneuploidy observed in sperm from the four proven fertile donors , as calculated using formulae developed by Hoegerman et al. (1996), ranged between 3.7 and 7.7%. The frequency of diploid sperm was 0.04%. This suggests that aneuploidy in controls identified by FISH is roughly three-fold higher than what had been found in studies of pronuclear chromosomes. Possible explanations which might account for this discrepancy include invalid FISH scoring criterion, non-independence of nondisjunction events and aneuploidy associated reduction in fertilizing ability. In any event, data on pronuclear chromosomes and FISH analyses strongly suggests that between 1.9 and 7.7% of sperm from normal donors are aneuploid.

To more clearly understand the cytogenetic make-up of sperm from OAT patients, we performed an extensive study for 14 chromosomes in patients undergoing ICSI. Examination of sperm from normal controls was compared to OAT males. Aneuploidy was determined using two- or three-probe FISH to differentiate disomy from diploidy.

For each OAT patient, estimates of total aneuploidy were calculated according to equations by Hoegerman et al. (1996). These formulae assume that numerical chromosome abnormalities are randomly distributed among cells and that the presence of an abnormal copy number for one particular chromosome has no effect on the probability of an abnormal copy number for

another chromosome. The equations estimate that the frequencies of aneuploidy for the unstudied chromosomes equals the mean frequency of aneuploidy for all studied chromosomes. These calculations are statistically valid only if the per chromosome abnormality frequencies are homogeneous; a condition which holds for the control data but not for the OAT patients. The only accurate way to determine total aneuploidy in sperm would be to simultaneously hybridize probes for all 23 chromosomes to a single sperm cell; an experimental condition currently not possible. Therefore, we have calculated the total aneuploidy in sperm from the OAT males in order to make a comparison to control means. Since patient-to-patient heterogeneity exists for all OAT patients, the OAT means should be considered as approximations. Using these equations, the total aneuploidy in sperm from the nine OAT patients ranged between 33 and 74% and the percent diploid sperm was between 0.4 and 9.6%. In comparison, the total aneuploidy in sperm from controls ranged between 4.1 and 7.7% with a mean of 0.04% diploid sperm.

The factor(s) responsible for the heterogeneity present both within and between semen samples from OAT patients is obscure. Heterogeneity is unlikely to have resulted from variability in decondensation and hybridization protocols as controls and patient samples were processed contemporaneously. Biological heterogeneity in the OAT population might account for some of the cytogenetic variability seen between patients but would be implausible to

generate the observed heterogeneity seen between chromosomes within patients. It is possible that mutations within a gene or genes associated with meiosis may predispose a particular chromosome to non-disjoin at a higher frequency than other chromosomes within the same gamete.

The current study showed a significant increase in autosome and gonosome aneuploidy in sperm from OAT patients. No separation technique was employed to isolate more motile sperm from non-motile sperm. It is possible that sperm selection methods to isolate sperm with increased motility and best morphology might also select for more normal haploid sperm. Overall, the frequencies of aneuploidy found using two- or three-probe FISH were consistent when considering the different experimental techniques. However, patient OE showed a frequency of disomy for chromosome 18 of 0.4% using two-probe and 2.51% for three-probe FISH. Patient OH had a frequency of nullisomy for chromosome 18 as 5.5% for two-probe versus 1.37% for three-probe FISH. Patient OI showed a frequency of nullisomy for chromosome 18 as 0 for two-probe and 3.5% for three-probe FISH. All data were included in the overall calculations of aneuploidy from the OAT patients.

Our data on controls showed an increase in disomy when compared to both nullisomy or sperm with no signal. In contrast, there was a significant increase in sperm from OAT patients with no signal or nullisomy versus that

found for the four proven fertile donors. This increase in no signal may reflect an experimental error associated with decondensation of sperm from OAT males or complete hybridization failure. Since normal controls and OAT samples were hybridized at the same time, it is unlikely that hybridization failure was a factor. On the other hand, the membrane of sperm from OAT males may differ in some way from controls. In fact, some sperm nuclei from OAT patients showed enlarged, overly decondensed sperm with degraded chromatin. These sperm cells were classified as unscorable. Colleu et al. (1988) reported that although the morphologic aspect of the chromatin remains the same in the nuclei of epididymal and ejaculated spermatozoa, human spermatozoa show a wide variation in extent of chromatin condensation even within a single ejaculate. The percent of stable sperm nuclei following SDS treatment was significantly lower in asthenozoospermia than in normozoospermia. Optimized sperm head decondensation, head swelling, and FISH protocols were extensively analyzed by Pang et al. (1996) to optimize experimental conditions.

These data show significant increases ($p<0.01$) in the frequencies of diploidy, autosomal disomy, autosomal nullisomy, sex chromosome number, and total abnormalities in sperm from OAT patients versus controls. The data suggests that meiotic errors occur at highly elevated frequencies in the germ cells of severely affected OAT patients. No patients undergoing ICSI in this study have ongoing pregnancies. The elevated levels of aneuploidy in sperm

from the OAT patients may be causally related to the lack of successful pregnancies following ICSI procedures.

The data reported by Tournaye et al. (1995), while impressive in their fertilization and implantation rates, shows an increased risk of having a fetus or liveborn with numerical chromosome abnormalities following ICSI.

Spontaneous abortions or miscarriages occur in 15-20% of all recognized human conceptuses and have provided tissue for pathological and genetic examination. Earlier embryonic losses are less amenable to direct study. Wilcox et al. (1988) has shown a high proportion of conceptions are lost between implantation and the first missed menses. It is just as likely that a high proportion of embryonic loss occurs between fertilization and implantation. However, collection of these products of conception (POC) are very difficult and no study has conclusively reported on the genetic diagnosis of this tissue. Preimplantation genetic diagnosis of the embryo is one way to directly correlate aneuploidy with implantation and ongoing pregnancy. In any event, Warburton et al. (1991) has catalogued chromosome abnormalities in spontaneous abortions. Numerical chromosome abnormalities predominate. Trisomy occurs in about 25% of all karyotyped early abortions and monosomy X is seen in about 7% of POC's. Almost no autosomal monosomies have been found in spontaneous abortions. The most common aneuploidy found in POC's is trisomy 16. It is

probable that natural barriers exist to eliminate certain chromosome aneuploidies due to the requirement of specific gene expression for normal development.

The aneuploidy found in the gametes of our OAT patients studied does not show any consistent pattern of chromosome loss or gain with the exception of elevated frequencies of sex chromosome aneuploidy. Data on autosomal aneuploidy provide no clear association between aneuploidy of a particular chromosome with fertilization and / or implantation. Results from Tournaye et al. (1995), In't Veld et al. (1995), and Hoegerman et al. (1995) support our data, suggesting that sex chromosomes non-disjoin at an elevated frequency in the germ cells of infertile males. We have also shown that particular autosomes also non-disjoin at higher frequencies, however, these aneuploidies in embryos are likely lethal.

The risk of chromosome abnormalities in the offspring of patients undergoing ICSI must be carefully evaluated. Data suggests that the frequency of chromosome abnormalities in somatic cells in males selected for infertility is inversely proportional to the sperm concentration (Kjessler, 1974). Chandley et al. (1979) analyzed an unselected group of 2372 infertile couples; 2.1% of males had somatic cell cytogenetic abnormalities; approximately half were sex chromosome abnormalities (47,XXY, 47,XYY, or 46,XY / 47,XXY). Yoshida et al.

(1995) reviewed the frequency of somatic cell cytogenetic abnormalities in 1007 infertile males. Sixty-five of 1007 males evaluated (6.5%) had karyotypic abnormalities; 41 of 65 (63%) were sex chromosome abnormalities. Thirty-one were 47,XXY, with seven being mosaic. Klinefelter syndrome, mosaic or full, accounted for nearly one-half of the karyotypic abnormalities found. The incidence of chromosome abnormalities was inversely proportional to abnormal semen parameters: 2.2% of normal controls had somatic cytogenetic abnormalities, compared to 5.1% of oligospermic males, 14.6% of azoospermic males, and 20.3% of males with non-obstructive azoospermia.

Deletion studies in somatic cells of infertile males (oligo- or azoospermia) within interval 6 of Yq11.23 suggest that a gene or genes controlling spermatogenesis likely resides within interval 6. However, less than 18% of infertile males show microdeletions. No deletion analyses have been reported in sperm from oligo- or azoospermic males. Somatic and germ cell mosaicism must be carefully evaluated.

Taken collectively, the direct relationship identified between somatic cell chromosome abnormalities in infertile males with abnormal semen parameters, the data of Tournaye et al. (1995) and In't Veld et al. (1995) showing increased chromosome abnormalities in the offspring of infertile males undergoing ICSI, and the current study showing increased aneuploidy in the sperm of severely affected OAT males, of which none achieved viable pregnancies, indicates that

there is an increased risk of numerical chromosome abnormalities in the offspring of males undergoing ICSI. The observed association between sex chromosome aneuploidy in the offspring of infertile males suggests a chromosome specific mechanism for numerical sex chromosome abnormalities as a result of paternal nondisjunction. The type and percent of aneuploid sperm found in infertile males may be correlated with the type and severity of male factor infertility.

Table 9. Age and semen parameters of OAT patients and controls

Subject	Age (yr.)	Volume (ml)	Count (X 10^6/ml)	Motility (%)	Normal form (%)
OA	35	3.0	6.4	32.6	1.0
OB	29	8.5	6.0	17.9	1.7
OC	39	4.4	10.0	25.7	4.4
OD	35	5.1	6.0	25.0	3.5
OE	37	4.0	15.0	27.2	2.0
OF	35	6.3	7.0	22.6	1.6
OG	36	3.0	2.1	27.9	1.0
OH	30	6.0	3.0	41.1	3.3
OI	33	3.4	2.0	30.0	3.0
FA	33	3.5	145.0	63.0	27.0
FB	33	4.5	108.0	65.0	30.0
FC	29	5.0	160.0	70.0	17.0
FD	30	3.7	79.0	80.0	20.0

Table 10. Frequencies, in percent, of numerical autosomal abnormalities in OAT patients and controls.

Subject	Disomy for Chromosome:												
	4	6	7	8	9	10	11	12	13	17	18	21	X/Y
OA	1.6	2.2	2.5	2.2	1.8	1.5	1.8	1.3	1.8	2	1.7	1.8	3.5
OB	0.9	1	0.7	0.7	1	1.4	1.1	0	1.2	0.9	2.3	0.6	1.9
OC	0.9	0.4	1.1	1.3	0.7	0.9	0.3	0.2	1.1	0.9	0.7	1.4	1.7
OD	1	1	0.7	1.3	1.2	1.1	2	0.3	1.5	1.5	2	2	1.6
OE	1.4	0.8	3	1.2	1.2	1.3	0.5	0.6	1.6	1.4	0.4	1.8	3
OF	1.5	1.3	0.8	0.9	1.1	1.3	0.6	0.5	1.3	1.2	2.5	1.7	3.2
OG	1.7	1	2.2	2	2.6	2.2	0	0	2.1	1.9	3.1	2.4	1.8
OH	2.4	1.7	2.6	3.5	2.6	2.9	5.4	1.6	2.6	3.1	0	3.4	2.2
OI	2.8	3	2.9	2.6	2.9	3.4	3.4	1.7	3.5	1.7	2.3	2.4	4.9
Control Means	0.15	0.13	0.31	0.23	0.2	0.2	0.13	0.05	0.2	0.2	0.11	0.18	0.15

Subject	Nullisomy for Chromosome:												
	4	6	7	8	9	10	11	12	13	17	18	21	X/Y
OA	1.2	2	1.5	1.2	1.9	1.4	1.3	1.3	1.2	1.4	1.8	1.5	1.5
OB	2.8	2.5	4	4.1	2.6	2.3	2.4	3.3	2.7	1.9	3.1	3.4	1.5
OC	0.5	0.9	0.8	0.8	0.9	0.4	1.1	5.7	0.6	1	4.8	0.5	1.9
OD	2.1	2.7	1.5	2.9	3	2.5	1.7	5.3	2.8	2.2	4.2	2.1	1.7
OE	1.3	0.9	3.4	2.7	1.6	3	3.7	3.3	1.8	1.4	3.9	1.7	0.9
OF	1.8	2	1.7	2	1.7	2.1	2.5	0	1.6	2.2	2.1	2	0.5
OG	3	2.6	4	2.5	1.8	2.3	7	1.1	2.1	4.2	1.3	2.2	0.8
OH	1.9	1.8	0	3.1	2.5	2.5	1.6	0.6	3.6	2.9	5.5	3.5	0.5
OI	1.5	2.2	2.3	0.6	2.1	1.9	0.6	2.8	2.1	1.2	0	1.9	2.6
Control Means	0.1	0.75	0.11	0.05	0.1	0.05	0.13	0.18	0.03	0	0.11	0.1	0.2

Subject	Aneuploidy (Defined as Disomy + Nullisomy) for Chromosome:												
	4	6	7	8	9	10	11	12	13	17	18	21	X/Y
OA	2.7	4.2	4	3.4	3.7	2.9	3.1	2.6	3	3.3	3.6	3.4	4.9
OB	3.7	3.5	4.7	4.7	3.6	3.6	3.5	3.3	3.9	2.7	5.4	4	3.4
OC	1.4	1.3	1.9	2	1.6	1.3	1.5	6	1.7	1.8	5.5	2	3.6
OD	3.1	3.7	2.2	4.2	4.2	3.7	3.7	5.6	4.2	3.8	6.2	4.1	3.2
OE	2.7	1.6	6.4	3.9	2.8	4.2	4.3	4	3.4	2.8	4.3	3.5	3.9
OF	3.3	3.3	2.5	2.9	2.8	3.4	3.2	5.3	2.8	3.4	4.6	3.7	3.7
OG	4.7	3.6	6.2	4.6	4.5	4.6	7	1.1	4.2	6.1	4.4	4.7	2.6
OH	4.3	3.5	2.6	6.6	5.2	5.4	7	2.2	6.2	6	5.5	6.9	2.6
OI	4.3	5.3	5.2	3.2	5	5.3	4	4.4	5.6	2.9	2.3	4.3	7.5
Control Means	0.25	0.2	0.43	0.28	0.3	0.25	0.25	0.23	0.23	0.2	0.23	0.28	0.35

Table 11. Raw data and fequencies (%) of sex chromosome and chromosome 18 abnormalities in OAT patients and controls.

	Signals Present	OA	OB	OC	OD	OE	OF	OG	OH	OI	Controls
Normal	X18	442	431	478	486	500	503	435	451	411	3984
	Y18	443	401	449	445	472	489	427	432	390	3977
Gonosomal Nullisomy	18	10	14	16	14	7	5	7	4	24	16
	1818*	3	0	2	2	2	0	0	0	0	0
	Sum	13	14	18	16	9	5	7	4	24	16
	Percent	1.32	1.53	1.78	1.55	0.84	0.46	0.75	0.39	2.54	0.2
Gonosomal Disomy	XX18	10	2	5	5	5	12	6	2	16	3
	YY18	11	8	5	4	11	7	5	5	13	0
	XY18	13	7	6	5	15	14	6	12	17	7
	XX*	0	0	0	0	0	0	0	1	0	1
	Sum	34	17	16	14	31	33	17	20	46	11
	Percent	3.45	1.86	1.58	1.36	2.88	3.02	1.82	1.96	4.87	0.14
Gonosomal Trisomy	XYY	0	0	1	0	0	0	0	0	0	2
	XXY18	0	0	0	0	0	1	0	0	0	1
	XXY1818*	0	0	0	2	1	1	0	0	0	1
	Sum	0	0	1	2	1	2	0	0	0	4
	Percent	0	0	0.1	0.19	0.09	0.18	0	0	0	0.05
Autosomal Nullisomy	X	11	13	21	25	12	13	13	7	14	0
	Y	12	11	18	13	17	17	9	6	19	11
	XX*	0	0	0	0	0	0	0	1	0	1
	Sum	23	24	39	38	29	30	22	14	33	12
	Percent	2.33	2.62	3.85	3.69	2.7	2.74	2.35	1.37	3.5	0.15
Autosomal Disomy	1818*	3	0	2	2	2	0	0	0	0	0
	X1818	7	13	2	9	13	12	13	2	11	0
	Y1818	8	11	2	8	11	11	6	1	9	0
	XXY1818*	0	0	0	2	1	1	0	0	0	1
	Sum	18	24	6	21	27	24	19	3	20	1
	Percent	1.83	2.62	0.59	2.04	2.51	2.19	2.03	0.29	2.12	0.01
Diploidy	XX1818	5	2	2	3	2	3	2	12	5	7
	YY1818	4	2	1	2	3	2	4	17	8	2
	XY1818	4	1	2	2	2	3	3	69	7	3
	Sum	13	5	5	7	7	8	9	98	20	12
	Percent	1.32	0.55	0.49	0.68	0.65	0.73	0.96	9.59	2.12	0.15
	SUM	986	916	1012	1029	1076	1094	936	1022	944	8017
No Signal		29	75	47	39	43	27	70	29	58	4

* These cells, with both autosomal and sex chromosome abnormalities, are listed in both catagories.

Table 12. Total aneuploidy and diploidy, in percent, in OAT patients and controls. Frequencies of total aneuploidy was estimated by caculating per chromosome aneuploidy frequencies as either A) the sum of disomy and nullisomy, B) twice the disomy frequency or C) twice the nullisomy frequency.

	Total Aneuploidy			
Subject	A	B	C	Diploidy
OA	55	59	50	1.4
OB	60	38	74	0.6
OC	43	33	51	0.4
OD	61	46	72	0.8
OE	58	47	67	0.7
OF	51	46	56	1
OG	66	57	73	1.4
OH	69	71	68	9.6
OI	65	74	54	2.6
Controls	5.9	7.7	4.1	0.04

Table 13. Results of assisted reproductive technologies using sperm from OAT patients.

Patient	No. of Mature Oocytes	No. of Fertilized Oocytes	Ferilization Rate (%)	Procedure	Outcome
OJ	16	15	93.8	ICSI	No pregnancy
OK	11	10	90.9	ICSI	No pregnancy
OL	11	6	54.5	ICSI	No pregnancy
OM	10	8	80.0	ICSI	No pregnancy
ON	11	10	90.9	ICSI	Pregnant
OO	3	2	66.7	ICSI	No pregnancy
OP	3	1	33.3	ICSI	No pregnancy
OQ	10	9	90.0	ICSI	No pregnancy
OR	10	6	60.0	ICSI	No pregnancy
OS	12	5	41.7	ICSI	No pregnancy

Chapter 5. Detection Of Aneuploidy Using Fluorescence In Situ Hybridization (FISH) In Sperm From Patients With Oligoasthenoteratozoospermia After Isolation of The Motile Sperm Fraction

Introduction

Therapy for the treatment of infertile males has changed dramatically over the past several years. Since the first report by Palermo et al. (1992) of a pregnancy following intracytoplasmic injection of a single spermatozoon directly into an oocyte, intracytoplasmic sperm injection (ICSI) has developed into the treatment of choice for severe male infertility. Using ICSI, high fertilization rates are achieved with sperm from men with severe oligospermia and pregnancy rates approach those of natural conception (Van Steirteghem et al., 1993; Nagy et al., 1994). Even immature spermatozoa, directly aspirated from the epididymis or testes, are used to successfully fertilize eggs, although at a lower frequency.

ICSI protocols use sperm which is free of seminal plasma. Several IVF centers perform sperm separation techniques which also enrich for more motile and morphologically normal sperm. Some investigators feel that higher fertilization and pregnancy rates are achieved when motile and morphologically normal sperm are used for ICSI. Conventional sperm separation methods, such as swim-up and glass-wool preparation yield few motile sperm with normal morphology. Some IVF centers use minipercoll centrifugation or migration

sedimentation methods to recover sperm best suited for ICSI. Sanchez et al. (1996) compared and contrasted minipercoll centrifugation and migration sedimentation as selection methods for sperm used for ICSI from cryptozoospermic or severe oligoteratozoospermic males. The study determined sperm count, motility, vitality, morphology and chromatin condensation. The number of spermatozoa obtained following minipercoll centrifugation was higher compared to migration sedimentation. However, the sperm quality was best following migration sedimentation selection. Therefore, sperm isolated by migration sedimentation were used for ICSI. Over a 13 month period, 159 ICSI cycles were performed. Seven hundred ninety of 1045 aspirated oocytes underwent ICSI. The fertilization rate was 70.4%. One hundred forty-six embryo transfers occurred with 56 pregnancies; 39.7% per transfer and 36.5% per cycle. However, there was a high spontaneous abortion rate; 18/58 (31%).

Liu et al. (1995) reported on ICSI results from 2732 cycles carried out in couples with severe male factor infertility. Ejaculated semen, epididymal sperm, and testicular spermatozoa were used. Sperm separation was by Percoll gradient centrifugation. The overall fertilization rate was 71% of intact oocytes. In 1828 of 1900 (96.2%) couples, ICSI resulted in normal fertilization and a clinical pregnancy rate of 37% per treatment cycle. However, no fertilization occurred in 76 of 2732 (3%) cycles from 72 couples. Of these, 22 of 26 couples achieved fertilization following subsequent ICSI treatment. Liu et al. (1995)

suggested that the total fertilization failure was mainly due to poor sperm viability or low sperm number available for ICSI. Poor quality oocytes were also suggested as a possible contributing factor. The authors suggested that repeated ICSI treatment may be useful or necessary to achieve successful fertilization and clinical pregnancy.

Oehninger et al. (1995) determined the efficacy and factors affecting ICSI outcome in 92 couples with severe male factor infertility. This was a prospective study carried out on couples with a prior failed IVF treatment cycle or having sperm parameters unsuitable for traditional IVF. Separation of the morphologically superior motile sperm was performed by Percoll gradient centrifugation or by swim-up. Of the 92 couples studied, the overall fertilization rate was 60.9%. Clinical and ongoing pregnancy rates per transfer were 31.9 and 26.8% respectively. Oehninger et al. (1995) suggested that sperm parameters did not correlate with ICSI outcome. Maternal age had no inverse affect on the fertilization rates but did have a significant impact in reducing pregnancy rates; 35-39 yrs: 22.9%; greater than 40 yrs of age: 5.9% clinical pregnancy per transfer.

Some IVF centers using micromanipulation are using immotile sperm for ICSI. In cases of congenital bilateral absence of the vas deferens (CBAVD) or other obstructive azoospermia, microsurgical epididymal sperm aspiration

(MESA) with IVF has shown fertilization and pregnancy resulting in live births (1990) but the results showed lower fertilization and pregnancy rates. Silber et al. (1994, 1995) has demonstrated normal fertilization and pregnancy rates using both epididymal spermatozoa and testicular sperm utilizing ICSI. They suggested that when epididymal spermatozoa cannot be retrieved, a testicular biopsy can be performed and the few barely motile spermatozoa can be obtained and used for ICSI. Silber et al. (1995) concluded that all cases of obstructive azoospermia can be treated using epididymal or testicular spermatozoa.

While the fertilization rates and ongoing pregnancies using ICSI are impressive, one must consider the potential transmission of genetic abnormalities to the offspring. Several aspects regarding ICSI require clarification. Why is the fertilization rate only 60-70%? Why do some morphologically normal spermatozoa and oocytes used in ICSI fail to fertilize? Why is there a high rate of spontaneous abortions in some couples undergoing ICSI? Do chromosome abnormalities in male gametes contribute to reduced fertilization percentages, reduced implantation rates, or failure to achieve a viable pregnancy? Do ICSI techniques using sperm from severely affected OAT males enrich for offspring with elevated frequencies of sex chromosome aneuploidy? Do artificial reproduction techniques that bypass barriers for normal fertilization contribute to aneuploidy in the offspring of infertile males undergoing

ICSI?

Molecular studies on the origin of trisomy have shown that approximately 90% of trisomies are of maternal origin (Koehler et al., 1996). However, studies of 133 informative XXY concepti showed 44% were of paternal origin and caused by XY sperm resulting from meiosis I nondisjunction (NDJ). Paternal errors of NDJ may be preferentially associated with live offspring with sex chromosome aneuploidy.

Cytogenetic studies of somatic cells from infertile males have shown an increase in the incidence of constitutional chromosome abnormalities as compared to normal fertile controls (Baschat et al., 1996; Chandley, 1979; Bourrouillou et al., 1985). Data suggest that the frequency of chromosome abnormalities in somatic cells in infertile males is inversely proportional to semen parameters (sperm count, morphology and motility).

Molecular studies in somatic cells of oligospermic and azoospermic males have shown between 3 and 18% of these male having deletions within interval six of Yq11.23. Corroborative data exist to suggest the presence of genes associated with spermatogenesis at different loci of Yq11. However, all oligospermic and azoospermic males do not have identified deletions within interval six. It is probable that other genetic mechanisms are also associated

with male infertility.

The possibility that aneuploid offspring may be relatively common following ICSI is suggested by the results of Tournaye et al. (1995) and cumulative data reported by Van Steirteghem (IX World Congress on In Vitro Fertilization and Assisted Reproduction, April 3-7, 1995, Vienna Austria). They reported on 1275 consecutive treatment cycles using ICSI performed in 919 couples. All men had male factor infertility. Results showed a diploid (2PN) fertilization rate of 47.7% and 66.4% per successfully injected metaphase II oocyte. The clinical pregnancy rate was 28.4% per started cycle or 31.3% per transfer. The mean maternal age was 32 years. Five fetuses with sex chromosome aneuploidy (47,XXX, 47,XYY, two 47,XXY, 46,XX / 47,XYY) and one fetus with trisomy 20 were diagnosed prenatally. An additional pregnancy yielded a Down syndrome newborn. The overall aneuploidy frequency in the offspring was 1.4% (7/491); sex chromosome aneuploidy was 1.02% (5/491). The frequency of observed sex chromosome aneuploidy in this study was three-four times higher than the frequency of sex chromosome aneuploidy (approximately 3/1000) seen in newborns from the general population (Thompson et al., 1991).

In't Veld et al. (1995) performed prenatal diagnosis for advanced maternal age on 12 patients whose pregnancies were established by ICSI in IVF clinics in

Belgium and the Netherlands. Four of 15 (27%) were diagnosed with chromosomal abnormalities. Three patients had a twin pregnancy. Cytogenetic results identified two XXY's, one 45,X/46,X,dic(Y)(q11)/46,X,del(Y)(q11), and two with Turner syndrome.

To more clearly evaluate the genetic make-up of sperm from oligoasthenoteratozoospermic males, Pang et al. (1996a) evaluated sperm from fresh ejaculate of nine severe oligoasthenoteratozoospermic patients and four proven fertile donors for aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y. The total aneuploidy for the nine OAT patients ranged between 33 and 74%. In contrast, the total aneuploidy in controls' sperm ranged between 4.1% to 7.7%. Six series of ICSI were performed on 5 of the OAT patients. Four series resulted in no establishment of pregnancy; the other two failed to establish ongoing pregnancies. These data showed significantly elevated frequencies of diploidy, autosomal disomy and nullisomy, sex chromosome number, and total aneuploidy in OAT patients. These chromosome abnormalities most likely contributed to the patients' infertility.

Jarvi et al. (1996) evaluated males presenting for infertility investigation for cystic fibrosis (CFTR) mutations or variants. Men were classified as CBAVD, oligospermia, obstructive azoospermia or testicular failure (no sperm production). CFTR mutation and variant (intron 8 thymidine tract) analysis found

77% of males with CBAVD, 35% of males with obstructive azoospermia, 26% of males with oligospermia, and 11% with testicular failure had at least one CF mutation or variant. The frequency of CF mutations was significantly higher ($p<0.001$) for CBAVD, obstructive azoospermia and oligispermic males versus men with testicular failure. The frequency of observed mutations and variants for the testicular failure group was similar to the expected frequency in the population (9%: 4% for typical CF mutations and 5% for variants). These preliminary results suggest that CFTR mutations could be transmitted to the offspring of some infertile males undergoing ICSI.

Little is known about the relationship between infertility and sperm aneuploidy. It has been recognized since the 1970's (Skakkebaek et al., 1973; Zuffardi and Tiepolo, 1982; Chandley et al., 1976; Ferguson-Smith, 1976; Hendry et al., 1976;Mi'ci'c et al., 1981; Koulischer et al., 1982) that approximately 15% of infertile men have severely impaired meiosis which often involves a failure of spermatocytes to progress from the first to the second meiotic division. Such errors can result in aneuploid gametes. However, the extent to which lesser disturbances of meiosis may contribute to either subnormal semen parameters and / or to aneuploidy in the remaining sperm is not well understood. Hoegerman et al. (1996) hypothesized that methods used to obtain sperm samples or sperm selection techniques (best motility, etc) might affect the aneuploid condition of sperm used for ICSI. Kearns et al. (1996)

hypothesized that the severity and type of infertility are correlated with the type and percent of aneuploidy found in somatic cells and sperm from infertile males. Data of Pang et al. (1996)· Tournaye et al. (1995), In't Veld et al. (1995), and Hoegerman et al. (1995) suggest that sex chromosomes preferentially non-disjoin at higher frequencies, leading to aneuploidy, in germ cells of infertile males. Autosomes also undergo increased levels of nondisjunction in male meiosis, producing sperm with elevated frequencies of autosomal aneuploidy. However, most autosomal aneuploidies in embryos are lethal. The most common aneuploidies found in newborns involve chromosomes 13,18, 21, X and Y.

To more clearly understand the relationship between aneuploid sperm and fertilization or pregnancy rates, we used three-probe, three-color FISH for chromosomes 1, 13, 18, 21, X, and Y on sperm from 10 OAT patients and four proven fertile donors. We examined the effect that sperm selection by swim-up may have on separating more motile and morphologically normal sperm from abnormal sperm. We determined whether the swim-up technique separates aneuploid sperm from haploid sperm and we present an analysis of the outcome of the ICSI procedure on these men who may be at risk for producing chromosomally abnormal concepti.

Materials and Methods

Subjects and Specimen Preparation

Semen was collected from ten OAT patients by masturbation on the day of ICSI. Sperm concentration and progressive motility were assessed by computer-assisted semen analysis (Hamilton Thorn IVOS System, Danvers, MA). Morphology assessment was performed on a fresh seminal smear according to strict criteria (Kruger classification system). OAT was defined by the criteria of both Kruger et al. (1986) for morphology and the WHO (1992) for concentration and motility. Semen parameters were considered abnormal if the sperm concentration was less than $20x10^6$ /mL, less than 50% showed progressive motility, and less than 14% had normal morphology. The ten OAT patients ranged in age between 32 to 47 years. Sperm counts were between 1.2 and 14.5 x 10^6/mL. Motility ranged between 17 and 50% and between 2 and 10% of sperm showed normal forms. The percent of dead sperm as determined by an Eosin vitality test ranged between 8 and 39%.

The four normal subjects used to determine control aneuploidy frequencies ranged in age from 30 to 45 years. Their sperm counts varied from 35.6 to 67.5 X 10^6/mL. Between 67 and 76% of sperm were motile and normal morphology, by strict criteria, was seen in 14 to 16%. The mean percentage of dead sperm from controls was 11%. All donors abstained from sexual activities and alcohol consumption for 3 days. All have at least one child. Semen was obtained by masturbation on the day of isolation of the motile sperm fraction.

Separation of the more motile fraction sperm was performed on OAT patients and controls using the swim-up technique (Veeck,1986). Controlled ovarian hyperstimulation and ICSI protocols were as previously described (Oehninger et al., 1990). Sperm cell nuclei from both pellet and swim-up fractions were decondensed within 3-5 hours following semen liquefaction as described (Pang et al., 1996b).

FISH

Simultaneous three-probe three-color FISH was performed using probe sets for either chromosomes X, Y, and 13 (Set I) or 1, 18, and 21 (Set II). Probes used for FISH were from loci D1Z3, 13q22 contig (RB1), D18Z1, 21q22.1 contig (D21S259-D21S341-D21S342), DXZ1, and DYZ3. For each normal control and OAT patient, 1000 sperm from the pellet and 300 sperm from the swim-up fraction were scored. Three-probe FISH was used to differentiate disomy due to nondisjunction from diploidy due to nonreduction. Simultaneous scoring of three chromosomes also provided an internal control to differentiate nullisomy from lack of hybridization.

Alpha satellite or contig DNA (Amersham Life Sciences and VYSIS, Inc.) was directly labelled with dNTP - fluorochromes using either nick translation or random priming. Twenty nanograms of each alpha satellite DNA probe was mixed in 60% formamide, 2XSSC, pH 7.0 (total volume = 10 μl) and denatured

for 5 min at 80°C. In separate tubes, contig DNA, 60 - 100 ng was mixed with human Cot-1 DNA which was dissolved in 10% dextran sulfate, 50% formamide, 2XSSC, pH 7.0 (TV = 10 μl). The DNA was denatured for 5 min at 80°C and preannealed for 5 min at 37 °C. Sperm nuclei were denatured in 70% formamide, 2XSSC, pH 7.0 for 5 min at 80°C. Slides were dehydrated in ice-cold ethanol (50, 70, and 95%, 3 min each), air dried, and prewarmed to 42 °C. The contig and alpha satellite mixes were added to the slides, covered with 22 x 22 mm coverslips, and sealed with rubber cement. All slides were hybridized in a moist chamber for 2 - 20 hr at 42 °C.

Stringency washes of 3 X 10 min in 50% formamide, 2XSSC, pH 7.0, followed by 10 min 2XSSC, pH 7.0 and by 10 min 2XSSC, 0.1% NP-40, pH 7.0, all at 37 °C were performed. Transition to antifade was accomplished by a 5 min PBS, pH 7.0 wash at RT. Coverslips were added over 13 μl antifade without counterstain.

Microscopy was performed using a Nikon epi-fluorescent microscope equipped with a 20x PlanApo objective, a 40x 1.3 na fluorite objective, a 60x 1.3 na fluorite objective, and a 100x 1.4 na plan apochromatic objective, one beam splitter, and one emission filter. Multiple fluorescent signal detection was accomplished using a Ludl filter wheel with six different excitation filters. Images were captured using a Photometrics Series 200 cooled CCD camera (grade 2

chip) controlled by a MacIntosh Quadra 800 computer using BDS Image and FISH analysis software.

Scoring criteria

Nuclei were scored only if they were not over-decondensed, did not overlap and were intact with clearly defined borders. A sperm was scored as disomic for a particular chromosome if it showed two signals for that chromosome and one each for the simultaneously probed chromosomes. For disomy to be counted, the distance between the two signals had to equal or be greater than the diameter of one fluorescent domain. Two spots separated by less than the diameter on one domain were scored as a single signal. The absence of signal for a single chromosome was scored as nullisomy for that chromosome. Sperm showing signal for none of the chromosomes of a probe set were scored as such but were not included in the calculation of nullisomy frequencies as this outcome may be an artifact resulting from unsuccessful hybridization due to inadequate nuclear decondensation. A cell was scored as diploid if there were two signals for each probed autosome and two sex chromosomes present.

Statistical Analyses

Chi-square analysis and Fisher's exact test were performed as appropriate. $p < 0.05$ was considered significant.

Results

Disomy and Nullisomy Detection in the Swim-up (SU) and Pellet Fractions from OAT and Controls

Per chromosome disomy frequencies ranged between 0.0 and 3.7% for patients' swim-up fraction and 0.0 to 2.1% from pellets (Table 15). Disomy in controls' swim-up fraction ranged between 0.0 and 0.3%, whereas pellets disomy ranged from 0.0 to 0.8%. Per chromosome nullisomy for patients ranged between 0.0 and 6.3% in swim-up and 0.1 to 3.1% in pellets. Per chromosome nullisomy in controls ranged between 0.0 and 0.3% in swim-up and 0.0 and 0.7% in pellets (Table 16). OAT patients showed significantly higher ($p<0.05$) disomy and nullisomy frequencies for chromosomes 1, 13, 18, 21, X and Y compared to normal controls. However, no significant differences were detected between the swim-up and pellet from either patients or controls. Patient-to-patient heterogeneity was found for all chromosomes analyzed.

Diploid Sperm in the Swim-up (SU) and Pellet Fractions from OAT and Controls

The frequency of diploid sperm in patients' swim-up fraction was between 0.0 and 1.7% and 0.0 to 1.4% in pellets (Table 17). In contrast, from 0.0 to 0.3% diploid sperm was found in swim-up fractions of controls and between 0.3 to 1.1% was found in pellets. The incidence of diploidy differed between the swim-up and pellet fractions for both OAT patients and controls. OAT patients

showed a significantly higher ($p<0.05$) frequency of diploid sperm in the pellet as compared to the swim-up fraction. Controls showed a significantly higher ($p<0.05$) percent of diploid sperm, when using the chromosome 1,18 and 21 probe set (Set II), in the pellet versus the swim-up fraction. For both probe sets, patient-to-patient heterogeneity was observed.

Aneuploidy Detection in the Swim-up (SU) and Pellet Fractions from OAT and Controls

Per chromosome aneuploidy for patients' swim-up fraction ranged between 0.0 and 10% and 0.3 to 3.9% in pellets (Table 18). Controls' per chromosome aneuploidy ranged from 0.0 to 0.6% in swim-up and 0.0 to 0.6% in pellets. The mean aneuploidy (sum of disomy and nullisomy) frequencies for chromosomes 1,13,18, 21 X and Y were significantly higher ($p<0.05$) in the OAT patients' swim-up fraction and pellet as compared to controls. No significant differences were detected between the swim-up and pellet from either patients or controls. Due to the heterogeneity in aneuploidy both within and between OAT patients, the mean data can only be used as estimates.

Total Aneuploidy in the Swim-up (SU) and Pellet Fractions from OAT and Controls

Using formulae developed by Hoegerman et al. (1996), the mean total aneuploidy in the swim-up fraction from patients was 40.3% and 34.2% in

pellets. In contrast, the mean total aneuploidy in controls swim-up was 6.81% and 8.81% in pellets.

Results of ICSI Using Sperm from the Swim-Up of OAT Patients (Table 19)

Eleven series of ICSI and 11 embryo transfers were performed in ten couples. The overall fertilization rate was 70% (range 33 - 94%). The overall pregnancy rate was 20%. Eight of ten couples had embryo transfer with no resultant pregnancy. One couple has an ongoing singleton pregnancy subsequent to transfer of 5 embryos. The remaining couple did not achieve pregnancy from initial transfer of 3 embryos. However, in the following cycle, transfer of cryopreserved embryos yielded an ongoing pregnancy.

Discussion

Fluorescence in situ hybridization (FISH) is a powerful technique to determine aneuploidy in unselected sperm. Hoegerman et al. (1996) developed formulae for the estimation of per chromosome aneuploidy rates, diploidy frequencies, and the estimation of the total frequency of aneuploid sperm. Use of these formulae and a comprehensive review comparing aneuploidy observed for the chromosomes of male pronuclei with data on sperm chromosomes using 2 or 3-probe FISH, suggests that between 1.9 and 7.7% of sperm from normal donors are aneuploid (Pang et al., 1996a,b; Hoegerman et al., 1996). Pang et al. (1996a) has shown that between 33 and 74% of sperm from nine severely

affected OAT patients were aneuploid and no ongoing pregnancies resulted from six series of ICSI involving these patients. Patient -to-patient heterogeneity was noted.

In an attempt to more clearly understand the relationship between aneuploid sperm and fertilization or pregnancy rates, we used 3-probe, 3-color FISH on sperm from ten OAT patients and four proven fertile donors. We examined the effect that sperm selection by swim-up may have on separating aneuploid sperm from chromosomally normal sperm.

Aneuploidy was significantly higher in the OAT patients as compared to the normal fertile donors. Using formulae by Hoegerman et al. (1996), the frequency of aneuploidy in the OAT patients' ranged between 35 and 52% (mean of 40.3% in the swim-up and 34.2% in the pellet). There were no significant differences in the aneuploidy frequencies between the swim-up and pellet fractions. The diploid sperm predominantly remained in the pellet for both OAT and normal controls. The frequency of disomy for chromosomes 1, 13, 18, 21, X and Y was significantly higher in the swim-up and pellet fractions from all OAT patients as compared to donors. The frequency of nullisomic sperm was also significantly greater in the swim-up and pellet fractions in the OAT patients when compared to fertile donors. The range of per chromosome disomic sperm in the OAT patients was between 0 and 3.7%. In contrast, the frequency of per

chromosome nullisomic sperm in the OAT patients was from 0 to 3.3%. The mean frequency of nullisomic sperm from the OAT patients was higher versus controls.

In this study and that of Pang et al. (1996a) the frequency of sperm with no signal or nullisomic for a particular chromosome in OAT patients was consistently higher in OAT patients versus normal controls. This increase may reflect experimental error associated with sperm decondensation and / or hybridization failure. This explanation is unlikely since normal controls and OAT samples were decondensed and hybridized at the same time. Inadequate sperm decondensation of the OAT samples is also unlikely because some sperm from the OAT patients' were enlarged and overly decondensed showing multiple FISH signals suggestive of chromatin degradation. Optimized sperm head decondensation and FISH protocols have been extensively evaluated and optimized by Pang et al.(1996b). The current results showed elevated levels of aneuploidy in sperm from OAT patients as compared to controls. However, the percent and type of aneuploidy was random which suggests that loss (or gain) of particular chromosomes may be associated with errors in meiotic checkpoints, causing spermatogenesis arrest, desynapsis, lack of proper chiasmata formation and random aneuploidy.

In this study, patient-to-patient heterogeneity was observed for the OAT

patients but to a lesser degree than previously reported by Pang et al. (1996a). The semen parameters for the nine OAT patients evaluated by Pang et al. (1996) were more severe and the percent of aneuploid sperm was higher (33-74%) as compared to the current study (35-52%). It is possible the type and percent of aneuploid sperm found in infertile males may be correlated with the type and severity of male factor infertility.

Trisomy is the most common type of chromosomal abnormality found in spontaneous abortions. The overall frequency of chromosome abnormalities in first trimester spontaneous abortions is at least 50% (Hassold et al., 1978). The overall miscarriage rate is approximately 15%. The most common abnormality found in abortuses is 45,X (Turner Syndrome), which is found in approximately 18% of chromosomally abnormal spontaneous abortuses (Thompson et al., 1991). In contrast, only 0.6% of chromosomally abnormal live births have this aberration. Monosomies for chromosomes 13 and 18 have never been seen in miscarriages or livebirths. Monosomy 21 has only been found in tissue from miscarriages. Aneuploidy for chromosome 1 has never been seen in products of conception or livebirths. Trisomies for chromosomes 13, 18 and 21 are found in spontaneous abortuses, however, many of these trisomic fetuses survive to term.

The frequencies of trisomic 13, 18, and 21 livebirths are approximately

1/5000, 1/3000, and 1/800 respectively. These trisomies are associated with advanced maternal age. The incidence of XXX is 1/1000; the frequency of XXY is 1/1000; and XYY is found in approximately 1/1000 livebirths. These trisomies may be associated with advanced maternal age. However, can paternal nondisjunction significantly contribute to offspring with numerical sex chromosome abnormalities? Molecular studies have shown 44% of XXY concepti were of paternal origin (Koehler et al., 1996). Studies of Tournaye et al. (1995) and In't Veld et al. (1995) have shown at least a 3-4 fold elevated frequency of sex chromosome aneuploidy in the offspring of infertile males undergoing ICSI. However, the observed numbers of sex chromosome abnormalities are low and larger studies must be performed in order to more clearly understand this association. In any event, this data suggests a possible chromosome specific mechanism for numerical sex chromosome abnormalities as a result of paternal nondisjunction.

Of the ten OAT patients' examined in this study, eleven series of ICSI and 11 embryo transfers were performed. Eight of 10 couples had embryo transfer with no resultant pregnancy. One couple has an ongoing singleton pregnancy subsequent to transfer of five embryos. The remaining couple did not achieve pregnancy from initial transfer of 3 embryos. However, in the following cycle, transfer of cryopreserved embryos yielded an ongoing pregnancy. The overall fertilization rate was 70% (range 33 - 94%). The overall pregnancy rate was

20%. No correlation was observed between sperm aneuploidy and successful pregnancy. Cytogenetic follow-up is proposed to evaluate the offspring for chromosome abnormalities.

In this study, the fertilization rate was 70% and the pregnancy rate was 20%. This reduced fertilization frequency and low pregnancy rate may be associated with aneuploidy. Aneuploidy may be associated with lack of implantation and the ability to carry a fetus to term.

Wilcox et al. (1988) has shown a high proportion of conceptions are lost between implantation and the first missed menses. Embryonic loss also occurs between fertilization and implantation. However, correlating such early fetal loss with cytogenetic abnormalities is complex because collection of products of conception is difficult and no study has substantially reported on the cytogenetic component of this tissue. Preimplantation genetic diagnosis of the embryo is one way to directly correlate aneuploidy with implantation and ongoing pregnancy with resultant karyotypical normal offspring.

In this study we showed that approximately 40% of sperm from ten OAT patients' are aneuploid. Patient-to-patient heterogeneity exists and only 2/10 (20%) males have successfully contributed to ongoing pregnancies. The swim-up separation technique failed to isolate more normal haploid sperm from

aneuploid sperm in all patients' evaluated. Different sperm separation techniques (i.e. - Percoll gradient) may enrich for haploid sperm. Additional studies are required.

Table 14. Age and semen parameters of OAT patients and controls.

Subject	Age (yr.)	Volume (ml)	Count (X 10^6/ml)	Motility (%)	Normal form (%)
OJ	34	3.5	14.50	18.0	3.0
OK	32	2.5	3.70	49.0	10.0
OL	36	5.3	2.70	30.0	2.0
OM	40	1.0	11.20	23.0	5.5
ON	36	2.0	9.60	38.0	10.0
OO	33	4.0	6.80	50.0	10.0
OP	38	2.0	1.20	17.0	4.0
OQ	34	1.6	1.40	24.0	5.5
OR	47	2.3	4.70	30.0	5.0
OS	42	5.8	2.10	27.0	5.0
FE	30	3.0	67.5	68.0	14.0
FF	45	2.0	36.90	67.0	16.0
FG	34	2.0	35.60	74.0	14.0
FH	35	5.5	50.60	76.0	15.0

Table 15. Incidence (%) of autosomal and gonosomal disomy in OAT patients and controls, in pellet and swim-up.

	Sex Chromosome		Chromosome 1		Chromosome 13		Chromosome 18		Chromosome 21	
Subject	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU
OJ	0.8	0.7	0.5	1.0	1.1	1.3	0.1	0.0	0.2	0.0
OK	0.6	0.7	0.3	0.0	0.7	1.0	0.3	0.3	0.1	1.0
OL	1.0	1.3	0.0	0.7	0.5	0.7	1.6	3.7	0.4	0.7
OM	1.3	1.3	0.6	0.7	1.1	0.7	1.3	2.0	0.4	0.3
ON	1.0	1.3	0.4	0.7	0.7	1.7	1.1	1.0	0.7	1.7
OO	1.2	0.3	0.5	0.0	1.1	0.7	1.2	0.7	0.5	0.3
OP	1.0	1.0	1.5	1.0	1.5	1.7	1.2	1.3	1.0	0.3
OQ	2.1	2.7	1.9	1.0	0.5	0.3	0.5	0.3	1.1	0.3
OR	0.4	0.0	0.1	0.3	0.5	1.0	0.2	0.3	0.2	0.0
OS	1.4	2.0	0.4	0.3	1.1	1.3	0.2	0.0	0.2	0.0
Mean	1.08	1.13	0.62	0.57	0.88	1.03	0.77	0.96	0.48	0.46
FE	0.3	0.3	0.2	0.3	0.3	0.0	0.1	0.0	0.0	0.0
FF	0.3	0.0	0.3	0.0	0.3	0.1	0.0	0.0	0.1	0.3
FG	0.7	0.3	0.5	0.0	0.4	0.1	0.4	0.3	0.2	0.0
FH	0.6	0.3	0.1	0.3	0.3	0.2	0.2	0.3	0.3	0.3
Mean	0.48	0.23	0.28	0.15	0.33	0.10	0.18	0.15	0.15	0.15

Table 16. Incidence (%) of autosomal and gonosomal nullisomy in OAT patients and controls, in pellet and swim-up.

	Sex Chromosome		Chromosome 1		Chromosome 13		Chromosome 18		Chromosome 21	
Subject	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU
OJ	1.3	1.3	1.2	2.3	2.3	2.0	0.8	0.7	0.5	1.7
OK	1.7	2.0	0.1	3.0	1.3	2.7	2.O	2.0	3.0	4.3
OL	2.4	1.7	0.5	0.7	3.1	3.0	1.6	6.3	1.7	1.3
OM	2.1	3.0	0.6	0.7	1.2	0.7	2.6	3.3	1.9	1.3
ON	0.5	0.7	0.8	1.0	0.7	0.3	1.4	1.0	0.3	1.0
OO	0.3	0.0	0.6	0.0	0.9	1.3	1.0	0.7	0.7	0.3
OP	1.1	0.7	1.7	1.0	2.0	2.0	0.8	0.3	0.7	0.0
OQ	0.8	0.7	0.9	1.3	2.1	3.0	0.9	0.7	2.0	1.7
OR	0.2	0.0	0.3	0.0	0.1	0.0	0.2	0.3	0.1	0.0
OS	1.1	1.3	0.1	0.0	0.4	0.3	0.4	0.7	0.3	0.0
Mean	1.15	1.13	0.68	1.00	1.41	1.53	1.17	1.60	1.12	1.70
FE	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF	0.1	0.0	0.1	0.0	0.3	0.0	0.2	0.3	0.4	0.0
FG	0.7	0.3	0.3	0.3	0.2	0.3	0.2	0.0	0.3	0.3
FH	0.0	0.0	0.0	0.0	0.2	0.3	0.0	0.0	0.1	0.0
Mean	0.25	0.08	0.15	0.08	0.18	0.15	0.10	0.08	0.20	0.08

Table 17. Incidence (%) of diploidy in OAT patients and controls, in pellet and swim-up

	Chromosome X, Y and 13		Chromosome 1, 18 and 21	
	Pellet	SU	Pellet	SU
OJ	0.0	0.3	O.1	0.0
OK	0.8	0.3	0.9	0.0
OL	0.7	0.3	0.1	0.0
OM	0.4	0.0	0.3	0.3
ON	0.3	0.7	1.4	0.3
OO	0.5	0.0	0.3	0.0
OP	1.2	1.7	0.5	0.7
OQ	0.4	0.0	0.4	0.0
OR	0.7	0.3	0.1	0.0
OS	1.4	0.0	0.4	0.0
Mean	O.64	0.36	0.45	0.13
FE	0.3	0	0.2	0.3
FF	0.4	0	0.1	0
FG	0.9	0	0.6	0.3
FH	1.1	0	0.9	0
Mean	0.68	0	0.45	0.17

Table 18. Incidence (%) of aneuploidy in OAT patients and controls, in pellet and swim-up.

	Sex Chromosome		Chromosome 1		Chromosome 13		Chromosome 18		Chromosome 21	
Subject	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU	Pellet	SU
OJ	2.1	2.0	1.7	3.3	3.4	3.3	0.9	0.7	0.7	1.7
OK	2.3	2.7	0.4	3.0	2.0	3.7	2.3	2.3	3.1	5.3
OL	3.4	3.0	0.5	1.4	3.6	3.7	3.2	10.0	2.1	2.0
OM	3.4	4.3	1.2	1.4	2.3	1.4	3.9	5.3	2.3	1.6
ON	1.5	2.0	1.2	1.7	1.4	2.0	2.5	2.0	1.0	2.7
OO	1.5	0.3	1.1	0.0	2.0	2.0	2.2	1.4	1.2	0.6
OP	2.1	1.7	3.2	2.0	3.5	3.7	2.0	1.6	1.7	0.3
OQ	2.9	3.4	2.8	2.3	2.6	3.3	1.4	1.0	3.1	2.0
OR	0.6	0.0	0.4	0.3	0.6	1.0	0.4	0.6	0.3	0.0
OS	2.5	3.3	0.5	0.3	1.5	1.6	0.6	0.7	0.5	0.0
Mean	2.23	2.27	1.30	1.57	2.29	2.57	1.94	2.56	1.60	1.62
FE	0.5	0.3	0.4	0.3	0.3	0.0	0.1	0.0	0.0	0.0
FF	0.4	0.0	0.4	0.0	0.6	0.1	0.2	0.3	0.5	0.3
FG	1.4	0.6	0.8	0.3	0.6	0.4	0.6	0.3	0.5	0.3
FH	0.6	0.3	0.1	0.3	0.5	0.5	0.2	0.3	0.4	0.3
Mean	0.73	0.30	0.43	0.23	0.50	0.25	0.28	0.23	0.35	0.23

Table 19. Results of assisted reproductive technologies using sperm from OAT patients.

Patient	No. of Mature Oocytes	No. of Fertilized Oocytes	Ferilization Rate (%)	Procedure	Outcome
OJ	16	15	93.8	ICSI	No pregnancy
OK	11	10	90.9	ICSI	No pregnancy
OL	11	6	54.5	ICSI	No pregnancy
OM	10	8	80.0	ICSI	No pregnancy
ON	11	10	90.9	ICSI	Pregnant
OO	3	2	66.7	ICSI	No pregnancy
OP	3	1	33.3	ICSI	No pregnancy
OQ	10	9	90.0	ICSI	No pregnancy
OR	10	6	60.0	ICSI	No pregnancy
OS	12	5	41.7	ICSI	No pregnancy

Chapter 6. The Development of a Technique to Correlate Aneuploidy and Sperm Tail Swelling Patterns Following Hypo-osmotic Swelling and Fluorescence In Situ Hybridization (FISH) In Sperm From Oligoasthenoteratozoospermic (OAT) Patients Undergoing ICSI

Introduction

Infertility affects approximately 15% of all married couples (Baker et al., 1986). In about half of these couples, male infertility is the major contributing factor. This underscores the need for an extensive andrological evaluation to be performed at an early stage of the infertility work-up (Bhasin et al., 1994). The severity of male factor infertility appears to correlate with low sperm count, poor morphology, and poor motility. However, deficits in the intricate steps required for normal fertilization, such as binding to the zona pellucida, acrosome discharge, and binding to the oolemma, also significantly contribute to male infertility (Liu and Baker, 1992).

Acosta (1992) has presented a clear distinction between "clinical" male infertility (as diagnosed by WHO semen criteria) and "assisted reproduction" male infertility. Clinical infertility quantitatively measures semen parameters, whereas assisted reproduction infertility qualitatively evaluates sperm fertilizing

ability.

Assisted reproductive technologies provide efficient means to overcome multiple sperm deficiencies with the primary measured outcome being fertilization and pregnancy. There is emerging evidence to suggest that a genetic basis for male factor infertility may exist in many men currently classified as idiopathic.

It is established that somatic cells from infertile men show approximately a 10-fold increase in the incidence of chromosomal anomalies as compared to normal controls (Chandley et al., 1972; De Kretser et al., 1972; Van Zyl et al., 1975; Chandley, 1979; Dutrillaux et al., 1982).

The introduction of intracytoplasmic sperm injection (ICSI) has revolutionized protocols used in in vitro fertilization centers. Even the most severely affected infertile males are able to successfully fertilize eggs, in vitro, using ICSI. The pregnancies resulting from ICSI have been closely scrutinized and there is preliminary evidence suggesting an increased frequency of sex chromosome aneuploidy in the offspring of infertile males following ICSI (Tournaye et al., 1995). To more clearly understand the male gamete contribution to aneuploidy in the offspring of males following ICSI, Pang et al. (1996) used FISH to determine aneuploidy in sperm from nine OAT patients.

Using formulae developed by Hoegerman et al.(1996), the total aneuploidy in controls sperm ranged from 4.1 to 7.7%. In contrast, the total aneuploidy for the nine OAT patients analyzed, was between 33 and 74%. Six series of ICSI were performed; four series failed to establish a pregnancy and two had first trimester spontaneous abortions. Pfeffer et al. (Pfeffer and Pang) determined aneuploidy using FISH in sperm from patients with OAT after isolation of the swim-up fraction. The total aneuploidy for OAT patients was approximately 40%. In contrast, total aneuploidy in controls was 6.8%. There were no differences in aneuploidy between swim-up and pellet fractions. Ten series of ICSI and eleven embryo transfers were performed in ten couples. Eight of 10 couples had embryo transfer with no resultant pregnancy. One couple has an ongoing singleton pregnancy subsequent to transfer of five embryos. The remaining couple did not achieve pregnancy following transfer of three embryos.

Since a functional membrane is a prerequisite for the fertilizing ability of spermatozoa (Rogers and Bentwood, 1982), assessment of this sperm property may be a useful indicator of fertility. The hypo-osmotic swelling test (HOST) assesses the functional integrity of the sperm membrane by evaluating the membrane's response to hypo-osmotic conditions. The HOST is relatively new but it may have clinical usefulness.

Casper et al. (1996) performed a nonrandomized, sequential comparative

study to determine the ability of HOST to differentiate live from dead sperm. No correlation was made between sperm tail swelling pattern and fertilizing ability; only dead sperm was differentiated from live sperm. Randomized sperm injection resulted in fertilization and cleavage rates of 26 and 23% respectively. No pregnancies were achieved. In contrast, injecting live, immotile sperm identified by HOST showed a 43% fertilization rate and a 39% cleavage rate. Three of 8 (37%) couples achieved a viable pregnancy. One pregnancy resulted in the birth of healthy twin boys, however, the other two were lost as spontaneous abortions. No chromosome analysis was performed on the products of conception.

To more clearly understand the biological significance of sperm tail swelling patterns, aneuploidy and fertilizing ability, we performed HOST and FISH on sperm from eleven OAT patients undergoing ICSI and four fertile donor controls.

Materials and Methods

Preparation of Spermatozoa

Human ejaculated spermatozoa were obtained by masturbation from both fertile donors (n=4) and OAT patients (n=11). Sperm count and motility were evaluated according to the WHO semen analysis criteria, using computer-assisted semen analyzer (CTS-60/200 system [Motion Analysis Co., CA]).

Morphology assessment was performed on a fresh smear according to strict morphology criteria (Kruger et al., 1986). Semen specimens were considered OAT if the sperm count <20 X 10^6/ml, progressive motility <50%, and morphology <14% normal forms. In specimens from men with severe OAT, manual semen analysis was performed. Donor's sperm counts varied from 68 X 10^6/ml to 120 X 10^6/ml (mean = 93.9 ± 23.2 X 10^6/ml). Between 65 and 80% (mean = 71 ± 6.4 %) of sperm were motile and normal morphology, by strict criteria, was seen in 16 to 23% (mean = 19.8 ± 3.0 %). All have at least one child. OAT patient's sperm counts varied from 0.05 X 10^6/ml to 17 X 10^6/ml (mean = 8.5 ± 6.3 X 10^6/ml). Between 5 and 41% (mean = 23 .1 ± 12.7 %) of sperm were motile and normal morphology, by strict criteria, was seen in 0 to 8% (mean = 2.2 ± 2.2 %).

All donors and OAT patients abstained from sexual activities and alcohol consumption for three days prior to semen collection. Semen was obtained by masturbation and, after liquefaction, sperm were immediately performed HOST and sperm pretreatment.

HOST and Pretreatment of Semen

The hypo-osmotic solution was prepared by mixing 7.35g sodium citrate and 13.51g fructose in 1000ml distilled water. The final osmotic pressure was 150 mosmol and the calculated ionic strength 0.15 M. A sample of sperm

suspension (1 ml) was mixed with 4 ml phosphate buffered saline (PBS). Following centrifugation (300 g, 5 min), pellets were resuspended in 10 ml prewarmed (37 °C) HOST solution, mixed well, and incubated at 37 °C water bath for 30 min. Following incubation, sperm and HOST mixture was centrifuged and the pellets were resuspended in 100-200 ml HOST solution. A drop of the suspension was smeared onto a clean slide, allowed to air dry, fixed with fresh fixative (3 parts methanol: 1 part glacial acetic acid), and short term slide storage was at 4 °C.

The air dried slides were incubated at 37 °C for 45 min in 2 mM dithiothreitol and allowed to air dry. The slides were denatured by 70% formamide/2XSSc solution at 80°C for 5 min. In order to evaluate sperm tail swelling patterns under the fluorescent microscope, denatured specimens were stained with Diff Quick (Baxter, FL), dehydrated successively in 50%, 70%, and 95% ethanol at room temperature. for 3 min each, and allowed to air dry.

FISH

Three-probe three-color FISH was performed using two probe sets as previously described. The first set probed for chromosome X, Y, and 18; the second set probed for chromosomes 1, 13 and 21.

RESULTS

Semen specimens from 11 OAT patients and four proven fertile donors were analyzed. The results of standard semen analysis and the distribution of the various tail swelling types observed after HOST are listed in tables 1, 2 and 3. To assess whether the differential sperm tail swelling patterns observed following HOST is useful in discriminating normal donor sperm from OAT sperm, a comparison between semen parameters and tail swelling patterns was performed (Table 22). The results showed significant differences ($p < 0.05$) in percent types A, B, C, D, E, and F between fertile donors and OAT patients. Type A was significantly higher in OAT samples, whereas types B->F were significantly higher in normal controls. The percent of sperm with tail swelling pattern G showed no significant differences between patients and controls.

The results of HOST and aneuploidy in OAT patients and controls are presented in Table 23. In fertile controls, aneuploid sperm was found in tail swelling patterns A, B, D, F and G. Most aneuploid sperm was in sperm with tail swelling pattern F, followed by types G and A. In contrast, for OAT patients, aneuploid sperm was found in all tail swelling types except type C. Diploid sperm was only found in types A and G for controls and OAT patients.

DISCUSSION

Data suggests that sperm tail swelling pattern's A, B, D, E, F and G may be aneuploid in OAT patients. No aneuploidy was found in sperm with a C type

tail swelling pattern in either OAT or normal controls. Diploid sperm in controls and OAT patients are only found in types A and G tail swelling patterns. Preliminary data suggests that there may be a correlation between HOST and aneuploidy for OAT patients. However, further studies are required.

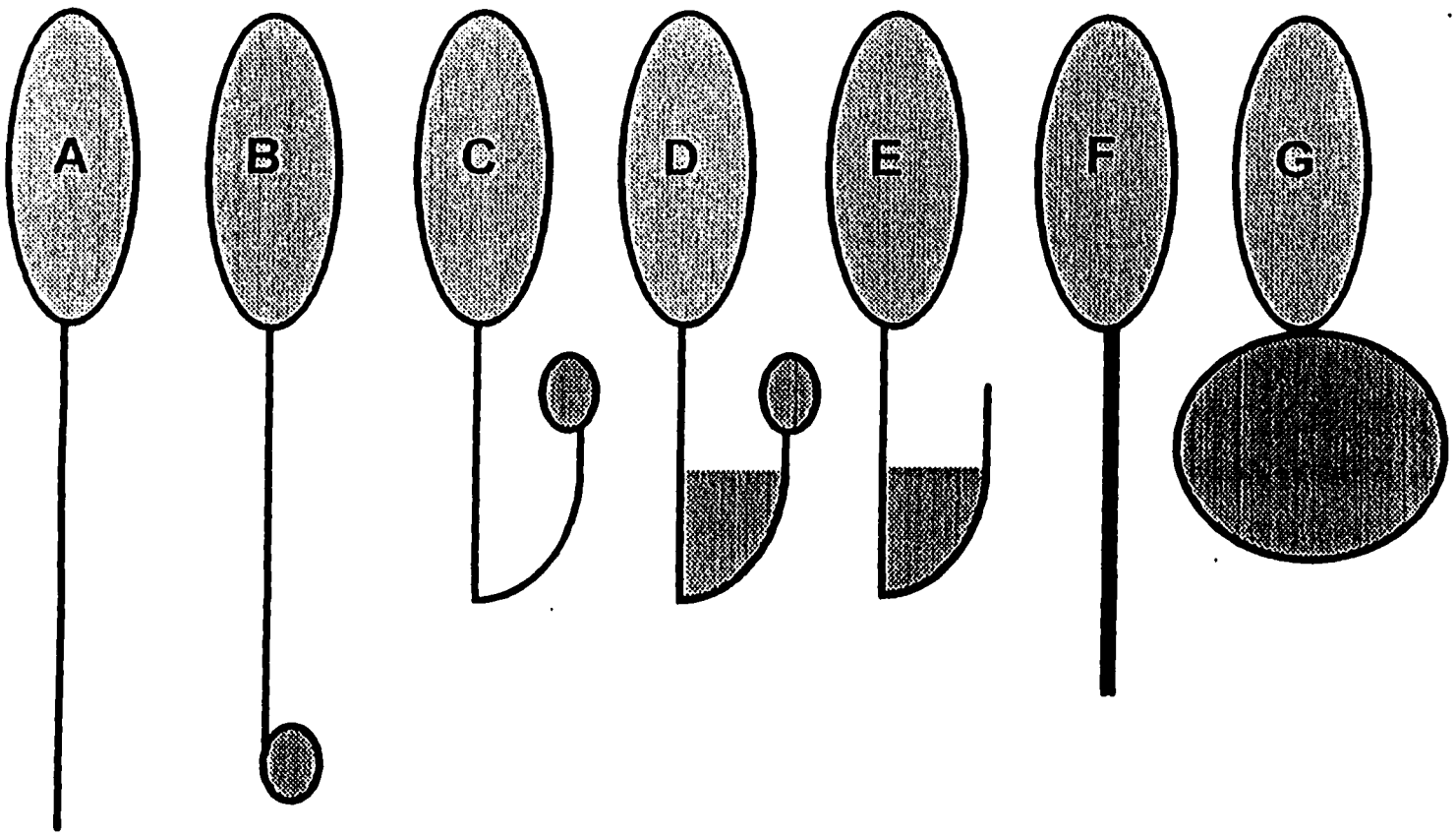

Figure . Schematic representation of typical morphological changes of human spermatozoa subjected to hypoosmotic stress: a; no change, b-g; various types of tail changes, b-d; tail-tip swelling

Table 20. Age and semen parameters of OAT patients and controls.

Subject	Age (yr.)	Volume (ml)	Count (X 10^6/ml)	Motility (%)	Normal form (%)
OT	37	3.2	17.00	41.0	4.0
OU	33	2.5	14.00	6.1	8.0
OV	35	1.9	2.00	34.8	2.0
OW	43	4.2	2.00	40.0	2.0
OX	30	3.7	10.70	27.2	2.5
OY	29	7.1	6.00	25.0	0.0
OZ	38	2.7	15.00	20.0	2.0
OAA	42	2.3	10.00	20.0	1.0
OAB	33	5.3	1.80	5.0	1.0
OAC	36	3.4	0.05	5.0	1.0
OAD	41	3.8	15.00	30.0	2.2
FI	31	3.8	68.00	80.0	19.0
FJ	32	3.9	120.00	69.0	16.0
FK	36	4.8	105.00	70.0	21.0
FL	35	5.1	83.00	65.0	23.0

Table 21. Sperm tail swelling patterns, total swelling (%), tail-tip swelling (%) in fertile donor and OAT patient.

	Tail Swelling Pattern (%)								
Subject	A	B	C	D	E	F	G	% SWELLING	% TIP SWELLING
FI	36.83	13.51	11.47	9.52	6.41	8.75	13.51	63.17	34.50
FJ	29.03	20.14	4.69	2.35	15.93	12.12	15.74	70.97	27.17
FK	23.99	19.63	5.65	4.66	6.80	12.61	26.66	76.01	29.95
FL	16.97	34.88	3.98	3.41	5.31	7.77	27.68	83.03	42.27
Mean	26.71	22.04	6.45	4.99	8.61	10.31	20.90	73.30	33.47
OT	52.66	10.46	3.60	2.49	4.89	3.34	22.56	47.34	16.55
OU	91.52	0.56	0.28	0.39	0.39	2.23	4.63	8.48	1.23
OV	62.57	8.74	3.83	1.38	2.46	2.85	18.17	37.43	13.95
OW	54.38	14.18	1.49	2.43	2.99	2.71	21.83	45.62	18.10
OX	70.32	8.73	1.84	0.78	1.26	3.59	13.48	29.68	11.35
OY	69.66	9.92	2.55	2.27	0.85	0.95	13.80	30.34	14.74
OZ	78.11	4.58	0.80	0.40	0.60	0.30	15.22	21.89	5.77
OAA	76.23	2.04	0.84	0.46	1.67	2.32	16.43	23.77	3.34
OAB	90.06	1.72	0.15	0.25	0.54	0.84	6.45	9.69	2.13
OAC	93.27	0.63	0.26	0.26	0.31	0.47	4.80	6.73	1.15
OAD	65.83	6.69	0.94	1.34	1.59	1.34	22.28	34.17	8.98
Mean	73.15	6.20	1.51	1.13	1.60	1.90	14.51	26.83	8.84

Table 22. Comparison of semen parameters and HOST between fertile group (n=4) and OAT group (n=11).

Variables	Fertile Group	OAT Group	P-value
Semen Analysis			
Concentration ($X10^6$/ml)	93.9 ± 23.2	8.5 ± 6.3	P = 0.000
% Motility	71.0 ± 6.4	23.1 ± 12.7	P = 0.000
% Normal Morphology	19.8 ± 3.0	2.2 ± 2.2	P = 0.000
HOST			
% Type A	26.7 ± 8.4	73.2 ± 14.3	P = 0.000
% Type B	22.0 ± 9.1	6.2 ± 4.6	P = 0.000
% Type C	6.45 ± 3.4	1.5 ±1.3	P = 0.001
% Type D	5.0 ± 3.2	1.1 ± 0.9	P = 0.002
% Type E	8.6 ±4.9	1.6 ± 1.4	P = 0.000
% Type F	10.3 ± 2.4	1.9 ± 1.2	P = 0.000
% Type G	20.9 ± 7.3	14.5 ± 6.8	P = 0.134
% Total Swelling	73.3 ± 8.4	26.8 ± 14.3	P = 0.000
% Tail-tip Swelling	33.5 ± 6.6	8.8 ± 6.5	P = 0.000

Table 23. Aneuploidy and diploidy frequencies in OAT patients and fertile donors related with tail swelling pattern.

Swelliing Pattern	Aneuploidy		Diploidy	
	Fertile Group	OAT group	Fertile Group	OAT group
A	0.80 (14/1751)	2.90 (323/11008)	0.11 (2/1751)	0.25 (28/11008)
B	0.10 (1/970)	0.50 (4/803)	0.00 (0/970)	0.00 (0/803)
C	0.00 (0/272)	0.00 (0/189)	0.00 (0/272)	0.00 (0/153)
D	0.46 (1/218)	0.65 (1/153)	0.00 (0/218)	0.00 (0/153)
E	0.00 (0/373)	0.95 (2/211)	0.00 (0/373)	0.00 (0/211)
F	1.71 (8/467)	8.92 (24/269)	0.00 (0/467)	0.00 (0/249)
G	0.85 (8/943)	6.90 (134/954)	0.11 (1/943)	1.02 (20/954)

Conclusions and Accomplishments

We have contributed to the development of formulae for the estimation of per chromosome aneuploidy rates, diploidy frequencies, and the estimation of the total frequency of aneuploid sperm cells. Using FISH, total aneuploidy can only be determined by simultaneous 24-probe, 24-color experiments; an impossible experimental technique at the present time. However, the developed formulae provides a close estimation of total aneuploidy in sperm following multi-probe, multi-color FISH. The accurate determination of aneuploidy frequencies of sperm will significantly improve our understanding of the cytogenetics of spermatogenesis.

We have provided a comprehensive review of the literature comparing aneuploidy observed in the chromosomes of male pronuclei, as seen after fertilization of hamster eggs by human sperm, with data on sperm chromosomes studied by FISH. Our analysis of data on the chromosomes of the male pronuclei showed the frequency of aneuploidy to be between 1.9 and 2.9%. In contrast, the mean frequency of aneuploid sperm observed following FISH was between 6.6 and 7.5%. This discrepancy suggests either that aneuploid sperm have diminished fertilized capacity, that aneuploidy is not randomly distributed among sperm cells, or that current FISH protocols and scoring criterion result in an overestimation of aneuploid sperm.

To optimize techniques to accurately determine disomy and nullisomy frequencies in sperm following FISH we developed specialized decondensation and FISH protocols. These experimental techniques significantly reduced the number of sperm cells observed with no signal (as compared to published data). In controls, the mean hybridization efficiency was 99.6%.

Using multi-probe, multi-color FISH and developed formulae, we determined total aneuploidy in sperm from proven fertile donors and OAT patients. In controls, the total aneuploidy ranged between 4.1 and 7.7%. For OAT patients, the total aneuploidy was between 43 and 69%. Diploidy was found in 0.04% of sperm in controls and ranged between 0.4 and 9.6% in OAT patients. No consistent chromosome gain or loss was found in OAT patients; patient to patient heterogeneity was present.

To more clearly understand the relationship between aneuploid sperm and fertilization or pregnancy rates, we used three-probe, three-color FISH to examine the effect sperm selection by swim-up has on separating haploid from aneuploid sperm. In this study, between 35 and 52% of sperm from OAT patients in the swim-up fraction and 36 to 49% from the pellet were aneuploid. No significant difference was observed. However, the percent of diploid sperm predominantly resided within the pellet for all OAT and proven fertile donors. These results suggest that the swim-up separation technique does not separate

aneuploid from normal haploid sperm. However, diploid sperm remains in the pellet fraction. Different sperm separation techniques (ie - Percoll gradient) may enrich for haploid sperm and further studies are required. Patient to patient heterogeneity was observed.

We compared semen parameters (count, morphology and motility) and aneuploidy for all OAT patients analyzed. The data suggests that the type and percent of aneuploid sperm may be inversely related to semen parameters.

In an attempt to optimize conditions for the selection of normal haploid sperm to be used for ICSI, we developed a technique to correlate sperm tail swelling patterns (HOST) and aneuploidy (FISH). We performed HOST and FISH on sperm from eleven OAT patients undergoing ICSI and four fertile donor controls. Data suggests that sperm tail swelling patterns A, B, D, E, F and G may be aneuploid in OAT patients. No aneuploidy was found in sperm with a C type tail swelling pattern in either OAT or normal controls. Diploid sperm in controls and OAT patients were only found in types A and G tail swelling patterns. Preliminary data suggests that there may be a correlation between HOST and aneuploidy for OAT patients. However, further studies are required.

Of the 30 OAT patients studied, 26 series of ICSI were performed. Sixty-eight percent of oocytes fertilized (165 embryos / 242 oocytes). Following fresh

embryo transfer, 1 preclinical abortion, 1 first trimester loss, and 2 term deliveries (2/26; 7.7%) occurred.

Our data show significant increases in the frequencies of diploidy, autosomal disomy, autosomal nullisomy, sex chromosome number, and total cytogenetic abnormalities in sperm from OAT patients versus controls. The data suggest that meiotic errors occur at highly elevated frequencies in the germ cells of severely affected OAT patients.

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