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The Expression Patterns of Estrogen-responsive Genes by Bisphenol A in the Wild Medaka (*Oryzias sinensis*)

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Abstract

Gene expression levels of choriogenin, vitellogenin and estrogen receptor were determined using Reverse transcription (RT)-PCR technique after exposure to estrogenic chemical bisphenol A in the Korean wild medaka (*Oryzias sinensis*). These genes have been known to be induced in male test fish when the fish are exposed to estrogenic chemicals. Therefore they can be suggested as a possible biomarker of endocrine disruption in fish, however, relatively little has been known about these genes expression by estrogenic chemicals in Korean wild fish. Mature male *Oryzias sinensis* were treated with bisphenol A at nominal concentrations of 0.02, 0.2 and 2 mg/L for 6 days and total RNA was extracted from the livers of treated fish for RT-PCR. When the five biomarker genes were amplified by RT-PCR in the same condition, mRNA induction level of each gene was elevated with different sensitivities. Conclusively, the results of this work indicated that measurement of vitellogenin and choriogenin using RT-PCR is effective as a simple tool for the screening of estrogenic chemicals and suggested that *O. sinensis* would be a suitable model fish for the environmental risk assessment of potential endocrine disruptors.

Keywords: *Oryzias sinensis*, Bisphenol A, Vitellogenin, Choriogenin, Estrogen receptor

The endocrine system plays an essential role in the development, growth, reproduction and behavior of human beings and animals[1]. Endocrine disruptors are mainly synthetic chemicals that when absorbed into the body either mimics or blocks hormones and disrupts the body's normal functions. There is increasing concern about endocrine disruptors, especially environmental estrogens or estrogen mimics, which have been suspected of modulating the endocrine system due to their ability to mimic natural hormones and to influence vital reproductive functions in humans and wildlife[2,3]. Among these endocrine disruptors, bisphenol A (BPA) began to attract considerable interest as a suspected endocrine disrupting chemical around 1996. Its current uses are as a primary monomer in polycarbonate plastic and epoxy resins. BPA is also used as an antioxidant in plasticizers and as a polymerization inhibitor in PVC. BPA is known to be an estrogen receptor agonist which can bind estrogen receptors leading to similar physiological effects as the internal estrogens[4]. It was found to be estrogenic in the MCF-7 human breast cancer cell culture assay[5].

In 1996, the OECD established a special activity on endocrine disruptors testing and assessment (EDTA) with the objectives of developing new and revised existing test guidelines to detect endocrine disrupting chemicals and harmonizing hazard and risk assessment approaches[6]. OECD EDTA is made up of national experts nominated by the national coordinators of the test guidelines program. The actual work on endocrine disruptors is carried out by the contributions of many scientists from member countries, industry and non-governmental organizations. They agreed to define the endpoints of endocrine disruption in fish as gross morphology, gonad histology and biological markers such as vitellogenin in the 2nd OECD expert consultation meeting on endocrine disruptors testing in fish, held in Tokyo, 15th-16th March, 2000[7]. Regarding the biomarkers of endocrine disruption in fish, recent researches have focused on vitellogenin, choriogenin, estrogen receptor and p450-aromatase, but the screening and testing methods using the biomarkers have not been fully validated yet. Recently, it was reported that choriogenin was more responsive to environmental estrogens than vitellogenin in atlantic salmon determined by ELISA system[8]. However, little study has been tried to compare the responsiveness to estrogenic chemicals with gene expression of specific subunits of the biomarkers in wild fish.

There are two species of wild medaka in Korean peninsula. One is *Oryzias sinensis* (called Chinese medaka) which inhabits the western Korea and the streams flowing into the west sea, while the other is *Oryzias latipes* (called medaka) which inhabits all

Figure 1. Wild medaka, *Oryzias sinensis* (upper) and Japanese medaka orange-red type, *Oryzias latipes* (lower).

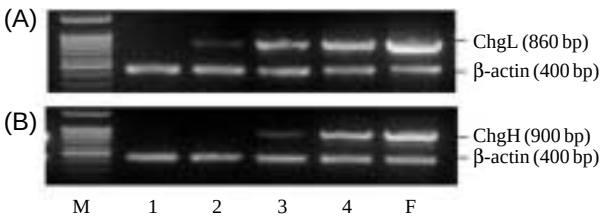

Figure 2. Choriogenin expression in the liver of male *O. sinensis* exposed to bisphenol A. RT-PCR was performed for 25 cycles. (A) Choriogenin L, (B) Choriogenin H, M: markers, 1: 0.02 mg/L, 2: 0.2 mg/L, 3: 2 mg/L, F: Spawning female.

over the Korean peninsula and has been known to be identical with Japanese medaka (*Oryzias latipes*)[9,10] (Figure 1). Japanese medaka has come to be widely used as a laboratory animal in various fields in biology, genetics and ecological toxicology because there are various mutants of this species[11]. The purpose of this study was to determine the effects of bisphenol A which was known to have estrogenic activity, on the estrogenicity of Korean wild medaka (*Oryzias sinensis*). The mRNA induction levels of choriogenin H and choriogenin L, vitellogenin I and vitellogenin II, and estrogen receptor in the *Oryzias sinensis* exposed to chemical were compared. To establish a rapid screening tool for endocrine disrupting chemicals by measuring mRNA expression in male medaka, Reverse transcription (RT)-PCR technique was used in this study. To measure the induction levels of the estrogen -responsive genes expression, male *O. sinensis* were exposed to bisphenol A ranging from 0.02 to 2 mg/L for 6 days. After the exposure to chemical for 6 days, amplified cDNA products of choriogenin H, choriogenin L, vitelllogenin I, vitellogenin II and estrogen receptor were observed. The estimated sizes of the amplified RT-PCR products of the biomarker genes

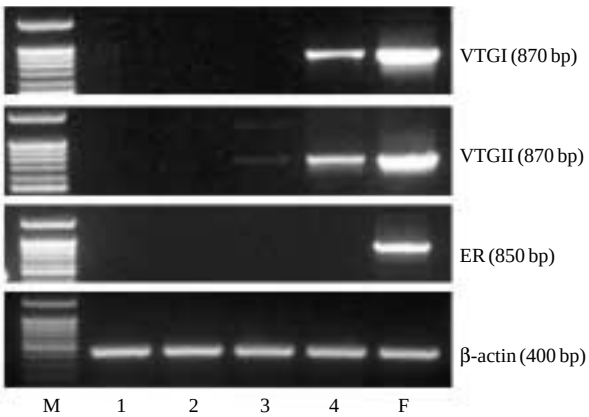

Figure 3. Vitellogenin, estrogen receptor and β-actin expression in the liver of male *O. sinensis* exposed to bisphenol A. RT-PCR was performed for 30 cycles. M: markers, 1: 0.02 mg/L, 2: 0.2 mg/L, 3: 2 mg/L, F: Spawning female.

were as follows; 900 bp of choriogenin H, 860 bp of choriogenin L, 870 bp of vitellogenin I, 740 bp of vitellogenin II and 850 bp of estrogen receptor, respectively. A pair of primers that could eliminate the amplification of contaminant genomic DNA and amplify critical single bands of each mRNA was selected.

Choriogenin L and H cDNA bands were started to be markedly induced at a concentration of 0.2 mg/L (Figure 2). 2 mg/L of bisphenol A was observed at which vitellogenin I and II genes expression was induced in the treated-males for 6 days as the lowest concentration (Figure 3). At a concentration of 0.2 mg/L, the bands were so fainted on the agarose gel that this concentration seemed hardly to induce vitellogenin expression. Amplified cDNA fragments of choriogenin and vitellogenin were not detected in non -treated group. However, β-actin cDNA fragments used as an internal standard were approximately equally expressed in treated and control groups. Transcriptional induction of the estrogen receptor seemed to be very weak by bisphenol A because its amplified cDNA fragments were not detected in this test condition (Figure 3). The sensitivity of estrogen receptor in *O. sinensis* could not be assured because this study did not cover various concentrations. However, mRNA of the estrogen receptor may be induced in a higher concentration of bisphenol A than that used in this test. As these results, choriogenin appeared to be more responsive than vitellogenin or estrogen receptor in RT-PCR method. Consequently, the induction of choriogenin and vitellogenin mRNA expression by bisphenol A showed a dose-dependent pattern, however, differential sensitivity of these genes in response to bisphenol A was observed.

Discussion

Relationships between chemical contaminant exposure and alterations in several biochemical processes in fish may allow the use of certain biochemical parameters as biomarkers of exposure and early responses to chemical contaminants[12]. RT-PCR method was used for the detection of these genes expression. RT-PCR represents sensitive technique, which allows analysis of gene expression in the various tissues of individual fishes at the transcription level[13]. Therefore, in our study we tried to investigate induction of the estrogen-responsive genes, vitellogenin I (VTGI), vitellogenin II (VTG II), choriogenin H (ChgH), choriogenin L (ChgL) and estrogen receptor (ER), in the wild male medaka (*Oryzias sinensis*), which inhabits Korean peninsula, exposed to estrogenic compound bisphenol A. Treatment of mature *O. sinensis* males with this chemical resulted in pronounced elevation of choriogenin and vitellogenin mRNA levels in the liver. However, the estrogen receptor mRNA level was significantly lower than choriogenin and vitellogenin.

Vitellogenin and choriogenin (also called egg envelope proteins) have been widely used as a biomarker for screening estrogenic endocrine disruptors in the aquatic environment[14-17]. Various experiments or surveys have been performed with wild fishes for the assessing of estrogenic activity by using vitellogenin as a biomarker in male fishes. The Japanese common goby (*Acanthogobius flavimanus*) was experimented using estradiol, nonylphenol and bisphenol A for the coastal water monitoring[18]. The carp (*Cyprinus carpio*) also was used for the test of bisphenol A effects on estrogen receptor-mediated vitellogenin production[19]. Vitellogenin induction in rainbow trout (*Oncorhynchus mykiss*) was investigated for comparison of the group of environmental estrogens[20]. However, most experiments have been performed in the protein levels such as western blotting, RIA or ELISA experiments[21,22]. On the other hand, little is known about the expression patterns of estrogen-responsive genes in the wild fishes exposed to environmental estrogens, although the measurement of specific mRNA levels for these biomarkers provides a particularly sensitive monitor of endocrine status in fish. It is proposed that *O. sinensis* is a suitable *in vivo* test model for the assessing the endocrine disrupting effects in the transcriptional level due to its small size, capacity to reproduce, ease of acclimation and breeding in the laboratory. Despite the widespread use of the Japanese medaka (*Oryzias latipes*) for various research fields including developmental science and toxicology, no study using wild medaka has been carried out with purpose of screening and testing of the hazardous chemicals[23-25]. In the present study, when the sensitivities of estrogen-responsive genes in the *O. sinensis* were compared at the same concentration of bisphenol A by performing RT-PCR, Chg was more responsive to bisphenol A than VTG. Regarding the comparative sensitivity among the biomarkers in the previous studies, egg envelop proteins were reported to be more responsive to environmental estrogens than vitellogenin in atlantic salmon[26-28]. However, in our study VTG subtype genes as well as Chg were expressed by bisphenol A in a dose-dependent pattern. Consequently, our results indicated that *O. sinensis* Chg and VTG can be a possible biomarker for screening of estrogenic endocrine disruptors. In addition, wild medaka *O. sinensis* would be a suitable model fish for the environmental risk assessment of potential endocrine disruptors.

Methods

Fish

The adult fish of the Korean wild medaka (*Oryzias sinensis*) were collected from canal of Gunsan, Chollabukdo and maintained at the Environmental Toxicology Laboratory, National Institute of Environmental Research in Korea. They were raised under constant light/dark cycles of 18/6 h and a temperature of $25 \pm 1^{\circ}C$. They were fed the artemia daily. *Oryzias sinensis* used in this study were both mature males and spawning females.

Preparation of Total RNA

Total RNA was carefully extracted from the livers of treated males and spawning females. For tissue disruption and preparation of total RNA, QIA-Shredder and RNeasy mini-kits (QIAgene Corp., Hilden, FRG) were used according to the manufacture's instrctions. Purified RNA samples were diluted at about 1 μg/μL for RT-PCR or stored at $-80^{\circ}C$ until further use.

Revere Transcription-PCR

Reverse transcription (RT)-PCR was performed using 1 μg RNA, 1 μL of AMV reverse transcriptase (34 units/μL), 1 μL of ribonuclease inhibitor (40 units/μL), 4 μL of 10 mM dNTPs and 1 μL of oligo dT primers in 20 μL total volume at $42^{\circ}C$ for 30 min and 98 $^{\circ}C$ for 5 min, and then 1 μL aliquots from the reverse transcription reaction mixture was added to 19 μL PCR reaction mixture. Twenty-five cycles or thirty cycles were then performed at 94°C for 30 sec, 60°C

Table 1. Primers for RT-PCR of the estrogen-responsive genes in this study.

Gene name	Primer sequences
Choriogenin L	F: 5′-GCCAAACCTGTAGTGCCATT-3′ R: 5′-CTGCTCCACTGACCTCCTTC-3′
Choriogenin H	F: 5′-CGCCATCTACTACTTTCCCG-3′ R: 5′-AATTTTGACCCATGATGAAA-3′
Vitellogenin I	F: 5′-CACTCATGGCTCTGAGGAA-3′ R: 5′-GCAGAGTAAAGACTCAGTTC-3′
Vitellogenin II	F: 5′-GGGGCATTGATTGCAAGCAGT-3′ R: 5′-CTCCTGAAGACCATGGTTAGG-3′
Estrogen receptor	F: 5′-ACTCCCCTTTACAGCCAGTCC-3′ R: 5′-TGGACCAGCTCCTTGTCTGCC-3′
β-Actin	F: 5′-TTCAACAGCCCTGCCATGTACG-3′ R: 5′-ATACCGCAGGACTCCATACCAA-3′

for 30 sec and 72˚C for 1 min. RT-PCR reactions were carried out using a program temperature control system (Perkin Elmer 2400). The primers used for RT-PCR of choriogenin subunits (L: low molecular subunit and H: high molecular subunit), estrogen receptor, and β-actin and vitellogenin were synthesized based on the previously known sequence. The sets of primers for RT-PCR are shown in Table 1. The housekeeping gene β-actin of medaka was used as an internal standard. Aliquots of each amplified cDNA fragment from total 20 μL PCR reaction volume were separated on the 1.0% agarose gel.

Expression of the Estrogen-responsive Genes

For the induction of the mRNA expression, the mature males with a mean weight of 250 mg/fish were exposed to bisphenol A (Sigma Corp.) at nominal concentrations of 0, 0.02, 0.2, 2 mg/L for 6 days. Bisphenol A was dissolved in ethyl alcohol and the control group fish were exposed to vehicle alone. Semi-static condition was applied for the exposure and the test solution was changed every 24 hours. After the exposure was ended, total RNA was isolated from the liver of males and amplified by RT-PCR method described above. Total RNA was also isolated from the liver of spawning female as a positive control.

References

1. Colborn, T. Endocrine disruption from environmental toxicant. In Rom, W. N. (ed) Environmental and Occupational Medicine, Third edition, Lippincott-Raven Publishers, Philadelphia (1998).
2. Jobling, S. *et al*. Altered exual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* **66**:272-281 (2002).
3. Iguch, T. *et al*. Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. *Environ Health Perspect* **114**:101-105 (2006).
4. Maricel, V. M. *et al*. Endocrine disruptors and reproductive health: The case of bisphenol A. *Molecular and Cellular Endocrinology* **254-255**:179-186 (2006).
5. Krishnan, A. V. *et al*. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinol* **132**:2279-2286 (1993).
6. Gelbke, H. P. *et al*. OECD test strategies and methods for endocrine disruptors. *Toxicology* **205**:17-25 (2004).
7. Endocrine Disruptors Testing and Assessment Task Force, 2nd OECD experts consultation on endocrine disruptors testing in fish (15-16th March 2000) Tokyo, Japan, April (2000).
8. Augustine, A. *et al*. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepathology* **2**:1-21 (2003).
9. Park, M. K. *et al*. Morphological Characters and Geographical Distributions of Korean Ricefish (Pisces, Adrianichthyidae), *Oryzias Korean J Ichthyol* **18**: 163-169 (2006).
10. Kim, I. S. *et al*. New record of the ricefish, *Oryzias latipes sinensis* (Pisces, Oryzidae) from Korea. *Korean J Syst Zool* **8**:177-182 (1992).
11. Papoulias, D. M. *et al*. An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethinylestradiol. *Aquat Toxicol* **48**:37-50 (2000).
12. Yamaguchi, A. *et al*. Short-term of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (*Oryzias latipes*). *Aquat Toxicol* **72**:239-249 (2005).
13. Lee, C. *et al*. Choriogenin mRNA induction in male medaka, *Oryzias latipes* as a biomarker of endocrine disruption. *Aquat Toxicol* **61**:233-241 (2002).
14. Yu, R. M. *et al*. Induction of hepatic choriogenin mRNA expression in male marine medaka: A highly sensitive biomarker for environmental estrogens. *Aquat Toxicol* **77**:348-358 (2006).
15. Maitra, S. *et al*. Circannual variation in plasma vitellogenin and gonadotropin II levels in relation to annual ovarian cycle in female mrigal, Cirrhinus mrigala. *Aquaculture* **265**:370-384 (2007).
16. Van den Belt, K. *et al*. Comparison of vitellogenin in zebrafish and rainboe trout following exposure to environmetal estrogens. *Ecotoxicology and Environmental Safety* **56**:271-181 (2003).
17. Christiansen, L. B. *et al*. Estrogenicity of xenobiotics in rainbow trout (*Oncorhynchu mykiss*) using *in vivo* Synthesis of vitellogenin as a biomarker. *Mar Environ Res* **46**:137-140 (1998).
18. Ohkubo, N. *et al*. Estrogenic activity in coastal areas

around Japan evaluated by measuring male serum vitellogenins in Japanese common goby *Acanthogobius flavimanus*. *Fisheries Sci* **69**:1135-1145 (2003).
19. Robert, J. L. *et al*. Effects of bisphenol A-related diphenylalkanes on vitellogenin production in male carp (*Cyprinus carpio*) hepatocytes and aromatase (CYP19) activity in human H295r adrenocortical carcinoma cells. *Toxicol Appl Pharmacol* **209**:95-104 (2005).
20. Lisette, B. *et al*. *In vivo* comparison of Xenoestrogens using Rainbow Trout vitellogenin induction as a screen ing system. *Environ Toxicol Chem* **19**:1867-1874 (2000).
21. Shao, J. Development and validation of an enzyme-linked immunosorbent assay for vitellogenin in Chinese loach (*Misgurnus angaillicaudatus*). *Environment International* **31**:763-770 (2005).
22. Kirby, M. F. *et al*. Ethoxyresorufin-O-deethylase (EROD) and vitellogenin (VTG) in flounder (*Platichthys flesus*): System interaction, crosstalk and implications for monitoring. *Aquat Toxicol* **81**:233-244 (2007).
23. Kimura, T. *et al*. Genetic linkage map of medaka with polymerase chain reaction length polymorphisms. *Gene* **363**:24-31 (2005).
24. Shima, A. *et al*. Medaka as a research organism: past, present and future. *Mechanisms of Development* **121**: 599-604 (2004).
25. Chen, P. *et al*. Comparisons of polychromatic and monochromatic UV-based treatments of bisphenol A in water via toxicity assessments. *Chemosphere* **68**: 1041-1049 (2007).
26. Lindholst, C. *et al*. Estrogenic response of bisphenol A in rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* **48**:87-94 (2000).
27. Arukwe, A. *et al*. Effects of xenoestrogen treatment on zona radiata protein and votellogenin expression in Atlantic salmon (*Salmo salar*). *Aquat Toxicol* **49**: 159-170 (2000).
28. Arukwe, A. *et al*. Differential biomarker gene and protein expressions in nonylphenol and estradiol 17beta treated juvenile rainbow trout (*O. mykiss*). *CompBiochem. Physiol Toxicol Pharmacol* **129**:1-10 (2001).