

Selecting Appropriate Animal Models and Experimental Designs for Endocrine Disruptor Research and Testing Studies

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Abstract

Evidence that chemicals in the environment may cause developmental and reproductive abnormalities in fish and wildlife by disrupting normal endocrine functions has increased concern about potential adverse human health effects from such chemicals. US laws have now been enacted that require the US Environmental Protection Agency (EPA) to develop and validate a screening program to identify chemicals in food and water with potential endocrine-disrupting activity. EPA subsequently proposed an Endocrine Disruptor Screening Program that uses *in vitro* and *in vivo* test systems to identify chemicals that may adversely affect humans and ecologically important animal species. However, the endocrine system can be readily modulated by many experimental factors, including diet and the genetic background of the selected animal strain or stock. It is therefore desirable to minimize or avoid factors that cause or contribute to experimental variation in endocrine disruptor research and testing studies. Standard laboratory animal diets contain high and variable levels of phytoestrogens, which can modulate physiologic and behavioral responses similar to both endogenous estrogen as well as exogenous estrogenic chemicals. Other studies have determined that some commonly used outbred mice and rats are less responsive to estrogenic substances than certain inbred mouse and rat strains for various estrogen-sensitive endpoints. It is therefore critical to select appropriate biological models and diets for endocrine disruptor studies that provide optimal sensitivity and specificity to accomplish the research or testing objectives. An introduction is provided to 11 other papers in this issue that review these and other important laboratory animal experimental design considerations in greater detail, and that review laboratory animal and *in vitro* models currently being used or evaluated for

endocrine disruptor research and testing. Selection of appropriate animal models and experimental design parameters for endocrine disruptor research and testing will minimize confounding experimental variables, increase the likelihood of replicable experimental results, and contribute to more reliable and relevant test systems

Key Words: alternatives; animal models; endocrine disruptors; experimental variables; *in vitro* systems; toxicology; validation

Since the early 1950s, scientists have increasingly reported endocrine system-related abnormalities in fish and wildlife linked to chemical exposures. In 1962, Rachael Carson heightened awareness of this connection when she described the effects of DDT on declining bird populations in her book *Silent Spring* (Carson 1962). More recent reports have documented gonadal and reproductive developmental abnormalities in fish and reptiles attributed to disruption of normal endocrine functions by chemicals (Colborn et al. 1996; EDSTAC 1998; IPCS 2002; NRC 1999). Such chemicals are now commonly referred to as endocrine-disrupting compounds (EDCs¹). Concern that EDCs could also adversely affect human health led to new laws enacted as the Food Quality Protection Act of 1996 amendments to the Federal Food, Drug, and Cosmetic Act, and amendments to the Safe Drinking Water Act (PL 104-170 1996a; PL 104-182 1996b). These laws require federal regulatory agencies to determine whether chemicals in food and drinking water have adverse endocrine effects, and to take appropriate actions to protect human health. The laws specifically direct the Environmental Protection Agency (EPA¹) to “develop a screening program using appropriately validated test systems and other scientifically relevant information to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such

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¹Abbreviations used in this Introduction: EDC, endocrine-disrupting compounds; EDSP, Endocrine Disruptor Screening Program; EPA, Environmental Protection Agency; ILAR, Institute for Laboratory Animal Research.

other endocrine effect as the [EPA] Administrator may designate.”

These new laws and heightened public health concerns have led to increased research efforts to broaden the understanding of the mechanisms by which EDCs disrupt normal endocrine functions. Testing has been initiated to determine the range of adverse effects that might result from exposure to such substances. The EPA and National Institutes of Health have funded and continue to fund significant endocrine disruptor biomedical research studies. To comply with statutory mandates, the EPA proposed its Endocrine Disruptor Screening Program (EDSP¹) in 1998, and initiated large-scale efforts to develop, standardize, and validate test methods for the EDSP (EDSTAC 1998; EPA 1998, 2004). The EDSP screening and testing methods will be used to prioritize and evaluate more than 80,000 existing chemicals as well as all new chemicals. The EDSP is designed to detect estrogenic, androgenic, and thyroid hormone effects, and will evaluate effects on reproduction, development, and growth. The EPA is also developing and validating screening and testing methods to determine whether EDCs cause adverse effects in ecologically important species, including amphibians, birds, fish, and invertebrates.

Due to the urgency to fill endocrine disruptor knowledge gaps and to meet statutory deadlines for implementing a screening program, traditional species and strains of animals and traditional laboratory procedures have typically been used for most research and testing efforts. However, subsequent research studies have shown that the use of many of these traditional standard laboratory animal models and procedures could, in many instances, result in difficulties in replicating results in different laboratories, and contribute to decreased ability to detect adverse effects from EDCs. Following reports that some chemicals can induce endocrine effects at very low doses, numerous laboratories attempted unsuccessfully to replicate these low dose and other chemically mediated research findings. Two factors likely contributed to the variation in results among laboratories. First, there are significant differences in response to EDCs among rodent species, stocks, and strains (EDMVS 2003; Spearow 2003; Spearow et al. 1999). Second, standard laboratory animal diets traditionally used for endocrine research and reproductive and development testing were found to produce high and variable levels of estrogenic activity due to significant phytoestrogen content (Boettger-Tong et al. 1998; Thigpen et al. 2004). Although the full implications of these and many other parameters on completed research and testing results are not yet completely understood, it is clear that diet and strain selection can have profound effects on the outcome of endocrine-related studies.

It is now obvious that animal model and experimental design parameters for endocrine disruptor research and testing must be carefully selected in order to minimize confounding experimental variables and to increase the likelihood of replicable experimental results. Although scientists might prefer to continue to perform their research

using traditional species/strains and laboratory animal husbandry procedures, it is imperative that such decisions for future studies be carefully reviewed and modified appropriately in light of current scientific knowledge. As the EPA and other regulatory authorities proceed with the development, standardization, and validation of endocrine disruptor testing methods, potentially confounding variables must be minimized to achieve optimal intra- and interlaboratory reproducibility (ICCVAM 1997; Stokes 2002). This will also likely require the selection of highly sensitive strains and species and avoidance of highly estrogenic diets in order to achieve optimal dynamic response ranges capable of detecting even weakly active EDCs.

Assessing human risks from chemical exposures normally involves extrapolating from data obtained from in vitro and in vivo test systems. However, such extrapolations involve uncertainties as to whether the same dose-related effects in animals will be observed in humans. Such uncertainties can be further reduced by increased understanding of the similarities and differences in the biological responses of animals and humans. Similarly, environmental risk assessments to protect thousands of species will be made based on extrapolating the results of testing from only one species each of mammals, fish, birds, amphibians, and invertebrates. To provide adequate protection of all of these widely diverse animal species in the environment, increased knowledge of the cross-species similarities and differences in susceptibility will be needed. The basis for the selection and use of many of the animal models for endocrine disruptor research and testing is provided in this issue.

This issue of *ILAR Journal* was conceived in keeping with the Institute of Laboratory Animal Research (ILAR¹) objectives to provide current scientific information that will facilitate high-quality animal research and testing, and that will support the refinement, replacement, and reduction of animal use where scientifically feasible. The first series of articles in the issue includes reviews and discussions of important laboratory animal experimental design issues for endocrine disruptor research and testing. In the next series of articles, authors describe commonly used laboratory animal and in vitro models currently being used or investigated for endocrine disruptor research and testing. Although it was impossible to cover every design consideration and animal model in a single issue, these expert reviews are intended to address most of the current major issues and provide descriptions of the most commonly used animal models.

Experimental Design Considerations for Endocrine Disruptor Studies

One of the driving forces for new laws requiring the screening of chemicals for endocrine disrupting effects was the fact that traditional toxicity testing methods had not previously identified many of the endocrine-related adverse effects of some chemicals, especially subtle effects on the

fetus. Although US laws established a deadline for implementation of an endocrine disruptor screening program, this date has now lapsed by several years, despite well-organized and focused efforts to meet the deadline. In the Commentary of Theo Colborn (2004), the author attributes this delay in part to the continued use of traditional toxicological endpoints and practices and a reluctance to apply new approaches. She advocates that testing systems must be designed to detect the time- and hormone-specific effects over a broad range of doses, including low-dose exposures. It will be necessary for such test systems to include nontraditional and/or more sensitive endpoints, such as those that have been used to characterize low-dose endocrine mediated effects of TCDD² and bisphenol A (Colborn 2004). The author emphasizes that it is crucial to evaluate the effects of exposures during highly sensitive prenatal developmental stages, and that it is essential to use appropriate diets and strains of animals (Colborn 2004).

Commercial laboratory animal diets contain high levels of the phytoestrogens diadzein and genistein from soybean meal, and coumestrol from alfalfa. In this issue, authors Colborn (2004) and Thigpen and colleagues (2004) attribute many of the difficulties in replicating low-dose endocrine disruptor studies to the failure to control and avoid high levels of phytoestrogens in the diet. For example, levels of phytoestrogens common in commercial diets have been shown to cause strong responses in control animals in the uterotrophic bioassay. This strong response could prevent detection of significant differences between the positive control animals fed diethylstilbestrol, a strongly estrogenic chemical, and the control animals fed only the commercial diet (Thigpen et al. 2004). These data suggest that standard laboratory animal diets could readily produce false-negative results not only in the uterotrophic assay, but also in other endocrine disruptor assays. Thigpen and coauthors review other research studies and endpoints that demonstrate the potential for standard laboratory animal diets with high phytoestrogen content to confound EDC experimental studies. Another common endpoint measured in EDC testing is alteration of the age at which vaginal opening occurs. However, these researchers (Thigpen et al. 2004) have determined that standard laboratory diets can accelerate vaginal opening, similar to EDCs with estrogenic activity. This has the effect of decreasing the dynamic response range, which could potentially reduce the ability to detect chemicals with weak effects. Because of the documented profound influence of phytoestrogens, they conclude that animal diets used for EDC studies should be free of agents that can modulate the endocrine system under study.

²National Toxicology Program 2004. Toxicology and Carcinogenesis Studies of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)(CAS No. 1746-01-6) in Female Harlan Sprague-Dawley Rats (Gavage Studies). Technical Report Series No. 521, NIH Publication 04-4455. US Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC (in preparation).

Another important consideration in endocrine disruptor studies is selection of the appropriate species and strain/stock of animal model (Colborn 2004; EDMVS 2003; Everitt and Foster 2004; Spearow 2003). Spearow and colleagues (1999) have shown that two inbred mouse strains were 16 times more sensitive to estrogenic substances than outbred CD-1 mice. Similarly, outbred CD-SD rats have been shown to be significantly less responsive to estrogenic substances than inbred F344 rats in studies for various estrogen-sensitive endpoints (Colborn 2004). Based on existing studies, Colborn emphasizes the importance of considering the strain/stock sensitivity for the specific endpoints of interest in endocrine disruptor studies, and the importance of selecting a sensitive strain/stock that will minimize the likelihood of false-negative results.

EDCs and dietary phytoestrogens can also significantly alter the expression of behavioral and related physiological effects. Lephart and colleagues (2004) review the influence of dietary soy isoflavones on consumptive, learning, memory, and anxiety-related behaviors, and discuss the influence of phytoestrogen on food and water intake, adipose deposition, and serum leptin and insulin levels. In one experiment, rats fed diets with phytoestrogens had reduced anxiety compared with rats given phytoestrogen-free diets (Lephart et al. 2004). Sex differences in learning and memory also occurred among rats on the two types of diets. In longer-term studies, Lephart and colleagues found that rats on the highest phytoestrogen diets displayed the lowest body and adipose tissue weights, whereas rats on the phytoestrogen-free diets had the heaviest body weights and adipose deposition (Lephart et al. 2004). These findings suggest the importance of controlling and characterizing dietary phytoestrogen content not only for endocrine disruptor studies, but also for all types of research and testing using animals.

Although differences in diet and strain/stock sensitivity are well-known sources of variation in endocrine disruptor studies, many other animal housing and environmental factors may also confound research and testing results. Everitt and Foster review several of these factors and provide examples where environmental factors have interfered with studies (Everitt and Foster 2004). For example, one recent report found that chemicals with potential EDC activity were released from plastic animal caging after treatment with harsh chemical cleaning agents (Howdeshell et al. 2003). Other potential confounding factors that should be standardized and controlled include room temperature, humidity, and the microenvironment in ventilated and microisolation caging systems (Everitt and Foster 2004).

The effects of exposure to potential EDCs during in utero development are often evaluated in endocrine disruptor studies. Vandenberg (2004) reviews the heightened sensitivity of the fetus to both endogenous hormones and EDCs during this critical period of organ and system development. Studies have demonstrated that the intrauterine proximity of female fetuses to adjacent male fetuses affects

a number of sexually dimorphic anatomical, physiological, and behavioral traits. For example, the anogenital distance at birth of a female fetus with two adjacent male fetuses will be shorter than that of a female fetus with two adjacent female fetuses. In fact, the anogenital distance adjusted for body weight provides a reliable estimate of the intrauterine position (Vandenbergh and Huggett 1995). In utero perturbations of anogenital distance have also been documented and may be a sensitive indicator of EDC effects. Accordingly, for some EDC studies, it may be important to observe or estimate intrauterine position for each fetus or pup, respectively, and to select and assign animals to experimental groups appropriately.

Animal Models for Endocrine Disruptor Studies

Assessing the potential adverse effects of EDCs on humans as well as on the diverse range of animal species in the environment requires the use of surrogate species from which extrapolations are made to one or more other species. Fortunately, such extrapolations are aided by the phylogenetic conservation of genetic and cellular structure and function, which often extends from lower simple organisms to higher, more complex organisms. Ecotoxicology assessments must include consideration not only of individual effects, but also of population effects that may adversely affect other species higher in the food chain. The articles in this section describe some of the models being developed for predicting ecotoxicology effects, followed by a description of the mammalian and in vitro models proposed for evaluating potential human health effects.

deFur (2004) reviews the use and role of invertebrate species as models for endocrine disruptor research and testing, and provides an overview of invertebrate endocrinology. The author notes that hormone systems are found in all invertebrate phyla, including arthropods, mollusks, nematodes, and many others. Invertebrates are essential components of every ecosystem on earth. Accordingly, the EPA plans to include an opossum shrimp (*Mysidacea*) or other invertebrate life cycle toxicity assay as part of its EDSP Tier 2 analysis. However, deFur notes that other invertebrate test systems will likely be necessary to characterize risks to the diverse range of invertebrate phyla and species. He also emphasizes that some invertebrate EDC assays may be useful as sentinels of potential vertebrate effects, but that further comparative endocrinology research is needed to understand the usefulness and limitations of this approach.

Birds are especially sensitive to the adverse effects of toxic substances, including EDCs. Accordingly, an avian two-generation test has been proposed by the EPA for inclusion in the EDSP Tier 2 analysis. Touart (2004) reviews the rationale for including birds in the EDSP, and discusses the attributes and basis for selecting Japanese quail as the animal model for the avian test. The author further describes

the basis for experimental design parameters incorporated in the two-generation test to ensure appropriate exposures during in ovo, juvenile, subadult, and adult life stages. Numerous endpoints are included to assess effects on survival, growth, multigeneration reproduction, and general toxicity; and the rationale for their inclusion is described. Touart (2004) acknowledges the need for development of improved husbandry and handling procedures to reduce both the occurrence of confounding behaviors and unintended morbidity and mortality. Additional work is also needed to determine the genetic strain of Japanese quail with the most appropriate attributes and sensitivity for endocrine disruptor testing.

Adverse effects on fish associated with exposure to environmental EDCs in wastewater treatment plant and industrial chemical discharges are now well documented (Ankley and Johnson 2004). These findings have led to additional laboratory research with fish to understand better the mechanisms and dose-relationship of EDCs to these adverse effects. In addition, EPA has proposed the inclusion of fish tests in both Tier 1 screening and Tier 2 multigenerational studies in their EDSP, and is currently standardizing and validating fish test systems. Ankley and Johnson (2004) review the structure and function of the hypothalamic-pituitary-gonadal axis in fish, its role in sexual development and reproduction, and relevant endpoints that can be measured to assess alterations by EDCs. The fathead minnow (*Pimephales promelas*), Japanese medaka (*Orizyes latipes*), and zebrafish (*Danio rerio*) are currently being evaluated as animal models for laboratory testing of EDCs. Ankley and Johnson (2004) discuss the advantages and limitations of each of these models, including their relative sensitivity and specificity for different classes of EDCs. They also review experimental design considerations unique to fish studies for both partial and full life cycle tests. These tests will be critical for providing information to support quantitative predictions of ecological risks of EDCs to fish populations, and they are expected to provide useful information for screening EDCs for generalized effects on vertebrate endocrine systems.

Endogenous estrogens are important for the proper function not only of the reproductive system, but also of neuroendocrine, skeletal, and cardiovascular systems (Walker and Korach 2004). However, adverse health effects from exogenous EDCs with estrogenic and/or antiestrogenic activity are of significant concern to both humans and animals. Discerning beneficial versus harmful effects of exogenous substances requires a thorough understanding of the types, distribution, and function of estrogen receptors. Fortunately, the availability of gene knockout techniques has provided the opportunity to create unique mouse models that can be used to increase our understanding of the specific functions of different estrogen receptors in various tissues. Walker and Korach (2004) review the two forms of estrogen receptors, ER-alpha and ER-beta, and the results of research conducted with knockout mice that lack functional receptors for

each type of estrogen receptor and that lack both functional receptors. They describe the phenotypic differences in male and female anatomy, behavior, and physiology observed in each knockout model, and the role of estrogen receptors in ensuring normal function of various body systems. These and future genetically modified mouse models can be expected to facilitate research to increase our knowledge of the effects of endocrine dysfunction at the cellular and molecular level further.

The laboratory rat has served as the traditional animal model of choice for regulatory developmental and reproductive toxicity testing conducted to support human health risk assessments. The rat has also been used extensively for developmental and reproductive physiology and endocrinology research, and has been more thoroughly characterized in these research fields than any other species (Gray et al. 2004). As would be expected, the rat became the default mammalian species of choice for the EDSP Tier 1 and 2 studies. In this issue, Gray and coauthors (2004) review the similarities and differences between rat and human reproductive function, noting that this function is highly conserved at the cellular and molecular levels among mammalian species. Three short-term rat assays are undergoing standardization and validation for the EDSP Tier 1 Screening Battery (Gray et al. 2004). The first is the 3-day rat uterotrophic assay, which is designed to detect estrogen agonists and antagonists based on the induction or inhibition, respectively, of increased weight changes in the uterus. The second is the 10-day rat Hershberger assay, which detects androgen and antiandrogen activity by measuring changes in the weight of male reproductive tissues. The third is the 21-day pubertal female rat assay where the age of vaginal opening is monitored, serum thyroid hormones and uterine and ovarian weight are measured, and histology is evaluated. This assay also provides information regarding potential EDCs with estrogenic and antithyroid activity, and those that may inhibit steroidogenesis (Gray et al. 2004). Other assays undergoing evaluation for possible inclusion in the Tier 1 Screening Battery include the pubertal male rat assay and an in utero-lactational assay. The latter assay involves exposure during both gestation and lactation and can therefore detect disruption during fetal and neonatal reproductive system development.

The rat was also selected as the animal model for the EDSP Tier 2 mammalian multigeneration test. As currently proposed, only chemicals that produce positive results in the Tier 1 screening battery of tests will be evaluated further in the more complex and extensive Tier 2 tests. These tests expose animals during all critical stages of development, and evaluate the reproductive function of animals that were exposed in utero. Gray and coauthors (2004) discuss the importance of using sufficient numbers of animals to achieve the statistical power necessary to detect reproductive effects in the F1 generation. Although these tests use large numbers of animals, Gray and coauthors (2004) suggest that the future addition of more sensitive endpoints and

more thorough evaluation of all animals on each study may allow for reductions in overall animal use. They also advocate the need to retain flexibility so that new, more sensitive assays and endpoints can be added to the screening and testing program in the future.

In vitro test systems are also proposed for the EDSP Tier 1 screening battery of assays. Information from both in vivo and in vitro tests will be considered in a weight of evidence decision-making approach to determine whether chemicals with positive results should be further evaluated in the more complex Tier 2 test systems. The in vitro test systems may also be used in conjunction with in silico systems, such as quantitative structure activity relationship models to prioritize chemicals for in vivo evaluations. In this issue, Charles (2004) reviews the various types of in vitro model systems currently undergoing standardization and validation for potential use in the EDSP. Current in vitro assays proposed for the Tier 1 Battery include an estrogen receptor binding or reporter gene assay, an androgen receptor binding or reporter gene assay, and a steroidogenesis assay with minced testis. An in vitro placental aromatase assay is also being evaluated for potential inclusion in the Tier 1 Battery. Charles (2004) describes the necessity to standardize and adequately validate the in vitro test systems in accordance with established validation criteria (ICCVAM 2003; Stokes 2003).

Many of the proposed in vitro tests require animal tissues or use receptors derived from animal tissues. However, recombinant technologies are now available that can produce receptors without the need for animal tissues, and cell lines have been established that eliminate the need to obtain cells directly from animal tissues (Charles 2004). Charles (2004) and Gray and coauthors (2004) emphasize that although properly validated in vitro test systems provide valuable mechanistic information helpful for prioritization and screening purposes, these systems currently have limitations that do not allow them to substitute for in vivo test systems. For example, in vivo systems incorporate chemical absorption, metabolism, distribution, and excretion that currently cannot be characterized adequately by in vitro systems. Nevertheless, in vitro test systems are viewed as an integral component of future endocrine research and testing programs.

Conclusions

Properly designed high-quality endocrine disruptor research studies and toxicity test systems should avoid or control factors that cause or contribute to experimental variation. Such studies and test systems should also utilize appropriate biological models that have been selected to provide optimal sensitivity and specificity to accomplish the research or testing objective. Reducing and controlling variables will not only contribute to greater reproducibility of data within and among laboratories, but may also allow for smaller numbers of animals per experimental group. These advan-

tages should help reduce the overall number of animals required for effective screening and testing programs and support meaningful research studies.

Clear and compelling evidence now confirms that two major confounding variables in endocrine disruptor studies are the use of traditional laboratory diets containing high levels of soy products and the selection of strains/stocks for animal models that have reduced sensitivity for the endpoints of interest. It is therefore imperative that diets and strains/stocks are carefully selected and justified for all current and future endocrine disruptor studies and test systems.

As new, potentially more sensitive technologies such as genomics and proteomics are incorporated into endocrine disruptor research studies and test systems, it is likely that many other experimental parameters will require additional control or modification to attain meaningful and reproducible results. Effective strategies to identify and minimize the impact of sources of variation in these animal-based studies will likely be enhanced by collaborative teamwork among scientists, laboratory animal veterinarians, animal scientists, toxicologists, and animal care specialists.

The recognition that endocrine-disrupting chemicals in our environment can cause profound adverse effects in animals has raised significant concerns about their additional potential to affect human health adversely. Major research initiatives now underway in the scientific community should provide the answers to the many questions about whether and how such chemicals may be affecting human health. Animal models are clearly essential for research and test systems necessary to evaluate the potential hazards of existing and new chemicals. Ensuring adequate protection of human health, animal health, and the environment will best be achieved by a flexible approach that integrates the use of animals, *in vitro* systems, and *in silico* models. Undoubtedly, good science, protection of public health and the environment, and good animal welfare will continue to be inextricably linked.

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