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Workshop 4.4

Enhanced one-generation reproductive toxicity study in rats for detecting endocrine-disrupting effects of chemicals*

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Abstract: An enhanced one-generation reproductive toxicity study in rats without adjusting a litter size during the lactation period is proposed as a rapid and reliable bioassay for providing the data concerning adverse and/or low-dose effects of suspected endocrine disruptors. In this study, pregnant females are treated with the test substance from gestation day 0 through lactation day 21, in principle. F1 offspring from one-half of the litters in each dose group are killed and necropsied at weaning, while those from the remaining litters are examined for sexual maturation, estrous cyclicity, and/or sperm production. A series of pilot studies with ethynylestradiol as a reference chemical have suggested that the exposure of estrogenic chemicals during the early gestation period is critical for detecting effects on fertilization and/or implantation of eggs and survival of implants, and that expression of some genes including AR in the prostate and IGF-1 in the uterus of F1 offspring may be sensitive markers for monitoring potential estrogenic effects of the test compound.

INTRODUCTION

Not a few chemicals are suspected of having endocrine-disrupting effects on living organisms. These include pesticides and/or their active metabolites (*o*,*p*′-DDT, *p*,*p*′-DDE, methoxychlor, and vinclozolin), phenols (bisphenol A and nonylphenol), and phthalates (DEHP and DBP). Some of these chemicals have been confirmed to interact with estrogen and/or androgen receptors by in vitro and in vivo screening assays [1–4]. Otherwise, the compounds are suggested to modulate the activity of key enzymes to synthesize steroid hormones [5–8]. As for many endocrine active compounds and/or suspected endocrine disruptors, however, adversity of their endocrine effects remains unclear at present. This paper focuses on the rapid and reliable bioassay for predicting adverse reproductive effects of chemicals on human health based on endocrine and other mechanisms for the future risk assessment.

AVAILABILITY OF A CURRENT TWO-GENERATION REPRODUCTION STUDY FOR DETECTING ADVERSE REPRODUCTIVE EFFECTS OF ENDOCRINE DISRUPTORS

The most common way to detect adverse effects of suspected endocrine disruptors may be conducting a two-generation reproduction study in rats according to the authorized guidelines [9–11]. Although the

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assay is made to examine reproductive toxicities of chemicals regardless of their underlying mechanism(s), this assay is expected to be able to detect adverse effects of endocrine disruptors. We have conducted a series of in vitro and in vivo experiments including a two-generation reproduction study to confirm the availability of these assays for use in evaluating endocrine-disrupting effects of pesticides [12]. Our results demonstrated that adverse reproductive effects of an estrogenic pesticide, methoxychlor, could be sufficiently detected by a current two-generation reproduction study, in which reproductive parameters in parental females such as estrous cyclicity, pregnancy rates, and numbers of implants and pups delivered as well as male reproductive endpoints of sperm counts were clearly affected dose-dependently (Table 1). Our results also suggest that the measurement of uterine weights of F1 and F2 female weanlings is useful to evaluate the potential estrogenicity of the test compounds. In addition, adverse effects of antiandrogenic pesticide, vinclozolin, have also been successfully evaluated by the two-generation reproduction study [13].

Table 1 Results of a two-generation reproductive toxicity study in rats with methoxychlor.

Findings	Dose levels (ppm) 10	500	1500
General toxicity on parental animals			
Reduced body weights/body weight gains	–	++	+++
Reduced food consumption	–	++	+++
Reduced accessory sex organ weights in males	–	–	+
Reproductive toxicity on parental animals			
Prolonged estrous cycle in females	–	+	++
Decreased sperm counts in males	–	–	+
Reduced fertility (pregnancy rate) in females	–	–	+
Decreased number of implantation sites	–	+	++
Decreased number of pups delivered	–	+	++
Toxicity on offspring			
Reduced body weights in pups	–	+	++
Reduced thymus weights in weanlings	–	+	++
Additional endpoints			
Decreased estradiol concentration in parental females	–	+	+
Increased uterine weights in female weanlings	–	++	+++

The observations described above suggest that the current two-generation reproduction study can be used for evaluating the adversity of treatment-related effects that are caused by endocrine active compounds and/or suspected endocrine disruptors. However, this assay may not always be the best assay to screen the adverse effect(s) of suspected endocrine disruptors. One of the disadvantages of a current two-generation reproduction study is the fact that the assay takes relatively a long time (approximately 36 weeks) to obtain the result, so that only the limited number of studies can be conducted. In other words, we can evaluate only a limited number of suspected endocrine disruptors closely. Secondary, a small number of offspring (usually 1/sex/litter) being examined at adulthood may cause (1) missing low incidence finding(s) and/or (2) overestimation of chemical effect(s) due to unexpectedly deviated data from an "odd fellow" in a certain litter of a certain group. For example, we experienced malformed offspring due to spontaneous mutation occurring in the breeding colony of the supplier during the course of conducting developmental and reproductive toxicity studies [14,15]. Matsumoto et al. [16] also reported the presence of spontaneous abnormalities in sperm production in a certain strain of rats. If the mutant characters and/or strain-specific spontaneous abnormalities became evident after maturation and if scientists might select the pups carrying the concealing abnormalities for post weaning examination as a representative of the litter without knowing the fact, these may lead sci-

entists to misinterpret the toxicity of test compound. Contrary, if the test substance actually induced a low incidence of abnormalities that became evident after weaning and if scientists might conduct the postweaning examination by using a normal pup as a representative of the litter, this may again cause a misinterpretation of the result.

AVAILABILITY OF AN ENHANCED ONE-GENERATION REPRODUCTIVE TOXICITY STUDY FOR DETECTING ADVERSE REPRODUCTIVE EFFECTS OF ENDOCRINE DISRUPTORS

Studies have shown that adverse reproductive outcome can be elucidated by the treatment of animals with endocrine disruptors during the period of sexual differentiation regardless of the underlying mechanisms [17–20]. Based on these facts and those described above, we propose an enhanced one-generation reproductive toxicity study in rats without adjusting a litter size during the lactation period as a candidate for rapid and reliable bioassay for providing precise adverse effect data. Animals in our enhanced one-generation study are treated with a suspected endocrine disruptor during the entire period of gestation and lactation (from gestation day 0 through lactation day 21), which is followed by postmortem examination of dams after weaning of pups and observation of F1 offspring at weaning, during sexual maturation, and at terminal sacrifice after sexual maturation (Fig. 1). The exposure period for the offspring can be extended if necessary. Essential endpoints include fertility and gestation indices of dams, numbers of implants and pups delivered, sex ratio and AGD of pups, viability index of pups during the lactation period, gross pathology of dams, and observations of F1 offspring such as organ weights at weaning and after maturation, sexual maturation (vaginal opening/preputial separation), estrous cyclicity, sperm analysis and gross- and histopathological examinations. Optional endpoints such as histopathological examination of dams and hormone measurements and/or quantitative analysis of mRNA expression in the target organs of offspring may be added flexibly according to the suspected endocrine mechanism(s) of the test compound.

Pilot studies have been conducted to confirm the availability of this assay by using ethynylestradiol (EE) as a reference chemical. We conducted a series of experiments in which the basic protocol (animal husbandry, dose levels of EE, administration route, endpoints, and the timing of each observation) was kept unchanged while only the treatment period was variable among the three. An administration route (subcutaneous injection) and dose levels (0, 0.01, 0.03, 0.10, 0.30, and 1.00 μg/kg/day) were determined according to the protocol used in OECD (Organization for Economic Cooperation and Development) validation exercises of rat uterotrophic assay [21]. The results demonstrated that the ef-

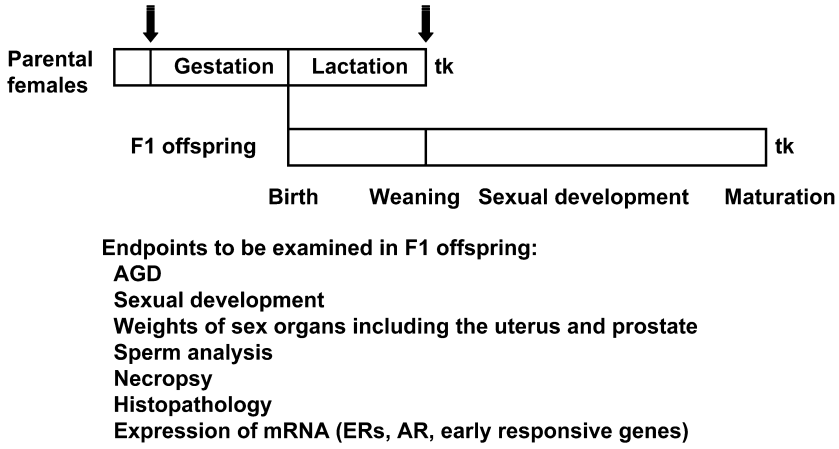

Fig. 1 Schematic explanation of a trans-generation assay.

fect of EE on maternal fertility (inhibition of pregnancy and reduction of litter size) could be detected when the chemical was administered to dams throughout the gestation and lactation period (Table 2), while the same treatment of dams during the organogenetic or perinatal period never disclosed this effect. The sensitivity of this endpoint in the gestational and lactational exposure study was almost equivalent to that of uterine weights in the uterotrophic assay. These results suggest that the exposure of estrogenic chemicals during the early gestation period (day 0 through day 5 of gestation) is critical for detecting effects on fertilization and/or implantation of eggs or survival of implants, and that animals should be treated throughout the gestation and lactation period in the future one-generation reproductive toxicity study for evaluating adverse effects of suspected endocrine disruptors with uncertain mechanism(s) of action. In the present studies, no clear adverse effects were observed in F1 offspring in terms of sexual differentiation, organ weights, female estrous cyclicity, and male sperm production. However, anogenital distances in female newborns were slightly increased, and expressions of mRNA in certain genes were up- or downregulated in all treated groups. Although toxicological meanings of these effects remain to be elucidated at present, the present results suggest that the expression of some genes including AR in the prostate and IGF-1 in the uterus may be sensitive markers for monitoring potential estrogenic effects of the test compound.

Table 2 Results of an enhanced one-generation reproductive toxicity study in rats with ethynylestradiol.

	Dose levels (μg/kg/day)				
Findings	0.01	0.03	0.10	0.30	1.00
Effects on dams					
Reduced fertility (pregnancy rate)	–	–	–	–	+++
Decreased number of implants	–	–	–	–	+++
Decreased number of pups delivered	–	–	–	++	[a]
Effects on F1 offspring					
Increased AGD in female newborns	+	+	+	[b]	
Down- or up-regulated mRNA expression[c]	+	+	+		

[a]Data not available because no dam in this group became pregnant.
[b]Excluded from evaluation because only a few pups were obtained in this group.
[c]Expression of mRNA was quantitatively analyzed in the prostate (ER-α, ER-β, AR and IGF-1) and the uterus (ER-α, ER-β, IGF-1 and IL-6) of F1 offspring at weaning and after sexual maturation.

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