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Review Article

Overview of EDSP Studies and Important Points for Evaluation of Potential Endocrine Mediated Effects

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Abstract:

To identify the chemicals which have potential to interact either with the androgen, estrogen, and/or thyroid system, EPA has developed two-tiered endocrine disruptor screening program (EDSP). Tier-1 contains 4 *in vivo* assays in rodent, 1 in frog and fish, whereas 5 *in vitro* assays. In 2009, EPA has adopted all the *in vivo* and *in vitro* guidelines for testing of chemicals which were identified as positive for endocrine mediated effects. Outline of each assays of Tier-1 and 2 is discussed. EPA view on dose range finding study for dose level selection is described. The phenomenon between maximum tolerated dose and endocrine-mediated effect is described in order to identify the potential of chemicals for endocrine effects. EPA has set performance criteria for each sensitive endpoint of every study. EPA wants that laboratories should make sure for following those performance criteria. However, if any endpoint is deviated from the set values of EPA, it does not invalidate the study. Hence, to ensure specificity for endocrine effects, the results of each Tier-1 assay is evaluated using a weight-of-evidence approach. Complete details of individual test can be finding from relevant guidelines; however, some of important points are discussed here.

Keywords: Tier-1 and 2, Maximum tolerated dose, weight-of-evidence, endocrine disruptor screening program (EDSP), Environmental Protection Agency (EPA).

OVERVIEW OF TIER-1 AND 2:

Since the publication of *Silent Spring* [1], there has been increasing consciousness that chemicals in the environment can exert profound and harmful effects on wildlife populations and that human health is in distinguishably linked to the health of the environment.

The last 20 years, in particular, have witnessed a growing scientific concern, media attention, and public debate, over the possible

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harmful effects that have the potential to interfere with the endocrine system.

Food Quality Protection Act (FQPA) mandated the US Environmental Protection Agency (EPA) to develop and retain a screening program to study the potential of chemicals to interfere with the endocrine system in humans [2]. Following this, EPA convened a federal advisory committee, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to evaluate the current state-of-the-science and help in developing an endocrine screening program [3].

EDSTAC [4] has numerous chemicals and mixtures that need to be considered for evaluation. To handle the immense number of chemicals in the list, EDSTAC recommends an initial sorting and prioritizing of the chemicals, followed by a tiered approach to identify endocrine-disrupting chemicals (EDCs) and quantify their effects.

Initial Sorting

It includes an evaluation of existing data on a chemical. On the basis of that information, a chemical is classified into one of four categories:

1. if sufficient data is available on chemicals (primarily polymers) which indicate that they are not likely to interact with the hormone systems (estrogen, androgen, thyroid) and therefore require no further analysis (such chemicals will be placed in a “hold box”);
2. if chemicals with insufficient data and therefore require tier 1 screening for hormonal activity;
3. chemicals with sufficient evidence of hormonal interaction and hence require tier 2 testing;
4. Chemicals with sufficient evidence of hormonal interaction and hormone-related effects and therefore require hazard assessment.

Priority Setting

Chemicals which are placed in the second category will be prioritized for evaluating on the basis of information available on exposure, effects, and statutory criteria.

Tier Testing

EPA developed a two-tiered structure for evaluating the chemicals and approached was named as the Endocrine Disruptor Screening Program (EDSP). The EDSP Tier 1 Battery consists of eleven assays (six in vivo and five in vitro) that proposed to discover the potential of a chemical to interact with the androgen, estrogen, steroidogenesis, or thyroid systems. The robustness of the Tier 1 battery is based on the strengths of individual assay. Tier1 screening battery is being designed to minimize false negatives effects, based on an assessment of the ability of the battery to detect known EDCs that act via estrogen, androgen, and testosterone (EAT). In connection with the point previously mentioned, the value of each individual assay must be considered by combining the strength of one assay over the limitation of other assay [4].

EDSP Tier 2 testing is designed to discover and establish a dose response relationship for any adverse effects which might be arising from the interactions with the endocrine system. Thus, the aim of the EDSP is to identify and differentiate the chemicals which have ability to interact from those that have minimum or no such ability. EPA intends to use a weight-of-evidence (WoE) approach to make this determination [5, 6].

The issue of dose–response relationships is the most debatable issue regarding EDCs. EDCs often act by interacting with the actions of endogenous hormones. These hormones are available at physiologically functional concentrations, so the dose–response effects for EDCs are often different than for

chemicals which are not acting through the endocrine system.

Research has clearly shown that EDCs can act at multiple sites via multiple mechanisms of action. Hormone synthesis, transport, and metabolism mechanism have been shown to be equally important for understanding [7]. However, the mechanism(s) of action are not understood properly. This makes it difficult to differentiate between direct and indirect effects and primary versus secondary effects of exposure to EDCs. It also indicates that considerable carefulness is essential in extrapolating from *in vitro* data to *in vivo* effects, in predicting effects from limited *in vivo* data, and in extrapolating from experimental data to the human situation [8]. A collective weight of evidence is essential in determining under what conditions observed effects resulting from exposure to EDCs occur via endocrine mediated mechanisms.

There are a number of complex issues that must be considered when evaluating the effects of endocrine disruptors [9]. Unluckily, many of the studies do not have good measures of exposure, which limits researcher's ability to draw firm conclusions. This problem is especially common for those chemicals that degraded rapidly in the environment or human body.

To draw conclusion for the chemicals which are having adverse effects on the endocrine activity, all of the available and relevant information needs to be considered in an organized and structured manner. Complete details of individual test can be finding from relevant guidelines; however some of important points are discussed here.

***In vivo*– animals models (Tier-1)**

Male Pubertal Assay (US EPA) [10]**:**

This assay have the ability to identify the chemicals which have potential to show androgenic, anti-androgenic, anti-thyroid activity, and changes in gonadotropins, prolactin, or in hypothalamic function which ultimately alter pubertal development. During 31 days of treatment from PND23, thyroid toxicant, chemicals which have potential to alter pubertal development, serum testosterone level, accessory sex organs and, pituitary, liver, adrenal, kidneys are evaluated.

Female Pubertal Assay (US EPA) [11]**:**

The chemical which have potential to show activity of anti-thyroid, estrogenic or anti-estrogenic, changes in follicle stimulating hormone, luteinizing hormone, prolactin, or in hypothalamic function which ultimately alter pubertal development. During 21 days of treatment from PND22, thyroid toxicant, chemicals which have potential to alter pubertal development, and organs weights (ovaries, uterus, liver, pituitary, kidneys, and, adrenal) are evaluated. This also tells about estrous cyclicity.

Hershberger Assay (US EPA) [12]**:**

The castrated male rats are used to detect the chemicals with potential to act as androgen agonists, and antagonists depending on the weight of androgen sensitive organ weights.

Uterotrophic Assay (US EPA) [13]**:**

The chemicals which are having estrogen agonist property are evaluated. Immature rat and ovariectomized methods are two methods are recommended by test guideline, however later is preferred. Immature method can show response to hypothalamic-pituitary-gonadal (HPG) axis, whereas ovariectomized animals does not have functional HPG axis.

Amphibian Metamorphosis Assay (US EPA) [14]**:**

The amphibian metamorphosis assay, is conducted in Xenopuslaevis, evaluates chemicals with the potential to interfere with the function of the Hypothalamus–Pituitary–Thyroid axis (HPT). This assay also evaluates general growth and development of tadpole when chemicals are administered through water.

Fish Short-Term Reproductive Assay (US EPA) [15]**:**

This assay detects alteration in HPG axis through changes in biochemical endpoints, spawning, morphology, and gonadal histopathology in sexually mature Pimephales promelas.

***In vitro* models (Tier-1)**

Estrogen Receptor Binding Assay (US EPA) [16]**:**

Chemicals which interact with the estrogen receptor respond in this assay. In this assay, 17 b-estradiol, endogenous hormone, is prepared from rat uterine cytosol. Chemical's receptor-binding affinity,which displaces the endogenous hormone, is measured. However, whether chemicalwill act as estrogen agonist or antagonist cannot be evaluated.

Estrogen Receptor Transcriptional Activation Assay (US EPA) [17]**:**

This assay is sensitive assay that identify the chemicals which bind and activate the estrogen receptor. This assay measures the agonist nature of chemicals. Luciferase enzyme, which is activated by chemicals which are estrogen agonist (estrogenic), is measured by quantifying the light emission reaction through luminometer. The amount of light produced is proportional to concentration or/and potency of chemicals.

Androgen Receptor Binding Assay (US EPA) [18]**:**

Using androgen receptor isolated from rat ventral prostate, this assay identifies the chemicals which interact with the androgen receptor. It measures the receptor-binding affinity of chemicals by evaluating ability to displace a bound reference androgen, typically radiolabeled R1881, a synthetic androgen. However, it has limitation that this assay cannot distinguish the agonist or antagonist nature of chemical.

Aromatase Assay (US EPA) [19]**:**

This assay identifies the chemicals that can inhibit the catalytic activity of aromatase through an interaction with the substrate binding site on the enzyme. This assay is capable to detect the conversion of androgen to estrogen in various target tissues or cell lines, or recombinant aromatase, and cyp450 reductase.

Steroidogenesis Assay (US EPA) [20]**:**

The chemicals that affect the steroidogenic pathway, from the sequence of reactions occurring after the gonatotropin hormone receptors through the production of testosterone and estradiol/estrone are identified. It measure cell viability, hormone production, and cytotoxicity in the human H295R adrenocortical carcinoma cell line.

***In vivo* – animal's models (Tier-2)**

Tier 2 testing consists of a group of in vivo tests designed to identify and characterize chemical induced interactions with androgen, estrogen, and/or thyroid for risk assessment. Tier 2 tests are designed to quantify dose-response relationships in a larger context of toxicity and potential adversity that may involve other biological systems to be used for risk assessment.

Following are the list of studies which are included in Tier 2 test:

Mammalian two-generation reproductive toxicity test (OECD) [21]**:**

Two-generation reproduction testing is designed to `provide general information concerning the effects of a chemical on the integrity and performance of the male and female reproductive system, and on the growth and development of the offspring.

Avian two-generation toxicity test (ENV) [22]**:**

The Japanese Quail Two-Generation Toxicity Test is used to characterize the nature and dose response relationship of chemical with endocrine bioactivity on birds. There are four

critical life stages during which endocrine mediated processes occurred and therefore could be sensitive for endocrine disruption: 1) in ovo, 2)offspring (F1) generation chick growth, 3) parental (P1) generation and F1 sexual maturation, and 4) P1and F1 egg-laying. Hence, two egg-laying cycles to assess effects on ecologically relevant endocrine dysfunction at each of these stages are essential.

Larval amphibian growth and development assay (OECD) [23]**:**

African clawed frog (Xenpouslaevis) is used as a surrogate to identify and characterize the adverse consequences of exposure. The chemicals which interfere with the normal development and growth of amphibians from embryo-larval development, through metamorphosis and early juvenile development are detected. Adverse effects identified may be caused by interaction of a chemical in amphibians, especially those active within the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-gonadal (HPG) systems.

Fish Multi-Generation Test (OECD) [24]**:**

This test measures the reproductive performance of medaka. This test measure chemical effect on reproduction and reproductive development in medaka exposed through multiple generations of their life-cycle.

Invertebrate two-generation test (OECD) [25]**:**

This test is designed to cover critical life states, in order to provide data on effects from endocrine and other mechanisms of action, and for assessment of adverse effects. Thus, tests for endocrine disruption often encompass two generations to address effects on fertility and mating, embryonic development, neonatal growth and development, and transformation from the juvenile life state to sexual maturity.

IMPORTANT POINTS FOR TIER-1 STUDY

Following points are place crucial role for in vivo rodent ED study:

Dose level selection:

Selection of proper dose for ED studies are challenging job for toxicologist. Test guidelines (TG) provide the following points for selection of dose levels:

- The highest dose level should be at or just below the Maximum Tolerated Dose (MTD) level but not more than limit dose of 1 g/kg/day.
- The MTD may be exceeded if abnormal blood chemistry values are seen at termination specifically blood urea nitrogen (BUN) and creatinine.
- The MTD may be exceeded if histopathology of the kidney (or any other organ where gross observations indicate damage) shows any damage.
- The high-dose should not cause the reduction greater than approximately 10% of the mean terminal body weight compared to the controls, and there are no associated clinical signs of toxicity.

Dose range finding (DRF) study:

Though guideline does not discuss to conduct dose range finding study for proper selection of dose levels for in vivo rodent studies, however they recommend providing justification for same. There are some studies which show the time of treatment commencement, place an important role for selection of dose levels. If a DRF is being performed in animals of 6-7 weeks, then selected doses can be suitable for Hershberger and Uterotrophic bioassay, however, in contrast to this, in pubertal assay, selected doses can cause more severe symptoms then expected as animals are 22 or 23 postnatal day old. Based on the author's experience and the potential differences between immature

and adult rats, the selected doses could cause severe symptoms, mortality, and MTD can be exceeded, if age is not taken in consideration while conducting dose range finding study specifically for in vivo ED studies.

If the data of previously conducted study is available with other route of administration, and not the one which is recommended by test guidelines (TG), then toxicologist prefers to conduct DRF. Because toxicokinetic of chemicals differ with the change in route of administration.

Number of Groups:

TG recommends conducting *in vivo* ED studies with only two dose levels. However, problem faced by toxicologist is, if the highest dose is established as MTD in presence of systemic toxicity. Then toxicity can lead to non-specific changes in endocrine related endpoints which can give false interpretation for chemical. Hence, it is better to conduct the DRF so toxicologist can avoid selection of such doses which can be established as MTD.

TG recommends lower dose should be half of the highest dose level. With two dose levels (low and high), dose relationship can't be decided. Whereas, third dose level will help for a better interpretation of dose–response relationships. Additional dose level will also ensures that there are sufficient groups below the MTD which will allow to interpret assay results properly (i.e., if the high dose is established as MTD then there are still two dose level based on which data can be evaluated for potential endocrine activity). While the addition of extra dose levels (e.g., three dose levels and a control) makes pubertal assay data applicable or useable for risk assessment purposes [26]. Addition of third dose level will also increase the cost of the study.

The consequence of over- or underestimating dose concentrations could result in an invalid study.

Systemic toxicity and Pubertal Development:

In male pubertal assay, at termination ≥6% decrease in body weight gain should be evaluated with care using a WoE approach in presence of available information or additional studies. Care should be taken to select dose levels where highest dose level should not cause >10% decrease in body weight gain from control animals on day of terminal sacrifice. Because, in routine toxicology studies also >10% decrease in terminal body weight is likely to observe.

Presence of systemic toxicity including toxic clinical sign (like cholinesterase inhibitor produces the signs at very low dose level) can cause the decrease in body weight and slows growth rate of animals. In the case where only two dose levels are used, and if high dose animals shows the toxic clinical sign, under such condition it is difficult for toxicologist to conclude the true endocrine-mediated effects.

Pubertal development and organs weights in the pubertal assays can be altered by changes in rate of body weight during growth period. Hence, it is difficult to interpret assay data and distinguish specific endocrine-mediated effects. There are some contradictory reports on the sensitivity of puberty onset to moderate decrease in the body weight. Laws *et al* [27] reported that 20–21% decrease in body weight did not significantly affect age at puberty onset in male or female rats, suggesting that the age at puberty onset is insensitive to changes in growth. However, other studies suggest that the age at puberty onset and body weight [28], and body weight alterations of approximately 15% could alter puberty onset in male rats [29]. These differences may be related to the rate at which the body weight reduction occurred (i.e., how

quickly it occurred and over what time frame/ages and for how long it appears).

Regardless of the cause for the differences, analysis of the pubertal onset data can be confused by numerous factors including body weight, toxic clinical sign, and secondary effect to systemic toxicity and therefore care must be taken in interpreting statistically significant effects to identify true endocrine-mediated effects from secondary effects due to systemic toxicity.

A important endpoint of the pubertal assays is age and body weight at puberty onset (PPS and vaginal opening). Puberty onset can be influenced by diet composition, growth hormone, etc. eg. [30-35]. Since attainment of puberty is a subjective evaluation hence to avoid interpersonal variation laboratory should train their personnel according to detailed standard operating procedure.

Estrous Cyclicity:

In author lab, vaginal opening in wistar rats generally takes place 5-6 days in SD rats, before terminal sacrifice on PND-42. Normal estrous cycle length is approximately 4-5 days. Hence, effect of test chemical on estrous cyclicity is challenging job. If estrous cycle evaluation begins in mid-cycle, then it may take 8 days or longer to observe two successive estrous cycles. It is common for young animals to cycle irregularly initially. It is well known that animals take about 8 weeks of age for normal cycles to begin). It is influenced by various factors such as stress, feed intake, and hormonal imbalance [36-38].

Each female should be characterized as "regularly cycling," or "irregularly cycling," or "not cycling" when conducting female pubertal assay. However, due to short monitoring interval, evaluation of a full estrous cycle is difficult. In addition, there are inter-animal differences in the duration of estrous cycle monitoring such that monitoring across the dose groups is often unbalanced. To perform a thorough assessment of estrous cyclicity, it requires at least 2 week period as required in the reproduction study design or female pubertal assay can be extended up to another 14 days from PND 42.

Thyroid hormone and Thyroid Microscopic:

In male and female pubertal assay, T4 and TSH should be determined, additionally serum testosterone in males. However, toxicologist should take care when evaluating the changes in hormonal level in absence of effect on thyroid weight and microscopy.

Variation in thyroid hormone level could be affected by number of factors including decrease in body weight or body weight gain [27], stress at the time of necropsy [39], or nutritional status of the animals [40]. The U.S. EPA has advocated that "the significance of changes in thyroid hormone levels in the absence of corroborative microscopy changes will be evaluated in the context of the overall toxicity of the chemical using the WoE approach including the thyroid toxicity data.

Any changes in observed in microscopy should be interpreted in combination with serum thyroid hormone levels. Because effects on thyroid are typically reversible if the effects on serum thyroid hormone are not continued. In at least one case from our lab, a chemical was designated as altering thyroid microscopy despite the lack of a statistically significant difference in thyroid values. In this case, the judgment of experienced toxicologist/pathologists had more utility than quantitative thyroid values.

Coefficient of variation (Performance criteria):

Coefficient of variation (CV) for pubertal assays has been established by EPA for maximum endpoints in control group. These criteria indicate that the study conducted was adequately sensitive to allow true conclusions on chemical effect. In hershberger bioassay,

maximum 3 of the 10 tissue weights should not exceed the defined CV. If 4 or more tissues exceed the CV, then ideally the assay should be repeated. According to TG, it is important for assays with negative result to meet the performance criteria. For uterotrophic assay, no specific CV is established; however, baseline uterine weights must be below specifications given in the TG.

Conclusion:

Hence, the points discussed above can be helpful for registrant as well as study director for designing the study and increase the acceptance percentage. This will also help to identify the true endocrine-mediated effects or secondary effect to systemic toxicity.

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