Male reproduction and environmental and occupational exposures: A review of epidemiologic methods

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Abstract

Concerns that chemical exposures in the environment have been detrimental to male sexual development and fertility have been heightened by reports of declining sperm counts over the past 50 years. Marked geographic variation has been found in semen quality and in the incidence of testicular cancer and certain urogenital defects. Debate continues over the existence, magnitude and significance of these trends, and how best to evaluate the hypothesis that *in utero* and childhood exposures to estrogenic compounds may be to blame. Epidemiologic methods for assessing the impact of hazardous substances on male reproductive health have been developed mainly in the area of occupational medicine, and this paper will review the currently recommended methods. These include questionnaires to determine reproductive history and sexual function; reproductive hormone profiles; and semen analyses such as sperm concentration, motility, and morphology. New research tools that show significant promise from the fields of clinical reproductive medicine and reproductive toxicology are discussed as possible additions to epidemiologic studies, including assays of sperm function and genetic integrity, and biomarkers of DNA damage. For population-based studies involving occupational groups or communities with environmental exposures, issues related to the cost, validity, precision and utility of these methods must be carefully considered.

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Resumen

Varios artículos publicados informan acerca de una declinación en la concentración de espermatozoides durante los últimos 50 años; lo anterior ha motivado una preocupación creciente en el sentido de que las exposiciones ambientales a diversos químicos actúen en detrimento del desarrollo sexual y de la fertilidad masculina. Se ha observado una marcada variación geográfica en la calidad del semen y en la incidencia del cáncer testicular y diversas malformaciones urogenitales. Persiste un debate acerca de la existencia, magnitud y significado de estos fenómenos y también acerca de la mejor forma para evaluar la hipótesis de que sus causas son las exposiciones *in utero* a compuestos estrogénicos. En este trabajo se revisan los métodos epidemiológicos recomendables para evaluar el impacto de sustancias peligrosas sobre la salud reproductiva masculina, varios de los cuales provienen del área de la medicina ocupacional. Se incluyen los cuestionarios para determinar la historia reproductiva y la función sexual; los perfiles de hormonas reproductivas, y los análisis de semen para medir concentración, morfología y motilidad. También se discute la posibilidad de utilizar para la investigación una serie de nuevas herramientas que provienen de la medicina y de la toxicología reproductivas, entre ellas los ensayos de la función espermática y la integridad genética y los biomarcadores de daño al DNA. Se deben tomar cuidadosamente en consideración los as-

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pectos relativos a la utilidad, validez, precisión y costos de estos métodos para la realización de estudios poblacionales, ya sea que involucren grupos ocupacionalmente expuestos y/o comunidades con exposiciones ambientales.

Key words: reproduction; fertility; male; environmental exposure; occupational exposure

Palabras clave: reproducción; fecundidad; masculino; exposición a riesgos ambientales; exposición ocupacional

In 1977, Whorton et al. documented a striking relationship between duration of occupational exposure to the nematocide 1,2-dibromo-3-chloropropane (DPCP) and diminished fertility among men working in a California pesticide factory.[1] This study sounded the alarm of the potential for chemically-induced injury to male reproductive health.[2] More recently, threats to male fertility have received greater attention following several reports of a decline in sperm counts over the past 50 years in some[3-10] but not all[11-13] populations, and evidence of marked geographic variation in semen quality.[10,14] The incidence of testicular cancer has progressively increased in many countries over the last century[15] and other disorders of the male reproductive tract such as hypospadias and cryptorchidism may have increased in some populations.[16,17] There is growing concern that occupational factors and environmental chemical exposures, especially *in utero* and childhood exposures to estrogenic compounds, may be correlated with these observed changes in male reproductive health and fertility.[18,19]

The perception that in recent years an increased emphasis has been placed on determining the frequency and origins of reproductive dysfunction in both females and males is indisputable.[20-24] In the lay press, the message seems to be that infertility and adverse reproductive events are on the rise[25-27] and that paternally mediated effects on pregnancy and offspring have been under-appreciated. In reality, the estimated 15 percent prevalence of infertility among married couples (i.e., those who are unable to conceive after 12 months of unprotected intercourse) has not increased significantly over the last four decades.[28] Late fetal deaths, stillbirths, and major birth defects –the most devastating of adverse reproductive events– occur infrequently, at a rate of less than 5 percent of pregnancies. However, it is now known that a disproportionate number of pregnancies end in spontaneous abortion; it has been estimated that up to 40 percent of all human conceptions are lost before the 28th week of gestation.[29,30] Rates of subfertility, fetal loss, and adverse outcomes clearly increase with age in women but there is no consistent trend with age of the father.[31,32] Therefore, the perception that infertility and adverse pregnancy outcomes are increasing may be due in part to changing social trends such as the deferral of childbearing among women in developed countries. Nevertheless, the significance of possible trends of semen quality and other reproductive disorders,[33] and the influence of occupational and environmental hazards, are active areas of international research.[34-37]

Because of the multifactorial etiologies of adverse pregnancy outcomes and infertility, the proportion of cases that are attributable to the male partner remains unknown. Recent estimates suggest that a "male factor" is present in at least 50 percent of infertile couples and that 30 percent may be caused by a "pure male factor."[38,39] Furthermore, advances in the availability and success of assisted reproductive technologies, e.g., intracytoplasmic sperm injection (ICSI) for treating male factor infertility, may reduce the chance that a couple will receive a complete infertility work-up to determine the underlying cause. It is unlikely that a primary health care provider or urologist will have the training or the access to information needed to perform appropriate risk assessment for individual patients.[24] Unfortunately, while many chemical and physical agents found in the workplace and environment are suspected reproductive toxicants, only four exposures (ionizing radiation, lead, the pesticide DPCP, and the disinfectant and fungicide ethylene oxide) are regulated by U.S. occupational safety standards in part due to their reproductive effects.

There are exceptional examples in which the investigation of an occupational or environmental exposure is initiated by some triggering event. These incidents may be exposure-driven (the discovery that workers or residents in a community have been systematically or accidentally exposed to toxic agents) or outcome-driven (an apparent cluster of adverse reproductive events in individuals with recognized exposure to known or suspected reproductive toxicants) or a combination of the two.[40] The investigations prompted by these "sentinel" events have been the impetus for many recent methodologic developments in reproductive epidemiology and toxicology. Reproductive

capacity or fertility status *per se* is assessed most often, but several other disorders or diseases of the reproductive system are also considered as outcomes. For example, testicular cancer and cryptorchidism are strongly correlated with infertility and suggest partially shared etiologies.[41] Moreover, extrapolation from "ecoepidemiologic" studies[19,42] of environmental hazards and reproductive problems in other species is currently driving some investigations in human populations. A growing number of reports demonstrate that common, persistent environmental contaminants, including endocrine disrupting chemicals, can influence reproductive function in wildlife and in laboratory animals. These effects have been seen in a range of species from invertebrates to mammals. Some of the most troublesome observations include the severe decline in several terrestrial and aquatic species,[43,44] demasculinization/feminization of males,[43,45,46] intersex conditions[47] and other anomalies like cryptorchidism.[48] Decades of careless production, use, and disposal of industrial chemical, on the one hand, and national or such as the international initiatives on the other hand, have raised public consciousness and stimulated scientific research regarding potential reproductive effects of environmental and occupational exposures.[49]

Researchers and clinicians interested in male reproductive health and fertility are utilizing increasingly sophisticated methodologies from the fields of toxicology, reproductive medicine, environmental and occupational medicine, and epidemiology. This paper aims to review the methods that have contributed to our current understanding of the impact of hazardous substances on male reproductive capacity. The second objective is to discuss new research tools that may in the future become available and feasible for larger scale epidemiologic studies.

Methods of Assessing Male Reproductive Capacity in Epidemiologic Studies

Reproductive capacity in the male is analogous to fecundability in the female, i.e., the physiologic capacity of an individual to produce a pregnancy, whether or not that capacity has been fulfilled. Assessment of male reproductive capacity requires the use of several complementary methods ranging from questionnaires that elicit reproductive history to sophisticated tests of semen quality and neuroendocrine function. A full battery of evaluations is most realistic when individuals are motivated by infertility to seek clinical diagnosis and treatment. For population-based studies involving occupational groups or communities with environmental exposures, issues related to the cost, validity, precision and utility of the available methods must be carefully considered.[20-22]

Table I provides a list of measures that are most commonly used and the reproductive functions they assess. The choice of appropriate methodologies to study the effects of reproductive toxicants is predicated by the investigators' understanding of several factors: the nature of the exposed population; the source, the levels and the known routes of exposure; the organ systems in which a toxicant exerts its actions; the hypothesized mechanisms of a toxicant's actions; and the techniques available to assess the effects of toxicants in the relevant organ systems. As shown in table I, the neuroendocrine system, the testes, the accessory sex glands and sexual function are the principal target sites for male reproductive toxicants.

There are several characteristics of the male reproductive system that make it simpler to evaluate than the female system. Male reproductive function is not dependent on a cycle, male germ cells can be obtained

Table I
ASSESSMENT OF MALE REPRODUCTIVE CAPACITY

Method of Assessment	*Endocrine System*	*Testes*	*Post-testicular Events**	*Sexual Function*
Follicle Stimulating Hormone (FSH)	X			
Luteinizing Hormone (LH)	X			
Prolactin	X			
Testosterone	X			
Inhibin-B	X			
Sperm density		X		
Sperm morphology and morphometry		X		
Sperm motility (% motile and velocity)		?	X	
Sperm viability (vital stain and HOS‡)			X	
Semen volume			X	
Semen pH			X	
Marker chemicals from accessory glands		X		
Sperm function assays§		X	X	
Sperm chromosome analyses#		X		
Nocturnal penile measurements				X
Personal reproductive history&	X	X		X

* Includes production of seminal plasma components and capacitation of sperm in the epididymis, vas deferens and accessory sex glands
‡ HOS= Hyperosmotic swelling
§ Includes acrosome reaction, hemizona assay (HZA) of sperm binding, and sperm penetration assays (SPA)
# Includes sperm chromatin stability assay (SCSA), Comet assay, and assessment of chromosomal aneuploidy and nuclear microdeletions
& Includes pubertal development, paternity (pregnancy timing and outcomes), sexual function (erection, ejaculation, orgasm, and libido)

Adapted from Schrader and Kesner (1993) and Schrader (1997)

by the millions, and the male gonads are more accessible for examination to diagnose or rule out abnormalities.[50] In humans the total duration of spermatogenesis, the process that results in the formation of spermatozoa from stem cells, is approximately 74 days. Therefore, is it feasible to conduct a prospective study with the expectation that recent exposures can be related to current measures of sperm quantity, quality, fertilizing capacity, and germ cell mutations. On the other hand, studying chronic or historical exposures may be more problematic. With the exception of live births, men may be less likely than women to accurately recall experiences such as time to pregnancy, pregnancy outcomes, or childhood illnesses in their offspring.[51,52] This needs to be considered when the only source of information on these outcomes is self-report, although the data can be improved by confirmation from existing medical or vital records, or validation by the female partner.

Reproductive history from questionnaires, medical records or vital records

The most common method of assessing fertility status of individuals involves interviewing both the man and his current partner about their reproductive history, including prior marriages and sexual partners. The questionnaire should elicit information about all previous reproductive events, specifically the number of pregnancies, time to pregnancy, interpregnancy intervals, and pregnancy outcomes for each partner. The most common applications of this type of data involve computing "indirect" epidemiological measures of reduced fertility or increased incidence of adverse events in comparison to a standard population or across a range of exposure levels. These measures include the standardized fertility ratio (SFR) which compares the observed number of live births to the expected number of live births based on person-years of observation. The expected number is calculated using the birth rates from an external population, for example, data collected by the National Center for Health Statistics for women of childbearing age in the U.S. Bias can occur if the analysis fails to take into account potential confounding due to age, time period, race, marital status, parity, frequency of intercourse, sterilization and contraceptive use.

More recently, the average time to pregnancy has been used to study the effect of environmental exposures in both male and female populations.[53,54] In the strictest sense, using delayed time to pregnancy captures the probability of nonconception. In truth, subclinical embryonic losses will contribute "misclassified" delays in time to pregnancy. Women respondents have remarkable long-term recall of the time to conception of recognized (clinical) pregnancies. A recent study found that retrospective data from a questionnaire conducted after a median duration of 14 years were almost identical to data obtained at the time of pregnancy.[51] Male respondents are able to give values for time to pregnancy, but data collected from men tend to be less complete and may be less reliable.[32,52,53] Among the limitations of this method are that people who have never achieved a pregnancy are excluded from the analysis. Unacknowledged or mistaken paternity may bias observed associations towards the null. Similarly, recall bias, although it is likely to be nondifferential across exposure groups, will lead to underestimation of associations. Potential confounding by all the factors listed above that can bias the standardized fertility ratio must also be considered. One methodologic modification is to restrict the study population or the analysis to couples who are "at risk" of pregnancy either because they are trying to conceive or are not using effective contraception.

Indirect measures of fertility continue to be widely used as functional parameters that are relatively easy to obtain for large populations. Despite their limitations, they have been shown to correlate with biological markers of reproductive capacity.[34,55] They can be used to monitor both male-and female-mediated effects, as long as potentially confounding characteristics of both partners can be measured and controlled.[32] Data from existing medical or vital records and population-based surveys can also be used to explore differences in rates of clinically recognized spontaneous abortion; sentinel phenotypes including congenital defects or cancers in offspring; and temporal or geographic variation in offspring gender ratios.[56]

All research studies of reproductive endpoints should include questions about history of urogenital disorders including infections, injuries or surgeries (see example in Table II). When feasible, a physical examination should be made by a trained clinician, to assess overall male habitus and maturation, including a focused evaluation of the male reproductive system. Routine biochemistry and a complete blood count should be performed to rule out medical conditions associated with fertility problems, e.g., abnormal renal or liver function.

Semen analyses

More direct assessment of male reproductive capacity can be accomplished by obtaining semen analyses. Standard measurements of sperm concentration, total

Table II

MEDICAL HISTORY QUESTIONS FOR USE IN MALE REPRODUCTIVE HEALTH STUDIES

"I have some questions about some medical conditions you may have and medications you may have taken."

MH1. Has a doctor ever told you that you had any of the following medical conditions?	*No*	*Yes*	*If Yes: Year diagnosed?*
a. Mumps	0	1	
If yes: Did it affect your testicles?	0	1	
b. Prostate infection (prostatitis)	0	1	
c. Testicle infection (orchitis)	0	1	
If yes: Which side? 1 = Left 2 = Right 3 = Both			
d. Epididymis infection (epididymitis)	0	1	
If yes: Which side? 1 = Left 2 = Right 3 = Both			
e. Infection of the seminal vesicles (vesiculitis)	0	1	
f. Blood in your ejaculate/semen	0	1	
g. Urinary tract infection	0	1	
h. Urethritis or discharge from the penis	0	1	
i. Chlamydia	0	1	
j. Syphilis	0	1	
k. Gonorrhea	0	1	
l. Genital herpes	0	1	

MH2. Have you ever had any of the following medical procedures?	*No*	*Yes*	*If yes: Specify*
a. Vasectomy	0	1	
b. Surgery in the pelvic area (of the prostate, penis, testes, bladder)	0	1	
c. X-rays in the pelvic area for diagnosis or therapy	0	1	
d. Testicle biopsy	0	1	
e. Hernia repair	0	1	

MH3. Have you ever had any other urological conditions (problems involving your genital area or urinary tract) or genital injuries for which you needed medical attention?

No= 0
Yes= 1
Don't know= 8
If yes, specify:

MH4. Have you had a high fever (over 102°F) during the past 2-3 months?

No= 0
Yes= 1
Don't know= 8
If yes, specify cause of fever:

MH5. Have you had any viral illness during the past 2-3 months?

No= 0
Yes= 1
Don't know= 8
If yes, specify:

MH6. Do you take saunas, steambaths, whirlpool baths, or spend time in a hot tube?

No = 0
Yes = 1
If yes MH6a. How often in the past 2-3 months did you take saunas, steambaths, whirlpool baths, or use a hot tube?
times/per week= 1
/per month= 2

MH7. Have you ever taken any of the following Medications/treatments at least 4 consecutive weeks?	*No*	*Yes*	*If yes: Are you currently taking? No*	*Yes*
a. Antibiotics	0	1	0	1
b. Non-steroidal anti inflammatories (Motrin, Advil, Ibuprofen)	0	1	0	1
c. Estrogen	0	1	0	1
d. Testosterone	0	1	0	1
e. Radioactive Iodine	0	1	0	1
f. Steroids (Prednisone, Cortisone)	0	1	0	1
g. Antacids, Mylanta, Maalox	0	1	0	1
h. Anti-ulcer medication (Tagamet, Zantac, Axid, Pepsid, Prilosec)	0	1	0	1
i. Anti-hypertensives or blood pressure medications	0	1	0	1
j. Diuretics	0	1	0	1
k. Seizure medications	0	1	0	1
l. Other *prescription* medications *currently*?	0	1	*Specify:*	
m. Other *over the counter* medications *currently*?	0	1	*Specify:*	

sperm count, motility and morphology have been the primary research tools for studying the effects of toxicants on the male reproductive system. Epidemiologic studies have successfully utilized semen quality as a marker of fertility[34,55]although not without problems, e.g., potential selection bias due to low compliance rates, inadvertent inclusion of vasectomized men, and substantial within-individual variability in semen parameters resulting in misclassification based on the static results of a single analysis.[57-61] Generally accepted normal ranges for the routine semen parameters established using World Health Organization (WHO) methods[38] and other well established criteria[62] are shown in Table III. Often in the absence of an unex-

Table III
SEMEN ANALYSIS REFERENCE RANGES FOR NORMAL VALUES

Volume	> 2.0 ml
Appearance	Whitish/Gray-yellow
Agglutination (scale 0 to 3)	0
Liquefaction	Within 30 minutes
Viscosity (scale 0 to 3)	0
pH	7.2 to 7.8
Sperm density	> 20 million/ml
Total count	> 40 million
Motility (@ 37° C)	> 50%
Progressive Motility	> 50%
WHO Morphology	> 50% normal forms
Strict Kruger Morphology	> 14% normal forms
Viability (vital stain)	> 75% alive
Round cells	< 1.0 million/ml
White blood cells (peroxidase positive)	< 1.0 million/ml
Acrosome reaction assay	Delta ≥ 5: Positive

Adapted from Bar-Chama and Lamb (1994) and Schrader (1997)

posed control group, the reference point for assessing male reproductive toxicity in exposed populations are these normal values.

Prior to beginning a study, it is incumbent on the investigators to establish a close association with a licensed andrology laboratory. Particularly when research is not the primary function of the laboratory, it will be necessary to ensure that consistent techniques are used, that one technician analyzes all the study samples when feasible, and that specimen aliquots are appropriately prepared and stored for specialized analyses. The study investigators must provide collection jars and storage tubes that are known to be free of any toxicants and have securely fastened labels. Collection instructions should state that the semen sample must be obtained by masturbation after a set period of abstinence (usually 2 to 5 days) and delivered to the laboratory within 1 hour from the time of ejaculation. It is important that each man record the duration of abstinence, time of semen collection, and any information regarding spillage on the label.

Certain semen evaluations must be conducted within one hour after the sample arrives at the andrology laboratory, including recording the temperature, turbidity, color, liquefaction time, volume and pH of the semen.[63,64] Sperm counts, preservation of seminal plasma, preparation of slides for morphology and morphometry, and viability assays should also be conducted at this time. If a mobile laboratory is set up for a field study, video recordings can be made for later assessment of motility parameters. Morphologic and morphometric analyses of sperm preserved on slides can be performed at a later time.

Only motile sperm are able to penetrate through cervical mucus, migrate through the female reproductive tract, penetrate the zona of the ova, and achieve fertilization.[39] In a routine semen analysis, overall quantitative motility is defined as the percentage of sperm that demonstrate any movement.[38,39] The forward progression of each spermatozoon is qualitatively graded as 'a'= rapid progressive motility; 'b'= slow or sluggish progressive motility; 'c'= non-progressive motility; and 'd'= immotility.[58] Motility and forward progression of spermatozoa analyzed visually by a technician is gradually being replaced by computer-assisted sperm analysis systems (CASA). CASA can provide useful information on both the pattern and vigor of motion of sperm cells, including curvilinear velocity, straight-line velocity, linearity, and amplitude of lateral head displacement.[64,65]

Over the past 30 years, several schemes have been presented for the assessment of normal and abnormal appearing sperm. Variations in sperm size and shape are not distinct entities, but rather represent a continuum. This provides a challenge within and especially among laboratories to establish a reliable system for morphological classification.[64] Insofar as debate continues regarding the most valid methodology for morphology assessment,[66] and to allow comparison with previous studies of reproductive toxicity which all utilized the WHO semen analysis guidelines,[38] some labs score all specimens by both the strict Kruger and WHO criteria. New methods have been developed that use transmission and scanning electron microscopy to evaluate the ultrastructural morphology of sperm organelles.[67] Significant ultrastructural abnormalities have been reported among infertile as compared to fertile men[68] and in radiation-exposed salvage workers from Chernobyl.[69] With recent advances in computerized image analysis, methods for objective assessment of sperm head size and shape have been introduced. The andrology laboratory at The National Institute for Occupational Safety and Health (NIOSH) has pioneered a protocol for assessing sperm head morphometry.[64,70] Individual sperm heads are outlined using a digitizing tablet; the software used allows for calculations of area, perimeter, length, width, width/length ratio, and $4\pi(\text{area})/\text{perimeter}^2$ (Pi factor). However, serious impediments remain in achieving agreement among different analysis systems, therefore, comparisons across systems should be avoided.

The viability and motility of spermatozoa typically reflect seminal plasma quality.[64] Alterations in sperm viability, as measured by eosin stain exclusion or by hypo-osmotic swelling[71] or alterations in sperm motility parameters,[65] suggest a problem with the accessory sex glands. Biochemical analysis of seminal plasma provides insights into glandular function by measuring marker chemicals secreted by each respective gland. For example, the epididymis is represented by glycerylphosphorylcholine (GPC), the seminal vesicles by fructose, and the prostate gland by zinc. Measures of semen pH and volume provide additional general information on the nature of seminal plasma, reflecting post-testicular effects. A toxicant or its metabolites may act directly on accessory sex glands to alter the quantity or quality of their secretions. Alternatively, the toxicant may enter the seminal plasma and affect the sperm or may be carried to the site of fertilization on the sperm membrane and affect the ova or conceptus. Seminal plasma can be analyzed for the presence of toxicants or their metabolites using atomic absorption spectrophotometry or gas chromotography/mass spectrometry.

As much as any other factor, uncertainty in the results of studies addressing threats to male reproductive health stems from debate about the definition of "normal" reproductive capacity and whether or not expected fluctuations are distinguishable from diminished reproductive capacity resulting from hazardous exposures.[72-75] Demonstrating a link between an exposure in a human population and an adverse reproductive outcome is rarely straightforward. Methodologic questions regarding intra-individual variation and the precision and reliability of assessment techniques can be addressed to some extent. More than one semen evaluation, usually required by clinicians for a definitive classification of fertility status,[39] is also desirable although less feasible in epidemiologic investigations. Individual semen samples can be split and replicate measurements made. The mean value from multiple aliquots can be used and intraclass correlations and coefficients of variation can be determined. It is more difficult, however to resolve questions about the validity of routine semen analyses. Which semen parameters are most predictive of fertility? Can threshold levels associated with impaired fertility be defined? Are shifts in sperm quantity and quality within populations related with measurable decreases in normal live births?[33,34,55] The uncertainties associated with traditional semen parameters has led to the development of assays to assess sperm functioning and genetic integrity which may be more sensitive and specific targets for toxicant actions.

Sperm function assays

Fertilization requires a series of intricate biochemical events that begins with sperm capacitation, followed by binding to the zona pellucida of the ovum and acrosomal discharge, binding to the oolemma and, finally, penetration into the ooplasm.[39,76] Abnormalities in any of the biochemical reactions by which sperm access and penetrate the ovum may be a source of infertility. There is considerable interest in determining the utility of including assessment of sperm functioning in epidemiologic studies of reproductive toxicity.[50] Although a variety of assays for evaluating sperm function have been developed recently, no single test is capable of evaluating all the steps involved in fertilization.[39] Certain sperm function assays may reflect toxicant effects at more that one site, for example, direct gonadotoxicity affecting spermatogenesis plus post-testicular effects on accessory sex gland secretions. A combination of tests can complement each other in providing a comprehensive evaluation of sperm functions. These include the penetration of sperm through cervical mucus (or viscous fluids simulating cervical mucus), the penetration of sperm into a zona-free hamster egg (sperm penetration assay or SPA), and the binding of sperm to the zona pellucida from a human ovum (hemi-zona assay or HZA). The acrosome reaction has been studied extensively as a predictor of fertilization success because it is a stable parameter of sperm function which, independently of oocyte quality, reflects the ability of the spermatozoa to capacitate.[77] The acrosome, a membrane-bound organelle covering the anterior two-thirds of the sperm head, contains numerous enzymes whose release is required for penetration of the zona pellucida. It is hypothesized that this release of enzymes is induced by one or more of the zona pellucida glycoproteins. In men with otherwise normal semen analyses, it has been shown that the failure of a significant proportion of sperm to undergo the acrosome reaction when appropriately stimulated is associated with lower *in vitro* fertilization (IVF) rates.[77] In addition, the acrosome reaction has a higher predictive value than standard semen analyses, including standard sperm head morphology.[77] A study by Menkveld et al. found that normal acrosomal morphology was strongly correlated with IVF success, independently of acrosome activity and normal sperm head morphology.[78]

Issues related to the reliability, validity and availability of the sperm function assays and the tests of genetic integrity described below have limited their assimilation into epidemiologic studies.[39,50] These concerns include the level of expertise needed to run some

of the more complex assays, the time and expense involved in performing the tests, the difficulties establishing standardization and quality control of the assays, and general doubts about the significance of isolated functional or genetic abnormalities in individual sperm cells.

Tests of genetic integrity or damage

Epidemiologic studies of large populations have demonstrated increased frequency of congenital anomalies associated with various paternal occupations.[79] Given the low frequency of even the most common anomalies, such studies require a population base of thousands of pregnancies in order to have a reasonable probability of detecting an increased risk. Numerous case-control studies of childhood cancers have found significant associations with paternal occupations and exposures,[80] but between-study variation with respect to case populations, control groups, and methods of data collection and analysis makes it difficult to interpret the findings. There has been considerable interest in developing more direct methods for use in epidemiologic studies to detect genetic damage in human germ cells that result from exposure to toxic agents.[81]

Environmental toxins may affect sperm DNA in various capacities, including disruption of the meiotic chromosome segregation (aneuploidy), fragmentation of the DNA, individual genetic mutations, disruption of the DNA structure (chromatin integrity), and production of DNA adducts. Assessing various DNA parameters in human germ cells is important for understanding whether a particular genetic alteration can affect the next generation, and to ascertain the level of toxicant exposures associated with specific germ cell end-points. If abnormalities are found, they may help to explain some of the subfertility and increased risk for spontaneous abortions noted in workers with particular occupational exposures.

The Comet assay detects genetic fragmentation by depicting DNA migrating out of the cell nucleus during electrophoresis.[82] Cells with undamaged DNA appear as intact heads without tails after specified electrophoresis times. DNA that has been fragmented will contain numerous strand breaks and will therefore migrate further than normal intact DNA. When visualized microscopically, the migrating DNA resembles the tail of a comet. After staining with ethidium bromide, the migrating DNA is quantified by measuring the intensity and extent of the fluorescence pattern. Increasing the duration of electrophoresis may enable detection of extremely low levels of DNA fragmentation. In addition, measuring the fluorescent intensity following DNA migration provides quantitative geometric measurements of the area and density of the dispersion DNA damage. The Comet assay has the significant advantage of being able to assess DNA fragmentation in individual cells.

The Comet assay has been used to measure DNA damage in individual human lymphocytes using relatively low doses of ionizing radiation and chemical genotoxins.[82] Refinements to the methods are being made to extend its applicability to other environmental exposures and lifestyle factors such as smoking[83] and other target tissues including sperm cells.[84-87] Anderson et al.,[87] for example, detected DNA fragmentation using the Comet assay with DBCP, two estrogens (β-estradiol and the phytoestrogen daidzein) and 1,2-epoxybutene (a metabolite of 1,3-butadiene) in a small study of six human semen samples.

Structural or numerical chromosomal abnormalities are relatively frequent in human germ cells and cause serious reproductive problems such as spontaneous abortion and congenital defects. Mikamo *et al*.[88] and Martin et al.[89] found that 1-2% of human sperm have an abnormal number of chromosomes and approximately 10% carry structural chromosome aberrations. Aneuploidy, i.e., abnormal chromosome number, can be detected using fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes.[90] Multiple probes can be employed to evaluate numerous chromosomes in a single cell.[91] The chromosomes usually evaluated when assessing sperm with FISH are the sex chromosomes as these appear to be most at risk for nondisjunction, suggesting that there is a chromosome-specific variation in nondisjunction frequencies.[92] FISH has been used to assess factors that may induce sperm aneuploidy in humans such as advanced maternal and paternal age, cancer chemotherapy, and radiation.[93-95] The techniques show promise for assessing lifestyle factors like tobacco, caffeine, and alcohol[96,97] as well as environmental exposures including pesticides and heavy metals.[98-100]

Following completion of spermatogenesis, sperm undergo extensive differentiation and maturation. During spermiogenesis DNA is tightly compacted and complexed to protamines. This chromatin structure is important for protection of the DNA and has a significant role in early human post-fertilization events and embryo development. Flow cytometric techniques have been developed to evaluate the chromatin structure of sperm in order to correlate the findings to fertility and as a biomarker of exposure to reproductive toxicants. The sperm chromatin structure assay (SCSA) measures the resistance of sperm DNA to *in*

situ denaturation (separating double stranded DNA into single strands) under thermal or chemical stress.[101] SCSA assesses flow cytogram-generated staining patterns, measuring a shift from green (native DNA) to red (denatured DNA) fluorescence in properly stained sperm chromatin. This shift is seen under conditions of "stress" to the sperm, such as low pH, and has been shown to correlate with toxic chemical exposures, drug exposures and diseases.[102-106] Limited numbers of studies in both animal (bovine) and human semen have suggested a relationship between sperm chromatin structure and fertility[107-109] and at least one study found that occupational exposure to lead was associated with decreased sperm chromatin stability.[110]

Unlike the cytogenetic assays (Comet and FISH), individual sperm are not evaluated with SCSA but rather thousands of cells, providing a representation of the whole ejaculate. There is a low variability of SCSA within individuals, with intraclass coefficients ranging between 67 and 90 in healthy volunteers.[101] Evaluation of semen samples collected in the same individual from two separate months showed highly repeatable results. This assay appears to be sensitive to early stages of chromatin alterations and is a potentially important method to assay for early events of toxicant-induced chromosome damage.

The cytogenetic and chromatin structure assays provide independent assessments of sperm quality that may or may not correlate well with other semen parameters. In infertility clinic populations, sperm density, total count, and morphology have shown low to moderate correlation with SCSA values.[101] However, in many studies in animals and humans, poor quality sperm chromatin structure was highly indicative of male subfertility.[109] Therefore, these assays may be of use in subfertile men who otherwise have "normal" semen parameters.

Efforts are also being made to develop biochemical markers of sperm DNA damage. Reactive oxygen species (ROS) are a group of potentially destructive molecules implicated in the oxidative damage of biological structures. These ROS, including the superoxide anion, the hydroxyl radical and hydrogen peroxide, may either be produced endogenously through cellular pathways of the mitochondria and lysosome, or induced exogenously in reaction to environmental assaults. Ultraviolet and X-ray radiation and oxidatively reactive compounds, such as those found in cigarette smoke, alcohol, and air pollution, have all been shown to induce the formation of harmful ROS.[111] Over the past decade, concern has been raised after numerous studies reported the reactivity of ROS to DNA nucleotides and suggested the potential for ROS to generate genetic mutations that may evolve to cancer or birth defects if germ cells are damaged.[112,113] 8-Hydroxydeoxyguanosine (8-OHdG) is one of many products of oxidative DNA damage and is currently used to evaluate ROS damage, including the analysis of 8-OHdG levels in the semen. DNA is enzymatically digested to excise damaged nucleosides and then analyzed with high performance liquid chromotography (HPLC) to quantify 8-OHdG levels. Shen et al.[113] used this assay to correlate a dramatic increase of 8-OHdG in semen of men exposed to cigarette smoke. In addition to damaging DNA, ROS are also known to oxidize cellular membrane fatty acid components. Sperm cells are especially sensitive to this lipid peroxidation because of the increased density of unsaturated fatty acids needed for sperm membrane fluidity, and the decreased intracellular space available in sperm heads for antioxidant protection.[114] Oxidatively damaged lipids remain in the plasma membrane and may be assayed through a biochemical technique that converts lipid peroxides into detectable malondialdehyde.[115] These assays allow analysis and characterization of oxidative stressors that may affect male reproductive ability and may help determine effective preventative antioxidant interventions, if indicated.[116]

DNA adducts are the complexes formed between a toxicant or its metabolites and DNA. The presence of these adducts affects DNA synthesis and repair, and may induce genetic mutations that cause cancer or other adverse outcomes.[117] A common source of adduct-generating compounds that has been widely studied is polycyclic aromatic hydrocarbons (PAHs).[118] PAHs are environmentally ubiquitous compounds formed from the industrial manufacture and combustion of organic compounds found in coal, tars, petroleum oils, and cigarettes. PAHs do not form DNA adducts innately but must first be metabolically activated by cellular P450-dependent monooxygenases. Arene oxides, quinones, diol epoxides and other PAH metabolites have all been discovered to form DNA adducts. Adducts can be detected through ^{32}P radiolabeling, but HPLC seems to be more effective at determining the presence of DNA adducts.

Reproductive hormone profiles

The profile recommended by NIOSH to evaluate endocrine dysfunction associated with reproductive toxicity consists of serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and prolactin. Because of the pulsatile secretion of LH, testosterone and to a much lesser extent FSH, as well as the variability in the evaluation of

reproductive hormones, it is recommended that three blood samples be drawn at set intervals in the early morning, and the results pooled or averaged for clinical assessment.[119,120] In epidemiologic field studies, however, multiple blood samples are impractical and may decrease participation rates.[121] Schrader et al.[121] determined serum concentration of FSH, LH, testosterone and prolactin as part of a longitudinal study of workers. They assessed the reliability of the measurements over time and compared the results from a single sample to the average from three blood samples drawn 20 minutes apart; samples were drawn between 8 a.m. and 8 p.m. on three occasions three months apart. The precision for these hormone measurements was very similar, although there was some decrease in the intraclass correlation coefficient for LH and prolactin. The measurements from samples drawn 20 minutes apart were highly correlated, and the major sources of variation occurred across individuals and over time (samples drawn three months apart); therefore, multiple measurements at short intervals on the same day do not increase precision. Alternatively, LH and FSH can be measured in urine, providing indices of gonadotropin levels that are relatively unaffected by pulsatile secretion. However, if an exposure can affect hepatic metabolism of sex steroid hormones,[122] urinary measures of excreted testosterone metabolite (androsterone) or estradiol metabolite (estrone-3-glucuronide) are not recommended. There are currently no assays available to measure prolactin in urine.

Future assessment of reproductive hormones may extend to inhibin, activin and follistatin, polypeptides that are secreted primarily by the gonads and act on the pituitary to increase (activin) or decrease (inhibin and follistatin) FSH synthesis and secretion. Within the gonads, these peptides regulate steroid hormone synthesis and may also directly affect spermatogenesis. Ongoing studies are investigating the utility of serum inhibin-B level as an important marker of Sertoli cell function and *in utero* developmental toxicity.[123,124]

Sexual function

Sexual function is attained through the integrated activities of the testes, the accessory sex glands, the endocrine control systems, and the neurological, behavioral, and psychological components of reproduction that are controlled by the central nervous system.[64] Assessments of libido, erection, ejaculation, and orgasm are difficult to make under normal conditions, therefore, detecting decrements associated with exposure to hazardous agents is very challenging. Questionnaires that require an individual to recall and report his sexual functioning may be confounded by psychological needs to guard a masculine image or to attribute pre-existing problems to occupational or environmental exposures. Therefore, there is ongoing research to develop objective means of evaluating sexual function, for example, monitors that quantify the frequency and quality of nocturnal erections.[64]

Conclusion

Clearly, initiating an investigation of occupational or environmental male reproductive hazards requires a team that includes urologists, occupational physicians, epidemiologists, andrologists, toxicologists, industrial hygienists, molecular biologists, technicians, and the men themselves as well as their labor union or employer, when applicable. Decisions regarding the study design and evaluations should be guided by input from these experts, characteristics of the population at risk, the relevant exposure(s), and the available resources. It is important that researchers consider the added-value of integrating some of the methods outlined in this paper, including tests of sperm function and genetic damage, into future epidemiologic studies of the impact of environmental and occupational exposures on male reproductive health.

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