Chapter 3
Medaka Screening and Definitive Test for Endocrine Disrupting Chemicals

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Part1 Validation with Reference Chemicals

Several works have been performed to verify the applicability of each test using mainly some reference chemicals recommended by the OECD Expert Consultation on testing in fish [1].

1. Materials and Methods

Medaka (*Oryzias latipes*) were originally purchased from a local fish farm in Kumamoto, Japan, and a breeding stock of medaka has been maintained in our laboratory. Female Leucofore Free strain (FLF) medaka were supplied from Bioscience Center of Nagoya University and has been maintained in our laboratory.

The following chemicals recommended by OECD [1] were tested as reference chemicals; ethynylestaradiol (EE_2, Wako Pure Chemical Industries Ltd., Tokyo, Japan), methyltestosterone (MT, Wako Pure Chemical Industries Ltd., Tokyo, Japan) and 4-*tert*-pentylphenol (PP, Tokyo Chemical Industry, Tokyo, Japan). EE_2 and MT were used for vitellogenin (VTG) assay and reproduction test (REP), and PP was used for fish partial life-cycle test (PLC) and fill life-cycle test (FLC). In short term PLC, 4-*tert*-octhylphenol (OP, Wako Pure Chemical Industries Ltd., Tokyo, Japan) was used as a screening chemical. 4-Nonylphenol (NP, Kanto Chemical Co., Inc., Tokyo, Japan) was used for all test types (FLC, PLC, short term PLC, REP, VTG assay) for hazard assessment (see chapter 3-2). Stock solutions were prepared by dissolving into dilution water or solvents (dimethyl sulfoxide or ethanol). The prepared stock solutions were delivered to each test chamber with a continuous-flow mini-diluter system modified from Benoit et al. [2].

The experimental conditions used in each test are briefly shown in Table 1 and the details of those are described in [3] for FLC, [4] for PLC and [5] for REP. Measurement of hepatic VTG is described in [6]. The draft protocols for FLC and PLC have been prepared (http://www.env.go.jp/en/topic/edcs/approach/2002.html) and submitted to the OECD.

2. Results and discussion

2-1. VTG assay and REP

The design of VTG assay is almost the same as REP; therefore, we show the results of the latter. REP using EE_2 has already been reported by us [5] (*see Appendix II- I*). We concluded that REP with medaka is applicable to the evaluation of estrogens from the observation of the estrogenic activity of EE_2 in medaka, as shown by induction of sex differentiation and VTG, and also its effects on reproductive potential.

Table 1. Experimental conditions used in each test

Item	Type of in vivo test				
	VTG assay	REP	PLC	FLC	Short term PLC
Species	Medaka (*Oryzias latipes*)	Medaka (*Oryzias latipes*)	Medaka (*Oryzias latipes*)	Medaka (*Oryzias latipes*)	FLF medaka (*Oryzias latipes*)
Age of test organisms at beginning of test	Reproductively matured fish	Reproductively matured fish	Fertilized egg	Fertilized egg	Fertilized egg
Duration	21 days	21 days	70 days	F0; 110 days F1; 70 days	45 days
Test type	Flow-through	Flow-through	Flow-through	Flow-through	Flow-through
Dilution water	Dechlorinated tap water	Dechlorinated tap water	Dechlorinated tap water	Dechlorinated tap water	Dechlorinated tap water
Water temperature	24±2℃	24±2℃	24±2℃	24±2℃ (28 ± 2℃ in reproductive phase)	24±2℃
Illumination quality	Fluorescent bulbs	Fluorescent bulbs	Fluorescent bulbs	Fluorescent bulbs	Fluorescent bulbs
Photoperiod	16-h light, 8-h dark	16-h light, 8-h dark	16-h light, 8-h dark	16-h light, 8-h dark	16-h light, 8-h dark
Test chamber size	Round glass jar (diameter 15 cm, depth 17.5 cm)	Round glass jar (diameter 15 cm, depth 17.5 cm)	Round glass jar (diameter 15 cm, depth 17.5 cm)	Round glass jar (diameter 15 cm, depth 17.5 cm)	Round glass jar (diameter 15 cm, depth 17.5 cm)
Test solution volume	1.8 L	1.8 L	1.8 L	1.8 L	1.8 L
Volume exchanges of test solutions	16 daily	16 daily	16 daily	16 daily	16 daily
No. of vessels per level	2	6	4	4	4
No. of fish per level	10 male	12 (6 male and 6 female)	60	60	60
No. of treatment	5 (plus controls)	5 (plus controls)	5 (plus controls)	5 (plus controls)	5 (plus controls)
Dilution factor	<3.2	<3.2	<3.2	<3.2	<3.2
Feeding regime	*Artemia* nauplii twice daily (*ad libitum*)	*Artemia* nauplii twice daily (*ad libitum*)	*Artemia* nauplii twice daily (*ad libitum*)	*Artemia* nauplii twice daily (*ad libitum*)	*Artemia* nauplii twice daily (*ad libitum*)
Pre-exposure period	None	21 days	None	None	None
Biological endpoints	survival, behavior, HSI, VTG	survival, behavior, sec. SEX, fecundity, fertility, GSI, HSI, VTG, gonadal histology	survival, behavior, sec. SEX, growth, GSI, HSI, VTG, gonadal histology	survival, behavior, sec. SEX, growth, fecundity, fertility, GSI, HSI, VTG, gonadal histology	survival, behavior, growth, GSI, HSI, VTG, gonadal histology

VTG assay; Vitellogenin assay, REP; Reproduction test, PLC; Partial life cycle test, FLC; Full life cycle test, short term PLC; Short term partial life cycle test, sec. SEX; secondary sex characteristics, VTG; Vitellogenin, GSI; Gonad-somatic index, HSI; Hepato-somatic index

Although there have been no reports on the reproductive effects of EE_2 exposure in fish other than medaka, there are a few studies on reproduction in fish exposed to 17ß-estradiol (E_2). Kang et al. [7] reported that the exposure of medaka to 463 ng/L E_2 for three weeks decreased fecundity, but that the exposure to 227 ng/L had no effect. When fathead minnows were exposed to E_2 for 19 d, the E_2 concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/L, respectively [8]. Therefore, medaka may be less sensitive than fathead minnow with regard to fecundity. There are several studies on VTG induction in fish exposed to EE_2. Although in our study [5] the lowest-observed-effect-concentration (LOEC) of EE_2 for VTG induction in medaka was 63.9 ng/L, Purdom et al. [9] found that a 10-d immersion exposure of male rainbow trout to EE_2 caused VTG induction at concentrations ranging from 0.1 to 10 ng/L. Furthermore, Jobling et al. [10] reported that exposure of adult rainbow trout to 2 ng/L EE_2 for three weeks caused significant induction of VTG. Länge et al. [11] reported that the LOEC of EE_2 for VTG induction over the full life cycle of fathead minnow was 16 ng/L. These differing sensitivities in terms of VTG induction may be caused partly by dissimilarities in the period and fish stage of exposure or by different methods of VTG assessment. Although medaka seems to be less susceptible to EE_2 than rainbow trout and fathead minnow in terms of VTG induction, further work is required to determine the relative sensitivity to EE_2 among various fish species.

We also have conducted REP with MT (unpublished). In MT exposure, we detected a significant decrease in the fecundity and fertility of medaka. In addition, male secondary sexual characteristics were observed in female medaka exposed to MT. We concluded that REP with medaka is applicable to androgens from the observation of the androgenic activity of MT in medaka, as shown by male secondary sexual characteristics, and its effects on reproductive potential. In other OECD recommended fish, Ankley et al. [12] reported that exposure of the fathead minnow to 200 µg/L of MT (nominal concentration) for 21 d caused significant decrease in fecundity. The authors suggested that MT might inhibit the natural process of endogenous androgens, which probably play a role in final maturation and/or release of eggs in female fish. The authors also reported on the easily discernible alterations in secondary sex characteristics, nuptial tubercles of both sexes, suggesting that nuptial tubercles in this species should be an excellent diagnostic endpoint for androgenic chemicals. These results suggest that both madaka and fathead minnow may be responsive to androgens in expression of the appearance of the secondary sex characteristics.

2-2. PLC and FLC

The design of PLC is the same as a part of FLC. FLC using PP has already been reported by us [13] but not published yet. In summary, for all of the endpoints monitored in parent medaka (F_0), the LOEC of PP for lethal and sublethal toxicity was 931 µg/L, whereas those for abnormal sex differentiation and VTG induction were 224 and ≦ 51.1 µg/L, respectively, and that for reproductive impairment was 224 µg/L. Typical gonadal sections of testis-ova are shown in *Figure 1*.

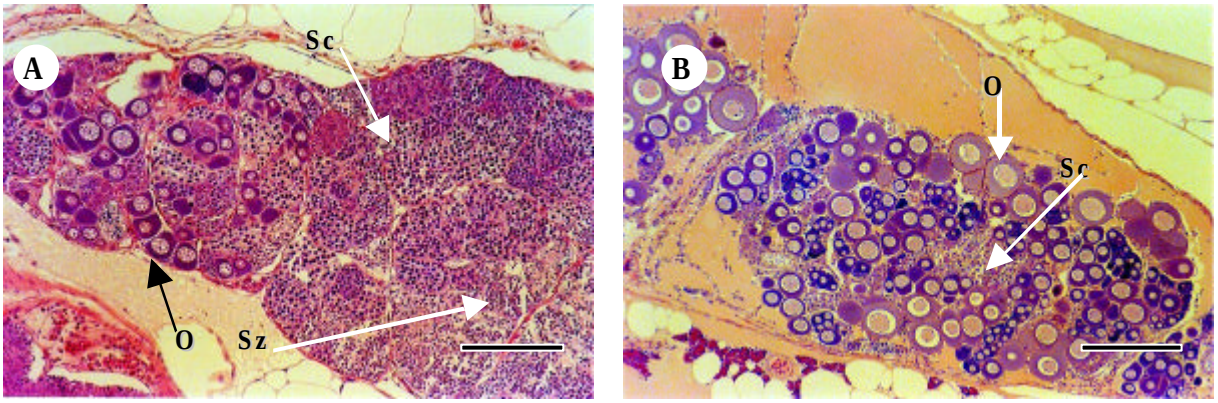

Figure 1. Longitudinal sections of testis-ova in the 224- µg/L (A) and 931-µg/L (B) 4-*tert*-pentylphenol treatment groups of F_0 generation medaka at 60-d posthatch. Each bar in (A) and (B) shows 200 and 400 µm length. (A): Oocytes (O) appear in clusters within the testicular tissue. Numerous spermatocytes (Sc) and spermatozoa (Sz) are still present in a compacted mass in this section. (B): A relatively advanced testis-ova. Almost the entire area is composed of oocytes (O), accompanying small testicular tissues interspersed with a few spermatocytes (Sc).

These results indicate that PP was about four times more sensitive for abnormal sex differentiation and reproductive impairment compared with lethal and sublethal toxicity. In the progeny (F_1) generation, the LOECs of PP for sublethal toxicity and abnormal sex differentiation, were 224 and ≦ 51.1 µg/L, respectively. We conclude that FLC with medaka is applicable to weak estrogens from the observation of the estrogenic activity of PP in medaka, as shown by abnormal sex differentiation and VTG induction, and its effects on reproductive potential. PP has already been reported to affect the sexual differentiation of fish. Gimeno et al. [14] exposed genetic male common carp (*Cyprinus carpio*) to PP from 50 d posthatch for 90 d at measured concentrations of 36, 90, and 256 µg/L, and then observed the effect on gonadal development. The authors observed not only the formation of oviducts, a female

permanent feature in these genetic male gonads at all tested PP concentrations, but also intersex gonads (testis-ova) as of 90 µg/L, demonstrating the phenotypic feminization of the male gonads by the estrogenic activity of PP. In our study, abnormal sexual differentiation in the F_0 generation of medaka was observed at 224 µg/L of PP, approximating to that in common carp, as described above. These results suggest that the effective concentrations of PP on gonadal development in medaka and common carp are similar and that medaka is as useful as other test fish in terms of the detection of abnormal sexual differentiation. In the progeny generation of medaka, we observed growth inhibition and feminization of the gonad at lower concentrations compared with its parent generation. Our previous study [3] showed that the exposure to NP over two generations of medaka induced the formation of hermaphroditic gonads at lower concentrations in the progeny than in the parent generation. We mentioned a possibility of maternal transfer of NP into the F_1 embryo as one of the possible explanations for the enhanced response in the progeny. Thus, the FLC for the testing of weak estrogens should be carried out over at least two generations, because some estrogenic chemicals may have adverse effects at lower concentrations in the progeny than in their parent generation. The mechanisms of this enhanced response of progeny to particular estrogens should be investigated by transgenerational exposure.

FLC using MT has been conducted by us, but not published yet [15]. In summary, although no dose-dependent effects of MT were observed on the embryo survival, hatching success, mortality after hatching and growth of the F_0 and F_1 fish at 60-d posthatch, we observed that all F_0 fish in the 27.7-ng/L treatment group showed male secondary sex characteristics, in which no fish with ovary could be discerned. In addition, several fish with ovary showed male secondary sex characteristics in the 9.98 ng/L treatment group in F_0 and F_1 generations. On the other hand, we observed swollen abdomens in the female fish at MT level of 9.98 ng/L in F_0 and F_1 fish. These swollen abdomens were induced by enlarged GSI, accompanying the declined fecundity and fertility in F_0 generation. These responses were most likely due to inhibition of the natural process of endogenous androgens, which probably play a role in final maturation and/or release of eggs in female fish, as reported by Goetz [16]. In respect to VTG, the levels of F_0 male medaka at the day after the end of the reproductive phase increased in all groups treated with MT at 0.348 – 9.98 ng/L, although no statistically significant difference was determined. In addition, VTG levels in the F_1 males exposed to 9.98 ng/L of MT were significantly increased compared with those of the controls. On the other hand, although the VTG

concentrations of females exposed to ≦ 9.98 ng/L of MT were not affected in F_0 generation, these were significantly decreased in the F_1 generation. In other OECD recommended fish, Ankley et al. [12] reported on the induction of plasma VTG in adult male and female fathead minnow exposed to MT for 21 d. We presume that some species and/or sexual differences are present with respect to VTG response in fish when exposed to MT. However, further work is needed to determine the applicability of this VTG parameter to andorogens in PLC and FLC. Overall, these results indicate that MT has androgenic effects which reduce the reproductive potential of medaka, and the FLC with medaka is applicable to the evaluation of androgens.

2-3. Short term PLC

We have conducted short term PLC with OP (unpublished). Overall, we detected abnormal sex differentiation (testis-ova) in genetically male (XY) medaka at 35-d posthatch while all gonads differentiated into ovary in genetically female (XX) medaka. We have already conducted the medaka PLC, and the abnormal sex differentiation was observed at 11.4 – 94.0 μg/L OP [17]. This result suggests that the effective concentrations of OP on gonadal development are similar between short term PLC and PLC. Consequently, short term PLC can detect an estrogenic activity such as induction of testis-ova, and its sensitivity may be almost the same as the PLC.

Referrences

1. Organization for Economic Co-operation and Development. 1999. Final Report of the Fish Expert Consultation Meeting, London, UK, October 28–29, 1998.
2. Benoit DA, Mattson VR, Olson DL. 1982. A continuous-flow mini-diluter system for toxicity testing. *Water Res* 16:457–464.
3. Yokota H, Seki M, Maeda M, Oshima Y, Tadokoro H, Honjo T, Kobayashi K. 2001. Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:2552–2560.
4. Yokota H, Tsuruda Y, Maeda M, Oshima Y, Tadokoro H, Nakazono A, Honjo T, Kobayashi K. 2000. Effect of bisphenol A on the early life stage in Japanses medaka (*Oryzias latipes*). *Environ Toxicol Chem* 19:1925–1930.
5. Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 21:1692-1698.
6. Yokota H, Morita H, Nakano N, Kang IJ, Tadokoro H, Oshima Y, Honjo T, Kobayashi K. 2001. Development of an ELISA for determination of the hepatic vitellogenin in medaka (*Oryzias latipes*). *Jpn J Environ Toxicol* 4:87–98.
7. Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effects of 17ß-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere* 47:71-80.
8. Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to warterborne 17 ß -estradiol. *Aquat Toxicol* 40:335–360.
9. Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. 1994. Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* 8:275–285.
10. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
11. Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17 ß - ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1216–1227.

12. Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short–term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276–1290.
13. Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. 2003. Fish full life-cycle testing for the weak estrogen 4-*tert*-pentylphenol on medaka (*Oryzias latipes*). *Environ Toxicol Chem* 22 (in press).
14. Gimeno S, Komen H, Gerritsen AGM, Bowmer T. 1998. Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. *Aquat Toxicol* 43:77–92.
15. Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. Fish full life-cycle testing for androgen methyltestosterone on medaka (*Oryzias latipes*). *Environ Toxicol Chem* (submitted).
16. Goetz FW. 1983. Hormonal control of oocyte final maturation and ovulation in fishes. In Hoar WS, Randall DJ, Donaldson EM, eds, *Fish Physiology*. Academic, New York, NY, USA, pp 117-170.
17. Seki M, Yokota H, Maeda M, Tadokoro H, Kobayashi K. 2003. Effects of 4-nonylphenol and 4-*tert*-octylphenol on sex differentiation and vitellogenin induction in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 22 (in press).