The Endocrine and Reproductive System: Adverse Effects of Hormonally Active Substances?

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ABSTRACT. Chemicals that have the intrinsic property to modulate or even disrupt the endocrine system are present in the human environment. Because it is the potency of such chemicals that determines the toxicologic relevance, assessment of the risk to human health must consider both the endocrine disrupting potential and the potency. Usually in vitro assays are applied to detect the potential of a hormone-like effect, and such data are considered useful to set priorities for additional testing and for mechanistic studies. However, such data allow only determination of relative potency of a chemical as compared with other xenobiotics, natural compounds, or endogenous hormones. Relevant information on the endocrine-disrupting potency can be taken only from in vivo assays, eg, the Hershberger (male reproductive organs) and uterotrophic (female reproductive organs) assays, the updated versions of the 28- and 90-day toxicity studies in rodents, and the 2-generation studies in rodents. With the use of this information and the concentration of these chemicals in humans, the potency of the effect as compared with endogenous hormone activity can be estimated. So far, the relative potencies of chemicals tested in in vitro systems as compared with estradiol are several orders of magnitude smaller, whereas potency of the phytoestrogen, eg, isoflavones such as genistein or daidzein, can even exceed that of estradiol, especially in infants who are fed soy-based formula as a sole source of nutrition. Although there are still open questions regarding in utero or early postnatal exposure, the low potencies and concentrations of manmade chemicals as compared with the endogenous hormones in humans make it unlikely that adverse effects occur at common exposure. Pediatrics 2004;113:1070–1075; endocrine disrupters, xenoestrogens, phytoestrogens, adverse effects, test systems.

ABBREVIATIONS. DDT, dichlorodiphenyltrichloroethane; PCB, polychlorinated biphenyl; OECD, Organisation for Economic Cooperation and Development.

Reports of decreased sperm counts and increased incidences of testicular cancer in men and breast tumors in women have aroused intense discussions in the general public and the scientific community. This became common knowledge in 1996 with the publication of the book by Colborn et al,1 Our Stolen Future: Are We Threatening Our Fertility, Intelligence, and Survival? A Scientific Detective Story.

Originally, Carlsen et al2 evaluated a total of 61 studies on sperm count and established that there was a decrease from 113 million to 66 million sperm/ml of semen between 1938 and 1990. On this basis, Sharpe and Skakkebaek3 presented the hypothesis that the dysfunctions and diseases of the male sex organs (as testicular cancer, malformations of the urethra, or cryptorchidism), which have been described with increasing frequency in the course of the past 3 to 5 decades, could be associated with chemicals that mimic the action of estrogen. In particular, medications that mimic the action of estrogen, phytoestrogens, estrogens in cow milk, and certain industrial chemicals have been considered. This assumption is supported by observations in wild animals. Dichlorodiphenyltrichloroethane (DDT), for example, accumulated in the bodies of birds of prey, which are at the end of the food chain, and led to reproductive disorders. Colborn et al4 described smaller penises in alligators and unfertilized eggs that had been laid as the result of an industrial accident in Florida, in which large quantities of the insecticide dicofol were discharged into a lake. In other regions, more female than male offspring were counted in several species of fish and the gulls that ate these fish. These observations and hypotheses resulted in a multitude of studies to identify hormonally active chemicals and to explain their mechanism of action and their significance for humans and the environment.

The different effects have been described and discussed by various authors and committees,5–14 as have the consequences for appropriate testing.15,16 A continuous flow of reports describe an association of chemical exposure of children to hormone-related disorders. These are birth defects, developmental disorders, declining proportion of male newborns, neuromental deficits in families of pesticide workers,17,18 testicular dysgenesis syndrome as a result of disruption of embryonal programming and gonadal development during fetal life by adverse environmental influences,19 and insufficient androgen action in the male fetus with subsequent undervirilization and hypospadias in the newborn as a result of intrauterine exposure to environmental hormonal disruptors.20 However, except for high-exposure scenarios such as Yusho disease after high exposure to polychlorinated biphenyls (PCBs), dibenzodioxins, and dibenzofurans,21,22 none of these suggested associa-
Substances that Mimic the Action of Hormones

In principle, an “endocrine disrupter” is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. In the case of xenoestrogens, it is the stimulation of mitotic activity in the tissue of the female genital tract. Many chemicals that humans ingest with their diet have estrogen-like effects, which have been identified in a multitude of test systems. Among them are industrial chemicals such as certain herbicides, fungicides, organochlorine insecticides, nematocides, organophosphates, pyrethroids, heavy metals, PCBs, and phthalates. Colborn et al compiled 45 chemicals, including the PCBs, dibenzodioxins, dibenzofurans, and DDT, to which they attribute an influence on the reproductive system in humans. Also included are the phytoestrogens, ie, naturally occurring plant substances.

Interaction with endogenous hormones may occur via different mechanisms. Xenobiotics can influence hormone synthesis—the release, the transport, the effect, the metabolism, and the excretion of hormones. Another group of natural and synthetic substances interferes with the hormones at receptors. Phytoestrogens such as coumestrol, daidzein, and genistein; medications such as diethylstilbestrol, ethyl estradiol, and tamoxifen; and industrial chemicals such as DDT, p,p'-DDE, and bisphenol A belong to this group. They bind to the estrogen receptor and interact with the binding of the hormone. A third group of substances, the DDT metabolite p,p'-DDE or vinclozoline metabolites, block the androgen receptors, ie, the receptors for the male hormone testosterone. Speculations that xenoestrogens may act through unique cellular receptors that do not bind endogenous endocrine modulators lack support.

Compounds that interact with a receptor trigger a cascade of events, which are regulated by the receptor. Substances that compete with the physiologic ligand, eg, a hormone at the receptor, and imitate its effect are called agonists; those that block the receptor are antagonists. The kinetics of interactions of xenobiotics with an endogenous compound at a receptor are well understood and are the basis for the evaluation of drug effects via a receptor (see textbooks of pharmacology). Interaction of a ligand with a receptor is described by:

\[ \text{Ligand} + \text{Receptor} \rightarrow \text{Ligand Receptor Complex} \]

Replacement of a physiologic ligand, eg, an estrogen from the receptor by a competitor, eg, a xenoestrogen, depends on its relative affinity to the receptor and its concentration. For example, replacement of the physiologic ligand from the receptor by a compound of 1000-fold lower affinity requires a 1000-fold higher concentration. Although this over-simplifies competitive interaction of compounds at a receptor, it demonstrates the need for information on the relative binding affinities of the compounds in question and their concentration in the organism.

The Scientific Committee on Toxicology, Ecotoxicology and the Environment of the European Commission has compared the potency of xenoestrogen concentrations detected in human blood as a surrogate for concentrations at the receptor with the potency of estradiol concentrations in blood. It seemed that the relative potency of p,p'-DDT, 4-nonylphenol or bisphenol A are approximately 1 million-fold lower than that of estradiol. Only the phytostrogen genistein showed a potency that exceeded that of estradiol. From this it was concluded that an interaction of the compounds at the receptor with physiologic consequences is unlikely.

In addition to the reproductive organs, the liver, kidney, adrenal gland, central nervous system, immune system, cardiovascular system, and bones are target tissues of the effect of steroid hormones. The corresponding receptors have been identified in these tissues. Moreover, there are indications of a concentration-dependent stimulation (low concentrations) or inhibition (high concentrations) of tumor growth by steroid hormones.

**Test Systems for Identification of Endocrine-Disrupting Chemicals**

A great variety of test systems are being used with different experimental modifications, which makes it difficult to compare the results directly. In most cases, standardization is required. The in vitro tests are able to identify the intrinsic hormone-like potential (hazard identification) of a chemical and its relative potency. However, they do not include toxicokinetics of the chemical and its metabolites, so results have to be verified by in vivo testing. Some of the tests are described briefly.

**In vitro Test Systems**

The recombinant yeast estrogen assay uses yeast cells that are transfected with the human estrogen receptor α gene, together with expression plasmids (estrogen responsive elements and lac-Z reporter gene encoding β-galactosidase). The cells are incubated with the test compound and a chromogenic compound. Active ligands that bind to the receptor induce β-galactosidase, changing the chromogen to yellow that is quantified by a spectrophotometer.

In the E-screen test, the human MCF-7 breast cancer cell line is incubated with the test compound for several days to allow proliferation. Thereafter, the number of cells is quantified, eg, by measuring optical density.

In the estrogen-R(α) competitor screening test, the human estrogen receptor is fixed to 96 wells. The test
compound and fluorescein-labeled 17β-estradiol are added. After incubation and washing, the fluorescence of 17β-estradiol attached to the receptor is measured.30

The placental aromatase assay uses aromatase cytochrome P450–mediated conversion of C19 androgens to aromatic C18 estrogens. Androgens have been shown to inhibit this reaction.31

The androgen binding or reporter gene assay and the thyroid receptor binding assay determine the relative affinity of androgen-like or thyroid hormone–like compounds to the specific receptors as compared with the endogenous hormones.

In vivo Test Systems

The traditional Hershberger test was originally designed to differentiate between anabolic and androgenic effects of drugs by determining the weights of the musc. levator ani and seminal vesicles in mice after treatment for several weeks. Other workers have used a wide range of treatment regimens to determine androgenic potential of chemicals in intact and castrated laboratory animals. The endpoints used are prostatic weight, seminal vesicle weight, and DNA/RNA contents of male reproductive organs.32

In the uterotrophid assay, juvenile female rats are treated with the test compound and uterine growth is monitored after several days of treatment. Several experimental designs are used, so the test requires standardization.33

The Organisation for Economic Co-operation and Development (OECD) Guideline 407 is a 28-day repeated-exposure test in rats.34 To better detect endocrine effects of the test chemicals, it is enhanced by determining weight and histopathology of hormone-regulated organs, analysis of epididymal sperms, estrous cycle, and battery of hormones.

The OECD Guideline 416 reproductive test in rodents permits an in-depth study of the growth, development, and sexual function of the F1 generation and includes the monitoring of the subsequent F2 generation through weaning.34 The recently revised protocol is considered to include parameters such as weights and pathologic examinations of the reproductive organs, detailed spermatogenesis, and sperm investigations of the parental generation and their offspring. Physical, sexual, and behavioral development and the learning and memory abilities of the offspring are also included.

RELATIVE POTENCY OF XENOESTROGENIC EFFECTS

The intensity of the effect of substances, particularly in comparison with the endogenous hormones or natural hormone-like chemicals, is critical for an evaluation. Sonnenschein et al35 studied the proliferative effect in estrogen-sensitive cells and found that the estrogenic potency of chemicals such as 4-octylphenol and bisphenol A was by >3000-fold and 30 000-fold, respectively, lower than that of endogenous estradiol. In view of the low amounts of these chemicals found in the organism, the resulting intensity of effect is very low. Safe36 calculated the estrogenic and antiestrogenic potencies of xenoestrogens that may be ingested daily relative to 17β-estradiol. A woman who takes a birth control pill ingests approximately 17 000 equivalents per day and during postmenopausal estrogen therapy ingests 3300 equivalents, whereas ingestion of estrogenic flavonoids in food is 102 and of environmental organochlorine estrogens is 0.0000025. Similar data have been obtained with antiestrogens.

Bolt et al37 compared the potencies of estimated daily intake of nonylphenol and bisphenol A (1 µg/kg body wt each) with that of 1 mg of the phytoestrogen daidzein/kg body wt and 0.5 µg/kg body wt ethinyl estradiol, ingested daily in a low-dose contraceptive pill. As compared with the daidzein intake, the estrogenic potencies of nonylphenol and bisphenol A were 125 to 250 and 1000 times lower, respectively, whereas that of ethinyl estradiol was 20 times higher than that of daidzein. Daidzein penetrates the placenta.38 Because soy is the major source of phytoestrogens in human food, which can amount to up to 8 mg/kg body wt in infants who are fed soy-based infant formula,39 the consequences of these exposures must be evaluated rather than those of the xenoestrogens.

Specific low-dose effects postulated by the experiments reported by the group of vom Saal40,41 on bisphenol A and nonylphenol could not be verified.16,42 Attempts to demonstrate potentiation of low-dose effects by the presence of several compounds also failed. McLachlan’s43 research group reported that, in an in vitro system with yeast cells, the effect of 2 separate weakly active estrogens was 1000 times higher in combination. Various other research groups have not been able to repeat these results.44 Finally, McLachlan45 officially retracted the results and any inferences drawn from them.

Considering this, a toxicologically relevant effect on the prenatal or postnatal phase from synthetic chemicals is not plausible and is unlikely. However, potential beneficial or adverse effects of phytoestrogens and other natural compounds with hormone-like effects must be considered in the toxicologic evaluation.

PHYTOESTROGENS IN THE HUMAN DIET

Phytoestrogens occur naturally in plants. Common classes comprise isoflavones, lignins, coumestans, or resorcylic acid lactones that are structurally similar to the mammalian estrogen and have a weak estrogenic potency ranging from 1/500 to 1/1000 that of 17β-estradiol. A major source of human consumption of phytoestrogens is soybean derived products, which contain the isoflavones daidzein and genistein. Because of the great differences in the consumption of such products, adults in the United Kingdom and the United States ingest approximately 1 mg of isoflavones per day; Asians ingest 50 to 100 mg/day.46 Infants who are fed soy-based formula as a sole source of nutrition ingest 22 to 45 mg/day.47

The biological effects of phytoestrogens in women have been investigated in several studies.46,48 These indicate that phytoestrogens in the diet have a beneficial effect with respect to breast cancer in women.
by prolonging the menstrual cycle. Mean cycle length in Western countries with high breast cancer risk is 28 to 29 days; it is 32 days in Japan, where breast cancer risk is 4-fold lower. Because breast cell division is 4-fold lower during the follicular phase of the cycle, the authors hypothesize that longer menstrual cycles result in longer follicular phases with reduced cell division during the premenopausal years. Other beneficial effects may be reduced serum cholesterol levels, low rates of cardiovascular disease and cancer, and, especially in postmenopausal women, promotion of conservation of bone mass.49

In neonates who are fed the recommended soy-based formula during the first 3 to 6 months of life, no clinical effects such as feminization have been reported so far. Essex50 assumed that in neonates, the hypothalamic-pituitary-gonadal axis is more active than in older children and adults, which compensates the estrogenic effects of the high intake of isoflavones. However, the author concluded that breastfeeding obviously is best for infants. If mothers do not breastfeed their infants, then a recognized cow milk formula is preferable. Soy-based formula should not be given routinely as prophylaxis to infants who are at risk for developing allergy or atopy. Parents who are vegans may use a soy-based formula because these contain no animal proteins.

**QUESTIONABLE CORRELATION BETWEEN DISEASES AND ABNORMALITIES AND ENDOCRINE-DISRUPTING CHEMICALS**

Several well-recognized institutions have evaluated the evidence for adverse health effects resulting from human exposure to endocrine disruptors.7,51,52 They conclude that analysis of the human data so far has failed to provide firm evidence of direct causal association between low-level exposure to endocrine disruptors measured in the general population and adverse health effects. The specific conclusions taken from the Scientific Committee on Toxicology, Ecotoxicology and the Environment7 and the International Programme on Chemical Safety52 are that temporal increases in the frequency of developmental abnormalities of the male reproductive tract, particularly cryptorchidism and hypospadias, have been reported, but no causative role for endocrine-disrupting chemicals has been determined. The reports on the declining proportion of male newborns during the last decades remain unexplained. Concerns have been raised about the influence of endocrine-disrupting chemicals on the timing of puberty, but the possible mechanisms of action and the role of other factors such as nutrition need to be clarified.

High accidental exposure of pregnant women to certain organochlorines, eg, PCBs, has led to delays in physical and mental development of the offspring. Some of these effects seem to result from altered thyroid or neurotransmitter function, but in most instances, endocrine mechanisms have not been demonstrated.

A number of studies report a decline (since 1939) in human sperm quality in several countries. Several reanalyses of these data have indicated possible bias and confounding and have reached different conclusions with respect to sperm quality, depending on the method used. Recent well-designed studies have shown large regional differences in overall sperm quality and time trends, both within and between countries. Even if there has been deterioration in semen quality, this would not necessarily be attributable to endocrine disruption.

Temporal increases in the incidence of testicular cancer have been reported in certain countries, but rates vary considerably among countries. The underlying reason(s) for the increased incidences has not been determined. However, exposure data on endocrine-disrupting chemicals for critical periods are lacking.

Exposure to certain pesticides and organochlorines has been linked to increases in the incidence of prostate cancer in a few limited studies, but most studies have found no association and the mechanism is unknown.

There has been a steady increase in breast cancer incidence rates in the past decades (in Europe). The available data associating breast cancer development with exposure to organochlorines do not support a causal relationship. Adult women who are currently at risk may have been exposed to endocrine-disrupting chemicals in utero or during infancy, childhood, and adolescence in the mid-20th century when contaminant levels of organochlorines were higher.

**CONCLUSION**

Although in vitro test systems allow estimation of the potentials and the relative potencies of endocrine-disrupting chemicals, the results have to be verified by in vivo studies, because toxicokinetics and metabolism affect the toxic potency of a substance. Using test systems such as the improved repeated-dose toxicity tests (28 days) in rodents (OECD 407) and the 2-generation reproduction toxicity test (OECD 416), either endocrine disrupters showed no effects or effects appeared at very high doses.

Considering the relatively low exposure of humans to endocrine-disrupting chemicals, there is neither scientific evidence nor plausibility that low-affinity compounds can displace high-affinity compounds from a receptor unless they reach sufficiently high concentrations. Such high exposure in humans has occurred only during high accidental exposure scenarios. Thus, the assumption that binding of a compound to a receptor per se results in biological consequences is not acceptable.

There is overwhelming experience from the therapeutic use of very potent synthetic estrogens during pregnancy to prevent abortion, for contraception, or to treat menopausal or postmenopausal disorders. In all of these situations, sufficiently high doses have to be given to obtain modulation of the endocrine system. No one would expect that a dose >1000-fold or more below the therapeutic dose is effective.

Overall, the science-based knowledge on the robustness of the endocrine system, the well-understood principles of substrate–receptor interactions, and the generally low exposure of humans to potentially endocrine-disrupting chemicals make it un-
likely that the last plays a causative role in diseases and abnormalities observed in children or the human population in general. An array of in vitro test systems to cover relevant endpoints of endocrine disrupters are available. They are useful for mechanistic studies and may be helpful in setting priorities. However, they do not include toxicokinetics of the chemical and its metabolites and may generate false-negative as well as false-positive effects, so the results must be verified by in vivo studies. Therefore, instead of developing in vitro screening tests, it is recommended to improve available long-term in vivo tests such as the enhanced OECD 407 test and the OECD 416 2-generation study test, which are considered most relevant to detect hormone-like effects of chemicals.

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