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Endocrine disruptors and estrogenic effects on male reproductive axis

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Abstract

Endocrine disruptors (e.g., polychlorinated biphenyls [PCBs], dichlorodiphenyl-trichloroethane [DDT], dioxin, and some pesticides) are estrogen-like and anti-androgenic chemicals in the environment. They mimic natural hormones, inhibit the action of hormones, or alter the normal regulatory function of the endocrine system and have potential hazardous effects on male reproductive axis causing infertility. Although testicular and prostate cancers, abnormal sexual development, undescended testis, chronic inflammation, Sertoli-cell-only pattern, hypospadias, altered pituitary and thyroid gland functions are also observed, the available data are insufficient to deduce worldwide conclusions. The development of intra-cytoplasmic sperm injection (ICSI) is beyond doubt the most important recent breakthrough in the treatment of male infertility, but it does not necessarily treat the cause and may inadvertently pass on adverse genetic consequences. Many well-controlled clinical studies and basic scientific discoveries in the physiology, biochemistry, and molecular and cellular biology of the male reproductive system have helped in the identification of greater numbers of men with male factor problems. Newer tools for the detection of Y-chromosome deletions have further strengthened the hypothesis that the decline in male reproductive health and fertility may be related to the presence of certain toxic chemicals in the environment. Thus the etiology, diagnosis, and treatment of male factor infertility remain a real challenge. Clinicians should always attempt to identify the etiology of a possible testicular toxicity, assess the degree of risk to the patient being evaluated for infertility, and initiate a plan to control and prevent exposure to others once an association between occupation/toxicant and infertility has been established. *(Asian J Androl 2008 Jan; 10: 134–145)*

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1 Introduction

Endocrine disruptors are estrogen-like and/or anti-androgenic chemicals in the environment that have potentially hazardous effects on male reproductive axis resulting in infertility and on other hormonal dependent reproductive functions causing erectile dysfunction (ED).

These chemicals are called "*endocrine disruptors*" because they (i) mimic natural hormones, (ii) inhibit the action of hormones, and/or (iii) alter the normal regulatory function of the endocrine systems. Besides reduced fertility and ED, testicular and prostate cancers, abnormal sexual development, alteration in pituitary and thyroid gland functions, immune suppression, and neurobehavioral effects are also possible due to such endocrine disruption in the male.

Data collected over the last 30 years have shown disturbing trends in male reproductive health. An earlier report from Scotland revealed that men born after 1970 had a sperm count 25% lower than those born before 1959–an average decline of 2.1% a year [1]. The lower

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sperm count was also associated with poor semen quality [2]. In contrast, Olsen *et al.* [3], used several statistical models and found an actual increase in average sperm numbers. Thus, while some environmentalists believe that the human species is approaching a fertility crisis, others think that the available data are insufficient to deduce worldwide conclusions [4, 5]. Newer tools for the detection of Y-chromosome deletions have strengthened the hypothesis that the decline in male reproductive health and fertility may be related to the presence of certain toxic chemical compounds in the environment [6]. These chemicals mimic or otherwise disrupt the estrogens or the androgen balance in the body by binding to hormone receptors during fetal and neonatal development and give rise to reproductive abnormalities, including low sperm counts in adulthood. Because of these effects, such endocrine disruptors are also popularly known as "*gender benders*". With discoveries of deformed frogs in Minnesota lakes and fertility problems in alligators found in Lake Apopka in Florida [7] attributed to embryonic exposure to pollutants, a myriad of environmental agents have been classified as male reproductive toxicants. This has been the subject of a number of reviews [8–12], suggesting that etiology, diagnosis, and treatment of male factor infertility remains a real challenge. However, the evidence that such environmental chemicals cause infertility is still largely circumstantial. There are many missing links in the causal chain that would connect receptor binding to changes in reproductive health with decreased fertility. The fact remains that one in six couples have trouble conceiving, with males equally responsible for their infertility. The most important recent breakthrough in the treatment of male infertility was the development of intra-cytoplasmic sperm injection (ICSI). This was made possible by many well-controlled clinical studies and basic scientific discoveries in the physiology, biochemistry, and molecular and cellular biology of the male reproductive system.

2   Background

Many environmental xenobiotic chemicals, such as polychlorinated biphenyls (PCBs), dichlorodiphenyl-trichloroethane (DDT), dioxin, and some pesticides have estrogenic effects [13–15]. Dibromochloropropane (DBCP) exposure impaired fertility in the absence of any other clinical signs of toxicity, suggesting that the male reproductive system was the most sensitive target organ. The potential hazards these chemicals may have on human health and ecological well-being include reproductive tract cancers, reduced fertility, embryo/fetal loss, birth defects, childhood cancer, other postnatal structural or functional problems, and abnormality in sexual development [14, 16–17].

However, the database for establishing safe exposure levels or risk assessment for such outcomes remains very limited. Declining semen quality is not the only indicator that suggests that human reproduction is at risk. A marked increase in the incidence of testicular cancer in young men has been associated with other abnormalities (including undescended testis, Sertoli-cell-only pattern, and hypospadias) which cause poor gonadal function and low fecundity rates.

The human male produces relatively fewer sperm on a daily basis compared with many of the animal species used for toxicity testing [18]. In fact, in many men over age 30, the lower daily sperm production rate already places them close to the subfertile or infertile range [18, 19]. A less dramatic decrease in sperm numbers, motility, and/or morphology in humans can have serious consequences for reproductive potential, even though it takes only one sperm to fertilize an egg. Problems in the production, maturation, and fertilizing ability of sperm are the single most common cause of male infertility. Although any discussion of gonadal function and toxicity is of special relevance to man, much of this understanding has been obtained from various experimental models and research using animal species. In addition, both intra-testicular and post-testicular events have been postulated and different mechanisms have been proposed to explain the presence of damaged DNA in human spermatozoa. Three among them, i.e. abnormal chromatin packaging, oxidative stress and apoptosis, are the most studied [20]. Higher levels of DNA damage means that sperm are less likely to undergo apoptosis which is a natural self-destruct process designed to rid the body of damaged cells. However, it is not clear whether increased damage arises because of chronological age or because of longer-term exposure to environmental factors that may cause such damage.

3   Male reproductive tract target sites

An endocrine disruptor can effect several potential target sites in the male reproductive tract. The most important being the testes, the male gonads, which usually exist in pairs and are the sites of spermatogenesis and androgen production. There are paracrine and autocrine regulations in various compartments of the testis that are under endocrine influences from the pituitary and hypothalamus. About 80% of the testicular mass consists of highly coiled seminiferous tubules within which spermatogenesis takes place. The remaining 20% consists of Leydig cells and Sertoli cells, whose main job is to establish normal spermatogenesis. Spermatozoa are the haploid germ cells responsible for fertilization and species propagation.

3.1 Sertoli cells

Within the testes are Sertoli cells, or "nurse cells," that form a continuous and complete lining within the tubular walls which envelope the developing sperm during spermatogenesis. These cells establish the blood-testis barrier by virtue of tight junctions. The luminal environment as controlled by these Sertoli cells is under the influence of follicle stimulating hormone (FSH) and inhibin. These Sertoli cells:

(1) provide nourishment for the developing sperm cells;

(2) destroy defective sperm cells;

(3) secrete fluid that helps in the transport of sperm into the epididymis;

(4) release the hormone inhibin that helps regulate sperm production.

The differentiation of Sertoli cells and the formation of a competent blood-testis barrier are essential to the establishment of normal spermatogenesis during puberty. Thus, many irregularities of spermatogenesis due to interference by endocrine disruptors may reflect changes in the function of the Sertoli cell population and not necessarily by pathology in the germ cells themselves.

3.2 Leydig cells

Leydig cells are the endocrine cells in the testis that produce testosterone from cholesterol via a series of enzymatic pathways and steroidal intermediates under the control of luteinizing hormone (LH) from the pituitary. These cells arise from interstitial mesenchymal tissue between the tubules during the eighth week of human embryonic development. They are located in the connective tissue between the seminiferous tubules.

3.3 Spermatogenesis

Spermatogenesis is a chronological process spanning about 80 days in man and 40–50 days in the rodent (depending upon species). During this period, the immature germ cells (relatively undifferentiated spermatogonia), develop into highly specialized spermatozoa in a cyclic manner. Spermatogonia undergo several mitotic divisions to generate a large population of primary spermatocytes, which produce haploid spermatids by two meiotic cell divisions. Spermiogenesis is the transformation of spermatids into elongated flagellar germ cells capable of motility. The release of mature germ cells is known as spermiation. Most of the testicular volume, which diminishes if testicular damage has occurred, consists of these germ cells,. During mitotic arrest, the gonocyte becomes acutely sensitive to toxic agents. Low-dose irradiation, e.g., may completely eradicate germ cells while causing little damage to developing Sertoli cells, thus creating a Sertoli-cell-only testes [21].

4 Role of endocrine disruption in male reproduction

Many estrogenic pollutants (endocrine disruptors), including agricultural products (phytoestrogens), industrial chemicals and heavy metals have significant reproductive consequences due to their multiple routes of exposure, their widespread presence in the environment, and their ability to bioaccumulate and resist biodegradation. In addition, many pharmacological and biological agents including radiation therapy affect male reproduction by disrupting hormonal balance (Table 1) as described below.

4.1 Environmental agents
4.1.1 Agricultural and industrial chemicals

A detrimental effect of agricultural and industrial chemicals on sperm concentration, motility, and morphology may be caused by impaired spermatogenesis secondary to various hormonal alterations [22]. Abnormal sperm morphology due to secretory dysfunction of the Leydig and Sertoli cells may impair the sperm-fertilizing capacity. Agricultural chemicals implicated in male reproductive toxicity include DDT (o,p-DDT), epichlorhydrin, ethylene dibromide, kepone, and dioxin [23]. DBCP, a nematocide widely used in agriculture, is a testicular toxicant that induces hypergonadotropic hypogonadism [24]. DDT, a commonly used pesticide, and its metabolites (p,p'-DDT, and p,p'-DDE) have estrogenic effects in males by blocking the androgen receptors [22, 23, 25]. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous undefined complex mixtures encountered in the environment because of industrial combustion and excessive use of tobacco products [26]. Methyl chloride, an industrial chemical, used in the production of organosilicates and gasoline antiknock additives has been extensively studied. Such organic solvents have been reported to induce changes in semen quality, testicular size, and serum gonadotropins [27]. Exposure to persistent organochlorine pollutants has been associated with human perturbations of the sperm X:Y chromosome ratio [6].

The levels of serum free/bound toxicant will influence the androgen-blocking capacity. The plasma/tissue concentration of an estrogenic toxicant depends upon the detoxification and elimination mechanisms in the organism. The fate and detoxification of these organochemicals have not been well defined, but these agents can disrupt the hypothalamic-pituitary-testicular axis affecting the endocrine and reproductive functions. Since environmental exposure is due to a mixture of various endocrine disruptors, the effect of their combined toxicity becomes more important. However, the long-term effects of such exposure, especially at low dose, on male reproductive axis and fertility have not been examined in detail in a well-designed study.

Table 1. Endocrine disruptors that affect male reproduction. CNS, central nervous system; DBCP, dibromochloropropane; DDT, dichlorodiphenyl-trichloroethane; HPG, hypothalamic-pituitary-gonadal; KTZ, ketoconazole; ROS, reactive oxygen species; LPO, lipid peroxidation; T, testosterone.

Class	Agent	Adverse effects
Environmental		
Organochemicals and pesticides	DBCP	[↓ fertility, ↓ libido,
	DDT	embryo fetal loss, birth defects,
	PCBs	cancer; estrogenic effects,
	Dioxins	poor semen quality]
	Methyl chloride	
Heavy metals	Lead	[↓ HPG-axis, ↓ spermatogenesis,
	Mercury	CNS effects, testicular damage]
	Cadmium	
	Cobalt	
	Chromium	
Ionizing radiations	α- and β-rays	[direct/indirect effect on gonads]
Biological		
	Hyperthermia	[↑ ROS, ↓ T biosynthesis, ↓ spermatogenesis,
		testicular damage, poor sperm morphology]
	Superoxide, and	[↑ ROS, ↓ antioxidants,
	Nitric oxide radicals	↓ sperm function]
	Oxidative Stress	[↑ ROS, ↑ LPO, ↑ cytokines, ↓ T,
		↓ sperm function]
Pharmacological		
Radiation therapy	X-rays, γ-rays	[germ cell and Leydig cell damage]
Drugs/Phytoestrogens	GnRH-analogs	[↓ HPG-axis, ↓ sperm,
	KTZ, Leuprolide	↓ libido, ↓ steroidogenesis]
	Cyclosporine	
	Lithium, Narcotics	
	Anabolic steroids	
	Ethanol, Nicotine	
	Flutamide, Gossypol	
	Marijuana	

4.1.2 Heavy metals

Heavy metals (e.g., arsenic, lead, boron, mercury, cadmium, antimony, aluminum, cobalt, chromium, lithium) have been found to exert adverse effects on the reproductive axis of human and experimental animals. More reports are available on lead-induced toxicity than any other heavy metal. Historically, the fall of the Roman Empire has been attributed to lead poisoning [28]. Men working in battery plants and exposed to toxic levels of lead demonstrated adverse effects on their reproductive capacity [29, 30]. In animals, lead exposure results in a dose-dependent suppression of serum testosterone and spermatogenesis [31, 32]. Although testicular biopsies reveal peritubular fibrosis, vacuolation, and oligospermia, suggesting that lead is a direct testicular toxicant [33], some mechanistic studies show that lead exposure can disrupt the hormonal feed-back mechanism at the hypothalamic-

pituitary level [9]. Animal studies suggest that these effects can be reversed when lead is removed from the system. Such detailed evaluations in humans need further investigation.

Boron is extensively used in the manufacture of glass, cements, soaps, carpets, crockery, and leather products. Oligospermia and decreased libido were reported in men working in boric acid-producing factories [34]. Boron has a major adverse reproductive effect on the testes and the hypothalamic-pituitary axis in a manner similar to lead toxicity. Cadmium, another heavy metal, is a testicular toxicant that is used widely in industries like electroplating, battery electrode production, galvanizing, plastics, alloys, paint pigments [35]. It is also present in soil, coal, water, and cigarette smoke. In animal studies, cadmium has been shown to cause severe testicular necrosis in mice that is also strain-dependent [36]. Cad-

mium-DNA binding and inhibition of sulfhydryl-containing proteins mediate cadmium toxicity directly or through transcription mechanisms. It can also induce the expression of heat shock proteins, oxidative stress response genes, and heme oxygenase induction mechanisms [37]. Clinical studies have associated cadmium exposure with testicular toxicity, altered libido, and infertility. Further studies are needed to delineate the specific gonadotoxic mechanisms involved in cadmium induced reproductive toxicity. Mercury exposure can happen during the manufacture of thermometers, thermostats, mercury vapor lamps, paint, electrical appliances, and in mining. Such exposure can alter spermatogenesis and has been found to decrease fertility in experimental animals.

4.2 Biological factors

Chronic disease states, aging, toxin exposure, physical injury, and exposure to many types of environmental contaminants enhance specific biological activity leading to hyperthermia and increased free radical generation leading to oxidative stress that can cause gonadal and gamete damage [38]. In addition, the generation of nitric oxide (NO) radicals and reactive nitrogen species (RNS) has recently been found to have an astounding range of biological influences–including vascular tone, inflammation with increased cytokines, and as a mediator of many cytotoxic and pathological effects [39]. NO generation in response to toxic exposure associated with hormonal imbalance can contribute to poor sperm motility and function leading to infertility [40].

4.3 Pharmacological agents

Radiation therapy and many pharmacologic drugs and chemotherapeutic agents are known to adversely affect male reproduction (Table 1).

4.3.1 Radiation

Radiation exposure (X-rays, neutrons, and radioactive materials) induces testicular damage that is generally more severe and difficult to recover than that induced by chemotherapy. Radiation effects on the testes depends upon the dose, number of doses and the duration of the delivered irradiation, as well as the developmental stage of the germ cell in the testes at the time of exposure [41]. Radiotherapy that is used as an alternative therapy for the treatment of seminomatous germ cell tumors and lymphomas can be gonadotoxic. In general, germ cells are the most radiosensitive. A direct dose of irradiation to the testes greater than 0.35 Gy causes aspermia. The time taken for recovery of the germinal epithelium increases with larger doses, and doses in excess of 2 Gy will likely lead to permanent azoospermia. At higher radiation doses (> 15 Gy), Leydig cells will also be affected [42]. Vulnerability of the testis to irradiation de-

pends upon the age and the pubertal status of the male. In addition to direct damage to the testes, whole body irradiation can also damage the hypothalamic-pituitary axis and affect reproductive capability [43].

4.3.2 Drugs and phytoestrogens

Many synthetic pharmacological agents, phytoestrogens and anabolic steroids affect normal endocrine functions. The use/abuse of these anabolic steroids mainly among athletes has grown to epidemic proportions. This has resulted in severe oligozoospermia and decreased libido. The hypogonadotropic hypogonadism due to feedback inhibition of the hypothalamus-pituitary axis is the most common cause of severe impairment of normal sperm production in this population [44]. These defects can be reversed within four months of discontinuation; however, sporadic azoospermia has been reported in some young men even one year after cessation of chronic anabolic steroid use [45].

4.3.3 Chemotherapeutic agents

As early as 1954, antibacterial agents were reported to be toxic to spermatozoa [46]. Antibiotics and cancer chemotherapy usually damages the germinal epithelium [47, 48]. Mechlorethamine, extensively used as nitrogen mustard during the second world war, causes spermatogenic arrest [49]. Many common cytotoxic agents cause a dose-dependent progressive decrease in sperm count, leading to azoospermia [50]. Postmeiotic germ cells are specifically sensitive to cyclophosphamide treatment, with abnormalities observed in progeny [51]. Chronic low-dose cyclophosphamide treatment in men may affect the decondensation potential of spermatozoa due to the alkylation of nuclear proteins or DNA. This is likely to affect pre- and post-implantation loss or contribute to congenital abnormalities in offspring [52]. Combination therapy with alkylating agents has been shown to improve survival in the treatment of Hodgkin's disease, lymphoma, and leukemia. However, such combination therapy has induced sterility in most adults, as revealed by complete germinal aplasia in testicular biopsy specimens [53]. Many antimicrobials (e.g., tetracycline derivatives, sulfa drugs, nitrofurantoin, and macrolide agents, like erythromycin) impair spermatogenesis and sperm function [46, 47].

In general, the severity of testicular damage is related to the category of chemotherapeutic agent used, the dose and duration of therapy, and the developmental stage of the testis. The recovery of spermatogenesis is variable and depends upon the total therapeutic dose and duration of treatment [54]. The effects of cytotoxic drugs on the testicular function of children are inconclusive, due to the relative insensitivity in detecting such damage with available technology; however, the prepubertal and

adolescent testes show damage to a lesser extent by chemo- and radiation therapy than the postpubertal or older testis [41]. This may be due to rapid turn-over and recovery of damaged cells by active spermatogenesis in younger gonads. The use of testicular biopsy, semen analysis, and assessment of the hypothalamic-pituitary-gonadal (HPG) axis can commonly achieve the evaluation of testicular toxicity.

5 Mechanism(s) of action of endocrine disruptions on HPG axis

Complex interactions are involved in normal gonadal function and hormonal communication. There are multiple loci that could be involved mechanistically in a toxicant's endocrine-related effects. Impairment of such hormonal control could occur as a consequence of altered hormone biosynthesis, storage/release and transport/clearance, receptor recognition/binding, and/or postreceptor responses.

5.1 Altered hormone bio-synthesis

A number of agents possess the ability to inhibit the biosynthesis of various hormones. Some of these agents inhibit specific enzymatic steps in the biosynthetic pathway of steroidogenesis (e.g., aminoglutethimide, cyanoketone, ketoconazole). Some fungicides block estrogen biosynthesis by inhibiting aromatase activity that converts testosterone to estrogen in the testis. Environmental estrogens and antiandrogens further alter protein biosynthesis induced by gonadal steroids through a series of signals at transcriptional and translational levels [55]. Both estrogen and testosterone have been shown to affect pituitary hormone synthesis directly or through changes in the glycosylation of LH and FSH [56]. A decrease in glycosylation of these glycoproteins reduces the biological activity of the hormones. Any environmental compound that mimics or antagonizes the action of these steroid hormones could presumably alter glycosylation.

5.2 Altered hormone storage and/or release

Steroid hormones do not appear to be stored intracellularly within membranous secretory granules. For example, testosterone is synthesized by the Leydig cells of the testis and released on activation of the LH receptor. Thus, compounds that block the LH receptor or the activation of the 3',5'-cyclic AMP (cAMP) dependent cascade involved in testosterone biosynthesis can rapidly alter the secretion of this hormone. The release of many protein hormones is dependent on the activation of second messenger pathways, such as cAMP, phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-trisphosphate (IP_3), tyrosine kinase, including Ca^{2+} channels. Interference with these processes consequently will alter the

serum levels (bioavailability) of many hormones. Several metal cations have been shown to disrupt pituitary hormone release presumably by interfering with Ca^{2+} flux [57].

5.3 Altered hormone transport and clearance

Hormones are transported from blood in the free or bound state. Steroid hormones are transported in the blood by specialized transport (carrier) proteins known as steroid hormone-binding globulin (SHBG) or testosterone-estrogen-binding globulin (TEBG). Regulation of the concentration of these binding globulins in the blood is of clinical significance because either increases or decreases in their level could affect steroid hormone bioavailability. For example, DDT analogs that are potent inducers of hepatic microsomal monooxygenase activities *in vivo* [58], could cause a decrease in transport of testicular androgen as a result of enhanced degradation. Similarly, treatment with lindane (gamma-hexachlorocyclohexane) has been reported to increase the clearance of estrogen [59].

5.4 Altered hormone receptor recognition/binding

Hormones elicit responses from their respective target tissues through direct interactions with either intracellular receptors or membrane-bound receptors. Specific binding of the natural ligand to its receptor is a critical step in hormone function. Intracellular (nuclear) receptors, such as those for sex steroids, adrenal steroids, thyroid hormones, vitamin D, and retinoic acid, regulate gene transcription in a ligand-dependent manner through their interaction with specific DNA sequences termed *response elements*. A number of environmental agents may alter this process by mimicking the natural ligand and acting as an agonist or by inhibiting binding and acting as an antagonist. The best known examples are methoxychlor, chlordecone (Kepone), DDT, some PCBs, and alkylphenols (e.g., nonylphenols and octylphenols), which can disrupt estrogen receptor function [60, 61]. The antiandrogenic action of the dicarboximide fungicide vinclozolin is the result of an affinity of this compound's metabolites for the androgen receptor [16]. Interestingly, the DDT metabolite p, p'-DDE has been found to bind also to the androgen receptor and block testosterone-induced cellular responses *in vitro* [25].

Many of the chemicals classified as environmental estrogens can actually inhibit binding to more than one type of intracellular receptor. For example, o,p-DDT and chlordecone can inhibit endogenous ligand binding to the estrogen and progesterone receptors, with each compound having IC50s that are nearly identical for the two receptors. Receptors for protein hormones are located on and in the cell membrane. When these hormones bind to their receptors, transduction of a signal across the membrane is mediated by the activation of

second messenger systems. These may include (a) alterations in G-protein/cAMP-dependent protein kinase A (e.g., after LH stimulation of the Leydig cell), (b) phosphatidylinositol regulation of protein kinase C, and inositol triphosphate (e.g., after GnRH stimulation of gonadotrophs; thyrotropin releasing hormone stimulation of thyrotrophs), (c) tyrosine kinase (e.g., after insulin binding to the membrane receptor), and (d) calcium ion flux. Xenobiotics thus can disrupt signal transduction of peptide hormones if they interfere with one or more of these processes.

5.5 Altered hormone post-receptor activation
Once the endogenous ligand or an agonist binds to its receptor, a cascade of events is initiated indicative of the appropriate cellular response. This includes the response necessary for signal transduction across the membrane, or in the case of nuclear receptors, the initiation of transcription and protein synthesis. A variety of environmental compounds can interfere with the membrane's second messenger systems. For example, cellular responses that are dependent on the flux of calcium ions through the membrane (and the initiation of the calcium/Calmodulin dependent cellular response) are altered by a variety of environmental toxicants. Interestingly, the well-known antiestrogen tamoxifen inhibits protein kinase C activity while the phorbol esters are known to mimic diacylglycerol and enhance protein kinase C activity [62].

Steroid hormone receptor activation can be modified by indirect mechanisms, such as a down-regulation of the receptor (temporary decreased sensitivity to ligand) as seen after 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) exposure (including the estrogen, progesterone, and glucocorticoid receptors) [63]. Consequently, because of the diverse known pathways of endocrine disruption, any assessment must consider the net result of all influences on hormone receptor function and feedback regulation.

5.6 Induction of oxidative stress
"Oxidative stress" is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived free radicals commonly known as reactive oxygen species (ROS). In addition, the generation of NO radicals and RNS has recently been found to mediate many physiologic, cytotoxic and pathological effects [39]. NO generation in response to toxic exposure may be associated with hormonal imbalance that can contribute to poor sperm motility and function leading to infertility [40]. Also, NO and superoxide radicals can combine to form highly reactive peroxynitrite radicals, which induce endothelial cell injury [64]. This may result in altered blood flow to the testis and may impair

testicular function.
The assumption that free radicals can influence male fertility has received substantial scientific support [65]. The proposed mechanism for loss of testicular and sperm function due to oxidative stress has been shown to involve excessive generation of ROS [66]. Free radicals can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding resulting in DNA strand breaks and cross-linking [67]. ROS can also induce oxidation of critical-sulfa-hyroxyl (SH) groups in proteins and DNA, which will alter cellular integrity and function with an increased susceptibility to attack by toxicants. Oxidative stress is theoretically the result of an improper balance between ROS generation and intrinsic scavenging activities. Adequate levels of superoxide dismutase (SOD), catalase, and probably glutathione (GSH) peroxidase and reductase normally maintain the free radical scavenging potential in the testes. This balance can be referred to as oxidative stress status (OSS), and its assessment may play a critical role in monitoring testicular toxicity and infertility [10].

6 Evaluation of male reproductive toxicity

Several methods are being evaluated for the assessment of the effects of toxicants on the male reproductive system (Table 2). Essentially, any risk assessment usually has four components: (1) hazard identification, (2) dose-response assessment, (3) human-exposure assessment, and (4) risk characterization. The hazard identification and dose-response data are developed from experimental animal studies that may be supplemented with data from *in vitro* studies. This information is then extrapolated and integrated to characterize and assess the risk to the human population. Table 2 lists how such effects of endocrine disruptors and other toxicants on specific components of HPG axis can be evaluated using both *in vivo* and *in vitro* tools.

6.1 In vivo systems
In vivo methods are important tools to study the integrated male reproductive system. The complete *in vivo* assessment of testicular toxicity involves multigenerational studies, now required by most regulatory agencies. These multigenerational studies have a complex design, because testicular function and spermatogenesis are very complicated processes. The spermatogenic cycle is highly organized throughout the testis. In the rat, it requires about 50 days. If a toxicant affects the immature spermatogonia, the effect may not be detectable as a change in mature sperm before 7 to 8 weeks. Effects on more mature germ cells would be detected sooner. To test the sensitivity of all stages of spermatogenesis, the

Table 2. Evaluation of effect of hormonal disruptors on the male reproductive axis. LH, luteinizing hormone; PRL, prolactin; FSH, follicle stimulating hormone; ABP, androgen binding protein; HPG, hypothalamic-pituitary-gonadal; RIA, radio immuno assay.

Potential sites	Effects	Evaluative tests
Testis	Necrosis	Weight, Histopathology
Leydig cells	LH/PRL	Receptor analysis, RIA
	T biosynthesis/secretion	*In vitro* production and hormone assay
Sertoli cells	FSH/Inhibin/Steroids	Receptor analysis, RIA
	Sertoli/Leydig cell function	*In vitro* tests (co-culture)
	Blood-testis barrier	Morphology
Seminiferous tubules	Spermatogonial mitosis	Germ cell count and % tubules without germ cells
	Spermatocyte meiosis	Spermatid counts and % tubules with luminal sperm
	Spermatid differentiation	Germ-cell culture, Morphology
Epididymis	Sperm maturation	Histopathology, Biochemical tests
Brain	Hypothalamic-pituitary axis	Pituitary cell-culture, Hypothalamus perfusion
		Histopathology, Hormone challenge
		Accessory sex-organ weights
Seminal Fluid	Daily sperm production	Spermatid counts, Semen evaluation
Blood	HPG axis	Hormones/ABP assays

exposure should last the full duration of the cycle. This cannot be achieved *in vitro,* because germ cell differentiation and the physical relationship of stages within the tubules are lost in cell culture systems. The germ cells are entirely dependent upon the Sertoli cells for physical and biochemical support. Complicated endocrine and paracrine systems control Sertoli cells, Leydig cells, and germ cells. Besides the loss of paracrine interactions, the altered metabolic activity of target or adjacent cells and difficulty in isolating and testing certain spermatogenic stages are other significant limitations of *in vitro* assessment of testicular toxicity [68]. In addition, for accurate identification of stage-specific lesions of the seminiferous epithelium, critical evaluation of morphological structures is very important. Because germ cells are continuously dividing and differentiating, the staging of spermatogenesis has proven to be an extremely sensitive tool to identify and characterize even subtle toxicological changes.

The most common approach to evaluate the effect of cytotoxic drugs on the testis in a clinic setting uses the tools (e.g., orchidometer) for measuring testicular size (Table 3). This is followed by semen analysis, endocrine assessment of the hypothalamic-pituitary-testicular axis by blood work, and analysis of testicular biopsy samples when indicated. Although research on testicular toxicology has been advanced significantly by the introduction of *in vitro* testing systems, the *in vivo* tools however, are still essential parts of the risk assessment process, and they are unlikely to be eliminated by *in vitro* models.

Food and Drug Administration (FDA) requirements for evaluation of reproductive toxicity or pharmacologi-

Table 3. Endocrine disorders associated with altered testicular size.

Clinical condition	Testicular size	
	Volume (mL)	Dimensions (cm)
Normal size (may include	20	4.3 × 2.8
obstructive or idiopathic	25	4.7 × 3.1
oligozoospermia)	30	5.2 × 3.3
Moderate hypogonadism (e.g.,	6	2.9 × 1.8
gonadotropin deficiency, or	10	3.4 × 2.1
maturation arrest)	15	4.1 × 2.5
Severe hypogonadism (e.g.,	2	2.0 × 1.2
Klinefelter syndrome)	4	2.5 × 1.5

cal testing of a new drug in humans involves a multicenter placebo controlled dose escalation format. Currently there is a focus on standardized semen analysis to note any changes. The studies entail refined statistical analysis for minimal variability and proper quality control [69].

6.2 In vitro systems

In vitro systems are uniquely suited to investigate specific cellular and molecular mechanisms in the testis and thus improve risk assessment [68]. These *in vitro* models can be used alone or in combination with each other to test hypotheses about testicular toxicity. An original toxicant, its metabolites, the precursors or selective inhibitors can be individually administered to isolated cell types to evaluate specific toxicity mechanisms and to note the interaction of adjacent cell types. Numerous *in vitro* model systems are described in the literature, including Sertoli-germ cell cocultures [70], Sertoli cell-enriched cultures [71, 72], germ cell-enriched

cultures [73], Leydig cell cultures [31, 70], Leydig-Sertoli cell cocultures [74], and peritubular and tubular cell cultures [70, 74]. These *in vitro* systems are the only way to directly compare human and animal responses and to screen a class of compounds for new product development. Though these *in vitro* systems are a valuable adjunct to the *in vivo* test system, they do not replace the *in vivo* data because they cannot provide all the facts essential for hazard assessment. Moreover, certain dynamic changes associated with spermatogenesis are difficult to model *in vitro*. For example, the release of elongated spermatids by the Sertoli cells (spermiation), which is commonly inhibited by boric acid and methyl chloride, can only be studied at present by specific *in vivo* systems.

In the wake of media coverage of possible reproductive health and cancer concerns, a few toxicologists have questioned whether these adverse health effects can be attributed to environmental endocrine disruption [75, 76]. Arguments for a demonstrable link between hormone-disruptive environmental agents and human reproductive health effects are supported by the fact that many pesticides and other agents with estrogenic or anti-androgenic activity operate via hormone receptor mechanisms. However, in the few studies of suspected weak estrogens, like the alkylphenols, some 1 000 to 10 000 times or up to 10^6 times more of the agent is required to bind 50% of the estrogen receptor than estradiol itself [61]. Of course, crucial to risk assessment is the need to know how many receptors must be occupied before activation of a response can ensue. For some hormones such as human chorionic gonadotropin (hCG), as little as 0.5% to 5% receptor occupancy is required for full activation of response. For other hormones (those that require protein synthesis for expression of effect), higher levels of receptor occupancy are needed. Fluctuations of hormone concentration and receptor activities, by design, absorb some environmental and physiological challenges to maintain homeostasis in adults. Only when the equilibrium control mechanisms are overwhelmed, the deleterious effects occur. An important question is whether homeostatic mechanisms are operative in the embryo and fetus.

Some investigators have proposed the use of *in vitro* assays to screen for estrogenic or other hormonal activity [77]. While steroid receptors bound to their ligand act as transcription factors for gene expression in the target tissue, simple *in vitro* screening assays based on binding to a receptor are not sufficient in themselves for measuring hormone activity. Binding of ligand to its specific receptor must be correlated with a physiologic response.

6.3 Sperm nuclear integrity assessment

Recent attention has focused on assessments of sperm morphology and physiology as important endpoints in reproductive toxicology testing [78]. Structural stability of sperm nuclei varies by species, appears to be enhanced by the oxidation of protamine sulfhydryl to inter- and intra-molecular disulfide bonds, and is a function of the types of protamine present. Chemicals may disrupt the structural stability of sperm nuclei, which depend upon their unique packaging either during spermatogenesis or sperm maturation. Decondensation of an isolated sperm nucleus *in vitro* can be induced by exposure to disulfide reducing agents, and the time taken to induce extensive decondensation is considered to be inversely proportional to the stability of the sperm nucleus. Human sperm decondenses most rapidly, followed by that of the mouse and of the hamster, while rat sperm nuclei demonstrates a slower rate of decondensation [79]. Such a *sperm DNA decondensation assay* is useful in the evaluation of some cases of unexplained infertility [80]. Evidence suggests that damage to human sperm DNA might adversely affect reproductive outcomes and that the spermatozoa of infertile men possess substantially more sperm DNA damage than do the spermatozoa of fertile men [80]. This is particularly relevant in an era where advanced forms of assisted reproductive technologies are commonly used (technologies that often bypass the barriers to natural selection), because there is some uncertainty regarding the safety of using DNA-damaged spermatozoa. However, sperm head morphology has shown low but significant correlations with the *sperm chromatin structure assay (SCSA)* variables [81]. Evaluation of damaged sperm DNA seems to complement the investigation of factors affecting male fertility and may prove an efficient diagnostic tool in the prediction of pregnancy outcome [20].

Other tests, called DNA stability assay or SCSA use direct evaluation of sperm chromatin integrity and may provide information about genetic damage to sperm and predict infertility [82, 83]. A shift in DNA pattern (from double stranded intact DNA to denatured single stranded) can be induced by a variety of mutagenic and chemical agents and evaluated either by DNA flow cytometry analysis or by sperm chromatin structure assay [84]. A modified *single cell gel electrophoresis (Comet) assay*, which uses a combination of fluorescence intensity measurements by microscopy and image analysis has been recently validated [20]. A shift in the DNA pattern can be evaluated by acridine orange staining, where double-stranded DNA is stained green and single stranded DNA is stained red. The data is expressed as *DNA Fragmentation Index (DFI)*.

DNA flow cytometry is a very useful tool that permits rapid, objective assessment of a large number of cells, but may not be readily available. Comet assay, when combined with centrifugal elutriation, can provide a useful *in vitro* model to study differences in metabolism and the susceptibility of different testicular cell types

to DNA damaging compounds. Thus, new findings through these systems should lead to greater knowledge about effects and mechanism(s) of a chemical or class of chemicals involved in testicular toxicity.

7   Summary

Some authorities suggest that the human race is experiencing increased incidences of developmental, reproductive, and carcinogenic effects. Some believe these adverse effects may be caused by environmental chemicals acting to disrupt the endocrine system that regulates many of these processes. This is supported by observations of similar effects in aquatic and wildlife species. However, the hypothesis that the reported increased incidence of human cancers, reproductive abnormalities, and infertility can be attributed to an endocrine disruption phenomenon is called into question for several reasons.

First, the secretion and elimination of hormones are highly regulated by the body, and mechanisms for controlling modest fluctuations of hormones are in place via negative feedback control of hormone concentrations. Therefore, minor increases of environmental hormones following dietary absorption and liver detoxification of these xenobiotics may be inconsequential in disrupting normal endocrine homeostasis. Second, low ambient concentrations of chemicals along with low affinity binding of purported xenobiotics to target receptors probably are insufficient to activate an adverse response in adults. Whether the fetus and the young are capable of regulating minor changes to the endocrine milieu is uncertain. Finally, the full data are not available for combinations of chemicals that may be able to affect endocrine function. At the same time, in the case of environmental estrogens acting as endocrine disruptors, it is known that competition for binding sites by anti-estrogens in the environment may moderate the estrogenic effects of some chemicals. Clearly, more research to fill in the data gaps is needed.

With few exceptions (e.g., diethylstllbestrol [DES]), a causal relationship between exposure to a specific environmental agent and an adverse effect on human health operating via an endocrine disruption mechanism has not been established. Short-term screening studies can be developed and validated in an effort to elucidate the mechanism. Through controlled dose-response studies, it appears that these compounds (e.g., alkyl phenol ethylates and their degradation products, chlorinated dibenzodioxins and difurans, and PCBs), can induce irreversible induction of male sex characteristics on females (imposex), which leadd to sterility.

In conclusion, a variety of external and internal factors can induce testicular toxicity leading to poor sperm quality and male factor infertility. Unfortunately, several

of these influences (e.g., glandular infection, environmental toxicants, nutritional deficiencies, aging, ischemia, and oxidative stress) disrupt the hormonal milieu and have been underestimated. Partial androgen insensitivity mainly due to an androgen-to-estrogen imbalance may contribute to lowered sperm production. The role of chronic inflammation on the reproductive organs is not completely understood as it may be asymptomatic and difficult to diagnose. There is a need to characterize all of the factors involved and to develop reliable animal models of testicular disease. No major advances have been made in the medical management of poor sperm quality. The application of assisted reproductive techniques such as ICSI to male infertility, regardless of cause, does not necessarily treat the cause and may inadvertently pass on adverse genetic consequences. Clinicians should always attempt to identify the etiology of a possible testicular toxicity, assess the degree of risk to the patient being evaluated for infertility, and initiate a plan to control and prevent exposure to others once an association between occupation/toxicant and infertility has been established.

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