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Review Article

Cross-Species Sperm-FISH Assays for Chemical Testing and Assessing Paternal Risk for Chromosomally Abnormal Pregnancies

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The father, like the mother, can transmit genetic defects to his offspring that are detrimental for normal development and a healthy life. Epidemiological studies have identified associations between several paternal exposures and abnormal reproductive outcomes, but these types of studies are inherently complex and expensive, and the risk factors for the paternal contribution to abnormal reproductive outcomes remain poorly understood. Several sensitive methods have been developed for detecting mutations and chromosomal damage directly in sperm. These assays are potential bioindicators for paternal risk factors for infertility, spontaneous abortions, aneuploidy syndromes, and genetic diseases in children. Among these methods, fluorescence in situ hybridization (FISH) has been adapted for the detection of numerical and structural chromosomal abnormalities in the sperm of an expanding number of species, including humans and rodents. Sperm FISH has identified several potential paternal risk factors such as age, drugs, lifestyles, and various environmental/occupational exposures. Here, we summarize the status of the development and usage of these sperm-FISH assays and suggest strategies for prioritizing chemical agents for epidemiological investigations to assess paternal risk for abnormal reproductive outcome. Environ. Mol. Mutagen. 45:271–283, 2005. Published 2005 Wiley-Liss, Inc.

Key words: aneuploidy; chromosomal structural aberrations; sperm; fluorescent in situ hybridization

INTRODUCTION

Abnormal reproductive outcomes result in major emotional and medical expenses to family and society [U.S. Congress, Office of Technology Assessment, 1986; Committee on Life Science, National Research Council, 1989]. Every year in the United States, about 2 million pregnancies are lost before the 20th week of gestation, about 7% of newborns have low birth weight, and 5% of babies are born with some birth defect [U.S. Bureau of the Census, 1992]. More than half of these birth defects have significant effects on the baby's health or viability.

The causes of reproductive abnormalities are diverse and not well understood [Hook, 1985; Sloter et al., 2004]. Chromosome abnormalities are an important factor [Chandley, 1991; Wyrobek, 1993], with about 1% of newborns having a numerical or chromosomal structural abnormality. About half of all spontaneous abortions and a substantial fraction of developmental and morphological birth defects are associated with *de novo* chromosomal abnormalities [Hassold, 1998]. Chromosomal abnormalities may arise during gametogenesis of either parent. They may also arise after fertilization, when they are likely to result in varying degrees of mosaicism, depending on how early in development they occur. Molecular analyses of the parental origin of abnormal chromosomes during development or after birth indicate that the relative parental contribution to various types of chromosomal

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abnormalities varies among different categories of defects. Autosomal trisomy appears to be predominantly maternal in origin (e.g., trisomy 21, 18, 16, 13), while sex chromosomal aneuploidies (e.g., 45, X, 47, XXY, 47, XYY, 47, XXX) have a substantial paternal contribution [Hassold and Hunt, 2001]. In addition, most *de novo* germinal point mutations and structural chromosomal rearrangements in offspring appear to arise during spermatogenesis [Chandley, 1991].

The human disease burden caused by parental preconception exposure to germinal mutagens is poorly understood except for a few examples. Of particular concern are cancer chemotherapeutics given before or during the reproductive years [Wyrobek et al., 2005]. Each year, more than 20,000 children or young persons of reproductive age in the United States are treated with cancer chemotherapeutic regimes, most of which contain known mutagens [Byrne et al., 1998]. As treatments become more effective and more patients regain their fertility after treatment, there is concern that chemotherapy-induced germ line mutations may increase the risk of abnormal reproductive outcomes for these cancer survivors. In addition, certain environmental, lifestyle, and occupational exposures have been shown to affect sperm quality [Wyrobek et al., 1983], raising concern that these exposures may also affect the genetic integrity of the germ cells. Further, decades of testing chemicals using rodent breeding assays (e.g., dominant lethal, heritable translocation, and specific locus tests) have shown that germ-cell mutagens can have many profoundly deleterious effects on reproduction, including infertility, lethality during development, and malformations or cancer among offspring [Shelby, 1996; Witt and Bishop, 1996]. However, these rodent breeding tests are very expensive and often require thousands of animals. This limits the numbers of agents that can be tested.

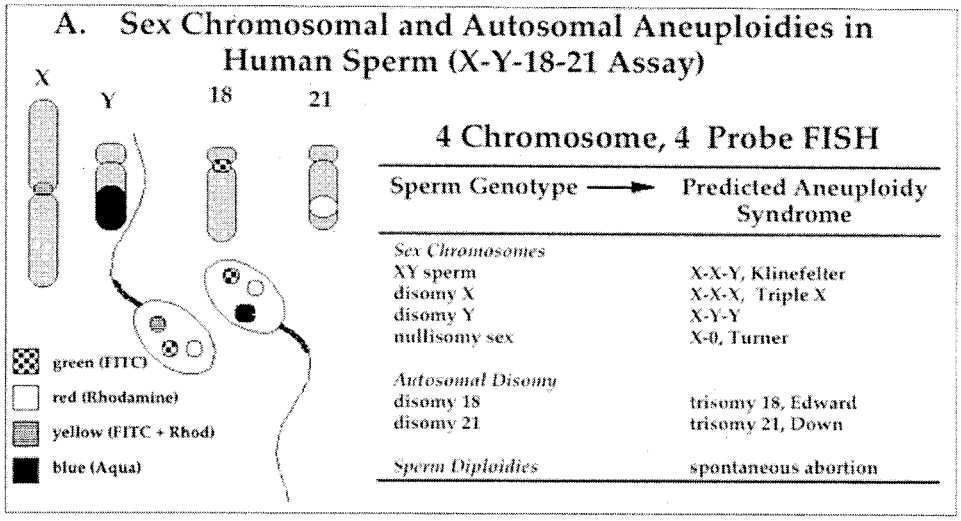
Evidence is growing that the risk of abnormal reproductive outcomes of paternal origin may be influenced by a number of factors, such as abnormal male reproductive physiology, predisposing genetic factors [Hassold and Hunt, 2001], past and present male environmental exposures [Olshan and van Wijngaarden, 2003], or random errors during sperm production [Crow, 2001]. Elucidating the relative contribution of each of these factors from epidemiological studies of affected offspring has been difficult because of the small sample sizes of offspring with specific defects and potential bias caused by prenatal selection against chromosomally abnormal embryos. This has provided an incentive for developing methodologies to detect damage directly in sperm.

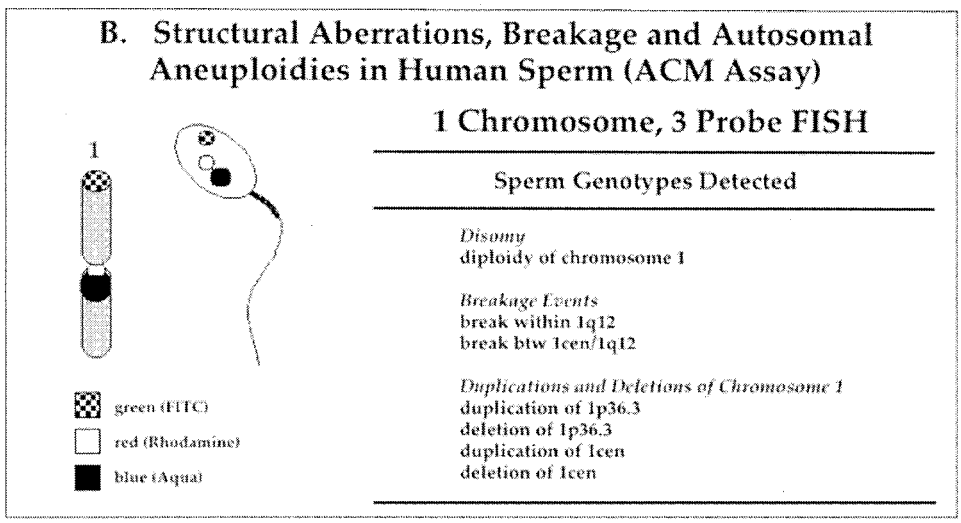
Direct Analysis of Human Sperm

Chromosomal analysis of male gametes provides a direct approach for studying the paternal contribution to abnormal reproductive outcomes. However, sperm chromosomes have been extremely difficult to study because sperm chromatin is highly condensed and poorly suited for analysis by conventional cytogenetic banding methods. The human-sperm/hamster-oocyte hybrid technique [Rudak et al., 1978] was one of the first approaches for the direct analysis of human paternal chromosomes. This technique provided evidence that chromosomal aberrations occur at frequencies of 5–7% in normozoospermic fertile males [Martin et al., 1986], that reproducible and significant variation occurs in frequencies of chromosomally abnormal sperm among healthy men [Genesca et al., 1990], and that men exposed to certain genotoxic agents (i.e., chemotherapy, radiotherapy) exhibit higher frequencies of sperm with chromosomal aberrations compared to controls [Martin et al., 1986, 1989; Brandriff et al., 1994; Robbins, 1996]. However, this technique is exceedingly difficult, labor-intensive, and inefficient, and this dramatically limits its utility for testing human male exposure to chemicals and the effects of lifestyle factors. First developed in the late 1980s [Pinkel et al., 1988], fluorescence in situ hybridization (FISH) has proven to be an efficient approach for labeling DNA of chromosomes in all interphase cells. It was adapted to sperm over a decade ago [Wyrobek et al., 1990; Holmes and Martin, 1993; Martin et al., 1994; Robbins et al., 1995; Shi and Martin, 2001]. Sperm-FISH assays have evolved from a one- to a two-, three-, and four-chromosome assay using multiple DNA probes, each specific for a different chromosome. The effectiveness of the technology has improved with the availability of chromosome-specific DNA probes for every human chromosome and with the broadly acknowledged emphasis on the importance of scoring criteria [Robbins et al., 1993]. Emphasis has also shifted from using any chromosome for which an effective DNA probe was available to selecting chromosomes with clinical relevance for human aneuploidy syndromes [Frias et al., 2003]. For example, Figure 1 illustrates a four-color FISH method [Van Hummelen et al., 1997] that simultaneously utilizes four chromosome-specific DNA probes to quantify the frequencies of sperm that are aneuploid for chromosomes associated with five major aneuploid syndromes among newborns (i.e., trisomy 21, trisomy 18, trisomy 13, Klinefelter, Turner).

Rodent Assays for Aneuploid Sperm

While multicolor sperm-FISH assays were developed for human sperm, similar methods were developed to detect sperm carrying numerical abnormalities and chromosome structural aberrations in laboratory animals. The mouse testicular sperm aneuploidy (mTSA) assay was developed to detect aneuploidy for chromosomes X, Y, and 8 in testicular sperm [Wyrobek et al., 1995]. The mTSA assay demonstrated increased sperm aneuploidy with advanced male age [Lowe et al., 1995] and elevated





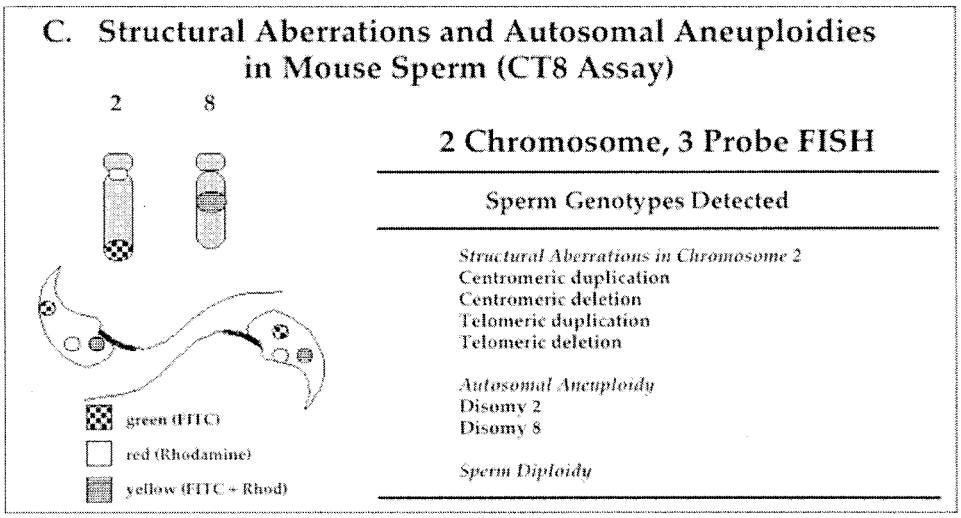


Fig. 1. Chromosome labeling strategies used in human and rodent sperm-FISH methods. **A:** Four-chromosome FISH assay for detecting aneuploidy in human sperm. **B:** ACM assay for detection of structural and numerical abnormalities in human sperm. **C:** CT8 assay for detection of structural and numerical abnormalities in mouse sperm.

frequencies of aneuploid sperm in specific Robertsonion translocation carriers [Baulch et al., 1996]. Subsequently, this approach was extended to the analysis of the more homogeneous pool of epididymal sperm, i.e., the mouse epididymal sperm aneuploidy (mESA) assay [Lowe et al., 1996]. Aneuploidy data obtained with the mESA method were consistent with those of previous testicular FISH methods and with the literature data for conventional germ-cell meiotic cytogenetic analysis. The spontaneous frequencies of aneuploid sperm in young adult mice and technical factors for the assay were described by Adler et al. [1996] and Schmid et al. [2001]. These include harmonizing scoring criteria, rigorous blinding of scorers using procedures that prevent the identification of treated and control animals based on sperm concentration, appropriate statistical evaluation of the dispersion characteristics of data in control and treatment groups, and replicating findings in repeated experiments using harmonized scorers.

A multicolor FISH assay was also developed to detect aneuploidy and diploidy in rat sperm using DNA probes for chromosome 4 and Y [Lowe et al., 1998]. A probe for a second autosome (chromosome 19) was subsequently added by de Stoppelaar et al. [1999], and efforts for developing a three-chromosome FISH assay utilizing both sex chromosomes to mirror the assays already available for human sperm are underway.

Human and Mouse Sperm-FISH Assays for Structural and Numerical Aberrations

Building on a strategy first described by Eastmond et al. [1994], a human FISH method was developed to detect both numerical and structural abnormalities in human sperm [Van Hummelen et al., 1996]. This early sperm-FISH method used probes specific for the centromeric and telomeric regions of chromosome 1 and a centromeric probe for chromosome 8 to control for ploidy. It allowed simultaneous detection of terminal duplications and deletions in chromosome 1p, as well as aneuploidy and diploidy for chromosomes 1 and 8. The assay was validated by comparing the frequencies of chromosomal structural abnormalities in the sperm of a reciprocal translocation carrier that were detected by this FISH method to those reported using the human-sperm/hamster-oocyte hybrid technique [Van Hummelen et al., 1997]. More recently, an improved sperm-FISH assay (ACM assay; Figs. 1B and 2i–k) was developed to detect a broader range of chromosomal structural aberrations in human sperm [Sloter et al., 2000]. The ACM assay uses DNA probes specific for three different regions of chromosome 1 to detect sperm that carry numerical abnormalities or structural aberrations (duplications, deletions, and breaks). Baseline frequencies were estimated for sperm with chromosomal aberrations involving chromosome 1 and were consistent with frequencies determined by the human-sperm/hamster-oocyte hybrid technique [Sloter et al., 2000]. The ACM method for detecting chromosomal breaks and rearrangements in sperm provides a direct approach for measuring exposure to chromosome-breaking agents and assessing genetic predisposition to such damage.

A FISH assay to detect numerical as well as structural chromosomal abnormalities was recently also developed for mouse sperm (Figs. 1C and 2p–r) [Hill et al., 2003]. This three-color FISH assay (CT8 assay) uses two DNA probes specific for the centromeric and telomeric regions of chromosome 2 plus a probe for the subcentromeric region of chromosome 8. The CT8 assay can detect sperm carrying several types of structural and numerical chromosomal defects such as duplications and deletions of the centromeric or telomeric regions of chromosome 2, disomy 2, disomy 8, and diploidy. The CT8 assay was validated by comparing the frequencies of chromosomal abnormalities in sperm of T(2;14) translocation carriers that were detected by the CT8 assay to those detected by the cytogenetic analysis of meiosis II spermatocytes using chromosome painting [Hill et al., 2003]. The CT8 assay may provide the first robust rodent screen for male germ-cell aneugens and clastogens, which includes agents that may lead to increased risks for chromosomally based developmental defects. An assay for detecting structural chromosomal aberrations is not yet available for rat sperm.

Fig. 2. Multicolor FISH images of labeled chromosomes in the sperm nuclei of humans, mice, and rats. Examples of sperm labeled by human sperm FISH are shown in panels **a–k** using an X-Y-8 assay in which chromosome X fluoresces green, Y fluoresces red, and 8 fluoresces yellow. Numerically abnormal sperm, such as disomy 8 (a and b), Y-Y-8-8 (c), and X-Y-8 (d) have three or more fluorescent domains. Using the X-Y-21 assay (f), chromosome X is yellow, Y is green, and 21 is red. Shown in f is an abnormal X-Y-21 Klinefelter sperm among a normal X-bearing and Y-bearing sperm. In the four-chromosome human sperm-FISH assay, X is yellow, Y is blue, 18 is green, and 21 is red. A field of normal sperm is shown in e. i–k illustrate sperm labeled with the A-M-8 FISH assay that detects both aneuploidy and structural chromosome damage. Here, probes are used for the alpha centromeric (green) and subtelomeric midi (red) region of chromosome 1p, plus a probe for chromosome 8 (yellow). Shown in g is a normal sperm (A-M-8). Shown in h is an abnormal sperm (A-M-M-8) carrying a duplication of the 1p midi telomere (red). In the human A-C-M assay, a third probe for the classical region of chromosome 1 is used together with the chromosome 1 alpha and midi probes. A normal sperm is shown in *i* with the alpha probe in red, the classical probe in blue, and the midi probe (M) in green. In addition to duplications and deficiencies of 1p, the ACM assay also detects chromosomal breaks between the alpha and the classical (j) and within the classical (k). **l–n** and **p–r** show examples of mouse sperm FISH. In the mouse ESA assay (l–n), X is yellow, Y is green, and chromosome 8 is red. Shown are disomy X sperm (l) and disomy 8 sperm (m). The mouse C-T-8 assay (p–r) detects both aneuploidy and structural chromosome damage and uses probes for the centromeric (C; green) and telomeric (T; red) regions of chromosome 2 plus a probe for chromosome 8 (yellow). A normal sperm (C-T-8) is shown in p. Shown in q is an abnormal sperm (C-C-T-8) carrying a duplication of the centromeric region of chromosome 2. Shown in r is an abnormal sperm aneuploid for chromosome 2. n, **o**, and **s** show examples of rat sperm FISH. In the rat ESA assay, Y is red and chromosome 4 is green. Shown are normal Y-4 sperm (n), disomy 4 (o), and disomy Y rat sperm (s).

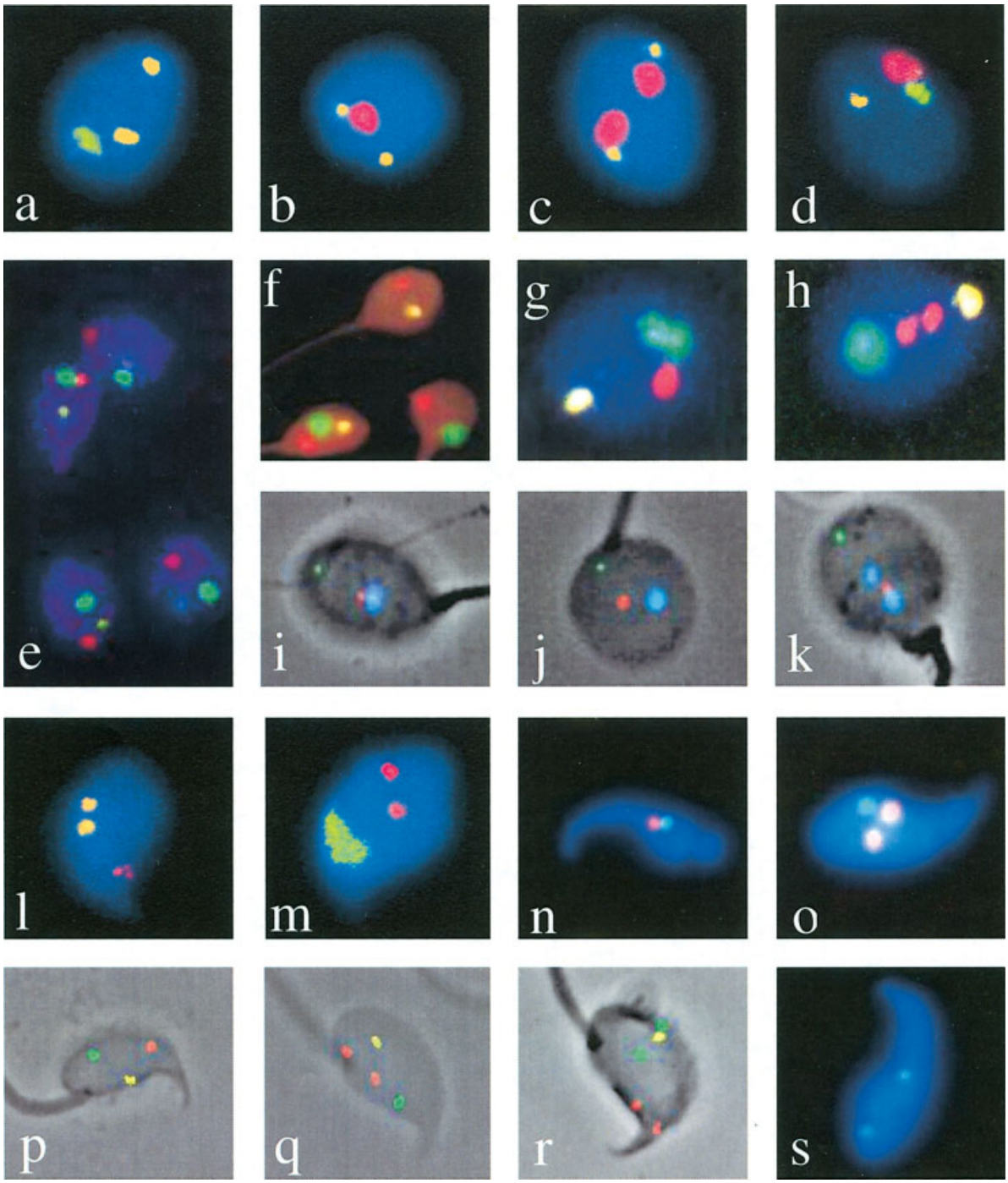


Figure 2.

VARIATION OF FREQUENCIES OF SPERM WITH CHROMOSOMAL ABERRATIONS IN THE GENERAL POPULATION

Understanding the sources of variation in the frequencies of defective sperm in the unexposed general population is critical for identifying potential germ-cell mutagens. Sperm-FISH has been applied to the investigation of how male age affects the baseline frequencies of defective sperm. Age-related increases in sperm carrying disomy Y and X were found in 14 donors aged 22–59 years [Robbins et al., 1995], but no age effect was found for disomy 8. Another study failed to identify an age effect in disomy frequencies of chromosomes 1 and 7 from 18 donors aged 21–49 [Lahdetie et al., 1997]. However, an age effect for disomy 1 and Y was observed in men aged 21–52 years [Martin et al., 1995]. In another study, using the human-sperm/hamster-oocyte hybrid technique,

TABLE I. Human Sperm FISH for Disomy and Diploidy

Chemical	Chromosomes in FISH assay	Number of exposed	Number of controls	Disomy[a]			Diploidy[a]			Reference
Lifestyle factors										
Caffeine	18, X, Y	26	19	X	1.2	NS	X-X-18-18	1.8	0.05	Robbins et al. [1997]
				Y	1.2	NS	Y-Y-18-18	1.8	0.05	
				XY	1.3	0.05	X-Y-18-18	1.3	0.05	
				18	1.4	0.05				
Alcohol	18, X, Y	32	13	X	1.4	NS	X-X-18-18	2.3	0.05	Robbins et al. [1997]
				Y	1.6	NS	Y-Y-18-18	1.7	0.05	
				XY	0.9	NS	X-Y-18-18	1.7	0.05	
				18	1.4	NS		1.4		
Smoking	18, X, Y	17	28	X	1.8	0.05	X-X-18-18	1.0	NS	Robbins et al. [1997]
				Y	1.5	NS	Y-Y-18-18	0.8	NS	
				XY	1.2	NS	X-Y-18-18	0.7	NS	
				18	1.5	0.05				
Smoking	8, X, Y	10	15	X	1.0	NS	X-X-8-8	2.0	NS	Rubes et al. [1998]
				Y	2.0	0.01	Y-Y-8-8	1.0	NS	
				XY	1.4	NS	X-Y-8-8	1.2	NS	
				8	1.4	0.01				
Smoking	13, X, Y	20	10	X	1.0	NS	NA	NA	NA	Shi et al. [2001]
				Y	1.5	NS				
				XY	0.8	NS				
				13	2.9	0.01				
Effects of medical drugs										
NOVP	8, X, Y	4	4	X	2.3	0.05	X-X-8-8	4.3	0.05	Robbins et al. [1997]
				Y	1.7	NS	Y-Y-8-8	2.1	NS	
				XY	4.7	0.05	X-Y-8-8	2.3	0.05	
				8	3.3	0.05				
NOVP	18, 21, X, Y	8	8	X	2.2	0.05	X-X-18-18-21-21	6.0	0.05	Frias et al. [2003]
				Y	2.4	0.05	Y-Y-18-18-21-21	4.0	0.05	
				XY	12.8	0.05	X-Y-18-18-21-21	9.0	0.05	
				18	7.1	0.05				
				21	3.2	0.05				
BEP	18, X, Y	5	5	X	1.0	NS	Total diploid	3.4	0.01	De Mas et al. [2001]
				Y	4.0	NS				
				XY	2.6	0.01				
				18	3.1	0.01				
BEP	1, 12, X, Y	1	1	X	1.2	NS	Total diploid	1.4	0.01	Martin et al. [1999]
				Y	2.2	NS				
				XY	2.3	0.01				
				1	1.5	NS				
				12	1.0	NS				
BEP	1,12, X, Y	4	4	X	1.0	NS	Total diploid	1.1	NS	Martin et al. [1997]
				Y	0.8	NS				
				XY	0.8	NS				
				1	0.6	NS				
				12	0.8	NS				
MACOP-B	1, 12, X, Y	1	5	X	0.6	NS	Total diploid	0.8	NS	Martin et al. [1995]
				Y	0.5	NS				
				XY	1.1	NS				
				1	1.3	NS				
				12	1.5	NS				
Diazepam	13, X, Y	2	2	X	2.3	0.05	Total diploid	1.5	NS	Baumgartner et al. [2000]
				Y	1.5	NS				
				XY	1.5	NS				
				13	2.2	0.05				
Occupational exposure										
Pesticides	18, X, Y	9	9	X	1.3	NS	X-X-18-18	1.9	NS	Recio et al. [2001]
				Y	2.4	NS	Y-Y-18-18	1.2	NS	
				XY	1.1	NS	X-Y-18-18	1.3	NS	
				18	1.1	NS				

TABLE I. *Continued*

Chemical	Chromosomes in FISH assay	Number of exposed	Number of controls	Disomy[a]			Diploidy[a]			Reference
Pesticides	13, 21, X, Y	20	20	X	0.9	NS	Total diploid	0.7	NS	Smith et al. [2004]
				Y	0.9	NS				
				XY	0.8	NS				
				13	0.9	NS				
				21	1.2	NS				
Pesticides	18, X, Y	13	16	X	1.7	NS	NA	NA	NA	Padungtod et al. [1999]
				Y	3.5	0.01				
				XY	1.9	NS				
				18						
Styrene	2, X, Y	18	13	X	0.7	NS	X-X-2-2	0.4	NS	Naccarati et al. [2003]
				Y	1.2	NS	Y-Y-2-2	0.4	NS	
				XY	0.6	NS	X-Y-2-2	1.8	NS	
				2	0.8	NS				
Acrylonitrile	X, Y	9	9	X	1.8	0.05	NA	na	NA	Xu et al. [2003]
				Y	2.3	0.05				
				XY	1.9	0.05				
Benzene	7, 8	15	12	7	3.2	0.01	Total diploid	2.3	0.01	Zhao et al. [2004]
				8	2.3	0.01				
Benzene	9, 18	14	16	9	3.4	0.01	Total diploid	1.9	0.01	Li et al. [2001]
				18	1.7	0.01				
Benzene	1, 18	15	14	1	2.0	0.01	Total diploid	1.0	NS	Liu et al. [2003]
				18	2.5	0.01				

[a]Chromosomes, fold increase, *P* value.

a higher frequency of numerical and structural aberrations was detected in the sperm of older men, and it was attributed mainly to increased nondisjunction, acentric fragments, and complex radial figures [Sartorelli et al., 2001]. The effect of age on chromosomally abnormal sperm remains ambiguous and is less pronounced in men than in women.

Examining geographic and ethnic effects, Shi and Martin [2000] found no differences in sperm aneuploidy between 10 nonsmoking, nondrinking Chinese men versus 10 nonsmoking Canadians. However, geographic or dietary differences were suggested as possible reasons for differences in the frequencies of XY and disomy X in sperm of nonsmokers from the Czech Republic versus nonsmokers from California [Rubes et al., 1998].

Recent findings show that men may vary significantly in their baseline frequencies for specific classes of chromosomally abnormal sperm, that variations can persist over years, and that there may be associations between aneuploidy frequencies in sperm and blood [Rubes et al., 2002]; the study also raises concerns about the long-term health consequences of persistently elevated levels of somatic and germ-cell aneuploidy. Clearly, additional studies with larger numbers of donors are needed to fully characterize the sources of the variations in baseline levels of chromosomally abnormal sperm among human populations and to better define how these variations may affect the statistical power to identify germ-cell mutagens.

STATUS OF CHEMICAL TESTING WITH HUMAN AND RODENT SPERM-FISH ASSAYS

The literature provides evidence that more than 50 chemicals or chemical mixtures including environmental, occupational, and medical exposures, can alter semen quality and possibly increase the risk for abnormal reproductive outcomes [Wyrobek et al., 1983; Wyrobek, 1993]. In the last 10 years, sperm-FISH assays have been increasingly employed to identify exposures that increase the frequencies of sperm with chromosomal abnormalities. The following is a synopsis of the current human and rodent data.

Human Sperm-FISH Studies

There is evidence that smoking, caffeine or alcohol consumption elevates the frequencies of sperm with chromosomal aberrations (Table I) is inconsistent. Robbins et al. [1997] found an effect of caffeine on disomy X and XY aneuploidy in sperm but not for 18 or Y. They also found a significant association between alcohol intake and the nondisjunction of X, but no association between smoking and general sperm disomies. An effect of cigarette smoking and alcohol consumption lifestyle on sperm aneuploidy was reported by Rubes et al. [1998] and Shi et al. [2001]. These conflicting results suggest that more carefully designed studies with larger numbers of donors

TABLE II. Chemicals Tested for Sperm Aneuploidies and Diploidies by Mouse Sperm FISH[a]

Chemical	Dose	Aneuploidy[d]	Diploidy[d]	Reference
Taxol	50 mg/kg[b]	−	−	Adler et al. [2002]
Vinblastine	0.5 mg/kg[b]	−	−	Schmid et al. [2001]
	1 mg/kg[b]	−	−	
	2 mg/kg[b]	+	−	
Etoposide	25 mg/kg[b]	+	+	Attia et al. [2002]
	50 mg/kg[b]	+	+	
Merbarone	30 mg/kg[b]	+	+	Attia et al. [2002]
	60 mg/kg[b]	+	+	
Diazepam	3 mg/kg[c]	−	−	Adler et al. [2002]
Diazepam	75 mg/kg[c]	−	−	Schmid et al. [1999]
	150 mg/kg[c]	−	+	
	300 mg/kg[c]	+	+	
Omeprazole	150 mg/kg[b]	−	−	Adler et al. [2002]
	300 mg/kg[b]	−	−	
Griseofulvin	500 mg/kg[a]	+	−	Shi et al. [1999]
	1000 mg/kg[a]	+	+	
	2000 mg/kg[a]	+	+	
Thiabendazole	100 mg/kg[a]	−	−	Schmid et al. [1999]
	300 mg/kg[a]	−	+	
Carbendazim	500 mg/kg[a]	−	+	Adler et al. [2002]
	1000 mg/kg[a]	−	−	
Trichlorfon	200 mg/kg[b]	+	−	Sun et al. [2000]
	300 mg/kg[b]	+	−	
	405 mg/kg[b]	+	−	
Acrylamide	60 mg/kg[b]	−	−	Schmid et al. [1999]
	120 mg/kg[b]	−	−	
Colchicine	1.5 mg/kg[b]	−	−	Schmid et al. [1999]
	3 mg/kg[b]	+	−	

[a]All these studies used the X-Y-8 sperm-FISH assay.
[b]Single i.p. injection.
[c]p.o. application, acute.
[d]Plus sign means statistically significant at $P < 0.05$; minus sign means not significant or inconclusive results.

will be needed to establish the relationships between sperm aneuploidy and common lifestyle factors.

Several medical exposures (Table I), including chemotherapy regimens, increase the incidences of chromosomal aneuploidies and diploidies in sperm of treated patients [Martin et al., 1997, 1999; Robbins et al., 1997; De Mas et al., 2001; Frias et al., 2003; Wyrobek et al., 2005]. Although the information is limited to a small number of chemotherapies, the increases in aneuploid sperm seem to diminish in semen with increasing time after exposure, suggesting that cancer patients may have only a transient risk for producing higher frequencies of aneuploid offspring after chemotherapy. A separate drug study, which applied a multicolor sperm-FISH assay utilizing probes for chromosome X, Y, and 13, detected significantly elevated levels of sperm disomies in two men who had chronically ingested 0.3 mg/kg/day diazepam for more than 6 months [Baumgartner et al., 2001].

Occupational exposure studies are summarized in Table I. Recio et al. [2001] investigated the effect of exposure to organophosphorous pesticides in men before and during the pesticide spraying season. They reported a significant association between organophosporous metabolite concentration in blood and increased frequencies of sperm aneuploidies. A study of sperm aneuploidy among Chinese pesticide factory workers using the X-Y-18 sperm-FISH assay reported an increase only in disomy Y [Padungtod et al., 1999]. A separate study of the effect of pesticides on seasonal outdoor workers using the X-Y-13-21 sperm-FISH assay found no differences in sperm aneuploidy or diploidy between exposed and control groups [Smith et al., 2004]; however, the exposure was of shorter duration, and thus at an overall lower level, than those of the study of Padungtod et al. [1999], in which men were in constant contact with pesticides.

Other occupational exposures that have been reported to increase the frequencies of sperm with chromosomal aneuploidies and diploidies in sperm of exposed men include acrylonitrile, which induced sex chromosome nondisjunction [Xu et al., 2003], and benzene, which induced aneuploidy and diploidy in sperm of men exposed to concentrations above 10 ppm [Li et al., 2001; Zhao et al., 2004]. Recently, benzene exposure has also been shown to increase the frequencies of sperm with chromosomal structural aberrations [Liu et al., 2003]. Finally, occupational exposure to styrene did not have an effect on numerical chromosome aberrations in sperm [Naccarati et al., 2003].

TABLE III. Chemicals Tested for Sperm Aneuploidies and Diploidies by Rat Sperm FISH[a]

Chemical	Dose	Aneuploidy[d]	Diploidy[d]	Reference
Carbendazim	50 mg/kg[c]	−	−	de Stoppelaar et al. [1999]
	150 mg/kg[c]	−	−	
	400 mg/kg[c]	−	+	
	800 mg/kg[c]	−	+	
Cyclophosphamide	6 mg/kg[b]	+	−	Barton et al. [2003]

[a]All these studies used the Y-4 sperm-FISH assay.
[b]i.p. injection, acute.
[c]p.o. application, acute.
[d]Plus sign means statistically significant at $P < 0.05$; minus sign means not significant or inconclusive results.

Mouse Sperm-FISH Assays

To date, 12 chemicals have been tested with the mouse sperm-FISH assay for sperm aneuploidy and diploidy (Table II), including four chemotherapeutics. Taxol was tested at the maximum tolerated dose and the increase of disomic sperm was at the borderline ($P = 0.06$) of statistical significance [Adler et al., 2002]. Vinblastine gave inconclusive results in repeated experiments in an interlaboratory comparison [Schmid et al., 2001]. Etoposide and merbarone, both topoisomerase II inhibitors, showed significant increases in the frequencies of diploid and hyperhaploid sperm [Attia et al., 2002].

Five pharmaceuticals have also been tested by mouse sperm FISH. Diazepam, a tranquilizer, significantly increased the frequency of disomic and diploid sperm in a dose-related manner [Schmid et al., 1999]. Griseofulvin, an antifungal drug, produced dose-related increases in the frequencies of diploid sperm, and while the frequencies of disomic sperm were significantly higher than the controls in every dose groups, the increases were not dose-related [Shi et al., 1999]. Thiabendazole, an antihelminthic drug, induced only diploid sperm. The antiulcer drug, omeprazol, gave negative results [Adler et al., 2002].

Among the pesticides tested with the mouse sperm-FISH assay, carbendazim induced diploidy but not aneuploidy [Adler et al., 2002], while trichlorfon induced a dose-dependent increase of disomy, but not diploidy [Sun et al., 2000]. Acrylamide, an important industrial chemical used mainly in sewage and waste water treatment plants, did not increase the frequency of aneuploid or diploid sperm [Schmid et al., 1999]. Two doses were tested, the higher one being the maximum tolerated dose. Colchicine, used as positive control, showed increased levels of disomic sperm, but only at the higher concentration [Schmid et al., 1999].

Rat Sperm-FISH Assay

Only two chemicals have been tested with the rat sperm-FISH assay (Table III). The pesticide carbendazim was evaluated in epididymal sperm after exposure during late pachytene of meiosis I. The results showed a clear induction of diploid sperm with a highly significant dose-response relationship, but aneuploid sperm were not induced [de Stoppelaar et al., 1999]. Chronic low-dose treatment of male rats with cyclophosphamide (6 mg/kg daily for 9 weeks) significantly increased the frequency of spermatozoa with chromosome 4 disomy and nullisomy [Barton et al., 2003], but neither disomy Y or diploidy was significantly increased.

Interspecies Comparisons of Effects of Chemicals on Male Germ Cells

To date, there are data for only one interspecies comparison on the effect of chemicals in male germ cells using FISH. Baumgartner et al. [2001] compared human and mouse sperm-FISH data, which indicated that human spermatocytes were 10- to 100-fold more sensitive to diazepam than those of mice. This interspecies comparison of relative sensitivity needs to be extended to other chemicals to determine whether this difference is specific to diazepam or more general.

ADVANTAGES, STATISTICAL POWER, AND CHALLENGES OF SPERM-FISH TESTS

The use of FISH methods to detect numerical abnormalities in the sperm of humans and mammals is gaining in popularity when compared with epidemiological surveys of human offspring or animal breeding studies. Sperm FISH can analyze large numbers of cells in a relatively short amount of time, provide a high level of sensitivity and statistical power, and small increases can be detected by analyzing sperm from relatively few donors. Table IV shows the number of sperm samples that would be necessary to detect an increase or decrease of 50% or 100% with the ACM assay [Sloter et al., 2000]. The size of the donor groups is dependent on the standard deviation among men in the normal population and the magnitude of the expected effects. Table IV shows that it is possible to detect a doubling of the frequencies of chromosomal breaks with a sample size of about 10 exposed men and a doubling of the frequency of disomic sperm with a sample size of about 6 exposed men (and equal numbers of unexposed men).

TABLE IV. Statistical Power of Human Sperm FISH (ACM Assay)

	Mean[a]	Sample size to detect % increase or decrease[b] 50%	100%
Segmental aneuploidies			
Duplications and deletions of the 1 pter region	10.8 ± 5.5	22	6
Duplications and deletions of the 1 cen-1q12 region	1.8 ±1.6	67	17
Chromosomal breaks			
Breaks between 1 cen and 1q12 and within 1q12	4.5 ± 3.2	41	10
Disomy and dipoidy			
Disomy 1 or diploidy	22.7 ± 11.2	21	6

[a]Mean ± SD per 10,000 sperm and data for 10 men (20–30 years, nonsmokers).
[b]Numbers of men in the exposed group with an equal number of controls.

There are three major challenges for the sperm-FISH assays that limit their general utility. First, only a few chromosomes are investigated in any one assay and it is possible that aberration rates may not be the same for all the chromosomes. To minimize this shortcoming, FISH assays are now using probes for up to four chromosomes simultaneously, each marked with a different fluorescent color [Frias et al., 2003]. In addition, the analysis of the same samples using different combinations of probes can further increase the proportion of the genome investigated. Second, the scoring criteria remain subjective and the control of technical factors remains critical to the reliability of the sperm-FISH assay, especially when small changes are observed between exposed and control groups. Interlaboratory comparisons have demonstrated the importance of harmonizing scoring criteria, rigorously avoiding scorer's bias, normalizing cell numbers, evaluating dispersion characteristics of the control and treatment groups, and replicating findings in repeat experiments. Third, visual microscopic scoring remains laborious and time-consuming. The development of reliable automated methods (e.g., flow cytometric analysis or computer-controlled microscopy) are urgently needed to improve the utility of these assays.

STRATEGIES FOR USING SPERM-FISH ASSAYS FOR CHEMICAL TESTING AND RISK ASSESSMENT

The growing evidence that environmental and occupational exposures can induce increases in the frequencies of sperm with chromosomal abnormalities raises the possibility that many naturally occurring or manmade chemicals, for which few data are available, also have the potential of inducing chromosomal damage in human germ cells. Because of the time and money necessary to conduct epidemiological studies, it would be cost-effective if we were able to prioritize chemical exposures that have a high potential of constituting a genetic hazard. The availability of sperm-FISH assays in a variety of species offers the possibility of implementing a multispecies approach that integrates rodent FISH assays and human somatic cell data to prioritize chemicals for epidemiology testing and for assessing the paternal risk of producing abnormal reproductive outcomes.

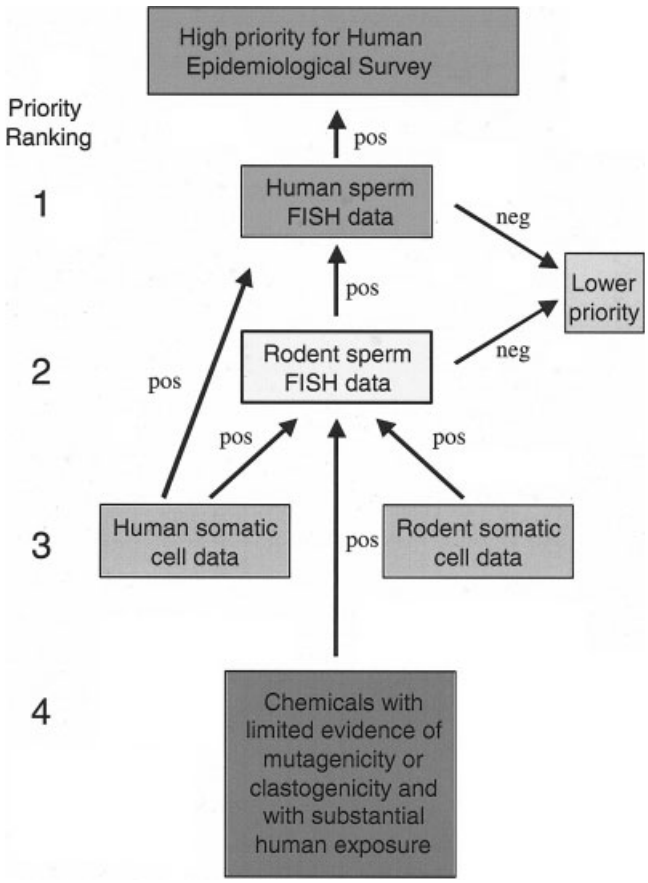


Fig. 3. Strategy utilizing rodent and human sperm-FISH assays for identifying and prioritizing chemicals for human epidemiological studies.

Figure 3 illustrates several strategies that employ multispecies sperm-FISH assay to prioritize chemical exposure for epidemiological evaluation. These strategies have the

common requirement that chemicals produce positive results in human sperm-FISH assays of exposed people (or where human samples are not available, in rodent sperm-FISH assays) before qualifying as candidates for human epidemiological studies. Depending on the toxicology data available, chemical exposures can be assigned into at least four priority rank groups.

One, chemical exposures that induce chromosomal abnormalities in human sperm by sperm-FISH assays would make them a candidate for human epidemiological evaluation of paternally mediated abnormal reproductive outcomes.

Two, chemicals that show an effect in the rodent sperm-FISH assays would become candidates for testing with the human sperm-FISH assays to confirm human sensitivity and to identify exposure parameters before proceeding with time-intensive and expensive human epidemiological studies.

Three, chemicals for which there are no data in human or rodent germ cells, but have produced positive mutagenicity or clastogenicity results in human somatic cells or rodent somatic cells (e.g., micronucleus tests), would be candidates for testing with the rodent or human sperm-FISH assays, depending on the availability of exposed human subjects.

Four, by far the largest group of chemicals has substantial human exposure but little or no prior testing data for mutagenicity or clastogenicity in any human or animal test system or have produced negative results in a somatic assay. One may consider prioritizing these agents by ranking their usage and potential human exposure and begin testing them with the rodent sperm-FISH assay.

This overall strategy offers an integrated approach to prioritize epidemiological efforts on chemicals that have a high potential to pose a genetic hazard to humans. It is not intended to eliminate chemicals from consideration for germ-cell effects. Obviously, chemicals with substantial human exposure remain a concern even when only negative data are available until a clear understanding is reached of the interplay between the chemical's mechanism of action, male germ-cell susceptibility, and postfertilization processing of sperm lesions on the formation of chromosomal abnormalities. The proposed strategy takes advantage of the rodent sperm-FISH assays to identify windows of germ-cell sensitivities to refine the experimental parameters to be used in subsequent epidemiological studies.

CONCLUSIONS

During the past decade, sperm-FISH techniques have evolved to provide considerable information about host factors and exogenous exposures that affect the chromosomal constitution of sperm and the risk for paternally mediated effects on abnormal reproductive outcomes. An integrated interspecies approach to germ-cell genotoxicity takes advantage of the strengths of the human and animal model sperm-FISH assays and has the promise of preventing and reducing the incidence of children with paternally transmitted chromosomal defects. Sperm-FISH assays may also help us to better understand the mechanisms of germ-cell stage sensitivity for the various types of genetic defects. Of particular concern are persistent effects on stem cells and the effects of chronic exposure of sensitive stages of spermatogenesis. The recently developed sperm-FISH assays (human ACM or mouse CT8) for the detection of chromosomal structural aberrations may help identify risk factors for chromosomal rearrangements found in post-meiotic germ cells. In addition, studies utilizing mice carrying specific mutations in DNA repair and meiosis genes may help us gain an understanding of the mechanisms of male germ-cell genotoxicity and germ-stage sensitivities.

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