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Biologic Markers in the Epidemiology of Reproduction

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Although most of the literature in reproductive epidemiology focuses on clinically recognized biological events, such as fetal loss or malformations, current and future studies are likely to involve a range of biologic markers, either as outcomes or to indicate exposure or susceptibility. Literature on effect markers such as semen analysis and early pregnancy loss is growing. Advances in molecular biology are providing new tools with which to investigate poorly understood disorders of reproduction. [See review by the National Research Council (NRC), 1989.] Wide-scale use of molecular genetic diagnostic tests is almost certain to be common in clinical medicine, yielding new insights into the etiology, mechanism, and risk of inherited conditions. Advances in DNA technology have allowed exploration of the previously speculative role of molecular mutation that results in germ line mutations, most likely leading to early pregnancy loss. Defects in genetic coding for products critical for embryonic or fetal development have been hypothesized to play a causative role in euploidic abortion in humans (Butler and McDonough, 1989).

The framework for employing biologic markers involves three separate but interlinked systems. A couple's reproductive success depends on a delicate physiochemical balance between and within the paternal, maternal, and fetal systems. Any disruption of this balance can result in a broad range of effects. The consequence of exposure of men and women to mutagens, teratogens, and carcinogens is described in Figure 15.1. Exposure of both genders may result in cancer or chromosomal damage, potentially increasing individual risk of cancer. Germ line mutation may lead to infertility, adverse pregnancy outcomes, or heritable alterations expressed in future generations.

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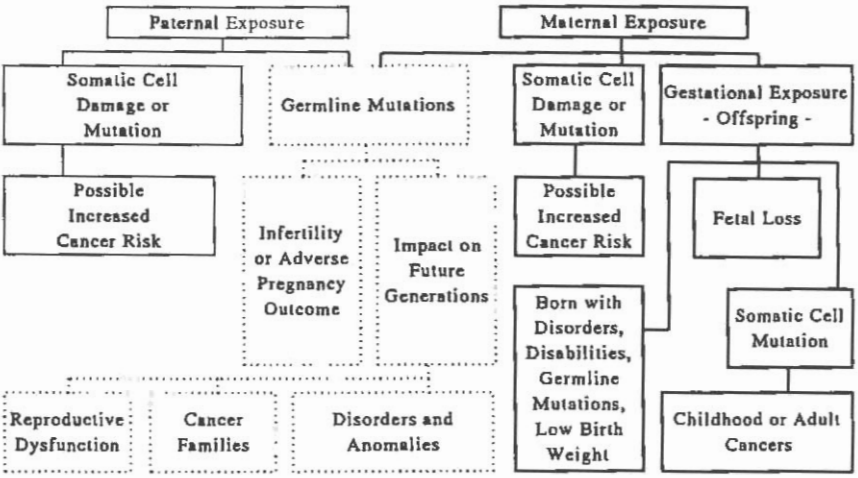

FIGURE 15.1 Consequences of exposures to carcinogens, mutagens, or teratogens.

Gestational exposure of the offspring may lead to such effects as fetal loss, disorders and disabilities, or childhood and adult cancers. To date, limited inquiries have been made to determine the impact of exposure on future generations. The identification of susceptible subgroups or, conversely, resistant groups in epidemiologic studies may lend itself to this backward tracing of genetic differences in the population. Technologies such as radioactively labeled DNA probes and polymerase chain reactions have proved fruitful in identifying genetic disorders and may help in understanding biologic differences in response to toxicants. In this chapter, we identify a framework for using practical and available biologic markers in epidemiologic research of reproduction. Markers of exposure, effect, and susceptibility are discussed for each gender. Figure 15.2 displays the interplay between these categories of markers.

Markers of Female Reproduction and Pregnancy Outcome

Markers of Exposure

In epidemiologic studies of reproduction, the use of biologic markers to assess exposure has been limited, but the need and the potential for such use exists. The need stems from the same weakness that has been recognized for most epidemiologic studies, namely, the classification of exposure or identification of early effects. In females, markers of exposure are not easily acces-

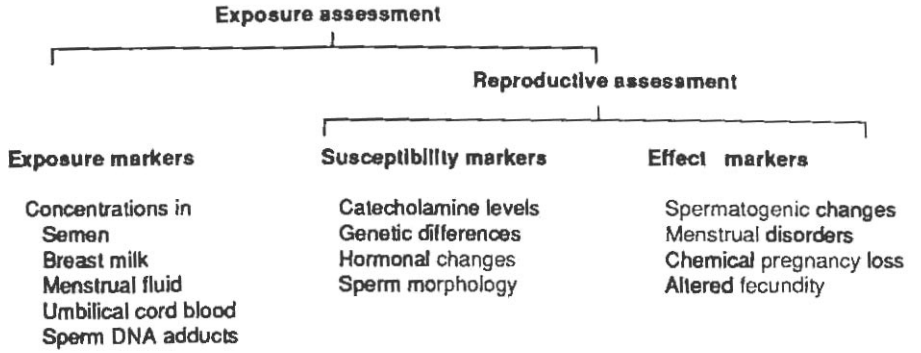

FIGURE 15.2 Exposure and reproductive health assessment.

sible from target tissues. Hence, general exposure-related markers in routinely collected biologic media, such as blood, urine, and breath, are often used. Specimens that are more difficult to collect, such as body fat, or more temporally limited, such as breast milk, umbilical cord blood, or menstrual blood, may be important exposure markers.

Depending on the research question, a unique issue in exposure assessment pertaining to reproduction will be the critical period of exposure. If, for example, fetal loss is the effect of interest, then the determining exposure marker might be collected just prior to conception or during early pregnancy. Exposure markers for teratogens generally are targeted during the period of organogenesis, up to days 55–60 of gestation, but for some anomalies, for example, of the central nervous system, eye, and genitalia, markers may need to be measured throughout the pregnancy. Figure 15.3 approximates a relationship between the time that an exposure marker may be associated with a particular effect. Time frames of exposure also can be represented by protein or DNA adducts. Hemoglobin, for instance, is a very accurate dosimeter of exposures during the preceding 4 months; albumin addresses the preceding month. In both cases, these dosimeters reflect cumulative dose. An example of the efficacy of the approach was described by Everson *et al.* (1988), who evaluated the association between DNA damage in the human placenta and maternal smoking and birth weight. The investigators evaluated the extent of an association between maternal smoking and birth weight assessed by questionnaire data, biochemical measures for smoking exposure, and molecular methods (Table 15.1). They found no association between birth weight and biochemical measures of smoking, but a clearly significant association between birth weight and the adduct levels. These findings were similar for birth length. Although these associations cannot be interpreted as evidence for a causal association between level of adducts and a decrease in

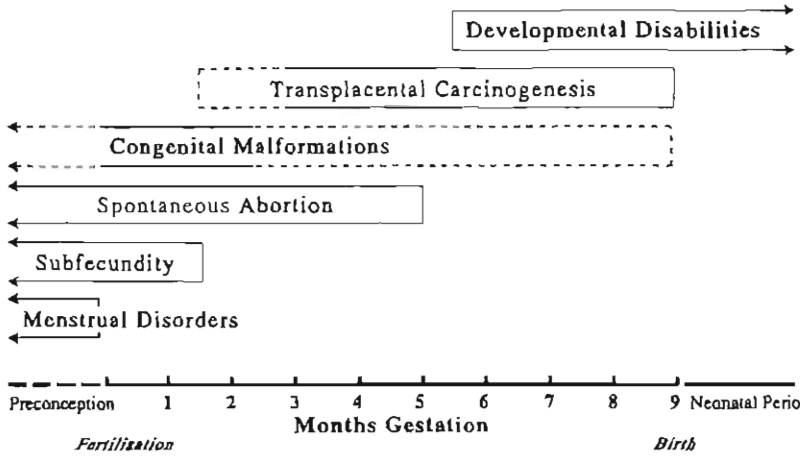

FIGURE 15.3 Reproductive outcomes associated with timing of maternal exposure. Solid lines indicate the most probable timing of exposure for a particular outcome; dashed lines indicate less probable, but still possible, timing of exposure; arrows suggest that a defined cutoff point for a specific outcome is not known. (Adapted from Selevan and Lemasters, 1987.)

birth weight or length, they do serve as a demonstration that the DNA adduct is an accurate dosimeter reflecting exposure to cigarette smoke that causes low birth weight (Everson *et al.*, 1988). The lesson of the study is the need to integrate various approaches in assessing how specific environmental agents might impair development of the fetus. Although widespread application will depend on further development and field testing, these exposure markers may be useful for depicting the time period surrounding conception and gestation.

Markers of Effect

Studies on the reproductive effects of toxic exposure of women are unique because two individuals are potentially at risk—the woman and, if she is pregnant, her developing offspring. Exposure to either may result in a wide range of adverse responses. Maternal exposure to a toxicant may cause infertility, genetic damage, menstrual disorders, illness during pregnancy, chromosomal aberrations, breast milk alteration, early onset of menopause, and suppressed libido. Adverse fetal effects include preterm delivery, fetal loss, perinatal death, lowered birth weight, altered sex ratio, congenital malformation, childhood malignancies, infant or childhood illness, inheritable genetic damage, and developmental disabilities. This section discusses the markers and monitors used in epidemiologic studies of menstrual cycle variability, fetal loss, and birth anomalies of the offspring.

TABLE 15.1 Association between Birth Weight and Smoking Exposure Comparing Results of Questionnaire and Biochemical and Molecular Methods for Assessing Intensity of Exposure to Smoking[a]

	Birth weight			Birth length		
Parameter for smoking exposure	R^2	Significance of model (P value)	Significance of adding smoking parameter to basic model (P value)	R^2	Significance of model (P value)	Significance of adding smoking parameter to basic model (P value)
None	0.41	.009	—	0.31	.05	—
Questionnaire data (Number of cigarettes smoked in 2nd trimester)	0.41	.02	.85	0.34	.06	.28
Biochemical and questionnaire summary of smoking exposure score	0.48	.006	.09	0.43	.01	.03
Level of adduct 1	0.52	.002	.025	0.47	.007	.01

Source: Reprinted with permission from Everson *et al.* (1988).

[a] All models present data for the 30 smokers for whom results of ^{32}P postlabeling assays and questionnaire data were available. Basic model includes terms for maternal age, maternal race, maternal years of education, and gestational age of the newborn. The R^2 indicates the proportion of variability of the dependent variable (birth weight or birth length) that is explained by the independent variables. Statistical significance of the model is increased as the independent variables explain a greater proportion of the variation in the dependent variable, but decreases if a larger number of independent variables are required to explain the same proportion of variability.

Menstrual Cycle Variability

In humans, between 3 and 4 million follicles are present in each ovary at birth; shortly after birth, the primary oocytes are arrested in late prophase of meiosis I. At puberty, gonadotropins stimulate meiosis and the number of follicles reduces to less than 400,000. During each ovarian cycle, follicles are recruited in groups, the leading follicle ovulates, and the remainder undergo atresia. After menopause, few if any follicles are present in the ovary; their absence is accompanied by reduced ovarian steroid synthesis. Reproductive senescence can occur if xenobiotics block oogenesis in the fetus or destroy oocytes, causing premature ovarian failure (Mattison, 1983). Susceptibility also depends on the developmental stage of the individual. In studies of girls receiving combination antineoplastics, prepubertal girls were generally less susceptible to ovarian damage than girls receiving similar therapy after puberty (Siris *et al.*, 1976; Lentz, 1977; Stillman, 1981; Chapman, 1983).

This sensitive and complex process requires the integrated function of the hypothalamus, pituitary, and ovary, representing several potential targets for damage. Primary ovarian failure can be evaluated using hormonal markers that indicate raised gonadotropins or lowered estrogens, followed by menopausal symptoms. One study found urinary hormones to be the most reliable in predicting ovulatory function in a small group of women (Kesner *et al.*, 1992). The use of urinary hormones blunts the effects of hormone pulsatility and may not be informative unless serially collected, preferably over several cycles (Scialli and Lemasters, 1993, in press). Hormonal concentrations in blood are unreliable measures of reproductive impairment because of erratic fluctuation over very short intervals. Less severe damage to the ovulatory process may be expressed in disorders of menstruation which, in turn, may be a surrogate of other events such as a decrease in individual fertility potential or a very early pregnancy loss in which menses may or may not be delayed. Several potential mechanisms associated with toxic exposure and disruption of cyclic ovarian function have been summarized (NRC, 1989). Agents that mimic the action of naturally occurring hormones such as polychlorinated biphenyls (PCBs) or DDT, for example, may alter (1) normal estrogen feedback between the gonad and brain, (2) hormone synthesis and storage, and (3) hormone release and metabolism.

Menstrual changes as effect markers related to exposures are relatively sparse, but studies suggest that menstrual patterns may be sensitive monitors of environmental influences. Although the characteristics of a normal cycle vary among women, variations in individual women are slight. The average age of menarche is 12.5 years with a range of 9 to 16 years (McFarland, 1980). The average duration of menses is between 2 and 7 days; the interval between menses ranges from 23 to 35 days with a mean of 28.1 days (Chiazze *et al.*, 1968; Goldsmith and Weiss, 1986). Regularity of intervals between menses for an individual generally falls within 5 days (Fogel and

Woods, 1981). The average menstrual blood loss is 30 to 100 milliliters (Goldsmith and Weiss, 1986). The estimated mean age at natural menopause is 50.5 years.

Menstrual abnormalities can be divided into three broad categories of (1) cycle length or rhythm, (2) characteristics of bleeding patterns, and (3) the presence of pain. Exposure to solvents such as benzene, toluene, and xylene have shown menstrual disturbances primarily associated with abnormal bleeding (Michon, 1965; Butarewicz *et al.*, 1969; Syrovadko *et al.*, 1973; Beskrovnaja, 1979). Exposure to perchloroethylene and other solvents in dry cleaners was associated with a significant excess of menstrual disorders including cycle length, menorrhagia, dysmenorrhea, and premenstrual syndrome (Zielhuis *et al.*, 1989). Of workers exposed to trinitrotoluene in explosive manufacturing, 83% experienced changes in their menstrual cycle with symptoms diminishing to 65% and 20% after 1 and 2 years postexposure, respectively (Gesev, 1967). The prevalence of menstrual irregularity and amenorrhea was shown to be higher in nurses who handled cytotoxic drugs than in controls and was most pronounced in women over age 30 (Shortridge, 1988).

Using menstrual disorders as monitors of reproductive toxicity presents many challenges. Often clinical signs arise only after the occurrence of major functional changes. Disruption in menses cannot discriminate between ovarian, hormonal, or other central nervous system target sites. Data collection procedures may be subject to recall bias. Studies requiring calendar event recordings are cumbersome, resulting in poor compliance. Biologic samples combined with questionnaire data can, however, be powerful tools for eliciting answers to female reproductive toxicity.

Fetal Loss

Exposure of the conceptus to a toxicant can result in diverse effects, depending on when the exposure occurred during embryonic–fetal development. Transport time of a fertilized ovum before implantation is 2–6 days. During this early stage of embryogenesis, exposures to chemical compounds may occur by penetration of chemicals into the uterine fluids. Absorption of xenobiotics may be accompanied by degenerative changes, alteration in the blastocystic protein profile, or inability to implant. The period of late embryogenesis is characterized by differentiation, mobilization, and organization of cells and tissues into organ rudiments. Embryogenesis is the period of greatest sensitivity to teratogens. Untoward responses during embryogenesis may culminate in loss of the embryo or other structural or developmental defect. The fetal period extends from embryogenesis to birth. The distinction between the embryonic and fetal period is somewhat arbitrary, approximating when the developing organism has a crown rump length of 30 mm or approximately 55–60 days after conception. The fetal period is characterized developmentally by growth, histogenesis, and functional maturation.

Toxicity may be manifested by a reduction in cell size and number. The brain is still sensitive to injury; myelination is incomplete until after birth. Growth retardation, functional defects, disruption in the pregnancy, behavioral effects, transplacental carcinogenesis, or death may result from toxicity during the fetal period.

Epidemiologic studies of fetal wastage are based primarily on relatively few methodologies, that is, biochemical assays, record searches, and interview and survey approaches. Wilcox and others (1987,1988) have described a practical biologic marker of human trophoblast cell development to assess very early "chemical" pregnancy loss. Urinary human chorionic gonadotropin (hCG) is measured with immunoradiometric assay using a detection antibody. It is measured with no cross-reactivity of human leuteinizing hormone (hLH) at almost 100% specificity. The lowest hCG concentration detectable was 0.01 ng/ml. Screening methods involved collection of daily urine specimens within a 15-day portion of each cycle, beginning 10 days before the onset of menstrual bleeding. The definition of early loss was hCG levels of 0.025 ng/ml for 3 consecutive days, a fairly conservative criterion. A decrease in hCG titers signals loss of the pregnancy. The use of hCG as a biomarker, however, does not distinguish if the pregnancy failure is of maternal or trophoblastic origin. To discern this difference, a multiple hormone profile such as estrone conjugates and relaxin can be used (Stewart *et al.*, 1990). Future research will be enhanced by the further development and improvement of hormonal markers in easily obtained biologic specimens, such as urine and saliva (NRC, 1989). These methods are needed for application in epidemiologic studies.

Approximately 15% of fertilized eggs have been estimated to be lost prior to implantation, leaving 85% available for implantation (Leridon, 1977; Schlesselman, 1979; Edmonds *et al.*, 1982; Jones *et al.*, 1983; Little, 1988). In a study using measures of hCG (Wilcox *et al.*, 1988), wastage of fertilized ova occurring postimplantation but subclinically was 22%. This early loss rate translates into 19% of the remaining conceptions (.85 × .22). Recognized losses constituted 9% of remaining conceptions or 8% of the potential total available ova (.85 × .09) (Hertz-Picciotto and Samuels, 1988). Thus, the "true" potential rate of spontaneous abortions among fertilized ova is high, approximately 42% (.15 + .19 + .08) for fetal losses occurring up to 28 weeks of gestation.

Congenital Anomalies

Numerous reviews are available on the etiology, mechanism, and types of malformations. This section highlights data on incidence rates, risk factors, and the use of congenital anomalies as events signaling the introduction of a new teratogen into our environment or serving as a barometer of change that has occurred in the germ line of previous generations.

Malformations may be single or multiple. Chromosomal defects gener-

ally lead to multiple defects whereas single gene changes or exposure to environmental agents may cause single defects or a syndrome (Warkany, 1971). A major malformation can be defined as a defect resulting in death, requiring surgery or medical treatment, or causing a substantial physical or psychological handicap.

The known causes of birth anomalies are genetic (10.1%), multifactorial inheritances (23%), uterine factors (2.5%), twinning (0.4%), or teratogens (3.2%) (Nelson and Holmes, 1989). The remaining 43.2% of anomalies are of unknown etiology but are probably multifactorial. Certainly one important mechanism of action of teratogens may be mutation associated with cell death. Many chemical mutagens are not direct acting but require metabolic activation by the cytochrome P450 proteins. Placental tissue, as well as the embryo, is capable of this oxidative metabolism. Studies have shown that the inducibility of the maternal and the embryonic genotype is important in the teratogenicity of polycyclic aromatic hydrocarbons (PAHs) because of the binding of the metabolites to DNA (Manchester, 1991).

Several environmental exposures have been associated with congenital anomalies in the offspring. These include maternal consumption of food contaminated with methyl mercury during gestation in Japan and Iraq, causing morphological, central nervous system, and neurobehavioral abnormalities (Bakir *et al.*, 1973; Amin-Zaki *et al.*, 1974). In Japan, the cluster of cases was linked to the consumption of fish and shellfish contaminated with mercury derived from the effluent of a chemical factory. Maternal ingestion of PCBs from contaminated rice oil in 1968 gave rise to babies with several disorders, including growth retardation, dark brown skin pigmentation, early eruption of teeth, gingival hyperplasia, wide sagittal suture, edematous face, and exophthalmos (Kuratsune *et al.*, 1972).

Several registries in the United States and other countries monitor births with anomalies and link information about exposures that occurred during the pregnancy. One reason these registries are important is that the offspring may be more sensitive to xenobiotics during the period of organogenesis than at any other stage in the life cycle (Wilson, 1973). If rates of a particular defect, syndrome of defects, or unusual anomaly suddenly appear or increase, presumably these monitors will serve as early harbingers of a change in exposure. Registries have provided useful clues about potentially hazardous occupations but have fallen short of providing early warning signals of the introduction of a new teratogen.

The incidence of malformations is dependent on the status of the conceptus: (1) live births, (2) spontaneous abortuses, or (3) stillbirths. Miller and Poland (1970) found 88% abnormal abortuses in 28-day-old embryos (73 of 83). The overall incidence by 20 weeks of gestation was 43% (223 of 498). In the earliest age group, multiple system defects and severe growth disorganization were found and became less frequent with each developmental stage. Birth defect incidence figures for live births are dependent on age of

diagnosis. The frequency of major congenital defects reported shortly after birth can triple on re-examination at 9 months of age. The incidence of all major defects combined fluctuates between 1 and 3%, with a frequency of about 2.2% (Nelson and Holmes, 1989). Rates for all minor defects combined ranges between 3 and 15% (Ekelund *et al.*, 1970; Warkany, 1971; Bloom, 1981). This large variation in reported rates for major and minor defects is created by differences in data sources, populations, definitions, and specialty of individuals performing the examinations.

One challenge in using congenital anomalies as effect monitors is deciding how to group for analysis (Källén, 1988; Kline *et al.*, 1989). Often, all malformations are combined or grouped in major and minor categories. The rationale for grouping all these together is that the majority arose during organogenesis. The advantage of this approach is the larger number of cases, thereby increasing statistical power. Assume, for example, a case-control study in which the combined incidence of major and minor defects was hypothesized to be 10%, in contrast to a specific single anomaly with a rate of 1%. With 300 cases and controls in each group, the study has approximately 80% power to detect a doubling effect with a two-sided alpha of 0.05, in contrast to less than 20% power for the single anomaly. If an exposure effect is specific to a particular type of malformation, that is, central nervous system, grouping all major and minor defects together may mask the effect. Alternatively, grouping may be done by organ system. Although this method may be an improvement, certain defects may dominate the class, for example, varus deformities of the feet when examining the musculoskeletal system. The optimal solution, given the limitation of power, is dividing the defects into pathogenically homogeneous groups (Källén, 1988). Other considerations should be given to the exclusion or inclusion of certain malformations, such as those likely to be caused by chromosomal defects, autosomal dominant conditions, or *in utero* positioning. When analyzing congenital anomalies, a balance must be maintained between precision and statistical power.

Markers of Susceptibility

Markers of susceptibility for female reproductive abnormalities are indicators that are inherited or acquired that increase the likelihood of an abnormality. Inherited markers might include maternal inborn errors of metabolism that influence the potential of the fetus to be carried to term, such as those pertaining to blood clotting or the oxygen carrying capacity of blood, that is, those factors necessary for the formation and maintenance of the placenta. Examples of acquired susceptibility markers are catecholamine as a marker of stress to distinguish sensitive subgroups and alterations in hormonal profiles due to an agent that competes for receptor sites. Bernstein

TABLE 15.2 **Adjusted Geometric Mean Estradiol,** Sex-Hormone-Binding **Globulin-Binding** Capacity, and **Human Chorionic Gonadotropin Levels** in **Sera** by Smoking **Status at** Time **of** Sampling[a]

Variable	Smoking status at sampling				P value[b]
	Nonsmoker		Smoker		
Sample size(n) for E_2	83		64		
E_2 (pg/ml)[c]	158.7		130.7		0.037
By cigarettes per day	None	1–5	6–10	11+	
n	83	16	19	29	
	158.7	152.5	125.5	123.5	0.025[d]
Sample size (n) for SHBG-bc	55		48		
SHBG-bc (μg/dl)[c]	5.93		5.20		0.153
By cigarettes per day	None	1–5	6–10	11+	
n	55	9	16	23	
	5.93	5.87	4.91	5.17	0.182[d]
Sample size (n) for hCG	66		48		
hCG (iu/ml)[c]	50.5		39.6		0.044
By cigarettes per day	None	1–5	6–10	11+	
n	66	13	15	20	
	50.5	47.3	40.9	34.4	0.013[d]

[a] E_2, Estradiol; SHBG-bc, sex-hormone-binding globulin-binding capacity; hCG, human chorionic gonadotropin.

[b] P value associated with analysis of covariance F test, with adjustment for data set and length of gestation.

[c] All geometric means adjusted for data set and length of gestation.

[d] P value for trend with adjustment for data set (because hormone and SHBG-bc levels were determined at separate times for each study) and length of gestation (Bernstein *et al.*, 1989).

et al. (1989) examined the impact of smoking on hCG, estradiol (E2), and sex-hormone binding globulin-binding capacity (SHBG-bc) as shown in Table 15.2. The decreased levels of E2 ($p = 0.025$), SHBG-bc ($p = 0.182$), and hCG ($p = 0.013$) in smokers were evident over the range of gestation studied, that is, 43–113 days. The authors believe that the smoking effect on E2 is explained largely in statistical terms by the associated hCG effect (that is, adjusting the E2 values for the associated hCG values accounted almost completely for the difference in E2 values between smokers and nonsmokers). Hence, the hCG marker measures a direct effect on the placenta, and the between-person differences in level of E2 mirror differences in hCG level. Key to using such susceptibility markers are understanding whether the markers are related to the exposure, to the outcome, or to both,

and understanding how to treat the markers in the design and analysis of the study.

If research is aimed at assessing the role of genes in a reproductive event, markers of susceptibility that depict preexisting or previously acquired mutation might be useful in assessing whether all study subjects are equally at risk. Hence, the impact of the particular gene being studied can be determined with minimum confounding or effect modification. Little research has been performed thus far on markers of susceptibility for untoward reproductive outcomes. One exception is based on the observations between abnormal fibrogens and spontaneous abortion (Weatherall, 1991). One fibrogen variant that shows a clear association with spontaneous abortions is the Metz variant. The Metz variant is characterized by a defective fibrinopeptide resulting from an arginine-to-cysteine substitution at position 16 in the alpha chain (Weatherall, 1991). Prenatal diagnosis by amniocentesis and chorionic villus sampling can be used to obtain specimens of susceptibility markers for cytogenetic, biochemical, or molecular studies. Once fetal DNA is available, it can be analyzed in several ways for genetic disorders that might influence whether the pregnancy will come to term. Mutations can be identified directly by restriction endonuclease mapping or indirectly using restriction fragment length polymorphisms (RFLPs). Chapter 14 should be reviewed for a more detailed discussion on these genetic markers.

Markers of Male Reproduction

Markers of Exposure

Unlike the female, the male provides greater accessibility to reproductive fluids, allowing the opportunity to evaluate relevant target biologic materials and obtain estimates of biologically effective dose. Markers of biologically effective dose that have been identified include DNA adducts in sperm, xenobiotics in seminal plasma, and various enzymes, metalloproteins, flavoproteins, and mucoproteins in seminal plasma (NRC, 1989). Additionally, markers most commonly thought of as effect markers, such as DNA mutations in sperm, also may be used to indicate exposure when used in studies comparing groups nominally classified as with and without putative exposure (Segg, 1991). In such studies, the best indicator of exposure might involve the combination of a nominal exposure indicator and the presence of altered parameters (that is, markers of biologically effective dose or early biologic effects). In this approach, it is necessary to consider confounding factors that can account for differences among groups. Certain changes in sperm parameters, for example, may be used as exposure or reproductive effect markers. Sperm DNA adducts may be markers of exposure whereas changes in sperm morphology may be markers of susceptibility or effects.

Markers of Effect

Semen Assays

Manifestation of male reproductive toxicity has been defined as alteration in sexual behavior, fertility, pregnancy outcomes of the partner, or modifications in other functions that are dependent on the integrity of the male reproductive system (*Fed Reg.*, 1988). Noticeably absent from this definition is reference to male-mediated effects, for example, childhood cancers, as expressed in the offspring. There is some debate about the proper placement of these events. Because the insult to the human germ cell is a prerequisite for male-mediated expression, we suggest that the definition should be inclusive based on where the biologic insult has occurred, not on where it is expressed.

Assessment of male reproductive toxicity has relied heavily on semen assays because seminal fluid represents a relatively easily collected and analyzed medium in which to observe biologic effects of exposure. Sperm assays provide a direct measure of male reproductive impairment and a possible indirect measure of transmission of genetic damage. Spermatogenesis is a renewing, synchronous process requiring at least 14 intermediate stages wherein the germ cell proceeds through mitotic division for cell proliferation, meiotic divisions to generate genetic diversity and to decrease the chromosome number by half, and differentiation steps prior to the release of immature spermatozoa from the testes. Any of the developing cell types, from spermatogonia, spermatocytes, and spermatids to immature and mature spermatozoa, may be susceptible to toxic exposure. The process of spermatogenesis requires approximately 74 days.

Given the site of insult and the specific sperm parameter affected, different adverse outcomes may be observed. The absence of sperm (azoospermia) or reduced sperm count, less than 20 million per milliliter semen (oligospermia), and sperm with low motility are associated with reduced fertility. For nonmutagenic events, the most likely outcome associated with insult to the spermatogonia (stem cells) may be cell death. Thus, the affected cell would be phagocytized early and would not matriculate through spermatogenesis to appear in the ejaculate. Although cell death also may occur in later stages, the speed and efficiency of phagocytizing processes are uncertain. In addition to decreases in concentration, one may also observe decreased viability, decreased motility, and degenerative cells. Perturbations of the biochemical milieu in which the mature cells are maintained may be reflected as alterations in motility, decreases in viability followed by cellular degeneration, and eventual declines in concentration (Lemasters and Zenick, 1985). Sperm with abnormal forms (teratosperm) or degenerative forms may be associated with basic interferences in cellular processes necessary for maintaining the integrity and viability of the cell. These abnormal forms are more likely to be associated with infertility or early fetal loss. There are large gaps

in our knowledge concerning the interrelationships among these various markers. In one of our clearest examples of an occupational exposure affecting male reproduction, workers exposed to a nematocide, 1,2-dibromo-3-chloropropane (DBCP) not only experienced sterility (Whorton *et al.*, 1977) but experienced an increased incidence of spontaneous abortions in their wives (Kharrazi *et al.*, 1980).

The most common biologic markers used in semen studies are sperm count, sperm velocity, percentage motile sperm, percentage viable sperm, and percentage sperm with normal morphology. A longitudinal study of 34 unexposed men was conducted to obtain data on normative values and statistical variation (Schrader *et al.*, 1988); from that study and another (Schrader *et al.*, 1987) these mean values (and standard deviations) are derived. Sperm count is measured as the total number of sperm ejaculated ($\bar{X} = 204.8 \pm 199.2$). Sperm concentration is reported in millions per milliliter ($\bar{X} = 47.4 \pm 22.9$). The percentage of motile sperm measured is defined as the proportion of sperm that fulfills at least minimum movement criteria ($\bar{X} = 59.8 \pm 20.9$). Sperm velocity (in μm/sec) is the swimming speed of sperm either along its swimming path or in a straight line from one set time to the next. Computer instruments are used to evaluate swimming speed and patterns. Percentage viable sperm is expressed as the portion of living sperm measured by a stain exclusion test ($\bar{X} = 71.4 \pm 7.4$) or by hypoosmotic swelling ($\bar{X} = 64.1 \pm 9.1$). The stain exclusion assays measure structural integrity whereas placement in a hypoosmotic solution to measure cell swelling assesses the functional integrity of sperm membranes (i.e., maintenance of ionic gradients). Sperm morphology is an assessment of the shape of the sperm head but also may incorporate the midpiece and tail. Morphology may be reported as the percentage normal ($\bar{X} = 80.2 \pm 9.5$) or described in terms of percentage macro, micro tapered, double head or tail, percentage immature, percentage amorphous, and so on. Alterations in sperm morphology may be considered markers of susceptibility for subfecundity, fetal loss, and heritable genetic abnormalities (Wyrobek *et al.*, 1984). Sperm motility and morphology are considered two of the most sensitive indicators of susceptibility for subfecundity (Jouannet *et al.*, 1988; Grunert *et al.*, 1989).

Although sperm count is the marker most frequently used in field studies, it lacks precision for detecting population differences; the average coefficient of variation within subject sperm count is high: 44% (Schrader *et al.*, 1988). Sperm velocity, on the other hand, has low overall between- and within-person coefficients of variation but the within-person variation is greater, indicating that fluctuations within a subject are almost as great as those among subjects. This finding suggests that multiple samples from the same individual may optimally stabilize the results. Parameters of morphology and viability have relatively good intraclass correlations and low coefficients of variation, allowing good precision for detecting trends and population differences.

These standard tests of sperm function may be of limited value, however, in predicting fecundity. For example, after intrauterine insemination, no significant differences were found in semen parameters among those who did or did not achieve pregnancies (Huszar and DeCherney, 1987). Biochemical markers of sperm quality are needed to identify deficiencies in sperm function. Huszar *et al.* (1990) have attempted to take this next step by evaluating sperm creatine kinase (CK) activity to predict sperm fertilizing potential of oligospermic men. A comparison was made of oligospermic men who were fertile (N=33) with an infertile group (N=66) with identical mean sperm concentrations, 11.9 million sperm/ml, and similar mean motility values of 23.7 and 23.0, respectively. Sperm CK was significantly lower in the fertile oligospermic group. These findings also were supported in normospermic men (Huszar *et al.*, 1988). Certainly future investigations will need to augment the standard tests of sperm parameters with biochemical measures that have the ability to signal defects of sperm development and fertilizing potential.

Survey Methods

Approaches for estimating effects of exposure on reproduction, other than the collection of biologic specimens, include measures of fertility or fecundity as biologic markers. The survey methods described in this section are equally applicable in evaluating subfecundity of an exposed female population. Often in studies of male workers, the spouses are the actual source of the interview data.

Terminology used in defining fertility can be confusing. Fertility statistics are based on the ability to deliver a live born child, whereas fecundity impairments are more inclusive and address the physiologic capacity to conceive. A couple is considered subfecund if they have difficulty conceiving or maintaining a pregnancy over a specified time period. Population statistics indicate that, for married women between the ages of 15 and 44 years, approximately 16% have nonsurgical fecundity impairments (U.S. Department of Health and Human Services, 1982). This rate is considerably higher in African-Americans (23%) than in Caucasians (15%).

In epidemiologic studies, three approaches may be used to assess subfecundity. The first approach asks specific questions that determine if the individual has identified a period of time, usually 1–2 years, during which the couple was trying to conceive. Dates for total time period(s) are obtained. The rate and length of time in days, weeks, or months that the couple was subfecund are compared for exposed and unexposed employees. Requiring a minimum of 1 full year of unprotected intercourse, however, may miss more subtle effects of exposures. This length of time requires the passage of about five sperm cycles and may be inappropriate for exposures that are acute or intermittent (Lemasters *et al.*, 1991).

Another statistical approach was employed by Wong *et al.* (1979) to

assess subfecundity of workers exposed to ethylene dibromide. This method compared the observed number of births for exposed person–years to an expected number estimated from maternal birth rates specific to maternal age, parity, race, and year of birth of the woman. Levine *et al.* (1980,1981) and Starr and Levine (1983) extended this method to examine the fertility experience, comparing preexposure to exposure time periods. The disadvantages of this approach are that important covariates such as contraceptive history and functional infertility are not considered. Expected rates are missing for some ages, cohorts, or parities, and there may be a lack of comparability of marital status between the study group and national statistics. Because standardized fertility ratios use only live births as the end point, other important reproductive events are ignored.

Determining the number of noncontracepting cycles that are required for a couple to conceive after complete termination of birth control is a third method (Baird *et al.*, 1986). This "time-to-pregnancy" approach incorporates a wider complement of reproductive experiences since it provides an estimate of the per cycle probability of conceiving a detectable pregnancy. Data are collected that may include information on menstrual periods, contraception, and frequency of sexual intercourse. Baird *et al.* (1986) have shown that the Cox proportional hazard model is quite robust to minor violations of assumptions and provides an estimated relative risk measure; the discrete proportional risk model, however, may be the preferred choice, depending on the specific question being asked.

The choice of approach in analyzing infertility or subfecundity is dependent on the study design. If a referent population is unavailable, the standardized fertility analysis might be considered with an understanding of design restrictions inherent in this approach. If considerable details are known on potential confounders and a referent group is available, the time-to-pregnancy approach is preferred. It is probably prudent to include more than one approach, for example, responses to specific questions about the couple's recognition of an infertility problem and the life-event calendar approaches inherent in the time-to-pregnancy analyses.

Markers of Susceptibility

The chromosomal constitution of human sperm can be used as a biomarker in studies to determine the potential impact of xenobiotics on the human genome and the future conceptus. An approach in analyzing this genetic information involves the fusion of human sperm with eggs of the golden hamster. The sperm chromatin decondenses in the activated egg and undergoes DNA synthesis, replicating the human and hamster genome. Abnormal chromosome spreads are verified by microscope analysis of the region surrounding the egg and by karyotyping the hamster chromosome complement to assess normality (Martin *et al.*, 1987). Structural aberrations are classified

sess normality (Martin *et al.*, 1987). Structural aberrations are classified according to an international system (ISCN, 1978). An example of the application of this test was shown with a significant dose-dependent increase in the frequency of sperm chromosomal abnormalities after radiotherapy in cancer patients (Martin and Rademaker, 1987). The range in the testicular radiation dose was 0.4 to 5.0 Gy and the frequency of sperm chromosomal abnormalities was 6 to 67%. Although quite laborious and not currently feasible for large population studies, this method nevertheless is promising for the identification of susceptible subgroups.

Another promising sperm assay currently being used to assess workplace exposures is the sperm chromatin structure assay (SCSA). This assay, originally developed and validated in livestock, uses flow cytometric analysis of acridine orange-stained sperm to prove structural integrity of the sperm chromatin; native DNA fluoresces green and denatured DNA fluoresces red (Evenson *et al.*, 1991). The derived measure is "alpha t," red fluorescence divided by the combination of red and green fluorescence showing good stability and minimal variability. It is speculated that, had the male workers at the Sellafield nuclear plant been followed from initial hire, an earlier "susceptibility" marker on sperm may have been observed prior to the reporting of an excess of childhood leukemia and non-Hodgkin's lymphoma in the offspring of fathers receiving a total preconceptual ionizing radiation dose of 100 mSv or more (Gardner *et al.*, 1990). Without development of some earlier warnings, it will always be too late to prevent disease.

Analyzing the effects of exposures on older or on younger individuals also may elucidate susceptible subgroups. For example, the older male is at greater risk for sperm chromosome structural abnormalities, but there is no relationship between age and numerical abnormalities in sperm (Martin and Rademaker, 1987). In addition, sperm parameters distinguish potentially high-risk groups in studies of environmental exposures. A prospective study of the effect of an exposure on fecundity, which is postulated to be related only to an effect on sperm motility, might use sperm count as a marker of susceptibility. A low sperm count due to normal variability may act as an intervening factor since these individuals also have lower probabilities of successful fertilization than do men with higher counts. The group of men with lower sperm counts might, therefore, constitute the only group with no built-in margin of safety.

The whole issue on margin of safety refers to the definition of subfertility, which is a sperm concentration of <20 million sperm per ml or <50 million sperm per ejaculate, <1.0 ml semen volume, <60% motile sperm, and <60% normal morphologic forms (Cunningham, 1978). Therefore, the group of individuals whose preexposed sperm count is borderline, that is between 60 and 70 million per ejaculate compared to the average of 200 million, has less biologic redundancy before their fertility potential is greatly

compromised. In summary, in this situation, an analysis requires examining crude versus adjusted rates by stratifying preexposure low- and high-count individuals and examining group differences.

Hormonal changes may serve as excellent markers of susceptibility but attention must be given to whether these markers correlate with exposure or disease. Spermatogenesis requires an intact hypothalamic–pituitary–testicular axis and hormonal alterations may provide a reading on the successful integration of this system. Luteinizing hormones (LH) stimulates Leydig cells in the testis to secrete testosterone. Testosterone diffuses from the interstitial space in the testis into the spermatogenic tubules. Testosterone in the tubules affects spermatogenesis, either directly or indirectly through the Sertoli cells (Overstreet *et al.*, 1985). Follicle stimulating hormone (FSH) also acts on the Sertoli cells to stimulate spermatogenesis. Repeated measurements of FSH, LH, and testosterone, both pre- and postexposure, may be useful for assessing the temporal effects of exposure that precede a more permanent effect on a couple's fecundity (NRC, 1989).

The possible effects of tobacco smoke on sperm concentration, morphology, and motility provides an interesting case study. Although study results have been mixed, it has been suggested that the effects of smoking on these semen parameters may be caused by the presence of intermediary factors such as increased levels of choline acetyltransferase inhibitors, catecholamines, prolactin, serum estradiol, or testosterone (Albin, 1986a,b; Klaiber and Broverman, 1988). Alterations in any of these measurements might be early indicators of susceptibility that precede direct effects on sperm values.

The challenge in using hormonal markers is to detect them sufficiently early in the exposure–disease process. The goal is to distinguish whether hormonal imbalances are a consequence of a primary effect on the hypothalamus or pituitary axis or are secondary to disruption of the testicular–hypothalamic feedback mechanism. Hence, markers may be useful for distinguishing (stratifying) a population with an environmental exposure to determine if there is effect modification or confounding due to a preexisting susceptibility factor. The ability to distinguish the marker as preexisting, that is, acquired prior to the exposure period of interest or inherited, is of utmost importance. Acquisition will be assessed by prospective studies, and inheritance may be assessed by family studies (i.e., pedigree studies).

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Molecular Epidemiology

Principles and Practices

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