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Germ-Cell Nondisjunction in Testes Biopsies of Men With Idiopathic Infertility

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Summary

Intracytoplasmic sperm injection (ICSI) has been used in combination with testicular sperm extraction to achieve pregnancies in couples with severe male-factor infertility, yet many of the underlying genetic mechanisms remain largely unknown. To investigate nondisjunction in mitotic and meiotic germ cells, we performed three-color FISH to detect numeric chromosome aberrations in testicular tissue samples from infertile men confirmed to have impaired spermatogenesis of unknown cause. FISH was employed to determine the rate of sex-chromosome aneuploidy in germ cells. Nuclei were distinguished as haploid or diploid, respectively. The overall incidence of sex-chromosome aneuploidy in germ cells was found to be significantly higher ($P < .00001$) in all three abnormal histopathologic patterns (range 39.0%–43.5%) as compared with normal controls (29.1%). The relative ratio of normal to aneuploid nuclei in the diploid cells of patients with impaired spermatogenesis was ~1.0, a >300% decrease when compared with the 4.42 ratio detected in patients with normal spermatogenesis. These results provide direct evidence of an increased incidence of sex-chromosome aneuploidy observed in germ cells of men with severely impaired spermatogenesis who might be candidates for ICSI with sperm obtained directly from the testis. The incidence of aneuploidy was significantly greater among the diploid nuclei, which suggests that chromosome instability is a result of altered genetic control during mitotic cell division and proliferation during spermatogenesis.

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Introduction

In the United States, ~10% of the population suffer from infertility (Office of Technology Assessment, United States Congress 1988). Male-factor abnormalities are the predominant cause in 30% of cases and are contributory in 50% (Office of Technology Assessment, United States Congress 1988). Most cases of male infertility are a consequence of altered spermatogenesis giving rise to an inadequate quantity or quality of sperm; yet the etiology of impaired spermatogenesis is frequently idiopathic, because the factors that contribute to this problem are often undefined. A genetic etiology has been suggested as playing a role in some cases of otherwise idiopathic infertility (Tiepolo and Zuffardi 1976; Reijo et al.1995; Mak and Jarvi 1996). Although it has been proposed that mutations in genes that regulate germ-cell migration, proliferation, and differentiation may be involved, few genes or genetic mechanisms have been identified. Consequently, relatively little effective therapy has been established for men with impaired sperm production.

Assisted reproductive techniques (ART), such as intracytoplasmic sperm injection (ICSI) with in vitro fertilization (IVF), have enabled the use of suboptimal semen specimens to achieve pregnancy (Palermo et al. 1992). Similarly, for individuals with maturation arrest, hypospermatogenesis, or other severe defects in sperm development, IVF and microinjection of ejaculated round cells or spermatid nuclei with no tails (Tesarik et al. 1995) may overcome infertility. A major concern associated with the use of ICSI for the treatment of couples with male-factor infertility is the safety of the procedure. In addition, the risk of selecting abnormal sperm with gene and/or chromosome aberrations remains unclear. Therefore, a better understanding of the potential risks associated with the success of this technique, and the development of alternative therapeutic options other than ART, require elucidation of the molecular mechanism causing infertility in these male patients.

There are a number of reports that show that infertile

men have a 10-fold–higher incidence of constitutional chromosomal abnormalities than fertile men (Egozcue et al. 1983; Rosenmann et al. 1985; Luciani et al. 1987). However, these studies involved on men with sperm in their ejaculate. Given that testicular tubular alterations may cause abnormalities in the meiotic process resulting in sperm aneuploidy, merely to analyze peripheral blood lymphocytes for chromosomal abnormalities in infertile men is insufficient.

FISH has been used to study chromosome nondisjunction in human sperm. FISH studies of human sperm have been performed to quantify chromosome-specific aneuploidy (Bischoff et al. 1994; Spriggs et al. 1996) and the effects of increasing age (Martin and Rademaker 1988; Griffin et al. 1995; Kinakin et al. 1997). In addition, FISH has been used to examine aneuploidy in semen from infertile men (Miharu et al. 1994; Moosani et al. 1995; Lahdetie et al. 1997; Guttenbach et al. 1997) and to correlate abnormal sperm morphology with chromosome aneuploidy (Martin and Rademaker 1988; Lee et al. 1996; Yurov et al. 1996; Bernardini et al.1997). In general, these studies provide only an overall estimate of aneuploid frequency, since they focus on the end result of the long process of spermatogenesis. Given that germ-cell degeneration can occur during mitotic cell division of spermatogonia or during meiosis, FISH done on mature sperm from semen samples limits the information that may be obtained regarding stage-specific male nondisjunction. Furthermore, in studies of male infertility, patients may not always be able to provide adequate numbers of sperm for analysis of nondisjunction. Thus, a more efficient study of nondisjunction in infertile men and evaluation of the incidence of chromosome aneuploidy associated with mitotic and meiotic stages of spermatogenesis requires direct analysis of testicular spermatogenic cells.

To test the hypothesis that an increased incidence of nondisjunction occurs in severe spermatogenesis defects, we performed three-color FISH on testis tissue biopsy specimens to evaluate the frequency of sex-chromosome aneuploidy among infertile patients with impaired spermatogenesis who required testicular sperm extraction (TESE) to obtain sperm for ICSI. Infertile patients who were confirmed to have normal spermatogenesis were used as controls. Using an internal control chromosome 18–specific probe to distinguish diploid and haploid nuclei, we determined the sex-chromosome aneuploidy rate among mitotic (diploid) and meiotic (haploid) spermatogenic cells. This is the first study to provide direct evidence of an increased aneuploidy rate in both mitotic and meiotic spermatogenic cells of patients who are candidates for TESE-ICSI-IVF because of their significantly abnormal spermatogenesis.

Samples and Methods

Testis Tissue Samples

Paraffin-embedded testicular tissue samples were retrieved from archival specimens. Tissue samples were obtained from patients confirmed, by histological examination, to have hypospermatogenesis ("hypo"; $n = 5$), maturation arrest at the spermatid stage ("late"; $n = 4$), and maturation arrest at the spermatocyte stage of spermatogenesis ("early"; $n = 6$). Testis tissue samples from infertile individuals who had undergone clinical evaluation and subsequently were confirmed to have normal spermatogenesis by histology were used as controls ("normo"; $n = 5$). Infertility in these control patients was a result of obstructed vas deferens or epididymis. Tissue samples were collected under a study protocol approved by the Baylor College of Medicine Institutional Review Board. Archival tissue samples were serially cut into 4-μm–thick sections and fixed on standard histology glass slides.

Tissue Pretreatment

We removed paraffin by placing the slides in Coplin jars with xylene for 10 min, followed by a 10-min incubation in 100% ethanol and air-drying. Tissue sections were pretreated in 0.5 mg proteinase K/ml at 43°C for 10 min and washed in 2 × SSC (sodium chloride and sodium citrate) for 3 min at room temperature. After serial 70%, 90%, and 100% ethanol dehydration incubations, for 2 min each, slides were allowed to air dry. Slides were used immediately for FISH when pretreatment was optimal (i.e., when nuclei appeared dark and flat).

FISH

Three-color FISH was performed, as described elsewhere, with direct-labeled chromosome-specific alpha-satellite probes, to identify chromosomes X, Y, and 18 (Hilsenrath et al. 1997). We applied the probe mixture to the target tissue section, using a 22-mm × 22-mm glass coverslip. The slide was then placed in an 80°C oven for 2.5 min to denature cellular DNA and probes simultaneously. After overnight hybridization at 37°C, the slide was washed in 0.25 × SSC (pH 7.0) at 67°C for 5 sec and rinsed in 1 × postwash detergent (ONCOR) for 1 min. Nuclear counterstain, 4,6-diamidino-2-phenylindole (DAPI II solution; Vysis), was applied. Cells were viewed with a Zeiss Axioskop microscope (Carl Zeiss) equipped with multiband pass filters.

Scoring Criteria

All the slides were scored in a blinded fashion, such that the observer had no knowledge of the pathologic diagnosis of each case. The chromosome-specific probes were identified by color, and the nuclei were analyzed for the presence of zero, one, two, or three or more signals, for each of the three probes. Nuclei containing signals that were of unexpected size or that appeared to be outside the nuclear membrane were eliminated from analysis. Signals were considered to represent split domains if (1) the size and intensity of each of the two signals was less than that of the signal for the other homologue and (2) the distance between the two signals was less than the diameter of either of the two signals.

Nuclei were grouped as haploid or diploid on the basis of cell size and the number of yellow signals for the control chromosome 18 probe (table 1). Although Sertoli cells were detected and had FISH signals, they were identified on the basis of the presence of their prominent nucleolus and were excluded from germ-cell scoring (fig. 1). In addition, spermatozoa were also detected but they did not contain any FISH signals. Typically, spermatozoa have highly condensed nuclei and require pretreatment in dithiothreitol to make nuclear DNA accessible to FISH DNA probes (Joseph et al. 1984; Coonen et al. 1991). However, to preserve the morphology of the primary and secondary spermatocytes, dithiothreitol pretreatment was not used; hence, spermatozoa did not contain any FISH signals and were not included in this study. Nuclei were grouped as "other" when chromosome 18 aneuploidy was suspected (tables 1 and 2).

Statistical Calculations

The distribution of signals scored within cases of the same pathological category and between groups was tested with the χ^2 goodness-of-fit test. All significance levels were set at $\alpha = .05$.

Results

A total of 9,309 germ cells in 146 tubules were analyzed. For each case, ~465 nuclei (range 436–633) were scored. Representative three-color FISH is shown in figure 1, illustrating detection of normal and aneuploid nuclei identified as either diploid or haploid. Since significant differences were not detected among cases within a designated pathological category, the scores were combined. Table 1 summarizes the frequency of haploid and diploid cells scored in all four histologic categories. Of 2,542 total cells scored for the five normal spermatogenic cases, 81.4% were haploid and 15.6% were diploid. The remaining 3.0% of nuclei were uninformative and likely represented aneuploid nuclei involving the control chromosome 18 probe. The overall frequency of haploid nuclei was lower in the abnormal cases (hypo, 62.9%; early, 58.9%; late, 70.1%) as compared with normal cases (81.4%). In contrast, the overall frequency of diploid nuclei was increased in all three abnormal groups (hypo, 23.5%; early, 31.6%; late, 17.0%) as compared with normal control cases (15.6%).

Significant differences ($P < .00001$) in the distribution of FISH signals between the normal and each of the three histologically abnormal groups were detected (hypo, $\chi^2_4 = 301.7$; early, $\chi^2_4 = 334.1$; late, $\chi^2_4 = 184.2$; table 2). The overall frequency of aneuploid nuclei in patients with normal spermatogenesis was 29.1% (2.9% diploid and 26.2% haploid). In all three abnormal cases, the overall frequency of aneuploid nuclei was significantly greater (hypo, 40.5%; early, 39%; late, 43.5%). The relative ratio of normal to aneuploid nuclei was determined among the haploid and diploid cells scored in all four patient groups (table 3). Among patients with normal spermatogenesis, the ratios of normal to aneuploid cells for the haploid and diploid germ cells were 2.1 and 4.42, respectively. The ratio of normal to aneuploid for the haploid cells was ~1.0 (range 1.04–1.20) among the three abnormal groups, a decrease of about onefold from the 2.1 ratio observed in the normal cases. Similarly, a greater than threefold decrease in the ratio (range 0.86–1.47) of normal to aneuploid diploid cells was detected among the patients with impaired spermatogenesis, regardless of histopathologic pattern.

Discussion

In the current study, a significantly greater incidence of sex-chromosome aneuploidy was detected in germ cells of infertile patients with abnormal spermatogenesis as compared with normal spermatogenesis controls. In all previous sperm FISH studies that have examined male nondisjunction and the incidence of chromosome aneuploidy, using semen specimens, nondisjunction at the mitotic stage could not be distinguished from errors in meiosis (fig. 2). In addition, the reported frequencies of chromosome-specific aneuploidy observed in semen specimens have been variable and do not account for aneuploid germ cells that fail to undergo maturation. Although it has been speculated that germ-cell degeneration is a process for removal of chromosomally abnormal germ cells during spermatocytogenesis and meiosis, there have been no studies to examine male nondisjunction and the actual incidence of chromosome aneuploidy associated with germ-cell degeneration. Therefore, this is the first study in which nondisjunction is evaluated among germ cells at different stages of spermatogenesis. Moreover, these results provide valuable insight about the possible risks associated with the use of ICSI after testicular sperm extraction for treatment of nonobstructive azoospermia.

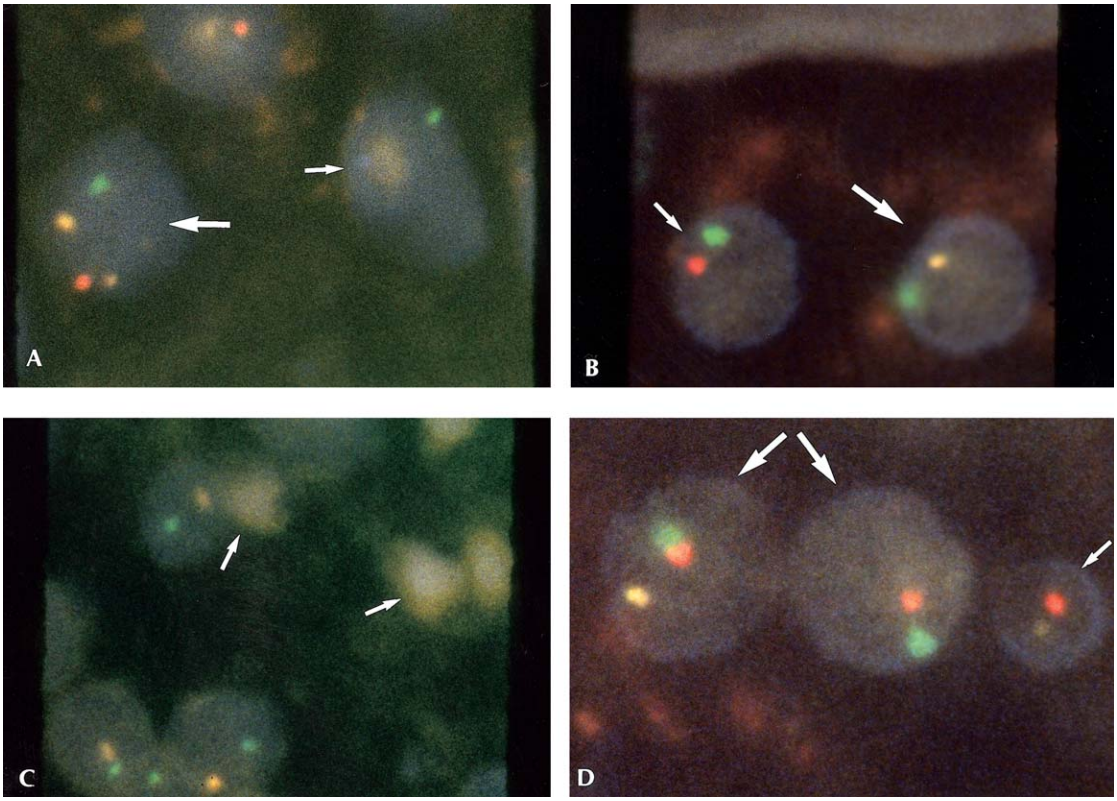

Figure 1 Three-color FISH detection of chromosomes X, Y, and 18 in archival testis tissue samples. Chromosome-specific X and Y probes were labeled with Spectrum-Green (green fluorescence signal) and Spectrum-Orange (red-orange fluorescence signal), respectively (Vysis). Combinatorial mixing of equal volumes of each chromosome 18 Spectrum-Green and chromosome 18 Spectrum-Orange probe produced a chromosome 18 probe with a yellow fluorescence signal. Representative nuclei are illustrated in panels A–D, as follows: *A,* Large arrow identifies a normal diploid cell with two yellow, one green, and one red signal(s). Small arrow indicates Sertoli cells, identified by prominent nucleolus. *B,* Large arrow identifies a normal haploid cell with one yellow and one green signal. Small arrow identifies an XY aneuploid cell with one of each red and green signal. Because of the three-dimensional nature of the cells, the yellow signal for the chromosome 18 probe is on another plane of focus and therefore is not seen in the cell. However, the two nuclei are equivalent in size and both were classified as haploid spermatocytes on the basis of the presence of only one yellow signal. *C,* Arrow identifying mature sperm in the seminiferous tubules containing no FISH signals. *D,* Small arrow identifies a normal spermatid with one yellow and one red signal. Large arrows indicate XY aneuploid haploid nuclei. In one cell, the chromosome 18 signal is on a different plane of focus and cannot be seen. However, the chromosome 18 probe is on the same plane of focus in the adjacent aneuploid haploid cell.

Table 2

Distribution of FISH Signals for Chromosomes X and Y in Normal and Impaired Spermatogenic Cases

Category	No. (%) of Normal Haploid Nuclei	No. (%)of Aneuploid Haploid Nuclei	No. (%) of Normal Diploid Nuclei	No. (%) of Aneuploid Diploid Nuclei	No. (%) of Other Nuclei
Normo	1402 (55.2)	667 (26.2)	323 (12.7)	73 (2.9)	77 (3.0)
Hypo	766 (32.3)	724 (30.6)	322 (13.6)	235 (9.9)	321 (13.6)
Early	768 (32.7)	617 (26.2)	441 (18.8)	301 (12.8)	224 (9.5)
Late	731 (35.7)	705 (34.4)	161 (7.9)	187 (9.1)	264 (12.9)

NOTE.—Among the haploid and diploid cell groups (table 1), normal and aneuploid nuclei were identified on the basis of the number of signals detected for the X and Y chromosomes. Normal haploid cells contained one signal for the control chromosome 18 probe and either an X- or Y-chromosome–specific signal. Aneuploid haploid cells also contained only one control probe signal but with two (XX, YY or XY) or no sex chromosome–specific signals. Normal diploid cells contained two signals for the control probe and one signal for each of the sex chromosomes. Aneuploid diploid cells were identified with two control probe signals, with only one (X or Y) or no sex chromosomes. Nuclei classified as "other" were identified as described in table 1.

The normal spermatogenesis control group in this study consisted of patients with obstructive azoospermia. On the basis of the present study, conclusions regarding the chromosome aneuploidy rate in normal, unobstructed spermatogenesis could not be made, because of the unavailability of such tissue. However, we recently evaluated testis tissue obtained from autopsy of two fertile men who died of heart failure. Our preliminary results show no significant difference in the frequency of aneuploid haploid and diploid germ cells or in the ratio of normal to aneuploid cells between the fertile and obstructed control cases. On the basis of these results, use of the latter group as controls representative of normal spermatogenesis is likely to be valid.

Previous studies have reported the aneuploid frequency for the sex chromosomes to be <1% in mature sperm (Bischoff et al. 1994; Griffin et al. 1995; Spriggs et al. 1995), significantly less than the 26.2% aneuploid haploid frequency observed among the control cases (table 2). Although nuclei in fixed tissue may be smaller in diameter and, thus, may result in more signal overlap as compared with fresh tissue (Munné et al. 1996), this is unlikely to account for the discrepancy between 26.2% and 1%. Other interphase FISH studies on fixed and fresh nuclei have shown no difference in signal detection (Bischoff et al. 1998). Alternatively, the increase in aneuploidy may be a result of the tissue sectioning procedure resulting in portions of cells being removed. However, partial removal of cells and/or signal overlap is likely to occur at random and account for the 3% aneuploid frequency observed among the diploid mitotic cells in the control cases. Therefore the discrepancy is more likely because of the physiological processes that occur during spermatid maturation and selection against abnormal sperm. With degeneration, genetically abnormal germ cells can be eradicated from the seminiferous tubules without proceeding any further in maturation (Johnson 1995). It has also been reported that a significant (36%–45%) loss of germ cells occurs during meiotic division in humans (Barr et al. 1971; Johnson 1982).

Table 3

Relative Ratio of Normal and Aneuploid Nuclei Observed among the Haploid and Diploid Germ Cells

	Ratio of Normal/Aneuploid Nuclei[b]			
Ploidy[a]	Normo ($n = 5$)	Hypo ($n = 5$)	Early ($n = 6$)	Late ($n = 4$)
Haploid	2.10	1.06	1.20	1.04
Diploid	4.42	1.37	1.47	.86

[a] Nuclei were identified as haploid or diploid on the basis of the number of control probe signals detected (table 1).

[b] Nuclei were scored as normal or aneuploid on the basis of the distribution of X and Y FISH probe signals detected. The ratio was calculated with the normal and aneuploid numerical values given in table 2.

Recent studies suggest that apoptosis is one major way that germ cells degenerate (Brinkworth et al. 1995; Blendy et al. 1996; Nantel et al. 1996; Lin et al. 1997). If this degeneration is physiological, however, the purpose and underlying mechanism is unknown.

The increased frequency of XY aneuploid haploid cells owes to meiosis I nondisjunction, possibly resulting from asymmetric pairing of the sex chromosome pair and unstable segregation (Muller et al. 1986; Griffin et al. 1995). In abnormal patients, the overall incidence of sex-chromosome aneuploidy showed a range of 39%–43.5%, with a significant increase in the number of aneuploid diploid cells (table 2). Although a relative increase in the number of aneuploid haploid cells should be expected, increase was observed only in the hypospermatogenic and late–maturation arrest conditions. Inability to detect increased numbers of aneuploid haploid cells in the early–maturation arrest cases is suggestive of possible different genetic mechanisms regulating spermatogenesis. Moreover, the relative increase in the number of aneuploid diploid cells indicates that mitotic nondisjunction rates are increased in all individuals with abnormal spermatogenesis. Increase in mitotic nondisjunction implies a possible role of alterations in checkpoint mechanisms, which regulate spermatogonial cell division.

There is a tendency to assume that ICSI for severe male-factor infertility is safe; however, there are a host of genetic defects in spermatogenesis that may affect male fertility, including chromosome nondisjunction. A major concern associated with the use of ICSI for treatment of couples with male-factor infertility is the safety of the procedure. Abnormal fertilization sometimes occurs, in which the resulting embryos have abnormal morphology and are not transferred. A greater concern is the presence of gene and/or chromosome aberrations in embryos with normal morphology. This study demonstrates substantially increased rates of X and Y nondisjunction in testicular tissue from patients with severe male-factor infertility, which has significance for couples who are candidates for ICSI to achieve a pregnancy. Bonduelle et al. (1996*a,* 1996*b*) and Liebaers et al. (1995) reported finding sex-chromosome aneuploid frequencies more than fivefold higher in the prenatal fetuses conceived by ICSI, when compared with the expected rate. This could have resulted from microinjection of aneuploid sperm. It has also been suggested that a significant subset of patients in the ICSI treatment group may have Klinefelter syndrome or 46,XY/47,XXY mosaicism. Alternatively, these patients with severe male-factor infertility may have had nondisjunction, as found in the present study, resulting in a sex-chromosome aneuploidy in the offspring. In another study, detection of sex-chromosome aneuploidy was reported in 5 of 15 fetuses (In't Veld et al. 1995). Preliminary studies suggest

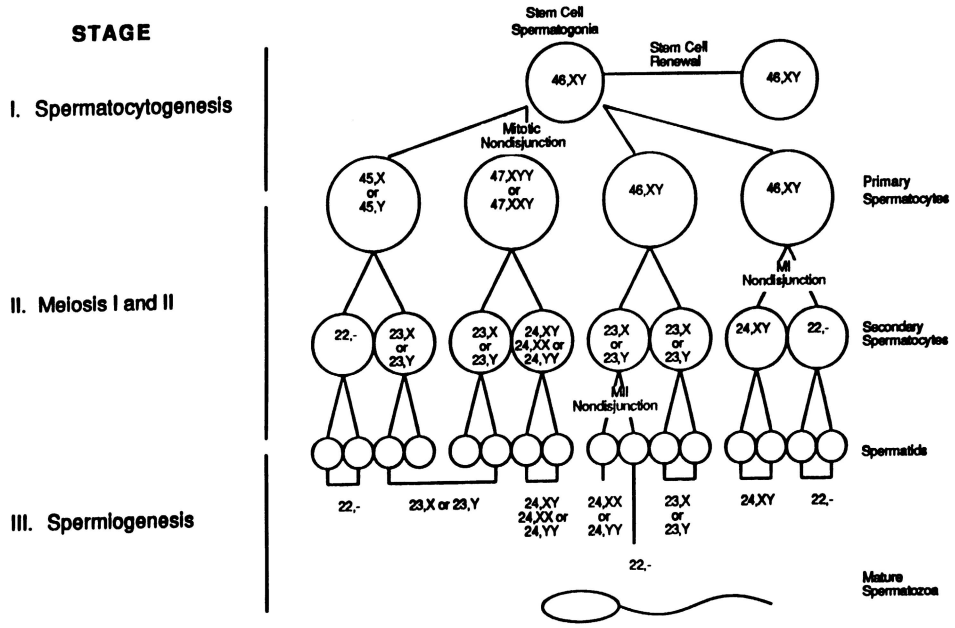

Figure 2 Germ-cell division and chromosome nondisjunction during the three stages (spermatocytogenesis, meiosis, and spermiogenesis) of spermatogenesis. Normal and abnormal segregation of the sex chromosomes is illustrated in germ cells undergoing meiosis I (MI) and II (MII) stage division. The figure further demonstrates that mitotic nondisjunction errors of diploid spermatogonial cells (primary spermatocytes) cannot be distinguished from meiotic stage errors present in secondary spermatocytes or in spermatids by FISH on mature sperm. Therefore, the analysis of sperm present in semen samples only is not sufficient for an in-depth understanding of this process, since it does not provide information on the fidelity of spermatogonial mitotic and meiotic cell division prior to germ-cell degeneration.

that there is no greater incidence of congenital abnormalities in the offspring generated by ICSI (Palermo et al. 1996), but, given the expected frequencies of many genetic defects in the population, larger, more comprehensive studies are required. This area remains controversial, and epidemiologists believe that there are too few data from too few clinics to determine whether ICSI is harmless (Kurinczuk et al. 1996).

The higher frequency of germ-cell nondisjunction reported among our patients with testicular failure is similar to that observed in ejaculated sperm from patients with less-severe male-factor infertility (Miharu et al. 1994; Moosani et al. 1995). The high frequency of mitotic nondisjunction in the testis of infertile patients is not a result of a gene recombination–related abnormality. Rather, the data suggest that it is a result of germline gene anomalies. As ART evolves, patients with severe male-factor infertility may be offered the options of using testicular sperm (Devroey et al. 1995; Silber et al. 1995) or, even earlier, less-mature germ cells (Fishel et al. 1995, 1997) in ICSI. Although the purpose and mechanism for physiological meiosis nondisjunction remain unclear, the results from the present study indicate that it is necessary to discuss with couples the possible aneuploidy risks associated with ICSI and to consider carefully preimplantation genetic diagnosis on the embryo.

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Table 1

Detection Frequency of Haploid and Diploid Nuclei by Three-Color FISH for Chromosomes X, Y, and 18

Category	No. (%) of Haploid Nuclei	No. (%) of Diploid Nuclei	No. (%) of Other Nuclei	Total No. Scored[a]
Normo	2,069 (81.4)	396 (15.6)	77 (3.0)	2,542
Hypo	1,490 (62.9)	557 (23.5)	321 (13.6)	2,368
Early	1,385 (58.9)	742 (31.6)	224 (9.5)	2,351
Late	1,436 (70.1)	348 (17.0)	264 (12.9)	2,048
Overall Total Scored	…	…	…	9,309

NOTE.—Nuclei were grouped as "haploid" or "diploid" on the basis of the presence of either one or two control chromosome 18 probe signals, respectively. Nuclei were grouped as "other" when chromosome 18 aneuploidy was suspected. These include nuclei with no yellow signals, and large (primary spermatocytes) and small (secondary spermatocytes) nuclei with one and two yellow signals, respectively.

[a] Significant differences were not detected among cases within each of the designated groups; therefore, the number of nuclei scored were pooled for cases within the same pathological category.

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