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Methods for Evaluating the Effects of Environmental Chemicals on Human Sperm Production

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Sperm tests provide a direct and effective way of identifying chemical agents that induce spermatogenic damage in man. Four human sperm tests are available: sperm count, motility, morphology (seminal cytology) and the Y-body test. These sperm tests have numerous advantages over other approaches for assessing spermatogenic damage, and they have already been used to assess the effects of at least 85 different occupational, environmental, and drug-related chemical exposures. When carefully controlled, seminal cytology appears to be statistically more sensitive than the other human sperm tests and should be considered an integral part of semen analysis when assessing induced spermatogenic damage.

Human sperm studies have complex requirements and, before sampling, careful consideration should be given to exposure details, group size and makeup, as well as animal and human data that indicate spermatogenic effects. Several study designs are possible and should include questionnaires covering medical and reproductive histories as well as known confounding factors. Animal sperm tests, such as the mouse morphology test, may be used to identify the toxic components of a complex mixture. Animal tests may also help assess the chemical effects on fertility and reproductive outcome in cases when human data are incomplete. Further efforts are needed in these areas to develop improved human sperm tests sensitive to induced spermatogenic damage, to develop improved animal models of induced spermatogenic damage, to understand the relationships among sperm changes, fertility, and reproductive outcome, and to develop sperm tests with express mutational end points.

Introduction

Studies with numerous chemical agents in a variety of mammalian species have shown that sperm anomalies can be used as indicators, and in certain instances, as dosimeters of chemically induced spermatogenic damage (*1,2*). Various other approaches have also been proposed to assess chemically induced spermatogenic dysfunction including testicular biopsies (*3*), questionnaire surveys (*4*) and blood levels of gonadotrophins (*5*). Sperm tests have the advantage that they are noninvasive, generally less expensive, require smaller sample sizes, and are sensitive to small changes (*1,2,5*).

A recent survey of the literature (*2*) showed that sperm tests have been more widely used to assess the effects of chemical exposures in man than was generally suspected; more than 100 papers involving some 85 different chemical exposures have been published. This paper briefly describes the methods and applications of the four most common human sperm tests, compares their relative sensitivities and suggests guidelines for undertaking a new human sperm study in men exposed to toxic agents. The paper also discusses the role of animal studies, the implication of semen findings for reproductive outcome, and future research needs in these areas.

Description of Human Sperm Tests

Human semen tests have a long history in the diagnosis of infertility (*5*). Thus, it is not surprising that the early attempts to assess altered spermatogenic function in men exposed to chemicals involved

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measuring changes in the sperm parameters commonly used in fertility diagnosis, such as sperm density (counts), motility, and morphology (seminal cytology). The following is a very brief description of these methods.

Sperm count is usually reported as the number of sperm per milliliter of ejaculate (or as the total number of sperm ejaculated) as determined by hemocytometer (*6*). The measurement is technically easy, and automated methods are also available. However, interpretation of results may be confounded by a number of factors, such as variable continence time before ejaculation and collection of an incomplete ejaculate (*7*).

Sperm motility is the swimming ability of the sperm and has been expressed in a large variety of ways (*6*). Although motility may be one of the best performance evaluations of spermatogenic function in relation to fertility, it is also very sensitive to time and temperature after collection (*8*). Thus, semen motility is very difficult to measure in a field study, especially when samples are collected at home. Considerable emphasis has been put on automated and quantitative methods for assessing sperm motility (*9*).

Sperm morphology (also referred to as seminal cytology) is the visual assessment of the shapes of ejaculated sperm. Although sperm-head shape is usually emphasized, some assessments also incorporate midpiece and tail abnormalities. In general, there has been little agreement in the definition of normal shapes or in the categories of abnormal shapes. This has resulted in much interlaboratory and interscorer variability (*10,11*). However, studies of MacLeod (*12*), David et al. (*13*), Eliasson (*14*), and others have shown that quantitative approaches to the visual assessment of morphology can be used with considerably success. These assessments are usually made by using smears that are air-dried, fixed and stained with a Papanicolaou method (*15*). Sperm can be systematically assigned to shape categories. In evaluating the effects of exposure, slides of controls should be concurrently analyzed with slides of exposed men in a blind-study design. Normal ranges have been established for several unexposed populations.

We have developed a human morphology test by describing 10 classes of sperm-head shapes (*16,17*) and classifying 500 sperm per individual. Through the intermittent use of coded standard slides, we have been able to assure constancy in the visual scoring criteria for sperm morphology over a period of many years. Our experience with this test shows that visual scoring criteria can be very objective (unpublished data). We have applied this method to men occupationally exposed to carbaryl (*17*) and anesthetic gases (*16*). The carbaryl workers showed higher proportions of sperm with shape abnormalities than controls but no dose response was observed and there was no difference in sperm counts. No effects of anesthetic gases on sperm were observed. In another study, men exposed to cancerchemotherapeutic agents showed drug-related decreases in sperm counts and increases in sperm-shape abnormalities (*18*).

The Y-body test scores the frequency of fluorescent spots in human sperm stained with quinacrine dye. Based on studies in somatic cells, it is thought that these spots represent Y chromosomes (*19*). The Y-body test scores the frequency of sperm with two spots, which are thought to represent sperm with 2 Y chromosomes due to meiotic nondisjunction (*20*). Unlike the other sperm tests (counts, motility, and morphology), the Y-body test has no direct counterpart in the mouse or other common laboratory animals. The Y-chromosomal fluorescence after quinacrine staining seems to be unique to man and certain apes (*21*). However, it should be noted that the field vole, *Microtus oeconomus*, has a unique distribution of heterochromatin, which allows visualization of the X and Y chromosome in spermatids and possibly testicular sperm (*22*). Studies with several chemical agents suggest that this system may be a useful animal model for studying the induction of sex-chromosomal nondisjunction in male germ cells.

For the analysis of Y-bodies in human sperm, air-dried smears can be fixed, stained and sperm scored under a fluorescent microscope (*19,20*). The number of sperm scored depends on the statistical precision required. Each sperm is scored as OY (containing no fluorescent body, presumably sperm with no Y chromosome), 1Y (those sperm presumably containing one Y chromosome) and 2Y (sperm presumably containing two Y chromosomes). We have developed the method so that we can repeatedly visualize approximately 50% of the sperm with a single fluorescent body (unpublished data). The Y-body test is very new, its relationship to chromosomal aneuploidy uncertain and only a few populations of exposed men have been analyzed (*2*).

Applications of Human Sperm Tests

The above methods have been applied to assess spermatogenic function in at least 85 different groups of chemically exposed men (*2*). Tables 1–4 categorize these agents into occupational and environmental chemicals (Table 1), experimental and therapeutic drugs (Tables 2 and 3), and recreational

drug use (Table 4). Details of the studies surveyed to generate these tables and the decision criteria used to classify each agent as one with adverse effects, suggestive of adverse effects or with no apparent adverse effects are published elsewhere (*2*). Several agents (not listed in these tables) have been reported to improve sperm quality in some cases (*2*).

Relative Sensitivities of the Human Semen Tests

Experience with agents like dibromochloropropane (DBCP) suggests that severe spermatogenic damage may occur at doses that show no other apparent clinical signs of toxicity. Therefore, for chemical exposures it seems unlikely that analysis of somatic cells (i.e., lymphocyte) can serve as a surrogate for effects on male germ cells.

The four human sperm tests described above are technically straightforward methods. The tests for counts, morphology, and Y-bodies are parameters that do not appear to be readily affected by postejaculation technical factors (*2*). Motility, however, is highly sensitive to time and temperature factors. In studies where home collection is used the motility test is not practical (*8*).

The statistical variations of the tests for counts, morphology, and double Y-bodies were recently

Table 1. Effects of occupational and environmental chemicals on human sperm.[a]

Agents with adverse effects	Agents suggestive of adverse effects	Agents with no apparent adverse effects
Carbon disulfide	Carbaryl	Anesthetic gases
Dibromochloropropane	Kepone	Epichlorohydrin
Dibromochloropropane + ethylene dibromide		Glycerine production compounds
Lead		Polybrominated biphenyls
Toluenediamine + dinitrotoluene		

[a]Table entries are based on studies of sperm counts, motility, morphology and double Y-bodies. The assignment of individual agents to columns is based on the data provided in the papers reviewed by the Human Sperm Reviewing Committee of the U.S. Environmental Protection Agency (EPA) GENE-TOX Program (*2*). These entries are generally based on few studies and may be expected to change as more data become available.

Table 2. Experimental and therapeutic drugs and agents or combinations of agents with adverse effects on human sperm.[a]

Acridinyl anisidide	Metanedienone
Adriamycin	Methotrexate
Aspartic acid	MOPP (Mechlorethamine + vincristine + procarbazine + prednisone)
Clorambucil	MVPP (Mechlorethamine + vinblastine + prednisolone + procarbazine)
Clorambucil + mechlorethamine + azathioprine	Norethandrolone
Clomiphene citrate	Norethindrone
Cyclophosphamide	Norethindrone + norethandrolone + testosterone
Cyclophosphamide + colchicine	Norgestrel + testosterone enanthate
Cyclophosphamide + prednisone	Norgestrienone + testosterone
Cyclophosphamide + prednisone + azathoprine	Prednisolone
CVP (cyclophosphamide + vincristine + prednisone)	Propafenon
CVPP (cyclophosphamide + vincristine + procarbazine + prednisone)	R-2323 + testosterone
Cyproterone acetate	Sulphasalazine
Danazol + methyl testosterone	Testosterone
Danazol + testosterone enanthate	Testosterone cyclopentylpropionate
Enovid	Testosterone enanthate
Gossypol	Testosterone propionate
Leutineizing hormone releasing factor agonist	VACAM (Vincristine + adriamycin + cyclophosphamide + actinomycin D + medroxyprogesterone acetate)
Medroxyprogesterone acetate	WIN 13099
Medroxyprogesterone acetate + testosterone enanthate	WIN 13099 + diethylstilbestrol
Medroxyprogesterone acetate + testosterone propionate	WIN 17416
Megestrol acetate + testosterone	WIN 18446

[a]Table entries are based on studies of sperm counts, motility, morphology and double Y-bodies. The assignment of individual agents to columns is based on the data provided in the papers reviewed by the Human Sperm Reviewing Committee of the U.S. Environmental Protection Agency (EPA) GENE-TOX Program (*2*). These entries are generally based on few studies and may be expected to change as more data become available.

Table 3. Effects of other experimental and therapeutic drugs on human sperm.[a]

Agents suggestive of adverse effects	Agents with no apparent adverse effects
Centrochroman	*Bromocriptine*
Cimetidine	Lysine
Colchicine	Methyltestosterone
Diethylstilbestrol	Niridazole
Methadone	Norethindrone + testosterone
Metronidazole	Orinthine
Nitrofurantoin	Tryptophan
Norethandrolone + testosterone	WIN 59, 491
Trimeprimine	

[a]Table entries are based on studies of sperm counts, motility, morphology and double Y-bodies. The assignment of individual agents to columns is based on the data provided in the papers reviewed by the Human Sperm Reviewing Committee of the U.S. Environmental Protection Agency (EPA) GENE-TOX Program (*2*). These entries are generally based on few studies and may be expected to change as more data become available.

Table 4. Effects of recreational drug use on human sperm.[a]

Agents with adverse effects	Agents suggestive of adverse effects	Agents with no apparent adverse effects
Alcoholic beverages (chronic alcoholism)	Tobacco smoke	None
Marijuana		

[a]Table entries are based on studies of sperm counts, motility, morphology and double Y-bodies. The assignment of individual agents to columns is based on the data provided in the papers reviewed by the Human Sperm Reviewing Committee of the U.S. Environmental Protection Agency (EPA) GENE-TOX Program (*2*). These entries are generally based on few studies and may be expected to change as more data become available.

compared for a group of control men (*17*). Sperm samples from approximately 25 men were required in both the exposed and control groups to detect a 25% change in the mean proportion of abnormally shaped sperm. Y-body analyses and counts required over 40 and 200 men, respectively, to detect a 25% change in means. This comparison suggests that the human sperm morphology test is statistically more sensitive to small induced changes than the other two tests. However, it is important to realize that a chemical exposure may preferentially affect any of these sperm parameters irrespective of its statistical sensitivity. At present, data are still insufficient to predict (a) which parameter would be most sensitive to an agent or (b) the interdependence of the parameters. Therefore, the conservative approach for assessing changes in human spermatogenic function should include tests for sperm count, morphology, Y-body, and, whenever practical, motility.

Guidelines for New Human Sperm Studies

Laboratory analyses of sperm tests represent only a small part of a human sperm study. Depending on the exposure under consideration, these studies typically require lengthy interactions with unions, management, local and state government, lawyers, physicians, hospital administrators, and human subject committees before any donors are contacted. The following criteria should be considered to determine if a human sperm study is warranted:

(a) Are there animal data available suggesting a spermatogenic effect of the exposure under consideration? (Animal data may exist in the literature or may be obtained using the short-term animal sperm tests.), or

(b) Are there human data that suggest that there may be a problem with infertility or pregnancy outcome that could be linked to the exposed male?

If the exposure under consideration meets these criteria, the following additional data should be obtained to aid in study design. (Though these points are generally self-evident, they are included because they have been often overlooked in human sperm studies.)

(a) What are the demographics of the exposure (size of the exposed population, geographic location, etc.)? The size of the exposed cohort is an important consideration since, as described above, the number of men sampled will be related to the statistical sensitivity for each semen parameter. The geographic dispersion of the exposed cohort is an important cost factor as well as a possible cause of sampling biases.

(b) Who was exposed (how many men, what are their ages and religious backgrounds, etc.)? Such factors can be expected to affect the participation rates.

(c) What are the details of the exposure (route, duration, dose, when it occurred in relation to the proposed time of semen collection, etc.)? It is well known from animal and some human studies that the occurrence of sperm anomalies is related to exposure dose. In addition, careful attention needs to be given to the time since the last exposure; since the effects of certain agents may be reversible, false negative results may appear if the time is too long.

(d) Can the exposed population be divided into dose groups? Every effort should be made

to group the exposed cohort by dose estimates, since a dose-related effect is extremely strong evidence for an identification of a human testicular toxin.

A questionnaire approach to assessing human problems in fertility and reproductive outcome should also be considered. This approach may be especially effective when large numbers of people of child-bearing ages have been exposed and the major exposures were many years ago. Human sperm studies are likely to be effective when smaller numbers of men are involved (see above section) and the major exposures are suspected to be recent or ongoing.

When considering sperm studies several study designs are possible. Since between-male variability in semen characteristics is high even among fertile and presumably healthy men, rather large numbers of cooperative subjects are required to establish differences between control and exposed groups in cross-sectional studies (each individual sampled only once). Longitudinal study designs may be more appropriate when fewer men are available for sampling. In this study design, repeated semen samples are collected from each man at different times in relation to the time of exposure and compared to assess chemically induced sperm defects. Since variation of sperm morphology within an individual is considerably less than variation among individuals (*23*), in principle, fewer people are required for induced changes to be detected. These studies, however, have some constraints: repeated samplings during a period of months and perhaps years are required; samples before exposure are needed (or within days of an acute exposure before any induced effects on morphology are seen); and the number of men needed for an effective study is unknown.

The effects of age, smoking, illness, medication, and other possibly confounding factors, especially those involving heat exposure, must be considered in the analysis of all human sperm data.

Possible Roles for Animal Tests

The availability of both animal and human sperm tests suggests several applications of animal studies in the assessment of chemically induced spermatotoxicity, antifertility effects, and heritable genetic abnormalities in man. First, animal sperm tests (such as mouse morphology) may be used to screen large numbers of agents to establish a ranking that sets priorities for identifying exposed men. Second, animal sperm studies may also be useful in evaluating an agent or the components of a complex mixture that are suspected of affecting human sperm (such as in an occupational or environmental exposure). Third, animal breeding tests may be used to study the relationship between changes in sperm parameters, fertility changes, and heritable consequences.

Since little is known of the quantitative relationships between induced sperm abnormalities and heritable genetic damage, indirect methods may be needed to assess the genetic risk to offspring of men who show induced sperm anomalies. By combining data from short-term mutagen bioassays (e.g., Salmonella/microsome assay, mammalian somatic cell mutation assays), which may demonstrate mutagenic potential, with data from animal and human sperm tests, which may demonstrate activity in the testes, we may be able to evaluate whether or not a mutagen is active in the testes. Further studies are needed to investigate this approach.

Genetic Implications of Chemically Induced Sperm Defects

Evidence from Human Studies

Although it is generally agreed that major reductions in sperm counts and motility are linked to reduced fertility, it remains unclear which sperm parameter(s), if any, is predictive of reproductive failure or heritable genetic abnormalities. Human data on this question are very limited. Infertility is seen in patients with 100% acrosomeless, round-headed spermatozoa (*24*), suggesting that some types of sperm shape abnormalities are associated with infertility. Human studies with DBCP showed the strong link between reduction in sperm counts and infertility (*25*). Regarding reproductive outcome, Furuhjelm et al. (*26*) reported that a group of fathers of spontaneous abortions showed significantly higher sperm abnormalities and lower sperm counts than fathers of normal pregnancies. This finding suggests a possible link between poor semen quality and frequency of spontaneous abortions (*27*). Clearly, more human studies are needed to compare exposure of the male parent, induced sperm defects, and reproductive outcome.

Evidence from Animal Studies

Most of the studies on genetic validation of induced sperm defects have been conducted with sperm morphology in mice. Several lines of evidence link induction of abnormally shaped sperm and heritable genetic abnormalities (*1*). First, it is clear that

sperm shaping and the production of abnormal sperm are polygenically controlled by autosomal as well as sex-linked genes. Second, in several studies using agents that induce sperm abnormalities, sperm abnormalities were transmitted to the male offspring of the exposed mice. Third, a brief survey of the literature suggests that the mouse sperm morphology test may be an effective prescreen for the more expensive tests of heritable germ cell mutations, such as heritable specific locus, heritable translocation, and dominant lethal tests in mice. False-negative responses with the mouse sperm morphology test for these tests seem to be very rare or nonexistent. However, data are needed for more chemicals before this relationship can be used with confidence. Spindle poisons that may cause nondisjunction in germ cells can also be identified with the mouse morphology test. Further studies (probably best done in mice) are needed to understand the quantitative relationships among dosage regime, appearance of abnormal sperm shapes in the semen, time between exposure and conception, fertility of the exposed male, frequency of genetically abnormal offspring, and fertility of the abnormal offspring.

Future Research Needs

Sperm tests have shown considerable promise in the assessment of spermatogenic damage induced by occupational and environmental exposures. But the available human tests are first-generational and carry many biases from their original applications in fertility diagnosis. More research is needed to adapt these available sperm tests and to develop the statistical criteria for the effective assessment of chemically induced spermatogenic damage. More work is also needed to develop improved animal models of human spermatogenic damage, to study the relationship among changes in sperm parameters, fertility, and reproductive outcome and to develop new indicators of reproductive toxicity in the male, especially indicators of heritable genetic damage.

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