

Critical Windows of Exposure for Children's Health: The Reproductive System in Animals and Humans

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Drugs and environmental chemicals can adversely affect the reproductive system. Currently, available data indicate that the consequences of exposure depend on the nature of the chemical, its target, and the timing of exposure relative to critical windows in development of the reproductive system. The reproductive system is designed to produce gametes in far greater excess than would seem to be necessary for the survival of species. Ten to hundreds of millions of spermatozoa are generated daily by most adult male mammals, yet very few of these germ cells succeed in transmitting their genetic material to the next generation. Although the number of oocytes produced in mammalian females is more limited, and their production occurs only during fetal life, most ovaries contain several orders of magnitude more oocytes than ever will be fertilized. Toxicant exposures may affect critical events in the development of the reproductive system, ranging from early primordial germ cell determination to gonadal differentiation, gametogenesis, external genitalia, or signaling events regulating sexual behavior. Although there are differences between the human reproductive system and that of the usual animal models, such models have been extremely useful in assessing risks for key human reproductive and developmental processes. The objectives for future studies should include the elucidation of the specific cellular and molecular targets of known toxicants; the design of a systematic approach to the identification of reproductive toxicants; and the development of sensitive, specific, and predictive animal models, minimally invasive surrogate markers, or *in vitro* tests to assess reproductive system function during embryonic, postnatal, and adult life. **Key words:** development, germ cell, oogenesis, reproductive system, spermatogenesis, toxicant. — *Environ Health Perspect* 108(suppl 3):491–503 (2000). <http://ehpnet1.niehs.nih.gov/docs/2000/suppl-3/491-503pryor/abstract.html>

Although many of the important organizational events required to form a reproductively competent adult occur *in utero*, others must occur throughout life. If exposures to exogenous agents, either naturally occurring or man made, have sufficient influence on essential events, adult reproductive competency is diminished or abrogated. The time periods during which the development of the reproductive system is most sensitive to perturbation are reasonably well characterized in the scientific literature. In this paper we define critical windows of development for the reproductive system as follows: Critical windows of development are limited temporal intervals characterized by the occurrence of sets of dynamic organizational events that constitute periods during which exposures may have the greatest potential to affect later reproductive competency.

Concern for reproductive hazards dates back at least to the Roman era, yet the etiology or mechanism underlying these undesirable effects remains elusive in most instances. Although it is clear that dysfunction of the reproductive system can indeed be induced by chemical agents, and that these agents have effects on many processes, more research is needed to elucidate the critical windows during which an exposure may adversely affect the reproductive system and children's health.

Reproduction is cyclical in nature, so the consideration of key periods of toxicant exposure is arbitrary and thus could start at various points in this cycle. Preconceptional exposures will be defined here as exposures that affect spermatogenesis in the adult male; in the female these exposures will be considered as those from the point at which oocytes progress from their arrested state in primordial follicles and proceed to primary and secondary follicular phases, ovulate, and become fertilized. During the first part of the prenatal period (from fertilization to genital ridge development), we are unaware of examples of specific exposures associated with selective deleterious effects on the reproductive system. In both sexes, critical windows of exposure for the establishment of the stem-germ-cell population in the gonads, as well as differentiation of the urogenital system, occur during the second part of prenatal development (from genital ridge development to birth). However, in the male, the major cell divisions (mitotic and meiotic) that result in the production of millions of spermatozoa daily are triggered only at puberty and continue throughout life. In the female, oogonial differentiation and all mitotic divisions, as well as the initiation of the first meiotic division, occur during fetal life. Consequently, in females the critical window for exposures to chemicals that affect gametogenesis is prenatal.

The pool of oocytes that is established and fixed during fetal life declines significantly after birth and more gradually after puberty until reproductive senescence in rodents, or until it is depleted and heralds menopause in humans. Postnatal exposures may accelerate this decline, impacting on fertility potential in adulthood. In addition, exposures that alter the regulation of ovarian function may alter its endocrine function, resulting in altered development of secondary sex characteristics during puberty and ovulatory competence at any time during adulthood.

Animal models, such as rodents and nonhuman primates, are frequently used for hazard identification and characterization of the potential risk to human reproduction. To use such models in studies designed to evaluate potential risk to children's health (with respect to their reproductive function as adults), it is important to understand and consider similarities and differences in reproductive development between the animal model and humans. The objectives of this paper are *a*) to review and discuss key events in the development of the reproductive system in animal models and humans (as summarized in Figures 1 and 2); *b*) to compare and contrast critical windows in human reproductive development with analogous events in experimental animals (where information exists); and *c*) to summarize the major health outcomes of concern that would be expected to be associated with exposures during the preconceptional, prenatal, and postnatal developmental windows.

Key Periods in the Development of the Reproductive System

Preconceptional Period

Animals. In the male, preconceptional exposures are defined as exposures affecting spermatogenesis in the adult. With the exponential

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proliferation of germ cells during spermatogenesis, the germ cells make up the majority of cells in the testis (1). The production of spermatozoa is a highly ordered process; the timing is species and strain specific. In rats, the process takes 48–52 days, depending on the strain examined, whereas it takes as few as 34.5 days in mice and as many as 64 days in man. The adult testis is the site of the highest ongoing mitotic cell division in the body. Spermatogonia undergo several mitotic divisions (3–6 depending on the species) to become spermatocytes. Primary spermatocytes proceed through prophase, metaphase, and anaphase; in anaphase the paired chromosomes move to opposite sides of the cell.

This differs from mitosis, where it is the sister chromatids that move to opposite sides of the cell. In telophase there is incomplete cytokinesis and the two daughter cells that result form a syncytium. At the completion of the first meiotic division, the primary spermatocyte becomes a secondary spermatocyte. There is a short interphase between the first and second meiotic division when no DNA synthesis occurs. During the second division, the secondary spermatocyte progresses from prophase through telophase; in anaphase there is separation of the sister chromatids, similar to mitosis. After the completion of the second division, the secondary spermatocytes are called spermatids.

Thus, one primary spermatocyte gives rise to four spermatids. The early spermatids are round cells that undergo a tremendous number of changes during a process called spermiogenesis: the acrosome is formed, chromatin condenses, excess cytoplasm is shed, and the flagellum develops. As germ cells develop, they move from the periphery of the seminiferous tubule (spermatogonia) toward the lumen (spermatids and spermatozoa). Spermiation is the release of spermatozoa into the lumen. Many chemicals perturb or arrest this process.

Although the first wave of spermatogenesis is under the control of both testosterone and follicle-stimulating hormone (FSH), the relative importance of FSH in maintaining spermatogenesis in the adult seems to be highly species dependent. In most species studied to date, testosterone, which is normally synthesized in Leydig cells under the influence of luteinizing hormone (LH), alone can qualitatively maintain complete spermatogenesis. In mice with null mutations for the FSH-β subunit, only a quantitative reduction in spermatogenesis has been noted, with no consequent effect on fertility. Hence testosterone appears to be the primary regulator of spermatogenesis, although many other factors, such as insulin-like growth factors, oxytocin, and transforming growth factor-β, clearly play a role in modulating this complex process.

Spermatozoa are released from the testis in an immature state (without motility or the ability to bind to or fertilize an oocyte). The maturation process takes place in the epididymis during a 5- to 10-day transit period. The epididymis is responsible for the maturation

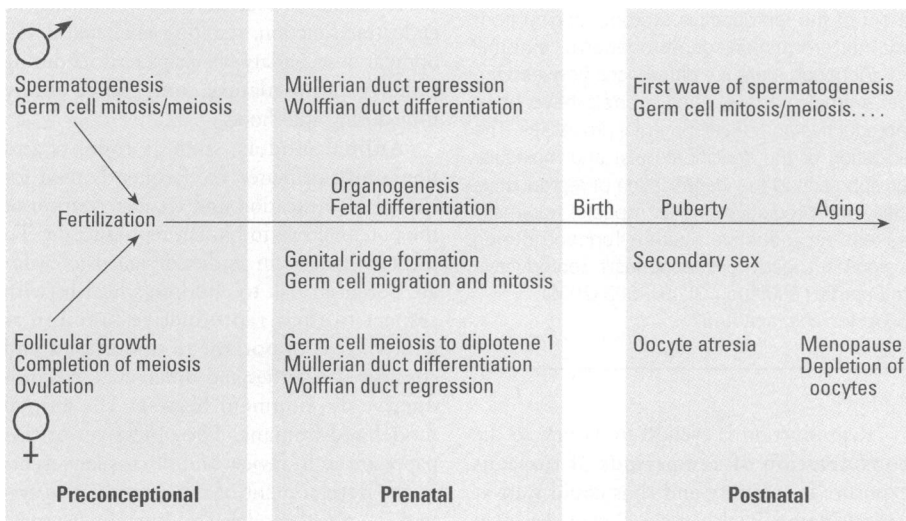


Figure 1. Critical windows of exposure: the reproductive system.

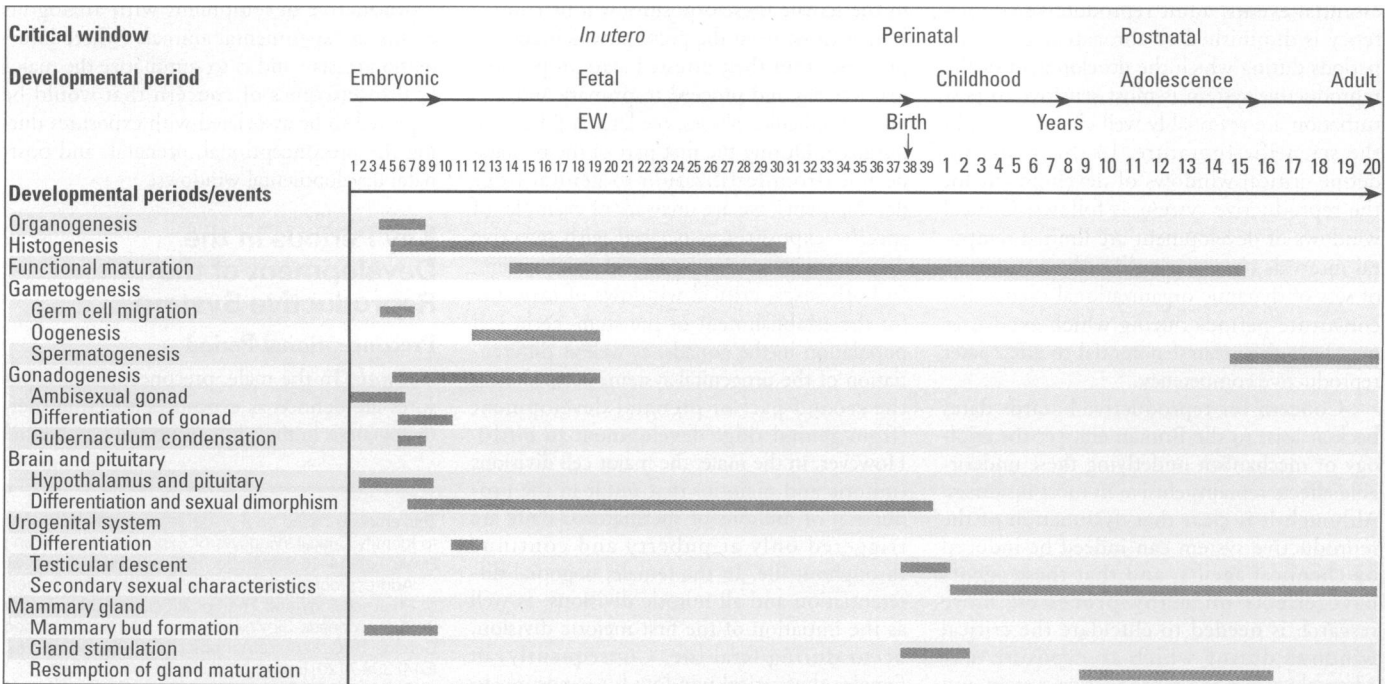


Figure 2. Key developmental events in human reproductive tissue, gland and organ development during the critical windows. EW, embryonic week.

of spermatozoa, as a conduit for spermatozoa to get to the vas deferens, and as a storage site (2,3). From the first meiotic division onward, germ cells are outside of the central compartment of the body, i.e., they traverse a blood–testis/blood–epididymis barrier. Although this barrier allows for the creation of a special environment that excludes proteins such as immunoglobulins and highly polar chemicals, it does not prevent the access of many chemicals to the germ cells.

At the time of ejaculation, spermatozoa, stored in the cauda epididymidis, vas deferens, ampulla, and ejaculatory duct, combine with secretions from the sex accessory glands (prostate, seminal vesicles, bulbourethral gland, and Cowper's gland). These glands provide the fluid environment for spermatozoa. The seminal vesicle provides most of the fluid and some of its components include ascorbic acid, prostaglandins, fructose, and clotting factors. Factors secreted by the prostate include citric acid, acid and alkaline phosphatases, polyamines, zinc, and various proteases. The secondary sexual glands and the epididymis are under the control of androgens. More specifically, these tissues are under the control of dihydrotestosterone (DHT), which is produced when testosterone is reduced in the 5 α position by steroid 4-ene 5 α -reductase. It is noteworthy that the proportion of the volume of semen contributed by spermatozoa and epididymal secretions is < 15% in most mammals; hence, most of the seminal volume is derived from the ductal system of the accessory glands. The presence of exogenous chemicals in these secretions may result in exposure of the female partner during mating.

In the female, preconceptional exposures may affect oocytes as they progress from their arrested state and begin proceeding through the primary and secondary follicular phases to ovulation and fertilization. Little is known about the intragonadal mechanisms involved in triggering an arrested primordial follicle to enter the primary follicle pool, although it is clear that the pituitary and higher brain centers are not required. Atresia is the predominant fate of primary follicles. If an oocyte does not undergo atresia, it will take three to four cycles (depending on the species) for a follicle entering the pool of primary follicles to become a Graafian follicle and be ovulated; gonadotropin-releasing hormone (GnRH), FSH, and LH play significant roles in folliculogenesis. The process of ovulation is carefully timed, and it requires accurate communication among the hypothalamus, the pituitary, and the ovary (follicular and luteal cells) using an array of signals. Although exposure to chemicals occurs during folliculogenesis, ovulation, oocyte transport, and fertilization, there is

little evidence that any such exposures result in specific toxic effects on tissues of the reproductive system.

Humans. In man the complete processes of spermatogenesis and sperm maturation are remarkably similar to those observed in other mammals, but it takes well over 2 months (64 days for spermatogenesis and 3–10 days for sperm maturation); the stages of spermatogenesis are arranged in a helical, as opposed to linear, arrangement in the seminiferous tubules. Among mammals, man is one of the species that produces the lowest number of spermatozoa per gram of testicular tissue; furthermore, his spermatozoa are of lower quality (more abnormal forms, fewer motile sperm, and with lower motility characteristics) than nearly any other mammal. The reasons why sperm production rates and sperm quality are so relatively poor in humans have not been established, but factors such as artificially elevated scrotal temperatures (due to clothing or certain lifestyles), exposure to toxicants, and genetics may play a role.

After sexual maturity in the female, folliculogenesis leads to a midcycle LH surge that induces the single follicle destined for ovulation to undergo events preparing it for possible fertilization. First, meiosis is resumed, with the completion of the first meiotic division and formation of one daughter cell and a second smaller cell with little cellular content, the polar body. The daughter cell enters into the second meiotic division and arrests in the second metaphase until fertilization. The follicle becomes highly vascularized and a protrusion in the follicular wall (macula pellucida) indicates the location of the rupture, resulting in the release of the oocyte with its surrounding granulosa cells. At ovulation, the oocyte (with its surrounding granulosa cells) is extruded onto the ovarian surface, where the cumulus mass can be retrieved by the fimbriated end of the fallopian tube. After ovulation, the walls of the follicular cavity in the ovary develop into the corpus luteum. If fertilization occurs, the lifespan of the corpus luteum is prolonged; otherwise, it disintegrates during the 14 days of the luteal phase of the menstrual cycle.

In addition to triggering ovulation, the midcycle LH surge induces luteinization of the theca and granulosa cells of the follicle wall. Normal luteal function depends on both normal folliculogenesis and successful follicular rupture. Vascularization of the follicle wall that occurs with ovulation provides the luteinized theca and granulosa cells with low-density lipoprotein cholesterol from the circulation, which is used to synthesize progesterone. Follicular growth, ovulation, and maintenance of pregnancy are controlled by the balanced secretion of FSH and LH. Both hormones act on the ovarian target cells

by means of cell surface receptors. In addition, FSH stimulates the aromatization of theca-derived androgens to estrogens by granulosa cells. The main endocrine action of LH is to stimulate progesterone production by granulosa cells. LH stimulation also induces follicular theca cells and stromal interstitial cells to produce androgens, which serve as precursors for FSH-dependent estrogen production by granulosa cells.

Prenatal Period

Animals. Chromosomal sex is determined at fertilization. The presently accepted hypothesis is that genetic sex is dependent on a gene on the Y chromosome, designated *Sry* (sex-determining region-Y-chromosome) [reviewed by Parker et al. (4)]. *Sry* has a DNA-binding motif termed the high-mobility group (HMG) box. *SOX* (homologous to *Sry* HMG box) genes have > 60% homology with *Sry*; one *SOX* gene, *SOX9*, is also involved in sex determination. The orphan nuclear receptor steroidogenic factor 1 (SF-1) regulates the expression of many genes involved in steroidogenesis, as well as the expression of Müllerian-inhibiting substance [(MIS) from Sertoli cells] [reviewed by Parker et al. (4)]. SF-1 is required for the development of testes and male sexual differentiation but may suppress ovarian development and female sexual differentiation. Other genes that are involved in sex determination include a tumor-suppressor gene, *WT1*, and a gene designated *DAX-1* (dosage-sensitive sex reversal, x-linked). *DAX-1* and *SF-1* may act in the same pathway.

Many of the critical steps in the formation of the reproductive system take place during embryogenesis. Establishment of the primordial germ cell lineage occurs early during development and is regulated by specific transcription factors and growth factors such as Oct-4 or Bmp-4 (5,6). In mammalian embryos, it remains unclear as to which specific inner cell mass cells in the blastocyst are specified to become germ cells. Initial gonadal formation involves the establishment of a primordial germ-cell lineage and the migration of these primordial germ cells to embryonic mesoderm of the primitive streak. Migrating germ cells move from the visceral endoderm of the yolk sac to the developing hindgut, through the dorsal mesentery, to finally reach the gonads. In the mouse on gestation day (GD) 8, 10–100 primordial germ cells can be identified; by GD 13, the gonads contain 10,000 germ cells [reviewed by Byskov and Høyer (7)]. The number of primordial germ cells depends on their mitotic activity; from GD 8.5–13.5 in the mouse, primordial germ cells replicate by mitosis at a uniform rate and in a similar manner in both males and females. Although some species have a

period of low mitotic activity, in others it is very short (rat) or nonexistent (mouse). The proliferative phases preceding meiosis are extremely sensitive to exposure to irradiation.

Sexual differentiation, or the establishment of a male or female sexual phenotype, occurs either during organogenesis or during the fetal period. Initially, the gonads are in an indifferent stage and the urogenital tracts of the male and female are indistinguishable. The time window during which gonadal sex can be influenced *in vitro* in the mouse is from GD 10–12.5 (7). *SF-1* plays a key role in sexual differentiation by regulating the synthesis and secretion of steroids and other morphogenetic substances that exert a major effect on the phenotype of the embryo. The bipotential gonad will differentiate in the male into a testis, with cordlike structures, and in the female into an ovary. Once sexual differentiation is initiated (at approximately GD 13.5 in the mouse), the subsequent kinetics of development are dramatically different in the male and female.

Testicular cords are formed in the differentiating testis; these contain germ cells that are in close approximation to somatic cells, which are the precursors to Sertoli cells. Germ cells enclosed in testicular cords are called prospermatogonia. In the fetal testis, prospermatogonia undergo waves of mitosis and morphological differentiation before a transient arrest in interphase (G_1) on GD 18 in the rat. Mitotic divisions are reinitiated just before birth. Sertoli cells proliferate throughout fetal development; their highest proliferative activity is on GD 16 in the fetal rat. The destruction of Sertoli cells or interference with their proliferation may have irreversible effects on the development of the testis and its potential to produce sperm during adulthood. In the rat, testosterone synthesis starts simultaneously with the differentiation of Leydig cells at the fetal age of 15.5–16.5 days; LH receptors appear at 15.5 days. Leydig cells proliferate slowly during early stages of differentiation. Steroidogenesis is one potential target for chemical action. Insufficient androgen will result in feminization of the testis, resulting in a gonad that lacks testicular cords. Testicular steroids are responsible for maintenance and differentiation of the internal (testosterone) and external (dihydrotestosterone) genitalia, and for priming the brain in the male direction at early developmental stages (8).

In the absence of testicular hormones, the pathway of sexual differentiation in mammals is female [reviewed by Byskov and Høyer (7)]. In the rodent fetal ovary, germ cells continue to undergo mitosis until they enter meiosis; the first germ cells to enter meiosis are localized in the inner part of the cortex. Meiosis is arrested in the late prophase of the first meiotic division, the

diplotene stage, at which time follicles begin to form. To survive beyond the diplotene stage, oocytes must be enclosed in a follicle together with supporting granulosa cells; a large percentage of the oocytes that enter meiosis do not reach the diplotene stage, but instead degenerate. In some species, such as mice and rats with immediate meiosis, ovarian steroidogenesis does not take place until after follicles are formed. MIS, which is secreted by Sertoli cells in the testis, can induce the formation of structures resembling testicular cords in the fetal ovary. Gametogenesis in the female may be especially sensitive to prenatal chemical exposures.

Genomic imprinting distinguishes genes as functionally nonequivalent on the basis of the parent of origin. Imprinting is an epigenetic mechanism by which the expression of certain genes (some 30 in humans and mice to date) becomes dependent on their parent of origin. Imprinted genes play key roles in growth and differentiation; the maternal and paternal genomes are both essential for normal embryo development to occur. One epigenetic feature that is consistently associated with imprinting is CpG methylation. Genomic imprints are erased during early embryo development and must be reestablished in germ cells during gametogenesis (9,10), making imprinting a potential target in gametes for toxicant action.

In the male, under the influence of testosterone from the Leydig cells, the Wolffian ducts differentiate into the epididymides, vas deferens, ejaculatory ducts, and seminal vesicles. Sertoli cells secrete MIS, which causes the Müllerian ducts to regress. Both testosterone and MIS are regulated by SF-1. In the female fetus, where there is an absence of testosterone and MIS (MIS secretion is not detected in the ovary until postnatal day 6 in the mouse), the Müllerian ducts differentiate into the oviducts, Fallopian tubes, uterus, and upper vagina, while the Wolffian ducts regress. Estrogen receptors are present in the Müllerian ducts in the mouse as early as day 15 of fetal life. The effect of MIS on the Müllerian ducts in the male can be prevented by exogenous estrogens. Wolffian duct differentiation is dependent on testosterone, whereas DHT, the 5α -reduced androgen, appears to be responsible for the differentiation of external genitalia in the fetus. Exposure to androgens will masculinize the female external genitalia. Other signaling molecules are also important in genital duct development. *Hoxa-10*, a developmentally important signaling molecule, is maximally expressed in the Wolffian duct and urogenital sinus in the day 18 mouse fetus; loss of *Hoxa-10* function is associated with diminished stromal cleaving of the seminal vesicles

and decreased size and branching of the coagulating gland (11). Signaling by *Wnt-7a* appears to be involved in the sexually dimorphic development of the Müllerian ducts (12).

Humans. The conceptus is properly called an embryo from the time the bilaminar germ layer appears during the second week after fertilization to approximately the eighth week of gestation, by which time most major organ and tissue development has occurred. By the end of the embryonic period in the eighth week, tissues and organ systems have developed and the major features of the external body form have developed. The period of development between the fourth and the eighth week, when all of the major tissue and organ systems begin to develop in the human embryo, is referred to as organogenesis. The fetal period extends from the ninth week until birth. During the fetal period, the fetus grows in weight from approximately 8 g to approximately 3,400 g. Weight is gained mainly in the third trimester, but the fetus increases in length mainly during the second trimester. Many organ systems, including the brain, peripheral nervous system, sensory systems, and the reproductive system, are not mature at birth.

There are striking similarities between the differentiation of the human reproductive tract and that of other mammals. The genital system arises in close conjunction with the urinary system; the primitive sex cords develop from cells of the mesonephros and coelomic epithelium that proliferate to form the genital ridges. Until the end of the sixth week, the male and female genital systems are virtually identical. Cortical and medullary sex cords and mesonephric and paramesonephric ducts are present in both sexes. This period is called the ambisexual (indifferent) phase of sexual development. In the sixth week a new pair of ducts, the paramesonephric (Müllerian) ducts, develop adjacent to the mesonephric ducts. The paramesonephric ducts have separate fates in the two sexes, and starting in the seventh week, the sexes diverge as the primitive sex cords pursue these separate fates. Germ cells from the yolk sac endoderm migrate along the dorsal mesentery of the hindgut to the gonadal ridge in approximately the sixth week of gestation.

Under the influence of testis-determining factor from the *Sry* gene, the primary sex cords differentiate into seminiferous tubules and the indifferent gonad develops into a testis. Sertoli cells are thought to develop from mesenchymal cells or mesonephrogenic blastema (13). The Sertoli cells surround the germ cells to form testicular cords (14). MIS plays a similar role in man as it does in other mammals, i.e., inhibition of the development of the Müllerian (paramesonephric) ducts;

without MIS, the Müllerian ducts would develop into the female reproductive system.

Leydig cells develop from mesenchymal cells in the testicular interstitium beginning at 8 weeks of gestation (15); these cells are stimulated by human chorionic gonadotropin to secrete testosterone beginning at approximately 8 weeks of gestation. At around 12 weeks of gestation, LH levels begin to rise and it is thought that the fetal pituitary begins to take over control of Leydig cell function (16). Serum testosterone peaks at approximately 14 weeks of gestation and after 24 weeks declines to female levels (17).

As in the male, the female primordial germ cells undergo mitotic division in the yolk sac until the time of gonadal differentiation (7). The migration of primordial germ cells into the gonadal ridge is complete by 7 weeks of gestation. The gonadal ridge grows and surrounds the primordial germ cells, and together they form the surface epithelium, the primitive cords, and the gonadal blastema. Gametogenesis begins during fetal life in females (oogenesis) but is suspended at an early stage and does not resume until after puberty. Female gonads begin to differentiate approximately 8 weeks after fertilization; this is about 1 week later than in males. The gonadal blastema begins to differentiate into medullary cords and stroma; the medullary cords mostly degenerate, whereas the cortex is preserved. The early ovarian cortex contains oogonia and epithelial cells. By the eleventh week of fetal life, interstitial connective tissues (stroma) intersect the epithelium leading to compartments containing single oocytes surrounded by a single layer of epithelial cells (primordial follicles). The ovaries descend into the pelvis by the twelfth week of fetal life.

Oogonia are transformed into oocytes by the initiation of meiotic division during the third to ninth months of fetal life. All of the oocytes a female will produce are present in the ovaries by 5 months of fetal life, at which time approximately 7 million primordial follicles have formed. This number is reduced by atresia to about 2 million by birth and 0.5 million by puberty. As in other mammals, in human fetal oocytes, meiosis proceeds only to the first meiotic prophase and then enters a stage of arrest in the diplotene stage of the first meiotic prophase.

The female phenotype is the default; it is generally believed that no additional factors are required for the development of female gametes and sexual characteristics. In the absence of MIS, the paramesonephric ducts give rise to the oviducts, uterus, and superior vagina. The caudal vagina forms from the posterior wall of the pelvic urethra. The caudal portions of the paramesonephric ducts fuse together to form a tube, the uterovaginal (genital) canal, which differentiates into the

uterus and the superior segment of the vagina. The cranial unfused segments of the paramesonephric ducts become the oviducts (fallopian tubes).

The early phases of external genital development are similar in the two sexes, and the sexes generally become distinct during the fourth month of gestation. As in other mammals, DHT induces male differentiation of the external genitalia, whereas female development occurs in its absence. The first step in external genital development is the appearance of a pair of cloacal folds on either side of the cloacal membrane. The fusion of the urorectal septum with the cloacal membrane in the seventh week creates the perineum. The urorectal septum also subdivides the cloacal folds into ventral urethral (urogenital and genital) folds and dorsal anal folds. A new pair of broad swellings, the labioscrotal swellings, develop laterally into the urethral folds. The urogenital membrane becomes patent in the seventh week. The genital tubercle elongates to form the phallus, which is divided by a coronary sulcus into a glans and a shaft. In the male, the scrotal swellings fuse in the midline to form the scrotum. In the female the labioscrotal swellings and urethral folds do not fuse. The labioscrotal swellings become the labia majora and the urethral folds become the labia minora. The testes and ovaries both descend under the control of a gubernaculum, which is a ligament that condenses within the subserous fascia during the seventh week. Testicular descent into the scrotum is probably under the control of both androgens and mechanical processes (14,18).

The mammary glands are modified apocrine glands that arise along mammary ridges on either side of the body. A pair of ectodermal thickenings called mammary ridges appears on the ventral surface in the fourth week of gestation. In the fifth week the mammary ridge at the site of each presumptive breast forms the primary bud of the mammary gland. In the tenth week these buds branch to form several secondary buds that then lengthen, branch, and canalize during the remainder of gestation to form 15–25 lactiferous ducts. At birth the lactiferous ducts of each mammary gland open into a superficial depression called the mammary pit, which is usually converted to a raised nipple within the first weeks after birth. It is speculated that the number of estrogen receptors in mammary tissue is determined at this stage and that this predisposes the breast for responses to environmental insults during adulthood, and thus possibly estrogen-dependent breast cancer. Hence, mammary bud formation is proposed to be a critical window for normal development and adult function.

GnRH release from the hypothalamus influences LH and FSH production by the

pituitary gland beginning in fetal life. The mean peak plasma levels of LH and FSH during gestation are higher in female than in male fetuses. The pulsatile release of LH and FSH secretion is controlled by neurons located in the arcuate nucleus of the hypothalamus, which secrete GnRH into the hypothalamic–hypophysial portal system.

Postnatal Period

Animals. In the male there are rapid mitotic divisions of both spermatogonia and Sertoli cells in the first few days after birth. Although some spermatogonia undergo mitotic divisions before the initiation of meiosis to eventually become spermatozoa, others continue to divide so that a pool of stem cells is maintained. New waves of spermatogenesis are initiated on an ongoing basis. In the rat, Sertoli cells stop dividing at the beginning of the third postnatal week and form tight junctions between adjacent cells, constituting the blood–testis barrier. After spermatogonia enter meiosis, they cross the barrier by a zipperlike action to enter the adluminal compartment. The last round of DNA synthesis occurs just before the first meiotic division. Thereafter RNA synthesis continues in spermatocytes and round spermatids but ceases as the sperm chromatin condenses in the final stage of spermiogenesis. Some of the RNAs produced in round spermatids are not translated until later in spermiogenesis. Instead these RNAs are bound to proteins (such as testis brain RNA-binding protein or p48/52); it has been suggested that germ-cell-specific mRNA-protein binding has a role in the stabilization and subsequent translation of transcripts throughout spermiogenesis (19,20). For any given species, there is a fixed ratio of Sertoli to germ cells; thus, agents that affect Sertoli cell proliferation during the first 2 weeks of postnatal life will set the sperm production rate per testis.

The neonatal hormone environment profoundly affects the ultimate sexually differentiated pattern of central nervous system (CNS) anatomy and neurochemistry, reproductive physiology, and behavior. Sexual differentiation of the CNS depends on exposure to gonadal steroid hormones (21–23). Morphological differences between the sexes have been shown at the light microscopic and ultrastructural levels in several nuclear regions of the CNS (24). Neonatal androgenization of female rats produces a diminished LH secretory pattern that is characteristic of males (25). Such androgenization results in delayed onset of puberty, decreased regularity of ovarian cyclicity, and cessation of cyclicity at an earlier age (26–28).

Sexually distinctive differences in patterns of behavior can be affected by perinatal exposure to gonadal steroids (29). Estrogen, and

to a lesser extent progesterone, act in the CNS to control lordosis behavior in rats. In female nonhuman primates, attractivity and proceptivity change with the stage of the menstrual cycle or as a result of sex steroid administration. The effects of hormones on receptivity are unclear. The lordosis response is diminished by androgenization. Other behaviors that show such sexually distinct differences include aggressive behavior, social play and open field behaviors, territorial marking, urination posturing, regulation of food intake, taste preference, and tests of performance and learning (21,22).

Studies of male mating behavior have been conducted in a variety of mammalian species. The repertoire of male mating involves precopulatory behavior, mounting, intromission, ejaculation, and postejaculatory behavior. In rodents, male sexual behavior is dependent on the production of estradiol from testosterone in specific brain nuclei. Important aspects of male mating behavior are controlled by the preoptic anterior hypothalamic area. Rhesus monkeys with bilateral lesions in this area do not attempt to copulate but continue to masturbate and achieve erections, indicating a deficit in mating behavior toward the female but with no obvious neuroendocrine physiological compromise (30).

Humans. Spermatogonia are the only germ cells present in the prepubertal testis, although there are reports of some preleptotene spermatocytes being present (31). Between birth and 10 years of age, there is a 6-fold increase in the number of spermatogonia; this occurs by mitotic division (14,32). At puberty there is an exponential increase in both the number of germ cells and the testicular volume (14). Immature Sertoli cells are the most common somatic cell type seen in the prepubertal testis (14). The total number of Sertoli cells increases in the prepubertal period and into early adulthood (14,33).

There is a postnatal rise in serum testosterone that occurs at approximately 3 months of age (34). It is not surprising, then, that there is a biphasic pattern of Leydig cell development with a nadir of testosterone-containing cells at approximately 1.5 years of age (16). The number of testosterone-producing Leydig cells progressively increases with time until it plateaus at adulthood.

Spermatogenesis is designed to generate haploid cells by reduction division and to increase genetic variation (3). Spermarche, defined as the age at which spermatogenesis begins, occurs early in puberty. The mean age of spermarche is typically determined by noting sperm in the urine collected from boys. In one longitudinal study the mean age of spermarche was 13.4 years, and ranged from 11.7 to 15.3 years (35). Adult levels of testosterone do not occur until 2 years after spermarche (36).

Because LH and FSH are involved in regulating spermatogenesis, the control of their pituitary release by the hypothalamus is important in maintaining testicular function. During puberty, inhibin, produced by Sertoli cells, is the major factor involved in the feedback inhibition of FSH. Testosterone exerts a negative feedback on GnRH release from the hypothalamus and is thus involved in the regulation of both LH and FSH secretion. Males release LH from the pituitary in a pulsatile manner, and this induces increases in circulating testosterone. Because of its longer half-life, the circulating levels of FSH exhibit little pulsatility. Neonatal exposure of the hypothalamus to androgen is necessary for the sexual differentiation of the LH secretory mechanism to respond to either androgen or estrogen.

In the newborn female, oocytes are enclosed within primordial follicles and are arrested in the diplotene phase of the first meiotic division. They remain in this condition until puberty; menarche is the beginning of menstrual function. Throughout postpubertal life, cohorts of follicles continue to be recruited. Some of these will mature to become Graafian follicles, with consequent ovulation; however, most follicles undergo atresia. Although approximately 2 million follicles are present at birth, essentially all are depleted by about 50 years of age, resulting in menopause. Phasic entry of cohorts of follicles into further folliculogenesis establishes menstrual cyclicity. During each menstrual cycle, only one oocyte of the cohort usually progresses to ovulation and to potential resumption of meiosis if fertilization occurs.

Follicular growth, ovulation, and maintenance of pregnancy are controlled by the balanced secretion of LH and FSH. The main actions of LH are to trigger follicular rupture and oocyte release, to stimulate progesterone production by granulosa cells, and to stimulate follicular theca and stromal interstitial cells to produce androgens. FSH stimulates follicular growth and the aromatization of theca-derived androgens to estrogens by granulosa cells. LH secretion in prepubertal females occurs in a basal fashion with irregular pulsatile occurrences. In cycling females, the ovarian steroid and pituitary profiles are intimately linked to produce the menstrual cycle.

Events occurring around puberty instigate the start of mating behavior. At puberty, males and females become aware of each other as sexual beings. Experience and hormones are important in the maintenance of copulatory behavior. In human males and females, androgen increases libido. Women treated with androgens frequently complain of excessive libido as a side effect, whereas women treated with antiandrogens complain of reduced libido. Sexual activity may occur when testosterone levels are relatively low,

although an interval of testosterone priming may be necessary. Stress reduces plasma testosterone in males and females by acting through the hypothalamic-gonadal axis; stress-induced depressed hypothalamic function affects both male and female mating behavior, decreasing and even eliminating it in some cases.

Environmental influences on reproductive function and mating behavior may occur through visual and olfactory cues. For example, circadian rhythms influence the timing of the periovulatory surge of LH and ovulatory rates. The LH surge tends to occur at night and in the morning (37,38). The best evidence of seasonal variation in human reproduction is from studies with Alaskan Eskimos, showing seasonal changes in birth rates corresponding to peaks in June and January (39). Humans do not reproduce in a rigidly seasonal manner, possibly because they have a reduced ability to respond to environmental cues or because they live in modified environments that attenuate the impact of such cues (40).

Macaques, which are seasonal breeders in temperate environments, will ovulate year-round when housed in conditions of controlled temperature and light (40,41). However, macaques that are reared indoors and exposed to varying photoperiods show the expected pseudoseasonal variation in ovulatory pattern, as if they were still housed outdoors; they do not lose their ability to ovulate year-round if then placed in a fixed photoperiod regime. This suggests that humans no longer demonstrate a reproductive response to changing photoperiods because of ontogenic and possibly evolutionary experience. Pheromones also may play important roles in human reproduction. However, the only confirmed pheromone-driven reproduction phenomenon is menstrual cycle synchronicity in females sharing the same living environment (40,42,43).

Until puberty, the mammary glands of males and females are essentially the same. In the female, the increase in estrogen levels at puberty resulting from pulsatile secretion of gonadotropins stimulates growth of the rudimentary mammary gland. When progesterone production starts as part of an ovulatory cycle, it stimulates the alveolar buds within the mammary lobules. After puberty, the changing estrogen and progesterone levels in the cycling female affect breast tissue development, with maximal activity in the luteal phase. Because the levels of estrogen and progesterone in the cycling female are under the influence of the hypothalamo-pituitary axis, the neuroendocrine system indirectly influences breast development. The mammary alveoli do not fully develop until pregnancy and lactation. Cortisol, insulin, and placental

lactogen contribute to alveolar development, but estrogen and progesterone are most important. During pregnancy, estrogen and progesterone cause the mammary gland ductal system to undergo further branching, and the alveolar cells undergo proliferation.

Health Outcomes of Exposure

Preconceptional Exposures

Animals. Because germ cells in the male are being generated continuously, treatment of adult males with radiation or with cytotoxic chemicals such as lead, ethylnitrosourea, or urethane or drugs such as cyclophosphamide can produce adverse effects on fertility and on progeny, with outcomes ranging from pre- and postimplantation loss (dominant lethality) to growth retardation and malformations (44,45). Exposure of the postmeiotic male germ cell to chemicals such as cyclophosphamide can also induce learning abnormalities in the progeny (46). The parameters that exposures may affect include the number of spermatozoa (producing oligospermia or azospermia), and their motility, morphology, or nuclear/chromatin structure (47). Other chemicals may adversely affect progeny outcome without any apparent effects on sperm numbers, motility, or external parameters of the male reproductive system itself.

The susceptibility of germ cells throughout spermatogenesis and epididymal sperm maturation to toxic chemicals is often chemical and/or stage specific; the susceptible stage can be assessed by the lag time between drug exposure and the effect on spermatozoa. Post-testicular damage to spermatozoa by chemical agents can occur during epididymal transit. For example, exposure of rats to cyclophosphamide for 4–7 days before conception, or after ligation of the efferent ducts, results in a marked increase in postimplantation loss (48). Ethylnitrosourea exposure of spermatozoa during epididymal transit also results in a high frequency of embryo death (dominant lethality) in the offspring of treated mice (49).

Germ cells that have completed their divisions and are differentiating (spermatids) seem to be particularly sensitive to agents that damage chromatin; this may be related to a loss of many of the enzymes associated with DNA repair during the later stages of spermatogenesis (50). For example, after exposure of mice to chlorambucil, a peak in mutation yield is observed when offspring are conceived from germ cells exposed as spermatids (exposure 10–18 days before conception) (49). Similarly, exposure to cyclophosphamide while germ cells are going through spermiogenesis results in dramatic increases in postimplantation loss (51). Germ cells at the spermatogonial stage are particularly susceptible to X-ray exposure in

both mice and man (longer exposures, 35 days or more before conception) (52).

In rats, the increase in postimplantation loss found after chronic low-dose exposure of the fathers to cyclophosphamide was reversed within 4 weeks of the termination of drug treatment (53); thus, cyclophosphamide-induced postimplantation loss was associated primarily with germ cell exposure during spermiogenesis. Presumably this reversibility occurred because the stem cells that were exposed either had the ability to repair the damage caused by alkylating drugs or died by apoptosis. In contrast, exposure to agents that result in more dramatic damage to stem cell nuclei, e.g., procarbazine, which causes sister chromatid exchange, had effects that were poorly reversible (54). The data that are available on the reversibility of the effects on spermatogenesis of exposure of men to anticancer drugs suggests that reversal in humans, at least with respect to effects on numbers of sperm in the ejaculate, may take years. The extent to which stem cells are affected is critical. It has been estimated that men are approximately 3 times more sensitive to the stem-cell-killing effects of radiation than mice (52).

The male genome is a focus for studies on the mechanism of the effects of paternal exposure to toxicants such as cyclophosphamide on progeny outcome, largely because such drugs interact with chromatin and some of their effects are transmitted to the F₂ generation (55). In rats it has been demonstrated that paternal exposure to drugs like cyclophosphamide damages male germ cells in such a manner that chromatin remodeling of the male genome is disrupted in the zygote, resulting in altered zygotic gene activation and embryo death (56). Another mechanism by which toxicants may affect germ cells leading to a disturbance in progeny development is disruption of the genomic imprinting of parental germ cells. An example of such a chemical exposure may be 5-azacytidine. The incorporation of 5-azacytidine into DNA prevents the 5' methylation of cytosine by DNA methyltransferase, inhibiting the usual hypermethylation of sperm DNA. The chronic exposure of male rats to 5-azacytidine results in oligospermia and azospermia as well as enhanced preimplantation loss among the offspring (57).

As an alternative to a direct effect on germ cells, preconceptional paternal exposures may adversely affect progeny outcome via the presence of chemicals in the seminal fluid. Many drugs are well absorbed after intravaginal administration. Thus, a drug in the seminal fluid may be absorbed and distributed throughout the female, and affect fertilization of the oocyte. There are drugs (methadone, morphine, thalidomide, and

cyclophosphamide) that have been reported to adversely affect progeny outcome by this type of mechanism on the basis of animal experiments (45,58–60). We speculate that the presence of a chemical in the seminal fluid may affect the completion of meiosis in the oocyte or male pronuclear formation in the fertilized zygote. One study demonstrated that the exposure of pig oocytes for 48 hr in culture to cyclophosphamide inhibited meiotic maturation (61). Unlike most mammals, humans may continue to have intercourse throughout pregnancy; hence, the conceptus may be exposed to a drug in the seminal fluid not only at the time of conception, but also at subsequent times during development. As a consequence, it is possible that animal experimentation underestimates the potential risk of drugs in semen to progeny outcome.

Relative to the male, few studies have been done to determine the effects of maternal drug exposure before conception on progeny outcome. A number of oocyte toxicants can induce aneuploidy with exposures around the time of oocyte maturation, ovulation, or fertilization (62). Exposure of oocytes to a chemical that is administered to the mother, or via the seminal fluid, may affect oocyte maturation and function. In a study in mice, the exposure of oocytes to X-rays 1–4 weeks before ovulation resulted in an increased incidence of embryo lethality, growth retardation, and malformations (gastroschises) among the offspring (63). It may be significant that these manifestations of developmental toxicity are similar to those that have been reported after exposure of the male gamete to a DNA-damaging agent (51,55). Disruption of the maternal or paternal parental genome may result in similar disturbances in zygotic gene activation, and thus in abnormal embryo development. Although immature (arrested) oocytes have been generally considered resistant to induced genetic damage, some studies with young female mice have shown that significant chromosome damage can be caused by γ -irradiation. The administration of high doses of cyclophosphamide affects mostly primordial follicles (64). Other potential targets for chemical insult in the mother include ovulation, transport of the ovum, and neuroendocrine regulation of ovulation.

Humans. There is relatively little information on the effects of preconception exposure to environmental toxicants on human reproduction. Not surprisingly, much of the information available is derived from patients receiving chemotherapy or radiation therapy for treatment of cancer.

Toxicants may affect fertility by decreasing the production of germ cells in the testis, resulting in azospermia or oligospermia. The sensitivity of the testis to cytotoxic therapies that decrease sperm numbers is proportional

to the relative proliferation of these cells (65). Sertoli and Leydig cells do not proliferate in the adult and therefore survive many cytotoxic therapies. Of the germ cells, spermatogonia are the most proliferative and are very susceptible to apoptosis induced by cytotoxic therapy. Spermatocytes and spermatids are relatively insensitive to cytotoxic agents (66). This is evidenced by the maintenance of relatively normal sperm counts for the first 2 months of cytotoxic therapy. After 2–3 months of therapy, the counts often decrease significantly, corresponding to the effects on spermatogonia, which in the 2- to 3-month interval will have become spermatozoa (67). If stem cells survive and differentiate, sperm production can recur. Often this takes 1 or 2 years after cytotoxic insult (52).

Genetic damage of the male germ cell is of concern because this damage can be transmitted to the next generation. That cytotoxic therapy can cause genetic damage in the germinal cells was demonstrated by fluorescence *in situ* hybridization analysis of men treated for Hodgkin's disease with novanthrone, oncovin, vinblastine, and prednisone chemotherapy (68). Aneuploidy of autosomal and sex chromosomes was shown in sperm of these patients, but the effects were transient and declined to pretreatment levels approximately 100 days after therapy. Therefore, risks for chromosomal damage were highest within one spermatogenic cycle (3 months) after the male was exposed to cytotoxic agents. Typically patients are counseled not to attempt a pregnancy until 6 months after therapy (66). A few studies have examined progeny outcome after treatment of the father with anticancer drugs or radiation therapy. In cases where exposed men have been able to father children, the limited data currently available have not shown an increased risk of birth defects or genetic disease after chemotherapy or radiation therapy (66,69). However, at present it is still difficult to assess the impact of the treatment of men with anticancer drugs on their offspring because the number of patients in most studies is low. Interestingly, no significant increase in genetic damage has been reported among 30,000 offspring of parents exposed to atomic bomb radiation in Japan (70).

The list of industrial chemicals thought to affect the reproductive capacity of human males includes carbon disulfide, dibromochloropropane (DBCP), and lead (71); DBCP is perhaps the most widely cited example of a man-made chemical that affects spermatogenesis. In men occupationally exposed to DBCP, spermatogenesis was either halted or severely compromised. Intervention was successful for some men, with resumption of normal spermatogenesis, whereas others with either higher exposure or

exposure over a longer time frame remained azoospermic (72). Dose-related disturbances in spermatogenesis resulting in abnormal sperm morphology and associated with infertility have been found among men with occupational lead exposures (73). Exposure to a chlorinated hydrocarbon insecticide, chlordane (kepone) has also been associated with oligospermia and altered sperm motility (74). Hence, human spermatogenesis is a sensitive target for man-made chemicals; if exposure is identified and eliminated, or reduced below a threshold effect level, then the effects on spermatogenesis may be reversible.

To date there is little evidence in the literature to suggest that preconceptional exposures of the mother will adversely affect the development or function of the reproductive system in her children.

Prenatal Exposures

Animals. During the first part of the prenatal period (from fertilization to gonadal ridge development), we know of no specific exposures associated selectively with deleterious effects on the reproductive system. In both sexes the establishment of the germ stem cell population in the gonads, as well as differentiation of the urogenital system, occur during the second part of prenatal development.

Cytotoxic drugs may affect mitosis in early germ cells in either the male or female fetus. The radiosensitivity of gonocytes in the fetal mouse testis increased from GD 14–18 (75). In the male mouse, the period of primordial germ cell migration (embryonic day 10) was the critical period for disturbance of postnatal testicular development and fertility after exposure to ethylnitrosourea (76). Exposure of the female rodent embryo or fetus to environmental agents such as ionizing radiation (77) and glycol ethers (78) depleted the pool of primordial follicles and thus significantly shortened the reproductive life span. Exposure of the female embryo to cyclophosphamide on GD 13 resulted in an increase in oogenesis on GD 17, with lesions of the synaptonemal complex, such as partial synapsis and desynaptic bivalents (79). Because oocytes cannot be replaced or repaired, the outcome of germ cell death in the female may be premature reproductive senescence.

One of the main mechanisms by which chemicals alter sexual differentiation is by dysregulating sex steroid signaling pathways. Clearly, chemicals with endocrine-disrupting activity have profound and predictable effects on the development of the reproductive system [reviewed by Barlow et al. (80)]. The most extensively studied compounds are those with estrogenic activity. In the female mouse reproductive tract there are estradiol receptors in the Müllerian duct from day 15 of fetal life (81). *In utero* exposure (GD 9–16 in the

mouse) to diethylstilbestrol (DES), the most widely studied synthetic estrogen agonist, resulted in malformations and adverse functional alterations in both the male and female reproductive tract and in the brain (82). DES seems to prevent total regression of the Wolffian duct in the female and results in persistence of Müllerian ducts in DES-exposed males. A high proportion of male mice exposed prenatally to DES were sterile and had epididymal cysts, undescended testes, and nodular lesions in the accessory sex glands (83). Although the mechanism by which DES disrupts development of the reproductive tract is still not defined, it is interesting that DES has been reported to down-regulate the expression of *Wnt7a* (84,85) and of *Hoxa-10* (86); fetal exposure to DES resulted in deregulation of *Wnt7a* and of *Hoxa-10* in the developing female reproductive tract. Thus, dysregulation of the expression of genes involved in signaling and pattern formation during development may underlie the teratogenic effects of DES on the developing reproductive tract. Other compounds with estrogenic activity for which developmental toxicity has been reported include other analogs of estradiol, pesticides, industrial chemicals such as methoxychlor, kepone, dioxins, bisphenol A, and phytoestrogens such as genistein and coumestrol [reviewed by Barlow et al. (80)]. In rats, cryptorchidism was induced with a suspected estrogenic contaminant, mono-*n*-butyl phthalate (87).

Androgen-receptor-mediated events are inhibited by pesticides such as vinclozolin, procymidone, or *p,p'*-DDE. These compounds block androgen-induced gene expression, delay puberty, reduce sex accessory gland size, and alter sexual differentiation in the male rat (88–90). Exposure of male rats (GD 12–21) to phthalates (which are estrogenic *in vitro* but not *in vivo*) causes malformations that appear to result from antagonism of androgens *in utero* (91). These malformations include hypospadias; cryptorchidism; agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; and interstitial cell hyperplasia of the testis. Flutamide, an androgen receptor antagonist, produced the same malformations in addition to inducing retained thoracic nipples, decreased anogenital distance, and a high incidence of inguinal testes. Treatment of pregnant sows with flutamide induced cryptorchidism (92). Inhibitors of 5 α -reductase, such as finasteride, also affect the masculinization of the urogenital sinus and external genitalia, events that are dependent on DHT (93). Prenatal administration of an aryl hydrocarbon receptor agonist [tetrachlorodibenzo-*p*-dioxin (TCDD)] produces a different spectrum of effects, including reduced ejaculated sperm

numbers in male rats (94). TCDD may also have direct effects on steroidogenesis.

Humans. Prenatal exposure to androgens, estrogens, and agents that mimic steroidal hormones can affect development of the reproductive system in both males and females. The classic example is the set of development effects due to exposure to DES *in utero*. Transplacental effects of DES were demonstrated initially in reports associating clear-cell adenocarcinoma of the vagina in adolescent women with prior exposure to DES *in utero* (95). Male offspring of mothers exposed to DES also have abnormalities of their reproductive tracts. This was demonstrated in a prospective double-blind study to evaluate the efficacy of DES for the protection of pregnancy; 840 pregnant women received DES and 806 pregnant women received placebo. Of the male progeny, 41 of 159 (25%) of those exposed to DES *in utero*, as compared to 11 of 161 (7%) of those exposed to placebo *in utero*, had epididymal cysts, hypotrophic testes, and induration of the capsule of the testis (96). Oligospermia (sperm concentration < 20 million sperm/mL) was found in 9 of 31 (29%) of men exposed to DES compared to 0 of 20 men exposed to placebo. To date there is no evidence for development of malignant lesions in male offspring exposed prenatally to DES. Although DES is no longer given to pregnant women, environmental toxicants that mimic gonadal steroids remain a concern today.

There is some debatable evidence that sperm counts have declined over a 50-year period between 1938 and 1991 (97); there may be a concomitant increase in the prevalence of cryptorchidism and hypospadias (97,98). Although the cause(s) behind failure of the testis to descend remain unknown, there is evidence from both epidemiological studies and animal experiments which suggests that man-made chemicals induce maldescent of the testis and may be inculpated as potential etiologic factors (99–102). An increase in the incidence of cryptorchidism has been reported for boys with higher levels of pesticide exposure as determined by place of residence (102). Lower circulating levels of testosterone have been demonstrated during gestation weeks 6–14 in boys with cryptorchidism (103).

Postnatal Exposures

Animals. There is a consistent decline in oocyte numbers from just before birth until the completion of the reproductive lifespan of the female. Postnatal exposures may affect this decline or may affect the development of secondary sex characteristics and the triggering of the ovulation process. Effects of toxicants on the imprinting of sexual behavior in the brain are probably mediated by

steroid receptors in specific nuclei of the brain. In addition, many chemicals that affect the tissues of the reproductive system are likely to do so by targeting the hypothalamic–pituitary complex. Decreased or altered function of these tissues results in reduced gonadotropin output and function of gonadal and reproductive tract tissues. An example of an agent that has multiple actions on male reproduction is estradiol. Although estradiol effectively reduces testicular weight, sperm counts, and the weights of the epididymis, prostate, and seminal vesicles, most of these effects are due to a decrease in serum LH and testosterone caused by the inhibitory action of estrogens at the hypothalamic–pituitary level in the male (104). However, estradiol receptors are also found in the testis, efferent ducts, epididymis, and prostate; mice bearing a null mutation in the estrogen α -receptor are infertile due, at least in part, to the inability of the efferent ducts to resorb fluid (105). Because steroid hormones and their receptors regulate the functions of many tissues and processes, it is clear that the consequences of exposure to endocrine disruptors are varied. One of the important targets for toxicant action in the reproductive system may be the timing of pubertal development. Extensive reviews are currently available in the literature on the effects of prepubertal exposure to endocrine-disrupting chemicals on sexual maturation in the rat (89,90).

In addition to targeting germ cells, toxicants can also target either Sertoli or Leydig cells. In young male rats, exposure to mono-(2-ethylhexyl)phthalate or 2,5-hexanedione causes the collapse of Sertoli cell cytoskeletal elements and a concurrent increase in germ cell apoptosis (106,107). Because Sertoli cells do not proliferate beyond postnatal day 16, the death of Sertoli cells has long-term consequences for spermatogenesis. Interestingly, Sertoli cell expression of Fas ligand, a key regulator of apoptosis, may be up-regulated by toxicants. Fas ligand in turn may induce the apoptosis of germ cells by interacting with its receptor, Fas. Fas gene expression is up-regulated by agents that cause germ cell injury, leading to the elimination of Fas-positive germ cells that can no longer be supported (106). Prolongation of the period in which Sertoli cells proliferate is also possible; neonatal treatment of rats with Aroclor 1254 (inducing hypothyroidism) increases testis weight and sperm production, presumably by increasing Sertoli cell numbers (108).

Leydig cells normally do not proliferate in the adult. However, unlike Sertoli cells, Leydig cells proliferate to reestablish their cell numbers if they are destroyed with ethane dimethane sulfonate, a chemical that selectively and effectively targets Leydig cells

(109,110). Chemicals such as reserpine or finasteride can induce Leydig cell hyperplasia after protracted exposures, especially in rodents [reviewed by Thomas (111)].

Epididymis-specific toxicants may affect spermatozoal maturation and storage. Exposure to one such chemical, chloroethylmethanesulfonate, results in a decrease in both testosterone levels and spermatozoal epididymal transit time (112). Another example of a drug acting on the epididymis is methyl chloride. The administration of methyl chloride can cause an increase in dominant lethal mutations (mutations in sperm that result in embryo lethality in the progeny) as a consequence of a selective inflammatory action of this agent on the epididymis (113).

In the first few days after birth, testicular androgen production is responsible for imprinting male sexual behavior and growth hormone secretion pattern. Subsequently, there is a rapid proliferation of Leydig cells between days 14 and 28 in the rat, followed by one more cell division between days 28 and 56. Androgen production increases slowly until day 28, and then rapidly, to plateau by day 56 (114). The presence of specific accessory sex organs is species dependent; however, they all depend on the presence of androgen. In response to the rapid increase in androgen production between days 28 and 56, there is rapid proliferation and differentiation of the various lobes of the prostate as well as the seminal vesicles. The differentiation of the epididymal epithelial cell types occurs between days 14 and 56 in response to androgens and to the arrival, from the rete testis, of germ cells and the contents of the fluid bathing them.

In female animals, experiments have revealed the effects of man-made chemicals on ovarian follicle counts without necessarily providing any evidence of an effect on circulating hormone levels, fecundity, or fertility. The effects of exogenous agents on ovarian follicle recruitment, growth, differentiation, and atresia are important to study.

Literally hundreds of scientific publications demonstrate that prenatal and neonatal exposures to several classes of exogenous estrogens or androgens affect sexually dimorphic CNS development in experimental animals (115,116). The estrogenic effects of synthetic compounds, in particular DDT and its analogs, have been extensively reported and persuasively shown to induce adverse effects if the exposures occur during a developmentally sensitive interval. Whereas part of the reproductive failure of sheep grazed on pastures exposed to estrogenic substances is due to alterations in genital tract development of the female offspring, alterations have also been reported in behavior and neuroendocrine end points [reviewed by Kaldas and Hughes

(117)]. Exposure of rats during development to common dietary phytoestrogens genistein and coumestrol at concentrations representative (scaled to body mass or caloric intake) of the levels consumed by women eating a traditional Asian diet, alters normal sexually dimorphic end points of hypothalamic anatomy and neuroendocrine functions; these effects are similar to those of DES or estradiol, albeit at a much lower potency.

Interference with normal brain-pituitary-gonadal function during the perinatal period (e.g., with a GnRH analog in monkeys) impacts adversely on subsequent reproductive, immunological, and behavioral functions. Studies in male monkeys suggest that the perinatal reproductive hormonal environment plays an important role in shaping later measures of peripubertal and adult reproductive status (118-125). Continuous GnRH agonist or GnRH antagonist treatment of neonatal male rhesus monkeys suppresses the pituitary-testicular axis and affects later peripubertal testicular function. Monkeys treated neonatally with a GnRH agonist exhibited subnormal sensitivity of the CNS to the excitatory amino acids aspartate and glutamate, suggesting that abolishing neonatal activation of the pituitary-testicular axis with a GnRH agonist permanently alters differentiation of central nervous system centers that are either involved in GnRH secretion or govern this process. Endocrine function and sexual behavior of the treated males were examined, with ovariectomized female monkeys receiving periodic estradiol treatment during the breeding and nonbreeding seasons. During the breeding season, there were no differences between treated and control males in the levels of LH and testosterone or in frequency of copulatory behavior. However, treated males masturbated less frequently than controls when the females were not given estradiol. During the nonbreeding season, treated males had lower testosterone levels than controls when the females were not receiving estradiol and copulated less when the females received estradiol, even though testosterone levels were comparable. These results suggest that the induction of reversible hypogonadotropic hypogonadism in neonatal male monkeys alters subsequent testicular development, peripubertal endocrine physiology, and sex-related behavior. Thus, perinatal activity of the pituitary-testicular axis appears to define a critical window of development for reproduction in male primates.

Synaptic reorganization of the brain continues throughout life and is sensitive to gonadal steroids. Treatment of sexually immature female rats with exogenous 17 α -estradiol or pregnant mare serum gonadotropin (PMSG) results in precocious puberty

and increased synaptic contacts per unit area in the arcuate nucleus (ARC/N) of the brain (126-129). PMSG presumably acts through the ovary to induce estrogen synthesis, which acts on the brain to increase the number of synapses per unit area of the ARC/N. Therefore, chemical mixtures that possess hormone-mimicking activity have the potential to alter the ontogeny of synapse formation in the ARC/N and affect the age of onset of puberty.

Humans. The testis is subject to damage from chemotherapeutic agents given during childhood. In fact, because adolescence marks an exponential increase in germ cell production and testicular size, this is likely to be a particular period of vulnerability to toxicants. Germ cells in the prepubertal testis are at least as sensitive to cytotoxic agents as in the adult (66). In one study of long-term survivors of childhood cancer, 6 of 13 patients treated prepubertally had a normal or near-normal reproductive state compared to 7 of 14 treated postpubertally (129). Interestingly, tests to detect abnormal testicular function, such as basal levels of gonadotropins and gonadotropin response to GnRH, are unlikely to detect testicular damage in prepubertal boys (130). This is illustrated by the case of a 6-year-old boy who received chemotherapy and had normal FSH levels prepubertally but then had abnormally high levels of FSH in early puberty (130). Leydig cell function is generally not affected by chemotherapy in the prepubertal male, as assessed by mean baseline and GnRH-stimulated LH levels and onset of puberty (131). In the same study, pubertal boys had an elevated LH response to GnRH stimulation that may have reflected a mild Leydig cell dysfunction in this older group. Direct irradiation to the prepubertal testis is more likely to cause Leydig cell damage than radiation treatment to the postpubertal testis (132).

A brief report of four pubertal patients (ranging from 10 to 18 years old) who received cyclophosphamide for treatment of nephritis suggests that this may be a window or sensitive period during which the testis is more susceptible to damage (133). Testicular biopsies from two of the patients showed significant effects on the seminiferous tubules, whereas biopsies from the other two patients were unremarkable. The two affected patients received more cyclophosphamide per body weight and were treated earlier in puberty than the two unaffected patients.

In contrast to the male, the number of germ cells in the female only decreases after birth. The decrease in the actual number of oocytes is very rapid during the period from birth to puberty. Postnatal exposures may affect this decline, potentially inducing premature reproductive senescence, or may affect the

development of secondary sex characteristics and the triggering of the ovulation process. Numerous animal experiments have demonstrated that the follicular reserve is affected by cigarette smoke and various industrial chemicals such as polycyclic aromatic hydrocarbons (134-136). However, few clinical studies have been done in human populations and these have tended to focus on populations with unique exposures. Only cigarette smoke has been clearly associated with an earlier onset of menopause. The effects of cigarette smoking also appear to be transgenerational.

Mammary tissue can respond to endogenous sex steroids produced by the neonatal gonad, but the importance of this target tissue in terms of consequences on later function or disease risk is unknown.

The susceptibility of elderly males and females to chemical insult deserves more attention but is not the focus of this paper.

Similarities and Differences between Humans and Laboratory Animals

As expected, there are many similarities regarding the major hallmarks of reproduction among most mammals examined. These include similarities in tissue architecture, both during development and in the adult, in homeostatic mechanisms, and in specific gene expression in various tissues of the reproductive system. In both humans and the animal models usually studied, gametes are produced in far greater excess than would seem to be necessary for the survival of species. Ten to hundreds of millions of spermatozoa are generated daily by most adult male mammals, yet very few of these germ cells succeed in transmitting their genetic material to the next generation. Although the number of oocytes produced in mammalian females is more limited, and this production occurs only during fetal life, most ovaries contain several orders of magnitude more oocytes than ever will be fertilized. Two of the most striking differences between laboratory animals and humans are the relatively poor quality of human spermatozoa and the high level of fetal loss exhibited by humans. Embryo lethality occurs at a very low incidence in outbred strains of rats and mice, yet fetal wastage has been estimated to vary from 43 to 78% of fertilized human ova; it is thought that approximately 20-30% of implanted fertilized eggs will be lost, whereas clinically recognized pregnancy loss is about 10-12%. Malformations are also relatively rare in the animal models studied most often (e.g., Sprague-Dawley rats), but the rate for major malformations in humans is typically 3-4%, and may be up to 8-12% when major and minor malformations are combined.

Another difference between humans and animals is the relative time allocation for the various phases of differentiation during embryonic and fetal life (Figures 1 and 2). The animal species frequently chosen as models have a short period to sexual maturity, a short gestational period, and large litter size. For example, organogenesis in mice and rats occurs within a week (approximately GD 8–15) compared to embryonic weeks 4–8 in humans. Gamete migration in mice and rats takes only a day or two, as opposed to weeks in humans. The spermatogenic cycles in experimental animals differ from humans in length but are nevertheless very similar physiologically. Similarly, differentiation of the gonads in rodents appears from GD 12 to 15, in contrast to weeks 8–12 in humans. Our current knowledge and understanding of comparative embryology and physiology enables us to use animal models to compare comparables. That is, we can study the effects of a toxicant or exposure on specific events within the critical window of development/exposure for that event in various model species.

That the timeline is condensed in many of our experimental animal models is a tremendous advantage; studies can take place over a much shorter time frame, reducing the time and investment required to find an answer. Experimental animals may be more sensitive to the developmental effects of exogenous substances because of the rapid rate at which cell proliferation, migration, and differentiation occur. In fact, there are few specific examples of toxicants to which experimental animals are relatively insensitive compared to humans; often the basis for this relative insensitivity is that there are differences in toxicokinetics or metabolism between the species.

The rat is the most commonly used animal model for basic and applied research in reproductive and developmental toxicology. One of the reasons is that there is a tremendous database in industry, regulatory agencies, and academia on the effects of toxicants in this species and on the normal variants in development. Yet many of the breakthroughs in terms of the molecules involved in regulating embryonic pattern formation and gene function are made today using transgenic null mutation mice. The development of an equivalent for rat to the embryonic stem cell/homologous recombination technology we now use in mice would be a boon to all toxicologists. It is likely that the rat genome will be sequenced within a few years of that for the mouse and human. The advances under way in genomics and proteomics will impact in the very near future on the realm of what is possible in reproductive toxicology. Using these approaches we should

soon have the ability to define the molecule or pathway targeted by a toxicant and to use this information to cross species barriers.

Objectives to Fill Gaps in the Knowledge Base

- Link and improve human exposure and outcome data
- Improve understanding of the basic mechanisms and the molecules regulating cellular events in the reproductive system
- Elucidate the specific cellular and molecular targets of known toxicants
- Develop surrogate markers of exposure, effect, and susceptibility that are minimally invasive
- Design *in vitro* tests to assess reproductive system function during embryonic, postnatal, and adult life
- Design a systematic approach to the identification of reproductive toxicants
- Develop sensitive, specific, and predictive animal models for key human reproductive and developmental processes of concern
- Validate existing test methods used in reproductive biology studies for potential application in regulatory reproductive toxicity testing.

Summary and Conclusions

There is clear evidence that there are preconceptual, prenatal, and postnatal exposures to environmental and therapeutic chemicals that can affect specific components of the male and female reproductive systems. The range of these effects encompasses infertility, which can result from arrested gametogenesis, to early embryo loss or malformations of the gonads and specific tissues of the reproductive system. Precise information is lacking regarding the risks to humans of low-level exposures to many environmental contaminants. Animal models are useful for hazard identification of known high dosages of chemicals, but the apical outcomes measured (genital tract development, fertility, and birth defects) are not designed to detect subtle changes in gene or cellular function that could lead to adverse health effects in humans after long-term or low-dose exposures. Even for those chemicals that have been identified as reproductive system toxicants, we frequently do not know the cellular or molecular mechanisms of action. Elucidation of the target molecules for reproductive system toxicants will permit the establishment of the biomarkers of effect that are needed for hazard characterization and risk assessment. Elucidation of the windows in time when components of the reproductive system are most susceptible to insult will be valuable in understanding the underlying pathology and in developing strategies to prevent exposures leading to adverse effects on reproduction.

Although it is the dose that makes the poison, there is no doubt that timing of the exposure may be as important as dose in determining the potential toxicity of a compound to the reproductive system. Furthermore, although early embryogenesis is clearly a critical window during which exposure to exogenous chemicals, both naturally occurring and man-made, may have profound and long-lasting adverse effects on reproductive structure and function, preconceptual and postnatal exposures may also adversely affect the reproductive system and progeny outcome.

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