https://doi.org/10.59415/ijfas.v10i1.234

ORIGINAL ARTICLE Open Access

ISSN 2278-1404

International Journal of Fundamental & Applied Sciences

OPTIMIZING ANIMAL MODELS AND EXPERIMENTAL DESIGNS FOR ENDOCRINE DISRUPTOR RESEARCH AND TESTING

K. S. Bhanuprakash[1]*, Deepa kumari G.B[2]

[1&2] Department of Biochemistry, The Oxford College of Science, Bangalore, India

Abstract

Growing evidence suggests that environmental chemicals can disrupt normal endocrine functions, causing developmental and reproductive abnormalities in fish and wildlife. This has raised concerns about potential human health risks. Consequently, US legislation now mandates the Environmental Protection Agency (EPA) to create and validate a screening program to identify chemicals in food and water that may act as endocrine disruptors. In response, the EPA proposed the Endocrine Disruptor Screening Program, which employs both in vitro and in vivo test systems to identify harmful chemicals for humans and ecologically significant species.

However, the endocrine system is highly sensitive to various experimental factors, such as diet and the genetic background of the test animals. To ensure accurate results in endocrine disruptor research, it is crucial to minimize or eliminate factors that contribute to experimental variation. Standard laboratory animal diets, for instance, contain varying levels of phytoestrogens that can mimic the effects of both endogenous and exogenous estrogens. Additionally, studies have shown that some commonly used outbred mice and rats exhibit lower sensitivity to estrogenic substances compared to certain inbred strains.

Thus, selecting appropriate biological models and diets that offer optimal sensitivity and specificity is essential for endocrine disruptor studies. This issue introduces 11 papers that delve into critical experimental design considerations and review current laboratory animal and in vitro models used in endocrine disruptor research. Careful selection of animal models and experimental design parameters will minimize confounding variables, enhance the reproducibility of results, and lead to more reliable and relevant test systems.

***Keywords*:** animal models; endocrine disruptors; experimental variables; in vitro systems; toxicology; validation.

1. Introduction

Since the early 1950s, scientists have increasingly reported endocrine system-related abnormalities in fish and wildlife associated with chemical exposures. In 1962, Rachel Carson brought significant attention to this issue in her book Silent Spring, where she detailed the adverse effects of DDT on declining bird populations (Carson 1962). More recent studies have documented gonadal and reproductive developmental abnormalities in fish and reptiles due to the disruption of normal endocrine functions by chemicals (Colborn et al. 1996; EDSTAC 1998; IPCS 2002; NRC 1999).

Corresponding author

Full Address :

Department of Biochemistry, The Oxford College of Science, Bangalore, Karnataka, India.

E-mail: *bhanu.cng@gmail.com*

These chemicals are now commonly referred to as endocrine-disrupting compounds (EDCs).

Concerns that EDCs could also adversely affect human health led to the enactment of new laws, specifically the Food Quality Protection Act of 1996 and amendments to the Federal Food, Drug, and Cosmetic Act, as well as amendments to the Safe Drinking Water Act (PL 104-170 1996a; PL 104-182 1996b). These laws mandate federal regulatory agencies to assess whether chemicals in food and drinking water have adverse endocrine effects and to implement measures to protect human health. The legislation specifically directs the Environmental Protection Agency (EPA) 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".

The laws specifically direct the Environmental Protection Agency (EPA) 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 other endocrine effect as the [EPA] Administrator may designate."

These new regulations and the growing public health concerns have prompted increased research to understand how endocrine-disrupting compounds (EDCs) interfere with normal endocrine functions. Extensive testing has been initiated to explore the range of adverse effects resulting from exposure to these substances. The EPA and National Institutes of Health have been funding significant biomedical research studies on endocrine disruptors. To comply with legal mandates, the EPA proposed the Endocrine Disruptor Screening Program (EDSP) in 1998, initiating large-scale efforts to develop, standardize, and validate test methods for this program (EDSTAC 1998; EPA 1998, 2004). The EDSP aims to prioritize and evaluate more than 80,000 existing chemicals and all new chemicals, targeting estrogenic, androgenic, and thyroid hormone effects, as well as impacts on reproduction, development, and growth. Furthermore, the EPA is developing methods to determine whether EDCs cause adverse effects in ecologically important species, including amphibians, birds, fish, and invertebrates.

Due to the urgency of filling knowledge gaps about endocrine disruptors and meeting statutory deadlines, traditional species, animal strains, and laboratory procedures have been used for most research and testing efforts. However, subsequent studies have indicated that these traditional models and procedures can lead to difficulties in replicating results across different laboratories and reduce the ability to detect adverse effects from EDCs. Reports have shown that some chemicals induce endocrine effects at very low doses, but attempts to replicate these findings have often been unsuccessful. Two main factors contribute to this variability: significant differences in response to EDCs among rodent species, stocks, and strains (EDMVS 2003; Spearow 2003; Spearow et al. 1999), and the high and variable levels of estrogenic activity in standard laboratory animal diets due to phytoestrogen content (Boettger-Tong et al. 1998; Thigpen et al. 2004). Although the full implications of these and other parameters are not entirely understood, it is clear that diet and strain selection profoundly affect the outcomes of endocrine-related studies.

It is evident that careful selection of animal models and experimental design parameters for endocrine disruptor research is crucial to minimize confounding variables and enhance the reproducibility of results. Despite a preference among scientists to use traditional species, strains, and laboratory procedures, it is imperative to review and adjust these decisions based on current scientific knowledge. As the EPA and other regulatory authorities proceed with the development, standardization, and validation of endocrine disruptor testing methods, minimizing potentially confounding variables is essential to achieve optimal intra- and interlaboratory reproducibility (ICCVAM 1997; Stokes 2002). This will likely necessitate selecting highly sensitive strains and species and avoiding highly estrogenic diets to achieve dynamic response ranges capable of detecting even weakly active EDCs.

Assessing human risks from chemical exposures typically involves extrapolating data from in vitro and in vivo test systems, which introduces uncertainties about whether the same dose-related effects observed in animals will occur in humans. These uncertainties can be reduced by increasing our understanding of the biological response similarities and differences between animals and humans. Similarly, environmental risk assessments to protect thousands of species will rely on extrapolating results from testing a few representative species. To ensure adequate protection of diverse animal species, it is essential to gain more knowledge about cross-species similarities and differences in susceptibility. This issue provides the basis for selecting and using many of the animal models for endocrine disruptor research and testing.

This issue of the ILAR Journal aligns with the Institute of Laboratory Animal Research (ILAR) objectives to provide current scientific information that supports high-quality animal research and testing, and facilitates the refinement, replacement, and reduction of animal use where scientifically feasible. The first series of articles includes reviews and discussions of key laboratory animal experimental design issues for endocrine disruptor research and testing. The subsequent series of articles describes commonly used laboratory animal and in vitro models currently employed or investigated for endocrine disruptor research. While it is impossible to cover every design consideration and animal model in a single issue, these expert reviews aim to address the most significant current issues and describe the most commonly used animal models.

2. Experimental Design Considerations for Endocrine Disruptor Studies

One of the key motivations for enacting new laws to screen chemicals for endocrine-disrupting effects was the recognition that traditional toxicity testing methods often failed to detect many endocrine-related adverse effects, particularly subtle effects on fetuses. Although U.S. laws set a deadline

for the implementation of an endocrine disruptor screening program, this deadline has been missed by several years, despite well-organized efforts to comply. In her commentary, Theo Colborn (2004) attributes this delay partly to the continued use of traditional toxicological endpoints and practices and a reluctance to adopt new approaches. She advocates for testing systems designed to detect time- and hormone-specific effects over a broad range of doses, including low-dose exposures, using nontraditional and more sensitive endpoints, such as those employed to characterize low-dose endocrine-mediated effects of TCDD and bisphenol A (Colborn 2004). She emphasizes the importance of evaluating exposures during highly sensitive prenatal developmental stages and using appropriate animal diets and strains (Colborn 2004).

Commercial laboratory animal diets often contain high levels of phytoestrogens such as diadzein and genistein from soybean meal, and coumestrol from alfalfa. Colborn (2004) and Thigpen et al. (2004) highlight that high phytoestrogen levels in diets have confounded many low-dose endocrine disruptor studies. For instance, the phytoestrogens in commercial diets can cause strong responses in control animals in the uterotrophic bioassay, potentially obscuring the differences between animals fed diethylstilbestrol, a strong estrogenic chemical, and those fed only the commercial diet (Thigpen et al. 2004). This suggests that standard laboratory diets could produce false-negative results not only in the uterotrophic assay but also in other endocrine disruptor assays. Thigpen and colleagues review research demonstrating that high phytoestrogen content in standard laboratory diets can significantly confound endocrine disruptor studies. Another common endpoint in endocrine disruptor testing, the age of vaginal opening, can be accelerated by standard diets similarly to EDCs with estrogenic activity, reducing the dynamic response range and potentially obscuring weak effects. Therefore, it is crucial to use diets free of agents that can modulate the endocrine system (Thigpen et al. 2004).

Selecting the appropriate species and strain/stock of animal models is another critical consideration in endocrine disruptor studies (Colborn 2004; EDMVS 2003; Everitt and Foster 2004; Spearow 2003). For example, research by Spearow et al. (1999) showed that two inbred mouse strains were 16 times more sensitive to estrogenic substances than outbred CD-1 mice. Similarly, outbred CD-SD rats were significantly less responsive to estrogenic substances than inbred F344 rats for various estrogen-sensitive endpoints (Colborn 2004). Colborn emphasizes that considering strain/stock sensitivity for specific endpoints in endocrine disruptor studies is essential to minimize the likelihood of false-negative results.

EDCs and dietary phytoestrogens can also significantly influence behavioral and physiological effects. Lephart et al. (2004) review the impact of dietary soy isoflavones on behaviors related to consumption, learning, memory, and anxiety, as well as on food and water intake, adipose deposition, and serum leptin and insulin levels. For instance, rats fed phytoestrogen-containing diets exhibited reduced anxiety compared to those on phytoestrogen-free diets (Lephart et al. 2004). Additionally, sex differences in learning and memory were observed among rats on different diets. Long-term studies found that rats on the highest phytoestrogen diets had the lowest body and adipose tissue weights, whereas those on phytoestrogen-free diets had the highest weights (Lephart et al. 2004). These findings underscore the importance of controlling dietary phytoestrogen content not only for endocrine disruptor studies but also for all animal research and testing.

While differences in diet and strain/stock sensitivity are known sources of variation in endocrine disruptor studies, many other animal housing and environmental factors can also confound results. Everitt and Foster (2004) review several such factors, providing examples of how environmental conditions have interfered with studies. For instance, chemicals with potential EDC activity were found to be released from plastic animal cages after treatment with harsh chemical cleaning agents (Howdeshell et al. 2003). Other 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 potential EDC exposure during in utero development are often evaluated in endocrine disruptor studies. Vandenbergh (2004) discusses the heightened sensitivity of the fetus to both endogenous hormones and EDCs during critical periods of organ and system development. Studies have shown that the intrauterine position of female fetuses relative to male fetuses affects various sexually dimorphic traits. For example, the anogenital distance at birth of a female fetus with two adjacent male fetuses is shorter than that of a female fetus with two adjacent female fetuses (Vandenbergh and Huggett 1995). Perturbations in anogenital distance due to in utero exposure have been documented and may serve as a sensitive indicator of EDC effects. Therefore, for some EDC studies, it may be important to observe or estimate intrauterine position for each fetus or pup and assign animals to experimental groups accordingly.

3. Animal Models for Endocrine Disruptor Studies

Assessing the potential adverse effects of endocrine-disrupting chemicals (EDCs) on humans and various animal species in the environment necessitates the use of surrogate species for extrapolation to other species. Fortunately, the phylogenetic conservation of genetic and cellular structures and functions across species—from simple organisms to complex ones—facilitates these extrapolations. Ecotoxicology assessments must consider not only individual effects but also population effects that can impact species higher in the food chain. The articles in this section discuss models for predicting ecotoxicology effects and describe mammalian and in vitro models for evaluating potential human health effects.

DeFur (2004) reviews the use of invertebrate species as models for endocrine disruptor research and testing, noting that hormone systems exist in all invertebrate phyla, including arthropods, mollusks, and nematodes. Invertebrates are crucial to every ecosystem. The EPA plans to include an opossum shrimp or other invertebrate life cycle toxicity assay in its EDSP Tier 2 analysis. However, DeFur notes that other invertebrate test systems will be necessary to assess risks to the diverse range of invertebrate species and emphasizes the need for comparative endocrinology research to understand the usefulness and limitations of invertebrate assays as indicators of potential vertebrate effects.

Birds are highly sensitive to toxic substances, including EDCs. Consequently, an avian two-generation test has been proposed by the EPA for the EDSP Tier 2 analysis. Touart (2004) reviews the rationale for including birds in the EDSP, focusing on the Japanese quail as the animal model for the avian test. The test design includes exposures during in ovo, juvenile, subadult, and adult life stages, assessing effects on survival, growth, reproduction, and general toxicity. Touart highlights the need for improved husbandry and handling procedures to reduce confounding behaviors and unintended mortality, and for identifying the most suitable genetic strain of Japanese quail for endocrine disruptor testing.

Adverse effects on fish from EDC exposure in wastewater and industrial discharges are well documented (Ankley and Johnson 2004). These findings have spurred additional laboratory research to better understand the mechanisms and dose-relationships of EDCs. The EPA has proposed including fish tests in both Tier 1 screening and Tier 2 multigenerational studies in their EDSP, and is standardizing and validating fish test systems. Ankley and Johnson (2004) review the hypothalamic-pituitary-gonadal axis in fish, its role in sexual development and reproduction, and relevant endpoints to assess EDC effects. The fathead minnow, Japanese medaka, and zebrafish are being evaluated as models for EDC testing, with Ankley and Johnson discussing each model's advantages and limitations.

Endogenous estrogens are vital for the reproductive, neuroendocrine, skeletal, and cardiovascular systems (Walker and Korach 2004). Exogenous EDCs with estrogenic or antiestrogenic activity pose significant health concerns. Understanding the roles of estrogen receptors is essential for discerning beneficial versus harmful effects. Gene knockout techniques have enabled the creation of unique mouse models to study these receptors. Walker and Korach (2004) review the two forms of estrogen receptors, ER-alpha and ER-beta, and describe phenotypic differences observed in knockout models, enhancing our understanding of endocrine dysfunction at the cellular and molecular levels.

The laboratory rat has traditionally been the model of choice for developmental and reproductive toxicity testing to support human health risk assessments. Gray et al. (2004) review the similarities and differences between rat and human reproductive functions, noting their high conservation at the cellular and molecular levels. Three short-term rat assays are being standardized for the EDSP Tier 1 Screening Battery: the 3-day uterotrophic assay for estrogen activity, the 10-day Hershberger assay for androgen activity, and the 21-day pubertal female rat assay for estrogenic and antithyroid activity. These assays, along with the proposed pubertal male rat and in utero-lactational assays, will detect disruptions in reproductive system development.

The rat is also the model for the EDSP Tier 2 mammalian multigeneration test, which will further evaluate chemicals that test positive in Tier 1. These tests cover all critical developmental stages and reproductive function of animals exposed in utero. Gray et al. (2004) emphasize the need for sufficient animal numbers to detect reproductive effects and suggest that more sensitive endpoints and thorough evaluations could reduce overall animal use. They also advocate for flexibility to incorporate new, more sensitive assays and endpoints in the future.

In vitro test systems are proposed for the EDSP Tier 1 screening battery, providing mechanistic information useful for prioritization and screening. Current in vitro assays include estrogen and androgen receptor binding or reporter gene assays, and a steroidogenesis assay with minced testis. An in vitro placental aromatase assay is also under evaluation. Charles (2004) discusses the need for standardization and validation of these systems according to established criteria. Although in vitro tests provide valuable information, they cannot fully replace in vivo systems, which account for chemical absorption, metabolism, distribution, and excretion. Nonetheless, in vitro test systems are seen as integral to future endocrine research and testing programs.

4. Conclusion

Properly designed high-quality endocrine disruptor research studies and toxicity test systems must rigorously control or eliminate factors contributing to experimental variation. These studies and systems should employ appropriate biological models selected for their optimal sensitivity and specificity to achieve the research or testing objectives. By minimizing and controlling variables, such research will enhance data reproducibility both within and among laboratories and may also reduce the number of animals required per experimental group.

5. References

Ankley GT, Johnson RD. 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J 45: 469-483.

Boettger-Tong HL, Murphy C, Cahiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Makela S. 1998. A case of laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. Environ. Health Perspect 106:369-373.

Carson R. 1962. Silent Spring. Boston MA: Boston University Press. Charles GD. 2004. In vitro models in endocrine disruptor screening. ILAR J 45:494-501. Colborn

T. 2004. Commentary: Setting aside tradition when dealing with endocrine disruptors. ILAR J 45:394-400.

Colborn T, Dumanoski D, Meyers JP. 1996. Our Stolen Future: Are We Threatening Our Fertility, Intelligence, and Survival? A Scientific Detective Story. New York: Plume/Penguin Books.

deFur PL. 2004. Use and role of invertebrate models in endocrine disruptor research and testing. ILAR J 45:484-493. EDSTAC [Endocrine Disruptor Screening and Testing Advisory Committee]. 1998. Final Report. Available online: http://www.epa.gov/scipoly/ oscpendo/history/finalrpt.htm. EPA [Environmental Protection Agency]. 1998.

Endocrine Disruptor Screening Program: Statement of Policy; Notice Endocrine Disruptor Screening Program: Priority-Setting Workshop; Notice. 63 FR 71541, December 28, 1998.

EDMVS [Endocrine Disruptor Methods Validation Subcommittee]. 2003. White Paper on Species/Strain/Stock in Endocrine Disruptor Assays. Available online: http://www.epa.gov/scipoly/oscpendo/assayvalidation/mtg_081803.htm.

EPA [Environmental Protection Agency]. 2004. Endocrine Disruptor Screening Program. Available online: http://www.epa.gov/scipoly/ oscpendo/edspoverview/index.htm [accessed 12 July 2004].

ICCVAM [Interagency Coordinating Committee on the Validation of Alternative Methods]. 1997.

Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. (NIH Publication No: 97-3981).

Research Triangle Park NC: NIEHS. Available online: http://iccvam.niehs.nih.gov/docs/guidelines/validate.pdf [accessed 29 August 2003].

Everitt JI, Foster PMD. 2004. Laboratory animal science issues in the design and conduct of studies with endocrine-active compounds. ILAR J 45:417-424.

Gray LE Jr, Wilson V, Noriega N, Lambright C, Furr J, Stoker TE, Laws SC, Goldman J, Cooper RL, Foster PMD. 2004. Use of the laboratory rat as an animal model in endocrine disruptor screening and testing. ILAR J 45:425-437.

Howdeshell KL, Peterman PH, Judy BM, Taylor JA, Orazio CE, Ruhler RL, Vom Saal FS, Welshons WV. 2003. Bisphenol A is released from used polycarbonate animal cages into water at room temperature. Environ Health Perspect 111:1180-1187.

ICCVAM [Interagency Coordinating Committee on the Validation of Alternative Methods]. 2003. Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. (NIH Publication No. 03-4508). Research Triangle Park NC:
NIEHS. Available online: http://iccvam.niehs.nih.gov/docs/guidelines/subguide.htm

IPCS [International Programme on Chemical Safety] 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. Damstra T, ed. Geneva: World Health Organization.

Lephart ED, Setchell KDR, Handa RJ, Lund TD. 2004. Models for evaluating behavioral effects of endocrine-disrupting substances: Phytoestrogens. ILAR J 45:443-454. NRC [National Research Council]. 1999. Hormonally Active Agents in the Environment. Washington DC: **National Academy Press**. PL [Public Law] 104-170. 1996a. Food Quality Protection Act (FQPA) of 1996. Available online: http://www.epa.gov/opppsps1/fqpa/gpogate/ pdf [accessed 12 July 2004]. PL [Public Law] 104-182. 1996b.

Safe Drinking Water Act Amendments (SDWA) of 1996. Available online: http://www.epa.gov/safewater/ sdwa/text.html [accessed 12 July 2004].

Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. 1999. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 285:1259-1261.

Spearow J. 2003. White Paper Presentation on Species/Strain/Stock inEndocrine Disruptor Assays. Available online: http://www.epa.gov/ scipoly/oscpendo/assayvalidation/mtg_081803.htm.

Stokes WS. 2003. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): Recent Progress in the Evaluation of Alternative Toxicity Testing Methods. In: Salem H and Katz S, eds. Alternative Toxicological Methods. New York: CRC Press. p 15-30.

Thigpen JE, Setchell KDR, Saunders HE, Haseman JK, Grant MG, Forsythe DB. 2004. Selecting the appropriate rodent diet for endocrine disruptor research and testing studies. ILAR J 45:401-416.

Touart LW. 2004. Factors considered in using birds for evaluating endocrine-disrupting chemicals. ILAR J 45:462-468. Vandenbergh JG. 2004. Animal models and studies of in utero endocrine disruptor effects. ILAR J 45:438-442.

Vandenbergh JG, Huggett CL. 1995. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 45:567-573.

Walker VR, Korach KS. 2004. Estrogen receptor knockout mice as a model for endocrine research. ILAR J 45:456-462.