

ASSESSMENT OF REPRODUCTIVE DISORDERS AND BIRTH DEFECTS IN COMMUNITIES NEAR HAZARDOUS CHEMICAL SITES. III. GUIDELINES FOR FIELD STUDIES OF MALE REPRODUCTIVE DISORDERS

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Abstract — Exposures to environmental toxicants can have detrimental effects on several aspects of human male reproduction: fertility, sexual function, hormone status, and pregnancy/birth outcomes. However, no simple prescreening methods are available for reliably identifying potential hazards; questionnaires alone are relatively imprecise and inefficient in the absence of field data. Multidisciplinary field studies are required that include detailed exposure information, health and reproductive histories, physical examinations, semen analyses, and possibly, hormone analyses. Semen analysis is a critical component of field studies for evaluating two aspects of male reproduction: 1) changes in sperm or seminal content, which may be indicative of adverse effects on the male reproductive system with possible implications for fertility potential; and 2) defects in sperm DNA or chromosomes, which may be associated with subsequent changes in viability during embryonic development and health risks to the offspring. Semen analyses may be tiered: 1) initially, each semen study may include conventional semen assays (concentration, motility, and morphology) as well as specific biomarkers indicated by the health effect of concern in the study cohort; and 2) archived samples (i.e., frozen, videotaped, or smeared) may be utilized in later second-tier analyses to further characterize specific findings. Before initiating any field study, it is cost effective to critically evaluate the suitability of the cohort by confirming exposure and determining that there are adequate numbers of male participants in each exposure category. Such evaluations must be based on the statistical sensitivities of the specific tissue biomarkers and health endpoints for detecting changes. This article summarizes the components of the ideal field study and identifies research needs for improving field studies of male effects and for understanding the mechanisms of male reproductive toxicity. Several promising semen methods currently under development are also discussed. © 1997 Elsevier Science Inc.

INTRODUCTION

Decades of animal and human research have convincingly documented that a variety of risk factors including exposure of a male to ionizing radiation or to certain chemical toxicants can diminish or destroy his fertility (e.g., 1,2) (Table 1). There is also convincing evidence, especially from animal studies, that exposures of the male to germinal mutagens before fertilization can have

detrimental effects on embryo viability and health of his children, which may be manifested at birth or later in their life.

Reproductive abnormalities occur commonly among humans, and frequencies of these events in the general population vary by abnormality. Various target sites in male and female germ cells and during development have been identified including (a) physical and functional defects of the male or female reproductive systems, (b) errors in differentiation occurring in the germ cells of the mother or father before fertilization, which would diminish the quality or quantity of gametes, and (c) developmental defects that occur after fertiliza-

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Table 1. Known or suspected risk factors for male reproductive abnormalities

Characteristic	Risk factors
Personal	Age of man
Medical conditions	Recent infection Trauma Autoimmune disorders High fever Mumps orchitis Diabetes Prostatitis Varicocele History of infertility
Medical drugs ^a	Estrogens Chlorambucil Cyclophosphamide Nitrofurantoin
Habits and recreational drugs ^a	Tobacco Alcohol Use of sauna or hot tub Recreational drugs (e.g., Marijuana)
Environmental agents ^a	
Chemical	Solvents Metals Pesticides Neurotoxicants
Physical	Heat Ionizing radiation

^aAdditional agents listed in Table 5.

tion. However, little is known about the relative paternal and maternal contributions to reproductive abnormality and few environmental causes have been identified.

In the past, concerns over the effects of environmental exposure on the human male reproductive system were typically precipitated by some triggering event (such as accidental exposures or clusters of reproductive abnormalities) and resulted in understandable anxiety within the affected community. A working group was convened by ATSDR to identify prerequisites and to discuss guidelines to be considered before contemplating a new field investigation of a possible link between male reproductive disorders and environmental exposure(s). This report includes (a) the male reproductive health effects of major concern; (b) the prerequisite information needed before considering a field study, especially related to exposures and the numbers of men who are exposed; (c) selection of study designs; (d) the utility of questionnaires; and (e) the selection and prioritization of relevant biomarkers.

The authors suggest that there are not any easy pre-screening tools for identifying a potential link between an environmental exposure and male reproductive defects in a field study setting. Sufficient time and effort should be encouraged to evaluate the prerequisites to determine whether a male reproductive study is warranted. If warranted, then a comprehensive study that

includes semen analyses and other biomarkers should be undertaken. Although, semen collection and analyses are a vital component of any comprehensive study, the individual semen biomarkers may be prioritized into phases or tiers, as will be discussed in this report.

REPRODUCTIVE HEALTH CONCERNS OF MALE ORIGIN

Environmental pollutants may adversely affect the reproductive system in men resulting in health effects that can be broadly characterized as:

- Infertility or subfertility in couples when males were exposed.
- Endocrine disruption or imbalance, altered sexual differentiation, and/or sexual dysfunction in exposed men or boys.
- Developmental defects in offspring: birth defects, stillbirths, miscarriages, fetal loss, childhood cancer, cytogenetic abnormalities, or specific genetic defects.

Concern over induced male reproductive disorders may be initiated by real or perceived human exposure to agent(s) known or suspected to be male reproductive or genetic toxicants or by an abnormal reproductive outcome that is potentially of male origin. The following are examples of events or situations that may trigger concerns about male toxicants as causes for specific abnormal reproductive outcomes.

Male infertility

Possible scenarios that would trigger a male fertility concern would be: previous reports (animal or human) indicating that a certain toxicant could have a detrimental effect on fertility, known mechanisms of the toxicant, reports from infertility workups that individuals from the population have poor quality semen (that is, cluster of individuals), or subfertility in the population. Public perception that chemical exposure is associated with male infertility may also develop after anecdotal accounts of failures or delays in initiating pregnancies. Potential genetic problems can also present as fertility problems.

Male neuroendocrine or sexual dysfunction

Scenarios leading to concerns of altered male neuroendocrine or sexual function would include: clusters of men with abnormal reproductive hormone profiles, a cluster of men with problems in sexual function, men with potential exposure to toxicants having endocrine properties (competitive or inhibitory), and men with exposure to toxicants shown to affect the neuroendocrine system (e.g., lead). Also, neuroendocrine disruptions while in utero may affect the morphology and function of the male reproductive system in the adolescent or adult male (3).

Male-mediated effects on the pregnancy or offspring

The primary concern of male-mediated adverse effects on pregnancy and birth outcomes and developmental abnormalities may occur when the toxicant exposure is known and the agent involved is known to cause genetic damage in germ cells or other cell types in animal model systems, if there is a cluster of males who have fathered adverse pregnancies, or if a cluster of adverse pregnancies has been observed in the population, especially when there are putative male exposures.

THE MALE REPRODUCTIVE SYSTEM AS A TOXICOLOGIC TARGET

The male reproductive system, of which spermatogenesis is only one part, involves interactions between somatic and germinal tissues as well as a complex hormonal axis with central nervous system controls. Exposures to environmental toxicants could affect any of these processes. Numerous test methods have been developed for detecting and assessing defects in various aspects of the male reproductive system of animals and human beings (4).

One major problem in studying the effects of toxicants on the male reproductive system is defining the timing of exposure in relation to the landmarks during germ cell development (i.e., in utero, childhood, and adolescence) and during spermatogenesis (i.e., in adults).

Spermatogenesis involves a continuously replicating cell population. Chemicals, whose toxicity depends on cell division, may have greater effect on the male germ cell than female germ cells. Human spermatogenesis requires approximately 75 d for a stem cell to differentiate into a sperm within the seminiferous tubules and about 12 d for the sperm to travel through the epididymis to the vas deferens. Thus, it is important to obtain the exposure history during the last 90 d prior to the specimen collection (in the case of semen studies) or prior to the data of fertilization (in the case of pregnancy or offspring studies).

Moreover, the patterns of spermatogenic cell sensitivities can be complicated and vary by toxicant. The different cell stages of spermatogenesis (stem cells, spermatogonial mitoses, meiosis, postmeiosis, posttesticular) may have markedly differing sensitivities for different agents, and in the extreme, only one cell type of spermatogenesis may be sensitive. In the latter case, only those exposures within the small window of time within these 3 months are important toxicologically. In the case of erratic exposures, grouping all men who were exposed at some time over the past 3 months may yield a considerable fraction of false positive exposure classifications. Further complicating the situation, a single toxicant may induce differing spermatogenic lesions leading

to different reproductive outcomes, and each outcome may have a different spermatogenic time window of sensitivity. Using the data for dibromochloropropane (DBCP), a nematocide, as an example, exposing stem cell and dividing spermatogonia would result in cell killing and reduced sperm count resulting in infertility, exposing postmeiotic cells would induce dominant lethality in the offspring, and exposing epididymal sperm would result in sperm motility defects resulting in infertility. However, what is true for DBCP may not necessarily be true for other agents. Ethyl nitrosourea is a potent mutagen primarily in spermatogenic stem cells of mice. In summary, whenever possible, it is important to determine exactly when in the last 3 months a man's exposures to the toxicant of interest occurred. Similar arguments can be made for obtaining detailed information on exposures to confounders and other risk factors.

ASSESSING MALE EXPOSURE

Assessing exposure is one of the most important prerequisites of conducting a field study of the effects of a potential toxicant on male fertility and male-mediated developmental abnormalities. However, as is often the case in environmental studies, exposure levels are unknown and biologic indicators of exposure are unavailable or impractical. In addition, large individual variabilities in susceptibility to toxicants are expected.

One of the key challenges in field studies is to distinguish between maternal and paternal exposure. It is well recognized that prolonged exposures of the mother can affect her germ cells before fertilization, can affect the fetus and embryo during development, and can continue to have effects on the child postnatally during lactation. Thus, an exposure assessment is needed for both the male and his female partner (5–7).

Community-based studies usually have relatively low exposure levels for study participants and the gradient between "exposed" and "unexposed" may be very small. Thus, it becomes more difficult to distinguish between the two groups. One option is to identify a subpopulation with higher exposures and to compare it with a relatively unexposed subpopulation. One could also identify a paternally exposed group of couples (exposed male with unexposed female sexual partner) and a non-exposed couple (unexposed male and unexposed female sexual partner) within a geographic area of interest. Rates for various adverse reproductive outcomes could be compared between the two groups. In addition, taking specimens during and after exposure could greatly contribute to the evaluation of any association.

Exposure information can be obtained by questionnaire, from industrial data, and from community data, as summarized below. A screening questionnaire can be

used to collect data on the exposure histories of men and their partners. Exposure data for pre-, peri-, and postconception periods need to be collected to address questions regarding the various possible mechanisms of toxicity that are relevant for male-mediated effects. Some of the advantages and limitations of various methods for assessing male exposures are described below.

Exposure by questionnaire

For occupational exposures, a questionnaire is an inefficient instrument for quantitative and precise identification of agents, yet it can be helpful in identifying groups of agents and categorizing men by their job tasks. For community exposures, questionnaires are also poor instruments for identifying exposure to agents. Geographic proximity to a point source toxicant is often used as a surrogate for quantitative exposure classification. In the case of Superfund sites, the toxic agents of potential importance are usually unknown, making qualitative or quantitative exposure assessments virtually impossible.

Exposure by industrial data

Certain types of industrial information may be available: hazardous waste measurements (air, water, and soil), bulk sampling of raw and finished products, area and personal breathing zone air concentrations, surface wipe samples, dermal patches, and biologic screening for parent compounds or metabolites in exhaled air, blood, and urine. Often, occupational test results can provide a quantitative measure of external exposure. Because industries can be the point source for community exposure, this type of data may be very useful. Occupational and environmental surveys can be conducted simultaneously in cases where an initial industrial release or spill leads to disease outbreak in a surrounding community. Workers may have been exposed either in the accident or in the clean-up efforts.

Exposure by community data

The following are examples of the types of information obtainable from community exposure data sources: public health monitoring of air, soil or water; data on hazardous material storage reported to fire departments; data from permits for transportation of toxic agents; local government records of past land use permits; and local utility district periodic measurements of water pollutants and electromagnetic field dosages. These data are typically hard to interpret on an individual basis and may not cover the precise time or location of suspect exposure. Again, proximity to a potential point source may provide the best estimate of an individual's exposure.

Personal dosimeters of external exposure

Practical personal dosimeters are available for only a few agents. Ionizing radiation can be directly measured

by external dosimetry and radiation workers throughout this country and the world have been monitored for decades. This measure was used to find an association between paternal occupation at a nuclear energy plant and a cluster of childhood leukemia cases. One can also use urinary metabolite assays to assess exposure to internal emitters.

Biologic dosimeters of internal dose

A biomarker of exposure is the measure of a toxicant or its metabolite or presence of adducts in body tissue or fluid (4). Its presence does not necessarily indicate or predict a health effect. For example, blood levels of lead have been used to assess lead body burden. High blood levels were associated with semen abnormalities, including asthenospermia, hypospermia, and teratospermia (8). However, the exposure-response relationship for semen effects at low lead levels is still under study. In communities, background blood lead levels are expected to be 5 to 6 $\mu\text{g}/\text{dL}$. This background requires community-based studies to utilize CDC-approved laboratories that are able to detect very low concentrations of blood lead.

STUDY DESIGN AND STATISTICAL CONSIDERATIONS

Several study designs may be used to investigate the male reproductive effects of environmental exposures.

- Case reports have been used for description of highly unusual clusters and exposure associations. Upon confirmation of these cases and evaluation of other potential cases, more elaborate epidemiologic study designs can be considered.
- Surveillance systems or registries have been used extensively for birth defects, childhood cancers, and, in other countries, for miscarriages. Exposure registries such as for lead can also be used.
- Oftentimes, a cross-sectional study is conducted because of logistic, financial, and feasibility considerations. It is important to assess the best timing for such a study with respect to the exposure chronology.
- Cohort or prospective studies are often the best source of information because serial examination of the male can best identify the sequence of the reproductive effect, the variability of the biomarkers, and whether there is a reversible effect and/or recovery period. Prospective studies are not subject to recall bias, which may occur when participants with specific exposures or outcomes recall information differently than those without the exposures or outcomes.

Studies of male reproductive effects ought to consider whether other risk factors are operating that might explain any observed effect. Suspected or known risk

factors are listed in Table 1, and these can be included in a study questionnaire (1,9–12).

Table 2 reviews the critical components of a field study questionnaire. Questionnaires are important for obtaining three broad types of information in studies of male reproductive effects: 1) information on the primary exposure or hypothesis being tested; 2) information on confounding risk factors including other exposures, personal habits, and medical histories; and 3) information on prior reproductive history and success. Questionnaires are an indispensable means of obtaining information about individual exposure, life style factors that may confound the linkage between exposure and effect (e.g., smoking, drinking, drug use, exercise habits), and health status that may identify hypersensitive individuals or elucidate medical conditions such as urogenital infections or diseases that are associated with infertility in themselves. Furthermore, individual reproductive histories may reveal altered reproductive function such as delayed or accelerated puberty, sexual dysfunction, or change in libido, independent of whether a particular male has ever attempted a pregnancy.

Certain biomarkers of male reproduction are well suited for field studies and for repeated analyses of individuals over time. This report evaluates the various design options available for field studies utilizing biomarkers of male reproductive health.

Table 2. Critical components of a field study questionnaire

Type of information	Components
Reproductive history	Reproductive histories with current and prior sexual partners Number of offspring and any pregnancy or birth abnormalities Verification by hospital, clinic or medical care providers Sexual development history Sexual history Age at first semen appearance Prior reproductive evaluations
Health questions	Urogenital disease Prior medical problems Prior medications, radiation or chemotherapy Prescription drug use Testicular trauma, disease, or surgery
Lifestyle factors	Use of tobacco, alcohol, caffeine Diet, stress, exercise Exposure to heat Street drug use
Exposure to chemical and physical agents	Specific chemical exposures Exposure to heat Exposure to ionizing and nonionizing radiation Details should include: duration, amount, dates, etc.
Other	Related to physical examination findings

One or multiple semen samples may be requested per donor, depending on the type of exposure and specific study design utilized. A chronic exposure over months to years would indicate that a cross-sectional study of the exposed men compared to a concurrent “control” group of men could be used, with only one semen sample per donor.

If a study is being proposed after an accidental spill, release, or exposure, a longitudinally designed experiment would be most useful. Repeated semen samples from each affected individual would be used to characterize the induction and persistence of sperm damage including the possible return to baseline values. A concurrent control group would be useful in a longitudinal design to detect seasonal and other environmental changes that may affect the final interpretation of results. It takes approximately 90 d for a stem cell to differentiate into a mature sperm in the ejaculate. A sampling procedure collecting specimens throughout the spermatogenic process will provide critical information on toxicant effects. Using this design, it is very important to sample the semen as soon after the exposure as possible (within the first week) to determine if the sperm in the epididymis or if the accessory sex glands are being affected. One sampling scenario that has been used (13) was sampling at the following times: 1 d, 1 week, 1 month, and 3 months after exposure. If a persistent effect is noted, a follow-up assessment (1 year after exposure) may show possible recovery.

An important prerequisite before initiating a field study is to determine whether there are sufficient numbers of potential participants. The numbers of men included in a study population depends on the assays and health endpoints to be evaluated. The workgroup members suggested that sample sizes be calculated to detect at least a twofold change for the critical semen assays and biomarkers to be used as described by Bloom (14).

The variation in the sample size requirements can be dramatic. For example, studies of specific mutations can require millions of children while studies of spontaneous abortions may require hundreds of pregnancies. By contrast sample sizes needed for semen biomarkers are typically smaller in the range of 10s to 100 (using a 5% significance test with 80% power to detect a 25% change in the mean value) (14,15).

It is important to identify a control or reference group and to categorize the study population into distinct exposure groups in order to assess whether there is an association with exposure (12). Clinical semen standards may be inadequate as reference values for men in the general population. The specific geographic region in which the study is considered may have different “normal ranges” for the traditional semen parameters. It is also crucial to use an experienced laboratory for all se-

men assays and to developed standardize measurements that can be compared among studies and among centers.

In summary, the following key prerequisites should be considered before initiating an *exposure-motivated* field study of male reproductive effects: (a) are the men indeed exposed to the substance(s) of concern, (b) is a suitable reference group available, and (c) are there sufficient numbers of highly exposed men and controls to meet the statistical requirements of the semen assays to be applied or health effect to be measured? The prerequisites that should be considered before mounting a *health outcome-motivated* study should be (a) can the existence of the cluster of health abnormalities be confirmed, (b) is the rate of a given health outcome statistically elevated as compared with the general population, and (c) are there sufficient numbers of men (or pregnancies and offspring) available for a confirmatory study?

METHODS FOR ASSESSING MALE REPRODUCTIVE DISORDERS

Field studies of male reproductive disorders may, depending on the specific health effect of concern, include reproductive histories, physical examinations, blood analyses, and semen analyses (Table 3). As stated earlier, they also need a convincing assessment of relevant exposure. The following is a discussion of the key aspects of the methods for monitoring male reproductive and genetic health disorders.

Reproductive history

The assessment of male fertility status and male-mediated abnormal reproductive outcomes requires information from both the man and his sexual partners. Men and women may have differing memories of reproductive events and, ideally, interviews with both members of the couple are needed to obtain reliable information on live birth and grossly abnormal birth outcomes. Females tend to be better at providing detailed information on pregnancy outcomes, maternal exposures before and during pregnancy, and episodes of infertility (16,17).

Currently, there is no standardized set of questions used by researchers to assess fertility status, history of abnormal outcomes, and the involvement of confounding factors. The workgroup discussed typical reproductive questions that would be contained in a standardized questionnaire. Table 2 lists suggested components of field study questionnaires. At the very least, one needs to ask information about the number of pregnancies, live births, timing of live births, fertility, recognized miscarriages, and birth defects for each sexual partner.

Physical examination and general health

The physical examination can be used to determine whether the reproductive tract is intact and normal and

Table 3. Questionnaires, databases, and laboratory methods to assess male reproductive toxicity and paternally mediated abnormal reproductive outcomes

Abnormality	Methodology
Sexual dysfunction	Questionnaire
Endocrine changes	Blood LH, FSH, testosterone (Prolactin, thyroid hormone ^a)
Semen abnormalities	Sperm count, motility, morphology defects Presence of toxicant in semen Genetic and biochemical biomarkers
Chromosomal anomalies in offspring	Chromosomal analysis (fetus or offspring)
Infertility	Questionnaire ^b Registries
Fetal loss	Vital statistics, medical records, questionnaire, urine HCG
Premature delivery, low birthweight	Medical records, questionnaire
Congenital malformations	Birth defects registries
Childhood developmental disabilities	Prospective assessments
Childhood cancer	Cancer registries, questionnaire
Intrauterine growth retardation	Medical records, questionnaire
Altered sex ratio	Medical records, questionnaire
Perinatal mortality	Medical records, questionnaire

FSH—Follicle stimulating hormone. LH—Luteinizing hormone. HCG—Human chorionic gonadotropin.

^aUsed infrequently.

^bExisting databases have been used in other countries (e.g., Scandinavian countries).

whether there are any abnormal secondary sex characteristics. It is important to examine individuals for physical abnormalities that may interfere with normal spermatogenesis, spermatozoa transport, ejaculation, or erection. Such conditions include: varicocele, small testicular size, prostate tenderness, hypospadias, cryptorchism, and secondary sex characteristics.

The physical examination is also used in conjunction with the health questions on the questionnaire to assess the general past and present health of a participant. Data need to be collected on a participant's general medical history and on other key risk factors that might be responsible for any association with reproductive defects (Table 1).

Biomarkers of neuroendocrine status, sexual function, and semen quality

Potential targets for the male reproductive toxicant include both somatic and germ cells. Biomarkers have been developed to monitor toxicant metabolism and transport of the toxicant to reproductive system, neuroendocrine effects, germ cell effects, accessory sex gland effects, and sexual function effects (4). Table 4 includes

Table 4. Biomarkers of physiologic reproductive toxicity in men related to effects on male fertility

Tissue or source	Type of measurement
Blood	Hormone levels
Sperm	Concentration and total count Morphology and computer-determined morphometry Motility (visual and CASA) Viability Agglutination Penetration and interaction: Cervical mucus Hamster eggs Nonliving human eggs Internal and surface domains Chromatin structure Biochemical measurements
Semen	Physical characteristics Chemical composition (normal and xenobiotic substances) Immature germ cells Nongerminal cells Cellular function measures: Sertoli cells Leydig cells Accessory glands
Testes	Histopathology
Urine (female partner)	Early pregnancy detection
Surveys and medical records	Standardized fertility ratio Time to conception

selected biomarkers of physiologic damage that would have most relevance for changes in fertility. The biomarkers of genetic damage and heritable mutations will be discussed later in this report.

Biomarkers of sexual function. Human male sexual function is dependent on the integrated activities of the testes and secondary sex glands, the endocrine control systems, and the central nervous system-based behavioral and psychologic components of reproduction (libido). Erection, ejaculation, and orgasm are three distinct, independent, physiologic, and psychodynamic components of sexual function. They normally occur concurrently in men (18–20).

If the primary concern in a field study is a decrease in sexual function, then some assessment of sexual function should be considered. Questionnaires perhaps with diaries of sexual activity frequencies are possible tools available for assessing problems of libido in field studies. Although there are clinical methods of assessing priapism, nocturnal emissions, etc., these hospital-based techniques (much like testicular biopsy) are best reserved for individual clinical evaluations and are not suitable for field studies. The analyses of the ejaculate provides indirect indications of ejaculatory dysfunctions. In some

cases, sexual function is altered due to a neuroendocrine imbalance and, therefore, hormone analyses should be considered, as described below.

It is difficult to assess the potential effects of a toxicant on impotence in field studies, but certain methodologies may be applied in studies of well-defined subpopulations. Impotence, the failure to achieve and/or maintain an erection, may have either a psychogenic or organic etiology. While organic impotence is characterized by reduced ability to achieve an erection at any time, whether awake or asleep, psychologically impotent men continue to experience spontaneous erections during rapid eye movement (REM) sleep. Exceptions to this principle have been noted by Krane and colleagues (20). A simple test is available to differentiate between the two etiologies. A device is available that is attached to the penis to detect changes associated with nocturnal erections including penile circumference, pulsatile blood flow, volume, or axial rigidity (the resistance of the penis to buckling when a known weight is applied to the glans penis). This test is typically conducted in the clinic during at least 2 consecutive nights. However, some clinicians have applied these tests for home use (21).

Assessment of anomalies of overall sexual function is difficult in field studies. Researchers usually rely on a man's recall of his sexual function and on his interpretation of what constitutes abnormal sexual function. This recall may be confounded by a reluctance to admit to sexual problems or by erroneous associations of sexual problems with workplace or environmental exposures. Questions to the female sexual partner may be needed for reference and confirmation.

Few reliable data are available regarding the effects of occupational or environmental exposure on sexual function because of the technologic and psychologic difficulties encountered when the present assays are applied in field studies. Various medicines have been shown to affect each of the three stages of male sexual function (22), indicating the potential for occupational and environmental exposures to exert similar effects. Antidepressants, testosterone antagonists, and stimulants of prolactin release are effective in reducing libido in men. Antihypertensive drugs that act on the sympathetic nervous system induce impotence in some men, but priapism in others. Phenoxybenzamine, an α -adrenoceptive antagonist, has been used clinically to block seminal emission but not orgasm. In contrast, anticholinergic antidepressant drugs block seminal ejection and orgasm but not emission. As a consequence, seminal plasma may leak uncontrollably from the urethra in individuals taking these drugs.

Recreational drugs may also affect sexual function (22). Ethanol may induce impotence while enhancing

libido. Cocaine, heroin, and high doses of cannabinoids may reduce libido. Opiates may delay or impair ejaculation.

The large and varied list of pharmaceuticals that have been shown to affect male sexual function suggest that some of the untested chemicals found in the workplace or environment may have similar properties. The potential for such effects may confound interpretation of occupational and environmental exposure studies. Therefore, further research is encouraged to develop efficient methods suitable for field applications in this area of male reproductive toxicology.

Biomarkers of neuroendocrine function. If the primary concern is an imbalance in the hormone profile that may or may not affect fertility and sexual function, then a careful analysis of reproductive hormones should be conducted.

There is a lack of consensus as to whether blood hormone measurements [luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone, both total and free] are a useful adjunct to field studies of male reproductive toxicity in which sperm analysis is already being done. Prolactin is also occasionally measured, but the implications of altered prolactin for the male are not clear. FSH appears to be elevated only in cases of severe oligospermia or azoospermia, and is otherwise not well correlated to sperm count, other semen parameters, or other measures of the health of the male. If blood analyses are done, they should be conducted in an analytic endocrine laboratory. The inclusion of split serum specimens and/or known standard specimens may allow for the evaluation of the validity and quality control of the analyses.

Considerable fluctuation in the blood levels of reproductive hormones is expected and, thus, results based on single samples per individual may be of little clinical value. However, group means may be potentially useful for the assessment of toxicant effect. The benefit of hormone analysis in a field study that already includes semen analysis has not been fully established (23).

Biomarkers of semen analysis. Given the inherent limitations of questionnaire data, physical examinations, and blood hormone analyses for identifying changes in fertility status, consideration is usually given to evaluating semen quality as a biomarker of male reproductive effects (5,11). Semen analysis provides direct information about sperm production (numbers of sperm, normalcy of shape) and function (viability, vigor, and normalcy of shape), both of which are necessary for fertility. Furthermore, recent technical advances in standardization of methods for semen analysis and collection of specific semen endpoints for analysis have improved the predictive value for identifying perturbations in male re-

productive function (24–28). Individual semen biomarkers, including new, possibly more sensitive and specific biomarkers, are discussed later. Thus, while it may be difficult to link exposure to infertility in a particular field population, it may be possible to link exposure to altered semen quality. This may be especially useful and important to do in the context of acute exposures where fertility measures would not be available for about a year or where fertility data would lack statistical power due to small numbers of people exposed. Semen biomarkers of physiologic damage related to changes in fertility status are listed in Table 4. Wyrobek et al. (1) reviewed more than 100 chemical agents and mixtures for their effects on sperm count, motility and morphology. Tables 1 and 5 underscore the fact that few data are yet available for environmental and occupational agents.

Semen analysis is relevant for two key aspects of male reproduction: 1) changes in sperm or seminal content may be associated with changes in fertility potential that may be relevant to the individual, and 2) defects in sperm DNA or chromosomes may be associated with detrimental effects on the viability of the embryo and subsequent health risks to the newborn. Thus, it must be understood that certain semen tests may be better associated with fertility changes while others may serve as biomarkers of potential heritable effects. Several reviews can be consulted on the methodologies for assessing human male reproductive, genetic, and developmental toxicities (2,4,5,25–26).

One of the challenges in studies involving semen collection is to achieve a high rate of participation. An average participation rate in occupational studies of semen quality has been 50 to 60% (29). The following measures can be used to gain participation: incentive payments, providing descriptive literature about the study to all participants, maintenance of complete confidentiality and privacy, and rapid follow-up if the specimen is not supplied at the agreed-upon time. For occupational studies it also helps to enlist the support of both labor and management and to provide on-site recruitment of workers. For environmental studies, it is beneficial to develop and enlist the support of a community advisory

Table 5. Occupational and environmental exposures with evidence of adverse effects on human sperm quality

Carbon disulfide
Dibromochloropropane
Dibromochloropropane and ethylene dibromide
Lead
Toluene diamine and dinitrotoluene
Carbaryl ^a
Kepon ^a
Ethylene dibromide
2-Ethylene glycol

^aSuggestive evidence only.

group. In general, the participation rate is substantially improved with outreach, education, and payment of participants (30,31).

An ideal semen biomarker for measuring toxicity to the male reproductive system should be a chemical, biochemical, or cellular factor that is a constituent of spermatozoa or semen (or other body fluid) that can be characterized objectively. The biologic mechanism represented should be sufficiently well understood that changes in the assay measurement can be directly related to changes in the biologic cell or tissue being monitored (e.g., germ cell, Sertoli cell, Leydig cell, accessory glands, epididymides, efferent ducts). Ideally, it should also be understood how changes in the biomarker measurement are related to changes in fertility status and changes in the chance of fathering an abnormal pregnancy (that is, the predictive value of the assay). With few exceptions, no biomarker has yet met all these criteria. In general, the mechanisms leading to biomarker changes are poorly understood at the biochemical or molecular level, even for changes in sperm counts. Only for values of sperm motion and morphology in the low-end extreme is there consensus among scientists on the predictive value of individual semen biomarkers. The following is an overview of the biomarkers of damage to male reproductive organs that have been selected for consideration for field studies (Table 4).

Sperm counts. The sperm count provides an index of the integrity and productivity of spermatogenesis. The number of sperm in the ejaculate is directly correlated with the number of germ cells per gram of testis. Azoospermia is the extreme of no sperm in the semen, and in the absence of efferent duct blockage it is an indication that type A spermatogonia have been lost and recovery is uncertain and unlikely (although not impossible as evidenced by the recovery of sperm counts in some DBCP-exposed men with azoospermia).

Sperm count is the most common variable measured in previous field studies assessing male fecundity. Some toxicants are known to affect the testis directly (that is, spermatogenesis) and thereby decrease sperm production. Exposure to DBCP reduced sperm concentration in the ejaculate to 46 million cells/mL in exposed workers compared to a median of 79 million cells/mL in unexposed men. Upon removing the workers from the exposure, men with reduced sperm counts experienced a partial recovery, while those who had been azoospermic generally remained sterile. Testicular biopsy revealed that the target of DBCP was the spermatogonia. This observation substantiates that the severity of the effect is most pronounced when stem cells are the target of toxicants (for references, see (1)).

Sperm count can be analyzed in whole semen or in a diluted specimen if that exact dilution is known. Sperm count is conducted using a chamber of known depth and a grid for counting. A hemocytometer or specialized chamber such as the Makler chamber may be used. Sperm counts can be measured in the field lab. Computer-assisted sperm analysis (CASA) sperm counts are not always accurate. CASA sperm counting must be validated across the entire range of sperm counts or manual counts should be conducted.

Abstinence, spillage, and vasectomy are all important confounders. At the time of collecting the semen specimen, each study participant should record the duration of abstinence, time of semen collection, and any information regarding spillage. Men who have had vasectomies must be excluded from the semen portion of the study. If the study participant indicates he has spilled (or lost) part of the specimen, try to get an indication of how much. If it is a significant fraction (e.g., greater than 10%), that specimen may be excluded from the sperm count assessment. If the man has lost some of the semen, it is best to request another specimen after the appropriate abstinence time.

Two or 3 d of sexual abstinence are typically requested from each man. Truth about abstinence time is important, and there should not be a penalty (e.g., withholding of payment) if the requested abstinence time is not met. Even though there is no way of verifying the abstinence time reported, an honest relationship with the donors should help assure veracity so that the abstinence interval can be used in the statistical analysis model as a confounder. Some studies have excluded specimens from men that fall below or above some reasonable range for abstinence time (e.g., <1 d and >7 d).

Statistical transformations are strongly recommended for sperm counts so that the data can be used for parametric analysis. For some variables an additional analysis based on categorization of the dependent variable might be considered. That is, in some populations, it is possible for a significant number of exposed participants to show an unusually low sperm count without having a significant effect on the average sperm count. Coding for sperm counts below a certain cutoff (e.g., 20 million cells/mL) and using parallel logistic regression analysis might detect a pattern when a *t*-test or a linear regression model would not.

Sperm motility. Adequate sperm motility is necessary but not sufficient for fertility. The visual assessment of sperm motion is being gradually replaced by CASA, which quantifies the pattern and vigor of sperm motion (32). Sperm motility reflects events in both the testis and epididymis.

Sperm motion can be characterized on three levels

of increasing comprehensiveness. The first level focuses upon the percentage of motile sperm, the percentage of progressive sperm, and the average straight-line velocity. The second level contains the distributions of the full set of kinematic parameters that characterize the pattern and vigor of motion. The third level focuses upon subpopulations of sperm with common kinematic signatures. Sperm motility includes both the pattern and vigor of motion. Different intracellular mechanisms control these two facets of motion. The straight-line velocity reflects both the pattern and vigor of motion and is the primal measure of sperm kinematics. The use of level 2 and level 3 procedures may increase the sensitivity of the sperm motion analyses.

The whole, undiluted semen is the medium for level 1 motility analysis. However, the determination of cut points for straight-line velocity and linearity of motion must be defined. Currently, semen must be diluted for analysis at levels 2 and 3, due to limitations of contemporary CASA instruments in tracking sperm the paths of which cross over each other. Current recommendations are that CASA assessment of percent motility be conducted using neat semen (undiluted) and that velocities be analyzed using at least a 1:1 dilution of semen with a physiologic buffer (33). The CASA methodology for level 1 analysis is well developed. CASA analysis at level 2 is reasonably well defined, but the choice of semen diluent is not yet optimal. CASA analysis at level 3 is still at a primitive level, but initial results are very promising. The significance of shifts in CASA-derived sperm motion measures is not fully understood.

A phase-contrast microscope equipped with a videomicrographic system is necessary to make video recordings of the fresh semen on site. The video images can be stored to videotape or converted directly to digital format for later analysis. Semen should be collected into standardized containers and transported to the host laboratory in standardized containers that stabilize temperature. Whether plastic or glass, the containers must be pretested to confirm that the interior of the container is inert in terms of sperm motion. Videotaping should commence within 1 h of semen collection. As with sperm concentration, abstinence should be controlled and researchers typically use semen from a range of abstinence times of 2 through 7 d. Chemical contamination of semen, temperature shock, or undue delay before taping are grounds for exclusion. There is evidence of dose-response relationships between exposures to toxicants and sperm motion parameters obtained by CASA in humans and rats. However, interpretation with respect to changes in fertility is limited by lack of data for this outcome.

The insights gained from joint motility and morphology studies are significantly greater than those

gained from either type of study alone. The technology of sperm motion analysis is ever improving, with more sophisticated algorithms and availability of high-speed computers that allow more frequent sampling and a more refined visualization of individual sperm.

Sperm morphology. Assessing the physical characteristics of sperm and especially of the sperm head provides indices of the integrity of spermatogenesis and spermiogenesis (34). Sperm morphology has been a traditional semen characteristic for assessing the fertility status of men. During the past 30 years several morphology schemes have been presented for the assessment of normal and abnormal sperm morphologies. While some sperm obviously fit into specific classifications, there are many sperm of transitional shapes and sizes that make repeatability of classification difficult for a technician and even worse between laboratories (35–38). The Genetox review of the use of sperm analysis for assessing the male reproductive effects of occupational, environmental, and therapeutic agents on human semen (1), showed that before 1983, more than 20 agents and complex mixtures had been evaluated by their effects on human sperm morphology.

With advances in computerized image analysis, several methods of sperm morphometry have been introduced (39–45). Morphometric analysis systems provide objective assessments of individual sperm head size and shape; however, comparisons between measurements from different analysis systems should be avoided (34). Several systems are under development, including those that measure only the most basic of nuclear parameters (e.g., area, width, length) to those that include measures of aspects of shape (e.g., eccentricity, sphericity) and nuclear texture (e.g., heterogeneity) [e.g., Moruzzi et al. (45)]. Boyle and colleagues (42–46) have shown a relationship between sperm head morphometry and fertility in veterans.

Sperm morphometry is now routinely used as part of the assessment of reproductive hazards to the male worker (5,15). Several field studies have employed sperm morphometry to evaluate the effects of occupational exposure on semen. For example, a study by Ratcliffe et al. (31) reported a significant reduction in sperm head width with ethylene dibromide exposure of male workers.

Sperm morphology and morphometry analyses are conducted on microscope slide smears of whole semen. A variety of staining procedures is available for morphologic categorization (27). Two staining procedures are widely accepted for sperm morphometry. The first stains the entire head (head morphometry) and the other stains the nucleus (nuclear morphometry). It is recommended that both types of morphometry be conducted. It is rec-

ommended that at least six slides be prepared from each semen specimen: two for sperm head staining (traditional morphology and head morphometry), two with a nuclear stain (nuclear stained), and two unfixed and unstained for repository use.

There are several limitations and remaining questions in the application of sperm morphology and morphometry analysis. First, in spite of years of awareness about the problem, there is still no firm agreement about what constitutes normal sperm morphology, as judged by visual criteria. Some hope that the advent of image analysis will bring about the physical measurements of sperm that will lead to research to address this question using objective sperm measurements. Second, the morphometric analysis of sperm components requires a better understanding of the effects of sperm fixation, pretreatment and staining conditions (47). Third, sperm morphometry remains poorly understood in terms of the specific parameters that can be measured, the pixel resolution that should be utilized, what aspects of a distribution to utilize in the statistical analysis, and the need for the development of special statistical approaches to utilize the large volumes of data that can be generated from measurements of even a small number of sperm. That is, one laboratory routinely makes more than 50 measurements on each of 100 sperm and then utilizes up to three aspects of each parameter distribution to describe each semen specimen (thus, more than 150 data points are generated for each semen specimen). Image analysis essentially is not limited in the numbers of measurements that can be made. The research challenges are to identify the parameters that are associated with types of infertility, those that may be associated with exposure to toxic agents, and those that may be indicative of an increased probability of fathering a defective child.

Other semen biomarkers

Creatine kinase assays. Creatine kinase (CK) in human spermatozoa is a promising biochemical marker of late sperm development (i.e., spermiogenesis). Increased sperm CK concentrations reflects residual cytoplasm in spermatozoa that did not complete the developmental step of cytoplasmic extrusion (48). A change in the biosynthesis of CK-isoform types occurs simultaneously with cytoplasmic extrusion. The value of both CK-activity and isoform ratios in predicting male fertility was demonstrated in clinical studies (49–53). Immature sperm populations with higher cytoplasmic content and diminished CK-M ratios were more susceptible to oxidative damage by lipid peroxidation. Increased rate of lipid peroxidation may be an inborn rather an acquired property of spermatozoa (54). The response of CK as biomarker has not yet been evaluated in field studies of

men exposed to potential reproductive toxicants, and further studies are encouraged.

Viability assays. Sperm viability is dependent on a multitude of cellular processes including membrane integrity. Tests of membrane integrity focus upon the tail or head of the sperm. Membrane integrity is most frequently assessed using the hypo-osmotic swelling test (HOS), which is a well-defined viability test with a morphologic endpoint involving the tail (55). Several supravital stains are available to assess the viability using the sperm head, notable Hoechst-3258. Sperm lacking membrane integrity take up the dye and their DNA fluoresces brightly when compared with that of viable sperm, which exclude the dye efficiently over the time scale of the test and do not stain brightly. Because the loss of sperm membrane integrity is closely associated with a loss of sperm motility, it is unlikely that viability tests will have more than a complementary role in reproductive toxicity testing.

Assays of sperm function. Sperm function is the primary concern in studies of reproductive toxicology (4). However, the assays for sperm function tend to be tedious, lack objectivity, are difficult to replicate, and usually, extremely difficult to employ in field studies. Several tests have been developed to assess sperm function, including the sperm mucus penetration assay and sperm acrosin-level measurements, which investigate properties of spermatozoa in isolation or in an environment that represents a challenge. Sperm function has also been evaluated using oocyte binding or oocyte penetration using either zona-free hamster oocytes or human hemizona or salt-treated human oocytes. These tests and their successors may play encouraging roles in clinical studies of infertility, but it is the opinion of the committee that these tests are too problematic for routine application in field studies.

No biomarkers of normal function of Leydig cells, Sertoli cells, epididymis, prostate, or seminal vesicles are suitable at present for inclusion in a field study of male reproductive toxicity. However, it is hoped that relevant biomarkers will be developed in the future because it is well recognized that these organs and cells play critical roles in sperm development and semen production.

Semen sampling considerations

This discussion will focus on the field application aspects of semen collection (25). The type of exposure and the study design will determine the sampling regimen. Optimally, a specimen should be analyzed within 1 h of production and insulated from temperature shock so that the spermatozoa remain viable for an adequate analysis. The laboratory technician should be requested

to record the time of collection and time of initial analysis for semen. These times may affect the interpretation of the results, especially motility.

Collection procedure. Standardized semen collection procedures are needed using specially provided containers that are proven to be inert in terms of a sperm motility effect. No sexual partners, condoms, or lubricants should be used. The entire volume of ejaculate should be collected and the specimen must not be subjected to extreme temperatures.

Number of specimens per man. Count shows more sample-to-sample variability within a donor than does percent motility, mean swim speed, or morphology. Serial semen specimens spaced at least 2 d apart can be used to obtain a reliable estimate of a person's semen parameters, but multiple samplings are typically not feasible in cross-sectional surveys.

Cooperation from potential study participants. Participation in the comparison group may be biased toward those with preexisting reproductive problems.

Expertise in specific semen samples. Semen samples of controls and test men can be split and mailed to the expert laboratories for analysis. Normal ranges and analyses for both traditional and computer-assisted semen assessment can vary considerably between laboratories. It is, therefore, important to select an experienced laboratory (preferably one with experience in field investigations).

PRIORITIZING THE BIOMARKERS TO BE APPLIED TO COLLECTED SEMEN

There is much confusion over what actually constitutes a normal population of men and how baseline data for biomarkers should be selected. Sperm count is used here as an example because it is the best understood and most studied of the sperm biomarkers. Early reports identified normal men as "fertile" when a man had fathered children recently or had a pregnant wife (56). More recent reports of baseline values are derived from cross-sectional analyses of a general population that is termed "normal" (57). These so-called normal populations include prevasectomy patients (58), and unexposed reference populations for epidemiologic field studies (12). Study groups for epidemiologic populations are expected to include individuals who are "subfertile" or "infertile." It should be recognized that each of the groups actually represents a different segment of the overall population of men and thus, it is not surprising that differing numbers were reported.

The authors suggest that once it has been deter-

mined that a field study of male reproductive disorders is warranted (i.e., based on the analyses of the prerequisites, as described above), such studies should always include semen collection. Sufficient monies should be prioritized to collect adequate numbers of semen samples and to provide for proper storage of information and frozen stored semen aliquots. However, practical and financial issues may be considered when selecting the specific semen assays to be analyzed. The working group suggests two phases or tiers of semen analyses:

Tier 1 semen analyses:

- certain conventional semen assays should be performed immediately on each sample collected, such as sperm count, visual motility, and visual morphology; and
- certain specific semen assays may also be performed immediately, depending on the health effect of concern. For example, the semen assays of genetic damage may be included if there is a genetic concern (i.e., spontaneous abortions, etc.).

Tier 2 semen analyses:

- remaining semen may be stored into aliquots so that additional assays may be performed in the future. It was the consensus of the authors that aliquoted and coded semen samples provide a critically valuable resource for further analyses of samples, which will become important as better and more efficient assays become available to study the effects of specific exposures and as exposure changes in a selected population.

Semen biomarkers for field studies should have standardized methodology for measurements and reporting. However, state-of-the-art bioassays are typically available only in the laboratories of active researchers and these biomarkers are usually undergoing continued improvement. In addition, biomarkers differ markedly in their degree of development and validation (5).

It is important to understand the constraints when results from one laboratory are compared with those of another. Sperm counts are so inherently variable and interlaboratory differences probably play only a minor role.

Motility is heavily dependent on technical differences among laboratories as well as differences in the criteria used in making visual assessments or in the type of instruments used to make automated assessments. More recently, studies analyzing video tapes on CASA systems among laboratories have been conducted to evaluate the repeatability of the system. The data are encouraging, although further work on the standardization of methods is required. The need for fresh specimens for assessing the motility variables has made such comparisons difficult. In addition, laboratories must adapt

common standards for CASA instrument operational settings as well as specimen preparation guidelines.

A considerable amount has been written about the interlaboratory assessment of sperm morphology. Differences in criteria for assigning a sperm to a specific shape category and for deciding what constitutes a normally shaped sperm have been responsible for the dramatic interlaboratory differences in scoring results that are reported (see morphometry discussion). This is illustrated by the differences in frequencies obtained using the WHO criteria vs. the strict criteria. There was an unrealistic optimism and hope that image analysis could solve these problems. Image analyses has brought a high degree of objective measurements to the problem, but in itself it cannot define a normal sperm or normal morphology.

The objective aspects of image analyses are highly advantageous for making comparisons of sperm measurements among different laboratories. Image analysis is very well suited for making specific morphometric measurements of sperm (e.g., length, width, eccentricity, etc.). Thus, any laboratories using similar methods for making sperm measurements (as well as the same slide preparation techniques and microscope settings) should then obtain similar values. However, biologists and clinicians generally differ in how they assess infertility and semen quality. Although computer scientists may successfully incorporate specific measurements into algorithms for classifying semen samples as normal or abnormal, one must accept that these algorithms will closely reflect the definitions of infertility used by the local clinicians. However, the availability of the Internet promises that classification algorithms developed at any center may be easily transferred among clinics for their evaluations and comparisons.

Few studies have reported on the extent of correlation of sperm measures. That is, Schrader et al. (57) showed that measures of sperm viability (stain exclusion and HOS) and sperm motility (percent motile) were moderately correlated (about 0.5 or 0.6). Lack of strong correlations among parameters suggests that the individual measures assess different characteristics of the sperm cells, and, therefore, would be both useful in a study of reproductive toxicity.

Based on the statistical analysis of data obtained from a longitudinal evaluation of a cohort of healthy men (57), it appears that sperm morphology, sperm velocity, the HOS assay, and the vital stain assay probably are the most useful semen parameters to measure, because they provide relatively precise information on both the individual and the population. Semen volume, sperm count, and sperm motility have lower precision and would appear to be less useful.

BIOMARKERS OF HERITABLE GENETIC DAMAGE

The role of the father in reproduction goes beyond sperm production and fertilization. The molecular analysis of chromosomal abnormalities and mutations in newborns has demonstrated conclusively that certain specific genetic defects preferentially involve the paternal chromosomes, especially aneuploidies involving the sex chromosomes (e.g., XYY, XXY, XO), de novo structural aberrations, and gene mutations (59). Detailed and long-standing studies of abnormal reproductive outcomes and gene mutations in offspring of atomic bomb survivors or childhood cancer have not yet identified ionizing radiation as a human germinal mutagen. Some investigators attribute this to the inefficiencies of the methods used to assess these effects and to the limited number of offspring that have been studied. On the other hand, there are numerous epidemiologic reports of associations between paternal exposure to toxicants (e.g., paints, solvents, occupation) and abnormal reproductive outcomes (2). However, it remains unclear whether these epidemiologic findings are (a) chance occurrences; (b) due to indirect exposure of the egg, embryo, or fetus via the mother; or (c) due to genetic lesions carried by the fertilizing sperm. The induction of genetic lesions in male germ cells has been best studied in animals where it has been known for over 4 decades that exposure of male mice to mutagens prior to fertilization results in any of a variety of genetic, chromosomal, and morphologic abnormalities and losses during development. A 1989 NRC report organized methods for the detection of genetic damage in germ cells and of heritable mutations in humans into categories depending on the tissue source of the measurement: testicular biopsy, offspring tissue or data, and semen (Table 6).

Studies of pregnancy outcomes and offspring

Studies of genetic defects inherited by offspring fall into three categories: 1) those based on questionnaires, medical record information, and/or data registries for spontaneous abortions, birth defects, etc., 2) those based on protein alterations in the offspring; and 3) those based on molecular changes in the DNA of the child. Conventional epidemiologic methods have identified various potential human reproductive and developmental toxicants (2). Using questionnaire-based techniques, studies may provide evidence of increased abnormal reproductive outcomes for group(s) of exposed individuals. It is essentially impossible when using these methods, however, to distinguish between induced and spontaneous abnormalities in individual families. In principle, longitudinal studies involving episodic exposure or follow-up studies

Table 6. Biomarkers of genetic damage and heritable mutations in the male germline^a

Tissue or Source	Type of Measurement
Semen (sperm)	Sperm cytogenetics (hamster technique) Sperm DNA and protein adduction Sperm aneuploidy and chromosome aberrations by FISH Sperm chromatin, single- and double-stranded DNA
Semen (immature germ cells)	Spermatid micronuclei Cytogenetics of ejaculated meiosis-I cells
Testes biopsy	Cytogenetic analyses of cells in mitosis, mitosis I, and meiosis II
Urine (female partner)	Early fetal loss detection
Offspring tissue	Cytogenetics DNA sequencing Protein mutations DNA restriction-length polymorphism RNase digestion Subtractive hybridization of DNA Denaturing gel electrophoresis of DNA Pulse-field electrophoresis of DNA
Offspring surveys and medical records	Sex ratio Miscarriages Offspring cancer Sentinel phenotypes

FISH—fluorescence in situ hybridization.

^aSource: National Research Council (4).

after the end of an exposure period may provide information at the level of the individual, but few epidemiologic applications have used this design and this strategy would work only for certain biomarkers (e.g., sperm biomarkers of cytogenetic damage, see below). Investigations of alterations in electrophoretic patterns of proteins also have not detect any mutagen-induced effects in offspring. Lastly, the applications of genomic alterations utilizing DNA molecular techniques for the detection of heritable mutations are still under evaluation.

Cytogenetic methods utilizing male germ cells

Although the utility of methods based on testicular biopsies is extremely limited for essentially every human exposure, these methods have provided valuable reference data on the frequencies of chromosomal abnormalities in germ cells [see review by Wyrobek (5)]. Most noteworthy is the dose–response relationship for the fraction of spermatocytes carrying reciprocal translocations detected at meiotic metaphase I after testicular irradiation. This finding demonstrates that the testis is not resistant to the chromosome breaking effects of ionizing radiation.

Semen contains immature and mature germ cells and increasing attention recently has been given to the development of markers of genetic damage utilizing

these cells. Several categories of genomic alterations in male germ cells are known or suspected to be associated with paternally transmitted developmental toxicity and genetic disease: gene mutations, structural abnormalities in chromosomes, abnormalities in the number of chromosomes, alterations in paternal genomic imprinting, and the production of DNA or protamine adducts in germ cells. There is currently no accepted procedure for detecting gene mutations directly in sperm.

The human sperm–hamster egg in vitro cytogenetic system has been used for over a decade to collect important baseline information on the fraction of sperm that carry abnormalities in the number and structure of paternal chromosomes (60). The importance of aneuploidy in sperm has been long appreciated as shown by the interest in the F-body tests, although this method has been brought into serious question. More recently, fluorescence in situ hybridization with DNA-specific probes has been developed as a valid measure of aneuploidy in sperm. This method has been used to establish baseline levels of aneuploidy for various chromosomes (61–66). This method has been used to detect increases in the fraction of aneuploid sperm in cancer patients who received chemotherapy (Robbins et al., in press) and in older men (65). Micronuclei in immature spermatids are found in the semen of most ejaculates and they might reflect chromosomal damage in germ cells.

The molecular mechanisms of sex-specific genomic imprinting are not well understood, and no biomarkers for imprinting are yet available.

Several studies have also attempted to use DNA adducts in semen to assess exposure of the male to germinal mutagens. However, there have been difficulties in retrieving DNA for analysis from sperm that is heavily crosslinked with disulfur bridges of protamine. DNA adducts have been measured in human sperm using the ³²P postlabeling method. In mice, it has been questioned whether protamine adducts rather than DNA adducts might play a larger role in the induction of dominant lethality of the offspring. Better methods (that is, non-isotopic) are needed for measuring the level of DNA and protamine adducts in human sperm.

Sperm chromatin stability

The sperm chromatin stability assay, which measures the relative amount of single and double strandedness in individual sperm nuclei, may provide information regarding genetic damage to sperm. To perform the assay, the sperm DNA is stressed thermally or chemically and then stained with acridine orange (67). Double-stranded DNA is stained green while single-stranded DNA is stained red (very little if any RNA is present in mature sperm). This analysis is performed with a flow cytometer. Animals exposed to known mutagens have an

increase in single-stranded DNA, indicating an increase in genetic damage (68–70). The fertility rate of bulls is correlated with the percentage of double-stranded DNA (71). A recent report indicates that the DNA stability assay is highly repeatable between ejaculates from the same man (72). This procedure has been developed in only a few laboratories at this time; however, the sperm can be frozen on dry ice and shipped to the analysis laboratory without affecting the results.

Interpretation of biomarkers of genetic damage in germ cells

It is unlikely that all or even most men of a group will respond in the same way to the same mutagen exposure, especially for agents requiring metabolism and for those that induce lesions for which repair pathways are available. However, we understand very little about this concept, and biomarkers of susceptibility differences to mutagenic agents are not known. Thus, further study is needed to characterize the susceptibility of males at the genetic level. This characterization will require the identification of specific genes involved in metabolism to mutagenic metabolites and in the repair of specific DNA lesions. Towards the end of spermatogenesis, spermatids lose DNA repair capability and DNA damage is not repaired until after fertilization.

The evaluation of the predictive value of measurements of genetic damage in male germ cells must consider the effects of egg and female factors, emphasizing again the importance of considering the couple as a unit when evaluating abnormal reproductive outcomes. There is no evidence that sperm carrying genetic defects have any selective advantage or disadvantage in fertilization. One of the least understood aspects of male factor in abnormal reproduction is the question of interaction between prelesions carried by the sperm and the capacity of the egg to alter the prelesions. This concern is based primarily on data from model systems. In mice, it has been shown that the genotype of the female is critical in determining the fraction of embryos that terminate in dominant lethality. Using the hamster-egg technique, the fraction of chromosomal breaks in human sperm has been found to be dependent on the repair status of the hamster egg. Unresolved breaks and DNA prelesions (e.g., adducts, apurinic sites) are most likely to be affected by the genotype of the egg. The fraction of sperm with abnormal numbers of chromosomes or with recombinant chromosomes is least likely to be affected by the genotype of the egg.

STUDIES ARE NEEDED TO UNDERSTAND THE MECHANISMS OF ACTIONS OF TOXICANTS

Primary research challenges in reproductive and developmental toxicology are to identify the mechanisms

Table 7. Mechanisms of male reproductive toxicity and male-mediated developmental toxicity

Categories of toxicity	Potential pathways
Toxicity to male endocrine system and sexual function	Toxicant metabolism Neuroendocrine effects Accessory sex gland effects
Toxicity to male reproductive tract and germ cells	Toxicant transport to reproductive tract ^a Germ cell effects
Genetic toxicity to male germ cells or genetic defects in offspring mediated via sperm	DNA, genetic, and cytogenetic effects in sperm Genetic, cytogenetic, and phenotypic effects on offspring due to paternal exposures
Indirect effects on the offspring occurring during pregnancy	Toxicant carried to egg by the sperm Exposure of pregnant women via semen during intercourse External exposure of pregnant woman by the male partner

^aIncludes effects on the epididymis, testis, and accessory glands.

leading to abnormal reproductive outcomes and to identify the physiologic factors, genetic factors, and environmental exposures that could increase the frequencies of abnormal outcomes. Understanding the mechanisms by which toxicants act is critical to the development of biomarkers to detect these different pathways of toxicity in exposed humans. Animal studies continue to be important in the characterization of toxicant effects and in studies of mechanisms. The selection and utility of animal models, however, requires a kit of biomarkers that measure the identical defects in animals and humans, that is, bridging biomarkers (5). Research involving in vitro assays is also expected to play an important role in defining certain biochemical mechanisms of action. Animal and in vitro assays were not evaluated in this report.

Biomarkers are playing an ever increasing role in the search for understanding mechanisms of action leading to abnormal reproductive outcomes. As listed in Table 7, the categories of mechanisms discussed by the authors included toxicity to male endocrine system and sexual function, toxicity to male reproductive tract and germ cells, genetic toxicity to male germ cells or genetic defects in offspring mediated via sperm, and indirect effects on the offspring occurring during pregnancy. A better understanding of the mechanisms promises to be very important in the future understanding of the causes of toxicity to the male reproductive tract and of male-mediated abnormalities in fetal development.

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