The Juvenile/Peripubertal Assays, also known as the "Female and Male Pubertal Assays"

Background

The female pubertal assay is a screening-level, apical, *in vivo* assay which detects effects of substances on pubertal development and thyroid function in the intact juvenile/peripubertal female rat. The assay detects compounds that display antithyroid, estrogenic, or antiestrogenic activity, or alter puberty via changes in luteinizing hormone, follicle stimulating hormone, prolactin or growth hormone levels or via alterations in hypothalamic function.

The male pubertal assay is also a screening-level, apical, *in vivo* assay. It was designed to detect effects of substances on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. The assay detects compounds that display antithyroid, estrogenic, androgenic, or antiandrogenic activity, or alters puberty via changes in follicle-stimulating hormone, luteinizing hormone, prolactin, growth hormone or hypothalamic function.

The female pubertal assay was recommended by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) for inclusion in the Tier 1 Screening battery of the Endocrine Disruptor Screening Program (EDSP); its counterpart in pubertal males was recommended for validation as part of a potential alternative combination of assays.

Description of the assays

Female pubertal

Fifteen female rats in each of two dose groups (maximum tolerated dose (MTD) and 1/4 MTD) are gavaged daily from post-natal day PND 22 through 42. Each pup is examined daily for onset and completion of vaginal opening (VO). Vaginal smears are taken every day post-VO to evaluate stage of the estrous cycle. At necropsy on PND 42, selected organs are weighed; certain organs are fixed for histological examination; and serum is collected for hormone analysis. Specifically, the following endpoints are required in the assay:

- Age and weight at vaginal opening
- Ovarian and uterine weights
- Ovarian and uterine histology
- Age at onset and regularity of estrus cyclicity
- Thyroid histology (follicular cell height and colloid area)
- Serum thyroxine (T_4) and thyroid-stimulating hormone (TSH) levels

- Growth (daily body weight change relative to controls)
- Liver, kidney, pituitary, and adrenal weights

All endpoints in this assay with the exception of the thyroid measurements are used in the mammalian 2-generation reproductive toxicity assay; the thyroid endpoints are standard and are commonly used for evaluation of thyroid activity.

Male pubertal

Fifteen male rats in each of two dose groups (MTD and 1/4 MTD) are gavaged daily from post-natal day PND 23 through 53. Each pup is examined daily beginning on PND 30 for onset and completion of separation of the prepuce from the glans penis (preputial separation, PPS). At necropsy on PND 53, selected organs are weighed; certain organs are fixed for histological examination; and serum is collected for hormone analysis. Specifically, the following endpoints are required in the assay:

- Age and weight at preputial separation
- Reproductive organ weights:
  testes, epididymides, seminal vesicles, ventral prostate, dorsolateral prostate, levator ani/bulbocavernosus muscle complex
- Testis and epididymis histology
- Thyroid histology (follicular cell height and colloid area)
- Serum thyroxine (T_4) and thyroid-stimulating hormone (TSH) levels
- Serum testosterone level
- Growth (daily body weight change relative to controls)
- Liver, kidney, pituitary, and adrenal weights

All endpoints in this assay with the exception of the testosterone and thyroid measurements are used in the mammalian 2-generation reproductive toxicity assay; the thyroid endpoints are standard and are commonly used for evaluation of thyroid activity.

Protocol Development

The protocols and the scientific bases for the two assays were described in detail in articles published in *Critical Reviews in Toxicology (CRT)*[1,2]. Those articles serve the function of the Detailed Review Paper which is prepared for assays being validated for the EDSP: they provide justification for the endpoints chosen for evaluation and discuss known limitations of the assays.

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[1] Goldman JM, Laws SC, Balchak SK, Cooper RL, Kavlock RJ. 2000. Crit Rev Toxicol 30(2):135-196. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations.

[2] Stoker TE, Parks LG, Gray LE, Cooper RL. 2000. Crit Rev Toxicol 30(2):197-252. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the EDSTAC recommendations.

Pre-Validation

The transferability of the assay from a research laboratory to a contract laboratory was examined. Furthermore, the sensitivity and the ability of the assay to detect the appropriate mode of action was tested by examining a number of chemicals representing different mechanisms of action and potencies.

Three separate studies were conducted:

1) *Single-Dose-Level Study (TherImmune 2000)*. The purpose of this study was to test the assays in a contract laboratory, using a variety of chemicals (6 in females, 6 in males) known to alter endocrine function (receptor agonist/antagonist, alter HPG and thyroid homeostasis). Each chemical was tested at a single (high) dose level. A subsidiary aim of the single-dose-level study was to examine the influence of two different rat strains on the sensitivity of the assay.

2) *Multi-Chemical Study (RTI 2003)*. Two dose levels of a larger variety of chemicals (6 in females, 8 in males) were tested in this study to determine the reliability of the assays in a contract laboratory.

3) *Multi-Dose-Level Study (TherImmune 2003)*. In this study, the sensitivity of the assay was further evaluated by using three dose levels of each chemical in a contract-laboratory setting. Fewer chemicals were used than in the "multi-chemical" study (3 in females, 3 in males). Some of the chemicals overlapped with the multi-chemical study (1 in females, 2 in males), but several were unique.

The first prevalidation study was conducted before the Endocrine Disruptor Methods Validation Subcommittee (EDMVS)[3] was fully operational, but the results from this study were reviewed and discussed with the EDMVS[4]. The results of the other prevalidation studies were presented to the EDMVS in Dec 2003. Results will be reviewed for the EDMVAC when the overall case for validation for these assays is presented.

Issues identified in discussions with EDMVS and resolved

The EDMVS raised significant issues concerning dose selection, effects of body weight on pubertal endpoints, ability to detect thyroid-active agents, effects of strain of rat used, and effects of phytoestrogens in feed. EPA considered the discussion about

[3] the predecessor of the Endocrine Disruptor Method Validation Advisory Committee (EDMVAC)

[4] First EDMVS meeting, Oct 2001, and second EDMVS meeting, Dec 2001.

dose-setting in its preparation for the validation study. Some members of EDMVS suggested that it is necessary to see how closely different labs can determine appropriate dose levels at which to test an unknown chemical; other members suggested that the focus of a validation study should be on the capabilities of the assay itself, not on the difficulties of dose-setting. EPA decided that in the validation study, dose levels would be the same across laboratories. The reasoning was that it matters less whether laboratories come up with similar high-dose levels in an interlaboratory comparison study than whether the high dose run for a test chemical is shown in the assay itself to be the maximum tolerated dose.

EPA's Office of Research and Development (ORD) conducted a special study to evaluate the effect of food restriction and body weight loss on the parameters measured in the pubertal assays. It demonstrated that the endpoints were not affected if a decrease in body weight was less than approximately 10% when compared to controls at the end of the assay. That is, based on ORD's results one can confidently conclude that effects on the endpoints measured in the pubertal assays are due to the test chemical rather than to body weight changes alone within this range. This information was presented to the EDMVS in July of 2002[5].

ORD also undertook several additional studies to investigate the range of applicability of the pubertal assays across modes of endocrine activity. It demonstrated that the pubertal assays will identify compounds that alter hypothalamic-pituitary control of the gonads. It also conducted several studies using thyroid-active agents, at the urging of the EDMVS. It showed that both the male and female pubertal assays detect alterations in thyroid function following exposure to compounds with different modes of thyroid-hormone-related activity.

In response to EDMVS' recommendation to write a white paper on the issue of rat-strain effects on pubertal assay endpoints, EPA prepared such a paper and presented it to the EDMVS in August 2003[6]. EPA concluded that although it appears that some strains of rats are differentially sensitive to endocrine disruptor (ED) effects, it is not possible at this time to determine which strain will be the most susceptible to which mechanism of action or endpoint. Because the pubertal assay has multiple endpoints, it was not possible to choose an optimal strain. While EDMVS did consider recommending that multiple strains be used, it decided that this would not be feasible for the multi-endpoint pubertal assays.

Other minor modifications and numerous clarifications have been made to the assays since publication of the original CRT article. Modifications made on the basis of the three contract studies described above include such things as measuring thyroid

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[5] Fifth EDMVS meeting, July 2002.

[6] Eighth EDMVS meeting, Aug 2003.

weight as a required endpoint (rather than an optional one) and changing the fixative for thyroid; clarifications include such things as specifying more clearly what anatomical structures to include in measurement of uterine weight.

Validation

Prior to conducting an interlaboratory validation study, EPA discussed the potential structure of such a study with the EDMVS[7]. The main issues discussed at that meeting were positive controls, negative controls, dose-setting, and phytoestrogen content of feed. Several other issues were also discussed; details can be found in the meeting materials posted on the Web.

Based on these discussions and its own internal deliberations, EPA decided that it was not likely to be appropriate to run positive controls for each mode of action every time the assay was performed for an "unknown" chemical, and thus it would not be necessary to include in the validation study a positive chemical for each mode of action to which these apical assays respond. Instead, it would be necessary to ensure only that all endpoints would be challenged, which could be done with fewer chemicals.

Many members of EDMVS were troubled by the lack of data showing that toxic chemicals that do not act directly on the endocrine system test negative in the assay. Based on these discussions and its own internal deliberations, EPA decided to include in the validation study a chemical that was presumed (but not known) to be endocrine-inactive despite known toxicity. Few chemicals have been tested apically for endocrine activity by methods that are thought to be reliable, and there are no chemicals *known* to be negative by all modes of action that are tested by the pubertal assays.

Some members of EDMVS suggested that the phytoestrogen content of feed be minimized in endocrine assays. Others noted that the influence of phytoestrogens in feed had been examined in the context of the uterotrophic assay, and that since preliminary indications are that the pubertal assay is similar in sensitivity to the uterotrophic assay it would not be necessary to restrict phytoestrogen levels below the level that had no effect in the uterotrophic assay. Based on this discussion and its own internal deliberations, EPA chose to place a cap on the phytoestrogen content of feed at approximately the highest level shown to have no effect in the uterotrophic assay.

The interlaboratory validation study (three laboratories each testing the same three chemicals (females; four for males) at identical dose levels) was initiated in July 2004. Draft reports were delivered to EPA in September and October 2005 and the results are scheduled to be presented to the EDMVAC at its next meeting.

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[7] Ninth EDMVS meeting, Dec 2003. Several other issues were discussed at the meeting but have been omitted here for brevity.

Other studies

Other studies which help to define the characteristics of the assay have been done since publication of the CRT article. As might be expected, the studies vary in the degree of similarity to the details of the method being suggested for the EDSP and since there are too few studies available to allow analysis of the effect of each variation, judgment will be required in determining how these studies can be used in the validation of the assay.