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MALE REPRODUCTIVE DISORDERS*

Workgroup Members

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Summary: *Five major disorders of male reproduction can be affected by toxicant exposure: infertility, sexual dysfunction, hormonal disorders, pregnancy and birth abnormalities, and semen abnormalities. However, no simple prescreening method has yet been validated; questionnaires alone are too imprecise and inefficient. Semen collection and analyses are critical components of field studies of male reproductive disorders for two key reasons: (1) changes in sperm or seminal content can be associated with changes in fertility potential of an individual and (2) defects in sperm DNA or chromosomes can be associated with*

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subsequent changes in viability during development and health risks to the offspring. Additional considerations for effective field studies are: (1) accurately timed and well-characterized exposure data for each study participant; (2) adequate numbers of participants as dictated by the statistical power of the biomarkers to be employed; and (3) proper semen specimen collection and storage, and standardized analyses. Semen analyses can be conducted in two phases (tiers): (1) conventional first tier semen assays as well as specific biomarkers indicated by the health effect of concern in the study population; and (2) later, second tier analyses of archived specimens, if indicated.

The male reproductive system involves a series of interactions between somatic and germinal tissues as well as a complex hormonal axis with central nervous system controls. Exposures to environmental toxicants can 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 (*1*).

A variety of risk factors, including exposure of a male to ionizing radiation or to certain chemical toxicants, can diminish or destroy his fertility and adversely affect embryo viability and the health of his children (Table 3-1) (*2,3*). These latter effects can be manifested at birth or later in the life of the offspring.

The workgroup on male reproductive disorders noted that there are not any simple prescreening tools for identifying, in a field study setting, a potential link between an environmental exposure and male reproductive defects. Sufficient time and effort are needed to evaluate the circumstances of a particular study site to determine whether a male reproductive study is warranted. If so, then a comprehensive study should be conducted, including semen analyses and other biomarkers. Individual semen biomarkers can be prioritized into phases (tiers), as is discussed later in this chapter.

Table 3-1.—Known or suspected risk factors for male reproductive abnormalities.

Characteristic	Risk Factors
Personal	Age of the participant
Medical conditions	Recent infection Trauma Autoimmune disorders High fever Mumps orchitis Diabetes Prostatitis Varicocele History of infertility
Prescription drugs*	Estrogens Chlorambucil Cyclophosphamide Nitrofurantoin
Habits and recreational drugs*	Tobacco Alcohol Use of sauna or hot tub Recreational drugs (for example, marijuana)
Environmental agents* Chemical	 Solvents Metals Pesticides Neurotoxicants
Physical	Heat Ionizing radiation

*Additional agents listed in Table 3-5.

REPRODUCTIVE HEALTH CONCERNS OF MALE ORIGIN

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

- Infertility or subfertility in couples when men were exposed.
- Sexual dysfunction in exposed men.
- Endocrine disruption, hormonal imbalance, or altered sexual differentiation in exposed men or boys.
- Developmental defects in offspring of exposed men (for example, birth defect, stillbirth, miscarriage, fetal loss, childhood cancer, cytogenetic abnormality, and specific genetic defect).

Concern over induced male reproductive disorders might 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. Various events or situations can trigger concerns about male toxicants as causes for specific abnormal reproductive outcomes.

INFERTILITY

Possible "scenarios" (examples of situations specific to individual communities) that would trigger a male fertility concern would be: (1) previous reports (animal or human) indicating that a certain toxicant could have a detrimental effect on fertility, (2) known mechanisms of the toxicant, (3) reports from infertility workups that individuals from the population have poor

quality semen (that is, a cluster of individuals), or (4) subfertility in the population. Public perception that exposure to hazardous substances is associated with male infertility can also develop after anecdotal accounts of failures or delays in initiating pregnancies. Potential genetic problems can also present as fertility problems.

Sexual Dysfunction and Hormonal Disorders

Scenarios leading to concerns of altered sexual or neuroendocrine function would include: (1) clusters of men with abnormal reproductive hormone profiles, (2) a cluster of men with problems in sexual function, (3) men with potential exposure to toxicants having endocrine properties (competitive or inhibitory), and (4) men with exposure to toxicants shown to affect the neuroendocrine system (for example, lead). Also, neuroendocrine disruptions while in utero might affect the morphology and function of the male reproductive system in the adolescent or adult (*4*).

Pregnancy and Birth Abnormalities

Male-mediated adverse effects on pregnancy and birth outcomes and developmental abnormalities are of particular concern (1) when the toxicant exposure is known and the agent involved is known to cause genetic damage in germ cells or other types of cells in animal model systems, (2) if there is a cluster of males who have fathered adverse pregnancies, or (3) if a cluster of adverse pregnancies has been observed in the population, especially when there are putative male exposures.

STUDY DESIGN CONSIDERATIONS

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 (gestation, childhood, and adolescence) during germ cell development and during spermatogenesis (adulthood).

Spermatogenesis involves a continuously replicating cell population, therefore substances whose toxicity depends on cell division might have greater effect on the male germ cells than on female germ cells. Human spermatogenesis requires approximately 75 days for a stem cell to differentiate into a sperm within the seminiferous tubules and about 12 days for the sperm to travel through the epididymis to the vas deferens. Thus, it is important to obtain the exposure history during the 90 days prior to the specimen collection (in the case of semen studies) or prior to the date 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 cell, spermatogonial mitoses, meiosis, post meiosis, and post testicular) might have markedly differing sensitivities for different agents and, in the extreme, only one cell type of spermatogenesis might be sensitive. In the latter case, only those exposures within the small window of time within the 3 months prior to specimen collection are important toxicologically. In the case of erratic exposures, grouping all men who were exposed at some time over the 3 months prior to specimen collection might yield a considerable fraction of false-positive exposure classifications. Further complicating the situation, a single toxicant might induce differing spermatogenic lesions leading to different reproductive outcomes, and each outcome might have a different spermatogenic time window of sensitivity. Using the data for the nematocide dibromochloropropane

(DBCP) as an example, (1) exposing stem cell and dividing spermatogonia would result in cell killing and reduced sperm count, which would in turn result in infertility, (2) exposing post meiotic cells would induce dominant lethality in the offspring, and (3) exposing epididymal sperm would result in sperm motility defects resulting in infertility. However, what is true for DBCP might not necessarily be true for other substances. 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 potentially confounding variables, such as exposures to other risk factors.

Another key challenge in field studies is to distinguish between maternal and paternal exposure. It is well recognized that prolonged exposure 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*).

Community-based studies usually have relatively low exposure levels for study participants and the gradient between "exposed" and "unexposed" might 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 identify a paternally exposed group of couples (exposed male with unexposed female sexual partner) and a nonexposed 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. 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 in the following sections. A screening questionnaire can be used to collect data on the exposure histories of men and their partners. Exposure data for time periods before, during, and after conception need to be collected to address questions regarding the various possible mechanisms of toxicity that are relevant for male-mediated effects (Table 3-1).

Table 3-2 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. In particular, individual reproductive histories can reveal altered reproductive function such as delayed or accelerated puberty, sexual dysfunction, or changes 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.

One or multiple semen specimens might be requested per donor, depending on the type of exposure and specific study design used. A chronic exposure over months to years might indicate that a cross-sectional study could be used, in which exposed men were compared to a concurrent "control" group of men, with only one semen specimen per donor.

If a study is being proposed after an accidental spill, release, or exposure to one or more hazardous substances, a longitudinal study design would be most useful. In this type of study, serial semen specimens from each exposed individual are used to characterize the induction and persistence of sperm damage, including the possible return to baseline values. A concurrent

Table 3-2.—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, or caffeine Diet, stress, and exercise Exposure to heat Use of recreational drugs
Exposure to chemical and physical agents*	Exposures to specific substances Exposure to heat Exposure to ionizing and nonionizing radiation
Other	Related to physical examination findings

*Details should include amount, duration, time periods, and the like.

control group is useful in a longitudinal design to detect seasonal and other environmental changes that might affect the final interpretation of results. It takes approximately 90 days for a stem cell to differentiate into a mature sperm in the ejaculate. A sampling procedure that collects specimens throughout the spermatogenic process can provide critical information on toxicant effects. Using this study 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 cells in the epididymis or if the accessory sex glands are being affected. For example, one study (*6*) sampled at the following times: 1 day, 1 week, 1 month, and 3 months after exposure. If a persistent effect was noted, a follow-up specimen was collected 1 year after the exposure to assess possible recovery.

An important prerequisite before initiating a field study is to determine whether there are sufficient numbers of potential participants. The number of men included in a study population depends on the assays and health end points to be evaluated. The workgroup suggested that sample sizes be calculated to detect at least a twofold change for the critical semen assays and that biomarkers be used as described by Bloom and colleagues (*7*).

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 might require hundreds of pregnancies. By contrast, the sample sizes needed for semen biomarkers are typically smaller, ranging between 10 and 100 participants (using a 5% significance test with 80% power to detect a 25% change in the mean value) (*7,8*).

It is important to identify a control (comparison or reference) group and to categorize the study population into distinct exposure groups in order to assess whether any adverse outcomes are associated with the exposure(s) of concern (*9*). Clinical semen standards might be inadequate as reference values for men in the general population. The specific geographical

region in which the study is contemplated may have different "normal ranges" for the traditional semen parameters. It is also crucial to use an experienced laboratory for all semen 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: (1) are the men indeed exposed to the substance(s) of concern, (2) is a suitable reference group available, and (3) 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 (1) can the existence of the cluster of health abnormalities be confirmed, (2) is the rate of a given health outcome statistically elevated as compared with that of the general population, and (3) are there sufficient numbers of men available for a confirmatory study?

METHODS FOR ASSESSING MALE REPRODUCTIVE DISORDERS

Field studies of male reproductive disorders might, depending on the specific health effect of concern, include reproductive histories, physical examinations, blood analyses, and semen analyses (Table 3-3). As noted previously, they also need a convincing assessment of relevant exposure. Potential targets for male reproductive toxicants include both somatic and germ cells. Biomarkers have been developed to monitor (1) toxicant metabolism and transport to the reproductive system, (2) neuroendocrine effects, (3) germ cell effects, (4) accessory sex-gland effects, and (5) sexual function effects (*1*). Table 3-4 includes selected biomarkers of physiological damage that would have the most relevance for changes in fertility. The biomarkers of genetic

Table 3-3.—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*)
Semen abnormalities	Sperm count, motility, morphology, presence of toxicant in semen, genetic and biochemical biomarkers
Chromosomal anomalies in offspring	Chromosomal analysis (fetus or offspring)
Infertility*	Questionnaire 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.
*Used infrequently. Existing databases have been used in other countries (for example, Scandinavian countries).

Table 3-4.—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)	Early pregnancy detection
Surveys and medical records	Standardized fertility ratio Time to conception

*CASA—Computer-assisted sperm analysis.

damage and heritable mutations are discussed in a subsequent section of this chapter.

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 might have differing memories of reproductive events and, ideally, interviews should be conducted with both members of the couple to obtain reliable information on live birth and grossly abnormal birth outcomes. Women tend to be better at providing detailed information on pregnancy outcomes, maternal exposures before and during pregnancy, and episodes of infertility (*10,11*).

Currently, there is no standardized set of questions that is used by researchers to assess fertility status, history of abnormal outcomes, and involvement of confounding factors. The workgroup discussed typical reproductive questions that would be contained in such a standardized questionnaire. Table 3-2 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.

Overall Health Status

The physical examination can determine whether the reproductive tract has normal structures and whether there are any abnormal secondary sex characteristics. It is important to examine individuals for genital abnormalities that might interfere with normal spermatogenesis, spermatozoa transport, ejaculation, or erection. Such conditions include: varicocele, small testicular size, prostate tenderness, hypospadias, and cryptorchism.

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 3-1).

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 psychological components of reproduction (libido). 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. The analyses of the ejaculate provide 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 in the next section.

Impotence, the failure to achieve or maintain an erection, can have either a psychogenic or organic etiology. It is difficult to assess the potential effects of a toxicant on impotence in field studies, but certain methodologies can be applied in studies of well-defined subpopulations. While organic impotence is characterized by a 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 (*12*). Physiologic studies conducted during sleep can help differentiate between the two etiologies, but are not practical for field studies.

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 could be confounded by a reluctance to admit to sexual problems or by erroneous associations of sexual problems with workplace or environmental exposures.

Because of the technological and psychological difficulties encountered when the present assays are applied in field studies, few reliable data are available regarding the effects of occupational or environmental exposure on sexual function. Various medicines have been shown to affect each of the three stages of male sexual function (*13*), 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 can leak uncontrollably from the urethra in individuals who take these drugs.

Recreational drugs can also affect sexual function (*13*). Alcohol can induce impotence while enhancing libido. Cocaine, heroin, and high doses of cannabinoids can reduce libido. Opiates can 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 substances found in the workplace or environment might have similar properties. The potential for such effects could 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.

NEUROENDOCRINE FUNCTION

If the primary concern is an imbalance in the hormone profile that might or might 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 both total and free testosterone) 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 hormone 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 analytical endocrine laboratory. The inclusion of split serum specimens or known standard specimens, or both, might 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 specimens per individual might be of little clinical value. However, group means might be 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 (*14*).

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 (*9,15*). Semen analysis provides direct information about sperm production (numbers of sperm and normalcy of shape) and function (viability, vigor, and normalcy of shape), both of which are necessary for and related to fertility. Furthermore, recent technical advances in the standardization of methods for semen analysis, and collection of specific semen end points for analysis, have improved the predictive value of this test for identifying perturbations in male reproductive function (*16,17*). Thus, while it might be difficult to link exposure to infertility in a particular field population, it might be possible to link exposure to altered semen quality. This could be especially useful and important to do in the context of acute exposures in which fertility measures would not be available for about a year or where fertility data would lack statistical power due to the small numbers of people exposed. Semen biomarkers of physiological damage related to changes in fertility status are listed in Table 3-4. Wyrobek and colleagues (*2*) reviewed more than 100 chemical agents and mixtures for their effects on sperm count, motility, and morphology. Tables 3-1 and 3-5 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 might be associated with changes in fertility potential, which could be relevant to the individual, and (2) defects in sperm DNA or chromosomes might 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 might be better associated with fertility changes while others could serve as biomarkers of potential heritable effects. Several reviews can be consulted on the methodologies for assessing human male reproductive, genetic, and developmental toxicities (*1,3,15,17,18*).

Table 3-5.—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*
Kepone*
Ethylene dibromide
2-ethoxyethanol

*Suggestive evidence only.

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% (*19*). 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 followup if the specimen is not supplied at the agreed upon time. In general, the participation rate is substantially improved with outreach, education, and payment of participants (*20,21*).

An ideal semen biomarker for measuring physiological 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) and 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 (for example, the germ cell, Sertoli cell, Leydig cell, accessory gland, epididymis, or efferent duct). Ideally, it should also be understood how changes in the biomarker values 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 of 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 of the predictive value of individual semen biomarkers. Following is an overview of the biomarkers of physiological damage to male reproductive organs that have been selected for consideration for field studies (Table 3-4).

Conventional Semen Biomarkers

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 per milliliter (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 remained sterile. Testicular biopsy revealed that the target of DBCP was the spermatogonia. This substantiates that the severity of the effect is most pronounced when stem cells are the target of toxicants (*2*).

Sperm count can be analyzed in whole semen or in a diluted specimen if the 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 can be used. Sperm counts are conducted in the field. 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 (for example, greater than 10%), that specimen might 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 three days of sexual abstinence are typically requested from each man. Truth about abstinence time is important and there should not be a penalty (for example, 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 ensure 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 (for example, less than 1 day and greater than 7 days).

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 considerable number of exposed participants to show an unusually low sperm count without having a notable effect on the average sperm count. Coding for sperm counts below a certain cutoff (for example, 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 (*22*). 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 kinematics 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 might 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, because of limitations of contemporary CASA instruments in tracking sperm whose paths 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 (*23*). 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 measures of sperm motion is not fully understood.

A phase contrast microscope equipped with a video-micrographic 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 each container is inert in terms of sperm motion. Videotaping should commence within one hour of semen collection. As with sperm concentration, abstinence should be controlled; researchers typically use semen from a range of abstinence time of 2 through 7 days. 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 substantially 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 which 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. 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 (*24-27*). The Genetox review of the use of sperm analysis for assessing the male reproductive effects of occupational, environmental, and therapeutic agents on human semen (*2*) showed that, before 1983, the human morphology effects of more than 20 agents and complex mixtures had been evaluated.

With advances in computerized image analysis, several methods of sperm morphometry have been introduced (*28-34*). Morphometric analysis systems provide objective assessments of individual sperm head size and shape; however, comparisons between measurements from different analysis systems should be avoided (*33*). Several systems are under development, ranging from those that measure only the most basic of nuclear parameters (for example, area, width, and length) to those that measure aspects of shape (for example, eccentricity and sphericity) and nuclear texture (for example, heterogeneity) (*34,35*). Boyle and colleagues (*36*) 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 (*8*). Several field studies have employed sperm morphometry to evaluate the effects of occupational exposure on semen. For example, a study by Ratcliffe and colleagues (*21*) 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 morphological categorization (*16*). 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 recommended 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 (*37*). Third, sperm morphometry remains poorly understood in terms of (1) the specific parameters that can be measured, (2) the pixel resolution that should be used, (3) the aspects of the distribution that is used in the statistical analysis, and (4) the special statistical approaches that are needed to utilize the large volumes of data 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 uses 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 is essentially unlimited 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 might be

associated with exposure to toxic agents, and those that might be indicative of an increased probability of fathering a child with a birth defect or developmental disorder.

Other Semen Biomarkers

Creatine Kinase Assays.—Creatine kinase (CK) in human spermatozoa is a promising biochemical marker of late sperm development (spermiogenesis). Increased sperm CK concentration reflects residual cytoplasm in spermatozoa that did not complete the developmental step of cytoplasmic extrusion (*38*). 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 has been demonstrated in clinical studies (*39-43*). 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 might be an inborn rather an acquired property of spermatozoa (*44*). 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 hypoosmotic swelling test (HOS), which is a well-defined viability test with a morphological end point involving the tail (*45*). Several supravital stains, notably Hoechst-3258*, are available to assess the viability using the sperm head. Sperm that lack membrane integrity take up the dye and their DNA fluoresces

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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.

Assays of Sperm Function.—Sperm function is the primary concern in studies of reproductive toxicology (*1*). However, the assays for sperm function lack objectivity, and tend to be tedious, difficult to replicate, and usually extremely difficult to employ in field studies. Several tests have been developed to assess sperm function, such as the sperm mucus penetration assay and measurements of sperm acrosin levels, which investigate properties of spermatozoa in isolation or in an environment that represents a challenge. Sperm function has also been evaluated through oocyte binding using zona-free hamster oocytes, or oocyte penetration using hemi-zona or salt-treated human oocytes. These tests and their successors might play encouraging roles in clinical studies of infertility, but these tests are considered 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

Optimally, a specimen should be analyzed within one hour of production and insulated from temperature shock so that the spermatozoa remain viable for an adequate analysis. Laboratory technicians should be directed to record the time of collection

and time of initial analysis for semen. These times can 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.—Sperm count shows more specimen-to-specimen variability within a donor than does percent motility, mean swim speed, or morphology. Serial semen specimens spaced at least 2 days 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.

Expertise in Specific Semen Specimens.—Semen specimens can be split and mailed to the expert laboratories for analyses. 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 Semen Biomarkers

There is considerable uncertainty in designating any reference population as "normal" 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 a normal man as fertile if he had fathered a child recently or had a pregnant wife (*46*). More recent reports of baseline values have been derived from cross-sectional analyses of a general population that is termed normal (*47*). These so-called normal populations typically include prevasectomy patients (*48*), and unexposed reference populations

for epidemiological field studies (*9*). Study groups for epidemiological populations are expected to include individuals who are "subfertile" or "infertile." It should be recognized that each group actually represents a different segment of the overall population of men and, thus, it is not surprising that differing numbers are reported.

Once it has been determined that a field study of male reproductive disorders is warranted (based on the analyses of the prerequisites as described previously) such studies should always include semen collection. The investigators must commit a sufficient level of effort needed to collect adequate numbers of semen specimens and to provide for proper storage of information and frozen stored semen aliquots. The working group suggests two phases (tiers) of semen analyses.

Tier 1.—Certain conventional semen assays should be performed immediately on each specimen collected, such as sperm count, visual motility, and visual morphology. Certain specific semen assays can 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 reason (such as occurrence of spontaneous abortions) for genetic concern.

Tier 2.—Remaining semen can be stored in aliquots so that additional assays can be performed in the future. Aliquoted and coded semen specimens provide a critically valuable resource for further study of semen parameters. Such studies 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.

Based on the statistical analysis of data obtained from a longitudinal evaluation of a cohort of healthy men (*47*), 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.

HERITABLE GENETIC DAMAGE

Certain specific genetic defects preferentially involve the paternal chromosomes, especially aneuploidies involving the sex chromosomes (for example, XYY, XXY, and XO), de novo structural aberrations, and gene mutations (*49*). Detailed and long-standing studies of abnormal reproductive outcomes and gene mutations in offspring of atomic bomb survivors or in children with cancer have not yet identified ionizing radiation as a human germinal mutagen. Some investigators attribute this absence of a finding 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 epidemiological reports of associations between paternal exposure to toxicants (for example, paints and solvents) and abnormal reproductive outcomes (*3*). However, it remains unclear whether these epidemiologic findings are due to (1) chance occurrences; (2) indirect exposure of the egg, embryo, or fetus via the mother; or (3) genetic lesions carried by the fertilizing sperm.

The induction of genetic lesions in male germ cells has been best studied in animals; for more than four decades it has been recognized that exposure of male mice to mutagens prior to fertilization results in any of a variety of genetic, chromosomal, and morphological abnormalities and losses during development. A 1989 report by the National Research Council (NRC) 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 3-6).

Table 3-6.—Biomarkers of genetic damage and heritable mutations in the male germline.*

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 (from 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

*Source: National Research Council (*1*).
†FISH-fluorescence in situ hybridization.

Cytogenetic Methods

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 (*15*). 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 has recently been given to the development of markers of genetic damage using 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.

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 because the DNA is heavily crosslinked with disulfur bridges of protamine. DNA adducts have been measured in human sperm using the 32P post-labeling 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, nonisotopic) 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, can provide information regarding genetic damage to sperm. To perform the assay, the sperm DNA is stressed thermally or chemically and then stained with acrinine orange (*50*). 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 (*51,52*). The fertility rate of bulls is correlated with the percentage of double-stranded DNA. A recent report indicates that the DNA stability assay is highly repeatable between ejaculates from the same man (*53*). Although this procedure has been developed in only a few laboratories at this time, the sperm can be frozen on dry ice and shipped to the analysis laboratory without affecting the results.

Interpretation

It is unlikely that all or even most men in a study group will respond in the same way to the same mutagen exposure. This is especially true for xenobiotics requiring metabolism and for those that induce lesions for which repair pathways are available. However, very little is understood 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 will require the identification of specific genes involved in the metabolism of certain substances 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 the 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.

STUDIES OF TOXICANT MECHANISMS

Primary research challenges in reproductive and developmental toxicology are to identify the mechanisms leading to abnormal reproductive outcomes and to identify the physiologic factors, genetic factors, and environmental exposures that could increase the frequencies of abnormal outcomes (Table 3-7). 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 the toxicant effects and in studies of mechanisms. The selection and utility of animal models, however, requires a kit of biomarkers (called bridging biomarkers) that measure the identical defects in animals and humans (*15*).

In vitro assays, currently under investigation, are also expected to play important roles in defining certain biochemical mechanisms of action. Animal and in vitro assays were not evaluated in this chapter.

Biomarkers are playing an ever increasing role in the search for understanding mechanisms of action leading to the abnormal reproductive outcomes.

Table 3-7.—Mechanisms of male reproductive toxicity and male-mediated developmental toxicity.

Categories of Toxicity	Potential Pathways
Physiologic toxicity to male endocrine system and sexual function	Toxicant metabolism Neuroendocrine effects Accessory sex gland effects
Physiologic toxicity to male reproductive tract and germ cells	Toxicant transport to reproductive tract* 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

*Includes effects on the epididymis, testis, and accessory glands.
†See Table 3-6.

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