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**Session 3**
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# 試　験　法

# Testing Methodology

# Ecotoxicology Test Methods for Endocrine Disrupters and Ecological Risk Assessment

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This presentation addresses priority scientific issues in support of future international environmental test guidelines for endocrine disrupters, emphasising the need for consideration of field data and the bigger picture of ecological risk assessment. While it has been known for many years that selected chemicals can cause endocrine disruption in mammals, research published in the past decade has led to an increased focus on endocrine disruption in wildlife as a concern in itself but also as an indirect indicator of potential human health issues.

Given the importance of the aquatic environment in this debate, the main part of this presentation will briefly review endocrine disruption in wild fish populations and how to best apply this knowledge to help establish future OECD fish test guidelines. The initiatives of the US Environmental Protection Agency and the OECD are supported in principle, since they both seek to identify an optimal screening and testing strategy for the aquatic environment including fish as sentinel species. Given current knowledge and global expertise, however, it is argued that the 3-tier OECD scheme for fish screening (Tier 1), partial life-cycle testing (Tier 2) and full life-cycle testing (Tier 3) is optimal since it will allows cost-effective identification of an endocrine mechanism and will provide flexible tools for use in different risk assessment scenarios. This approach will be illustrated by the evaluation of oestrogens and anti-oestrogens in candidate OECD fish test guidelines, including a juvenile rainbow trout screening assay (Tier 1), a fathead minnow adult reproduction test (Tier 2) and a fish full life-cycle test (Tier 3). The need to address androgens and thyroid active chemicals in fish test guidelines remains a major challenge for the scientific community, especially if we are to avoid the potentially redundant and unethical use of laboratory animals for endocrine disrupter screening. Nevertheless, the OECD proposal for oestrogens assessment represents a useful model for other classes of endocrine disrupter.

For aquatic invertebrates where life-cycle tests are often more feasible, similar issues pertain. Namely, invertebrate endocrine disrupter tests should use environmentally relevant exposures, be sensitive to weakly active compounds, include endpoints specific to the endocrine mechanism of concern and provide data for developmental and reproductive health protection. Finally, it will be argued that more effort should be invested in characterising potential exposures of aquatic animals to endocrine disrupters and their metabolites so that any testing requirements best support the ecological risk assessment of synthetic chemicals and natural substances.

# A Comparison of the Reproduction and Full Life-Cycle Tests with Medaka for Hazard Evaluation of Endocrine Disruptors

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The international proposals for endocrine disruptor screening programs have called for a series of screening and testing methods to be developed and validated. In this regard, the OECD Expert Consultaion on Endocrine Disruptors (EDs) testing in fish agreed on a tiered testing scheme and proposed several *in vivo* tests for each tier. Among them, a test encompassing complete life cycles of species (full life-cycle test, FLCT) was requested at tier 3 testing, because this test provides a definitive hazard data for evaluating the effects of EDs on development and reproduction in fish. It was also recognized that there was need to develop more shorter and pragmatic tests in tier 2 testing, such as an adult reproduction test and a partial life-cycle test, for prompt hazard identification and characterization of a large number of chemicals that have been suspected to be endocrine-active. Since the proposed tests must be effective for precise detection of endocrine disrupting effects to intact fish, there is need to include suitable endpoints with high sensitivity and selectivity as well as ecotoxicological significance. We have been working on the development of the FLCT and the reproduction test utilizing medaka. In this symposium, we present the test results with ethynylestradiol ($EE_2$) as a reference estrogenic substance and compare the sensitivity among the endpoints in each test.

In the reproduction test, pair-breeding medaka (6 pairs in each concentration) were exposed to waterborn $EE_2$ at mean measured concentrations of 32.6, 63.9, 116, 261 and 488 ng/L for 21 days under flow-through conditions. The fecundity and fertility of paired medaka decreased with increasing $EE_2$ concentrations, resulting in a significant difference in the fecundity of medaka at 488 ng/L compared with the controls. In contrast to the above reproductive parameters, the hepatic vitellogenin (VTG) induction in exposed males at the end of exposure occurred significantly at$\geqq$63.9 ng/L in comparison to control males. Furthermore, an intersex condition (testis-ova) of the gonad was also observed in male medaka exposed to $EE_2$ concentrations of$\geqq$63.9 ng/L, indicating that $EE_2$ can alter the gonadal development of male medaka even if exposure begins after the period of sexaul differentiation. Overall, these data indicate that the physiological and histological endpoints (i.e. VTG and testis-ova induction) were about 8 times more sensitive than those on reproductive ability (i.e. fecundity and fertility), suggesting that VTG measurement and gonadal histology in the medaka reproduction test are sensitive and specific endpoints for environmental estrogens. In this study, however, there was evidence that male medaka which were induced high VTG and testis-ova did not necessarily impair the reproductive ability for at least 21-day exposure period. These data are also very significant in selection of endpoints for hazard assessment on the ecologically relevant effects of EDs.

The full life-cycle test was initiated with embryos less than 24 h postfertilization and monitored through embryological development, hatch, posthatch survival, growth, sexual differentiation, and reproduction under flow-through 100-d exposure to mean measured $EE_2$ concentrations of 0.345, 1.17, 3.36, 10.1, and 30.6 ng/L. Except for the sexual differentiation and reproduction, no effects were observed on hatching success in embryological stage, and on survival and growth in the fish after hatching. The sex ratio estimated from the appearance of the secondary sex characteristics of the fish at 60-day posthatch skewed toward female in the 30.6-ng/L treatment. Additionally,

their gonadal histology revealed that 35 % of the fish in this treatment had testis-ova. Due to complete skewed sex ratio of the fish in the 30.6 ng/L treatment, effects on fecundity and fertility were monitored at measured concentrations of 0.345, 1.17, 3.36, and 10.1 ng/L from 71-d to 100-d posthatch. The mean total number of eggs spawned in the 10.1 ng/L treatment was significantly reduced relative to that of the controls. The mean fertility in this treatment was also 69% of that in the controls, although no statistically significant differences were determined. Thus, exposure to $EE_2$ resulted in a reduction in both fecundity and fertility at a lower concentration than that for sexual differentiation. No induction of the hepatic VTG in exposed males at the end of reproductive phase was observed in all treatments examined. Consequently, this study establishes that the lowest effect concentration (LOEC) of $EE_2$ on the sexual differentiation and reproduction of medaka were 30.6 and 10.1 ng/L, respectively, and hence the reproduction-related endpoints (i.e. fecundity and fertility) were most sensitive to the life cycle toxicity of $EE_2$ to medaka. The LOEC on the reproduction in this study was about 50 times lower than that determined in the above adult reproduction test, suggesting that continuous exposure of medaka to environmental estrogens throughout the life cycle would be necessary for determining the effective concentrations on the reproduction.

In conclusion, the short-term reproduction test with medaka can be valuable for determining the estrogenic effects of synthetic chemicals on not only the reproduction but the gonadal development of fish. However, this test would be lesser sensitive to the reproductive toxicity of estrogen agonists to medaka than a more prolonged exposure test including the period of sexual differentiation (i.e. FLCT). Therefore, the FLCT provides a definitive hazard data for evaluating the ecotoxicological effects of environmental estrogens to wild fish populations.

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# Screening of Estrogenic Chemicals Using Uterotrophic Assay

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For a long time, since the creation of diethylstilbestrol in the 1930's, synthetic Estrogenic chemicals were known to exist. Bisphenol A has been also claimed to be estrogenic since then. The endocrine disruption by such chemicals could be possible, and yet, it is still not clear which of any hormonal effects cause actually hazardous adverse effects to us. Nevertheless, there seems to be two major reasons why some scientists have worried about such kind of a possibility. Firstly, it is because the biological effects of these Estrogenic chemicals are mainly mediated through the estrogen receptor(s) that are known to be redundant for their ligands. This "Receptor-mediated toxicity" is a relatively new concept among toxicologists, and suggests many new aspects of toxicity, such as much lower dose range, uncommon dose-response relationship, complicated signal cross-talks, and possible direct and indirect effects on cell proliferation/differentiation/apoptosis in fetus, neonate, infant, prepubertal, adult and aged individuals. Secondly, the regular toxicity testing strategies, such as multigenerational reproduction study, tend to be insensitive to potent estrogens in a dose range above those used in humans for therapeutic and contraceptive purposes.

Not a small number of chemicals are already tested for the ability to bind to estrogen receptors *in vitro*. Since the antagonists also bind to the receptor, reporter gene assay is used to distinguish agonists from antagonists. Roughly speaking, potencies of genistein and other phytoestrogens are $10^{-3}$, and bisphenol A is $10^{-4}$ of 17beta-estradiol in such assays. Many chemicals are less effective than genistein and bisphenol A in these assays.

The reason for the necessity of *in vivo* assay would be to take into account for unexpected signal cross-talks, metabolism (activation/deactivation), and possible discrimination of hormonal effects from toxicity via other mechanisms for the hazard identification, that is difficult for *in vitro* systems to handle. The insensitivity to Estrogenic chemicals in the regular toxicological methods using intact rodents can be attributed to the feedback mechanisms as a part of the homeostasis.

It was well before the discovery of estrogen receptor that the uterotrophic assay was developed as a bioassay to sensitively detect estrogenic agonistic compounds. This assay utilizes an organism with estrogen-responsive target organ(s) and without the feedback mechanism. Two systems are commonly used, i.e. immature female and ovariectomized adult female rodent models.

The strategy for the risk assessment of endocrine disruptors has not been well established yet because of the lack of definitive scientific back up to identify adverse effects of such a category. Now, it is said that there are more than fifty thousands of chemicals to be considered as possible candidates for endocrine disruptors. While endocrinological and toxicological sciences are searching for the logic and methods to identify the hazardous endocrine disruptors, the "prioritization" of the chemicals for future analysis has been encouraged. *In silico* screening for receptor binding, in vitro receptor binding assay/reporter gene assay, and *in vivo* uterotrophic assay are the elements for the tentative screening system for the prioritization of these chemicals. The settings, such as dose and route, for the uterotrophic assay will be based upon *in silico* and *in vitro* assay data.

The "low-dose issue" can be a key factor in the field of endocrine disruptor issue. The strategies for screening and risk assessment will be directly involved, so that the basis for these strategies will be the scientific knowledge of its mechanisms. A systematic monitoring on receptor signaling and gene expression would be performed. The

"toxicogenomics" based on the cumulative data of spatiotemporal expression of genes during development, maturation and aging will eventually provide the solution to the endocrine disruptor issue. Meanwhile, the uterotrophic assay seems to be the most sensitive and robust *in vivo* system. Even though it is not clear whether this assay will survive along with the low-dose issue in terms of its sensitivity, it can be a data-generating model for the knowledge base; the immature uterotrophic assay which potentially holds more biological endpoints than the ovariectomized system might be utilized for risk assessment and perhaps only in conjunction with the toxicogenomics.

# Tier 2 Testing for Endocrine Active Chemicals: Is the Current Multigeneration Study Design Adequate?

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The current, rodent multigeneration reproduction study is a highly complex, labor intensive study to determine the adverse effects of agents on mammalian reproduction and provide more limited information on development. The study design has undergone a number of changes to improve the ability to detect agents that have endocrine-like activity. Many of these changes have been the addition of more hormonally sensitive end points, such as developmental landmarks of sexual differentiation at puberty. It is a requirement of Tier 2 tests for endocrine active chemicals (EACs) that they provide "definitive" information on the hazard identification, to confirm or refute observations noted in Tier 1 screens and assays, and also to provide appropriate dose response information for risk assessment. At the least, such a study would provide information on end points over which there has been hypothetical concern regarding changes in the human population (e.g. reduced sperm count, cryptorchidism and male reproductive tract malformations, including hypospadias). In these instances, it is important that there is a period of exposure that covers the developmental window for sexual differentiation in rodents, but more importantly examines animals after birth for the development of endocrine-mediated abnormalities. The multigeneration reproduction study is the only regulatory study currently meeting these criteria.

There are now several examples of false negative results, particularly with antiandrogens (e.g. linuron, butyl benzyl phthalate), where well-conducted multigeneration studies (and prenatal/ teratology studies) have indicated no endocrine activity using conventional designs, but where reproductive tract malformations (and other effects) have been observed using an *in utero* protocol where the total litter complement was examined as adults. This difference is due to only a small number of offspring (typically 1/sex/litter) being examined at adulthood and thus missing low incidence phenomena. This is in stark contrast to the prenatal study where every pup from each litter is examined for either skeletal or visceral changes specifically to detect malformations. Unfortunately, when the animals are examined in this study they are too immature to show gross changes or have not been treated throughout sexual differentiation. The multigeneration study may also produce false positive results (e.g. changes in prostate weight), particularly where the end point examined exhibits a high degree of variability in the control population and the selection of only one animal/sex/litter may significantly bias the outcome and not be reflective of the whole litter.

To provide the definitive information necessary for hazard characterization of EACs the multigeneration study must be either significantly improved to increase the number of offspring examined at adulthood with all the logistical issues that ensue, or a new study design formulated specifically for the purpose of providing the information required.