Kor. J. Env. Hlth., Vol. 31, No. 3, pp 227~233(2005)

Several Human Pharmaceutical Residues in Aquatic Environment may Result in Endocrine Disruption in Japanese Medaka (*Oryzias latipes*)

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(Received May 3, 2005/Accepted Jun. 8, 2005)

Abstracts : This study was conducted to determine the endorcrine disruption effects of the several major pharmaceutical residues in water using adult Japanese medaka (*Oryzias latipes*). Four frequently used pharmaceuticals including caffeine, ketoconazole, acetaminophen, and diltiazem were investigated for the vitellogenin (Vtg) induction in the medaka using Western blotting and ELISA. 17β,-estradiol was used as a positive control. Vtg was qualified and quantified through Western blot and ELISA. Following SDS gel electrophoresis, the dominant protein band was identified to molecular weight approximately 205 kDa in whole body samples of vitellogenic female. With female medaka exposed to 17β-estradiol, no significant difference in total protein induction was noted. In contrast, three to five day exposure of male fish to 17β-estradiol resulted in 63.07‰, increase of total protein comparing to that of control males ($p<0.01$). Vtg induction in male fish was observed with all the test pharmaceuticals: At concentrations greater than 1 ppm of diltiazem, 2 ppm of caffeine, 4 ppm of acetaminophen, and 10 ppm of ketoconazole, Vtg induction was monotonously increased in a dose dependent manner. This study is one of the first reports suggesting potential endocrine disruption mechanism of common human pharmaceutical products in aquatic ecosystem. Although the effect concentrations obtained from this investigation are environmentally unrealistically high, endocrine disruption should be considered as one of the important consequences of pharmaceutical pollution in aquatic environment, and warrants due attention in future researches.

Keywords : pharmaceuticals, endocrine disruption, caffeine, ketoconazole, acetaminophen, diltiazem, *Oryzias latipes*

Introduction

Recently, it has become a major concern that endocrine disruptors may have adverse effects on wild life and humans (Colborn *et al.*, 1993). Although some endocrine active compounds have been known for their protective effects on cancer, cardiovascular disease, brain function, alcohol abuse, osteoporosis and menopausal symptoms (Kurzer and Xu, 1997), there are many endocrine disrupting chemicals (EDCs) that may adversely affect reproduction in animal (Kim *et al.*, 2003, Hwang and Kim, 2002).

There are increasing concern about the occurrence, fate and toxicity of pharmaceutical products in the aquatic environment (Jones *et al.*, 2001). Although these compounds have been detected in a wide variety of environmental samples especially in water media, such as sewage effluent, surface waters, ground water and drinking water, we do not have clear understanding of the extent of aquatic environmental occurrence, transport, and ultimate fate of the pharmaceuticals after their intended use, i.e., stimulating specific physiological responses in humans, plants and animals (Gilliver *et al.*, 1999).

In addition, little is known about the potential consequences of the pharmaceutical contamination in the aquatic environment. Potential concerns from the environmental presence of these pharmaceuticals include abnormal physiological processes and reproductive impairment, increased incidences and reproductive impairment, increased incidences of cancer (Dupont *et al.*, 1987), and the emergence of antibiotic-resistant bacteria. (Sumpter *et al.*, 1995) Until now, many studies have been conducted to investigate the ecological risk of pharmaceutical residuals in water, and most of the test endpoints were mortality and reproduction in individual levels. Endocrine disruption capacity of pharmaceuticals in aquatic biota has rarely been investigated.

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In the present study, we evaluated estrogenicity of four pharmaceutical products, i.e., caffeine, ketoconazole, acetaminophen and diltiazem, using Oryzias latipes vitellogenin (Vtg), an egg yolk precursor, (Jobling *et al.*, 1996) as an endpoint. The four pharmaceuticals were chosen because of their frequent use in Korea and many reports on their occurrences in water. This study is, to our knowledge, one of the first studies which report the estrogenic potency of these pharmaceuticals.

Materials and Method

Fish Maintenance Conditions

A commercial orange-red variety of adult medaka (body length 2.5-3.5 cm) was generously provided by Dr. Sung K. Lee, who is work at Korea Institute of Toxicology. Groups of reproductively active fish (20 males and 20 females per group) were acclimated for 4 d in an aerated chamber (30 L) before the experiments. Experimental animals were maintained in balanced salt solution, made from nanopure water. All new fish were checked daily for a week for signs of illness and maturity. Healthy medaka were kept in dechlorinated tap water at 25 ± 1°C under a 14:10h-light/dark photo-period and fed a commercial food (Tetramin(R)) twice a week. Water was renewed once a week.

Induction of Vitellogenin by 17β-estradiol

We used the fish groups where more than 90% individuals had plasma Vtg levels below the detection limit by ELISA (Enzyme-Linked Immunosorbent Assay). 17β-estradiol (E2: Sigma E-8875) was used as a positive control. E2 was dissolved in ethanol (1 mg/m*l*) and diluted to 10 ng/m*l* in rearing water just before use. Male and female fish were exposed to 10 ng/m*l* E2 for 3-5 days. After exposure of male fish to E2 (10 ng/m*l*). Vtg reached a maxmum on the three to five d. After the exposure whole body samples were collected, processed, and analyzed for Vtg using ELISA.

Pharmaceutic Chemicals Exposure

To evaluate the estrogenic potency of the chemicals, male and female fish were exposed to each chemicals for 3-5 d. Aqueous solutions of chemicals were exchanged every 2 d.

Male and female fish were exposed to 2, 4 and 8 ppm of caffeine (Sigma C-0750); 1, 10 and 100 ppm of ketoconazole (Sigma K-1003); 4, 8 and 16 ppm of acetaminophen (Sigma A-3035); and 0.5, 1 and 2 ppm of diltiazem (Sigma D-2521).

Whole Body Sampling

Fish were kept on ice for 1-2 min, and whole body (Tyler *et al.*, 1999) extracted and measured the volume. Whole body was dissolved in sampling buffer, 20 mM tris (pH7.5) containing 1 mM EDTA, 150 mM NaCl, and 25 KIU/m*l* approtinin (Wako). When the samples were used for ELISA, whole body was diluted to 1 g/10 m*l* using 1% BSA in TBS (20 mM Tris, 150 mM NaCl; pH7.5). Whole body was homogenized (8,000×g), 3 min, 4°C) in 1 g/10 m*l* of ice-cold sampling buffer. The homogenized whole body samples were immediately centrifuged (24,000×g, 10 min, 4°C) and the supernatant were kept frozen at −70°C until use.

Protein Quantitative Analysis (Bradford assay)

Protein sample were pipeted for 5-20 μ*l* into tube. PBS was added to make a total volume of 100 μ*l* and 1 m*l* for Bradford buffer. Absorbances were determined at 595 nm with spectrophotometer after 2 min. The standard curve was remained linear only from about 5 μg to 20 μg of BSA.

SDS-PAGE

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS gels were used separaring gel 10%, stacking gel 4%. The preparations of gels, running buffer, and sample buffer were prepared normally. A standard protein to measured molecular weight has been high molecular weight range marker (205, 116, 97, 84, 66, 55, 45, 36 kDa) (Sigma M-3788). The proteins were electrophoresed during 1 h at constant voltage (100V).

Western Blot Analysis

SDS-PAGE samples were electroblotted by use of a Western blot System (KS-8460, Marysol) to a transfer membrane (nitocellulose membrane, Millipore ITVH-0010). The proteins were transferred into a nitrocellulose membrane during 5 h at 70 V. The membrane was blocked for 1.5 h in blocking

buffer (Added 1% BSA) while shaking, and incubated while shaking with primary antibody (1:1000; mouse anti-striped bass Vtg monoclonal antibody; Biosense) for 1 h. After washing, the membrane was incubated on shaking with secondary antibody (1:2000; Peroxidase-Labeled Affinity Purified Antibody to Mouse IgG KPL) for 1 h. DAB substrate reagent (InnoGenex A-0401) was used for staining protein bands of the antigen.

Preparation of Monoclonal Antibody-coated Microtiter Plates

A sandwich ELISA was developed to determine Vtg levels using 96-well microtiter plates (EnbioTec, Japan). The wells were coated with 50 μl (10 μg/ml) of monoclonal antibody in TBS and incubated at 4°C overnight. After washing three times with TBST (TBS with 0.05% Tween 20), the wells were blocked with 100 μl of blocking buffer (TBS with 1% BSA) overnight. The Ab-coated microtiter plates were stored at 4°C used within 4 wk.

Quantification of Vtg by ELISA

The quantification of Vtg was testing by ELISA using Medaka vitellogenin ELISA system (EnbioTec, Japan). The microtiter plate was set up with sufficient wells for running all blanks(zero standard), standards and samples as required. We recommend that all standards and samples are assayed duplicate. The well coated with mAb were washed three times with TBST, then 50 μl of standards (purified-Vtg) or samples were added and incubated for 1 h at 37°C. After washing three with TBST, 50 μl of the HRP-labeled at a 1:2000 dilution and incubated for 1 h at 37°C. The wells were then washed three times and pipette 50 μl for 20 min of room temperature (20-28°C) equilibrated substrate TMB (100 mM sidium phosphate, 50 mM sodium citrate, 0.05% H_2O_2; pH5.0) into all wells. The wells were added 50 μl of stop solution. Absorbances were determined at 450 nm with spectrophotometer.

Assay Performance and Statistics

Vtg Con.[1](‰) = C/D × 1,000

A : ELISA(Enzyme-Linked Immunosorbent Assay) testing value

B : Protein quantitative analysis value

C : Vitellogenin = Values calculated that injected A into a ELISA standard curve

D : Total protein = Values calculated that injected B into a Protein quantification standard curve

[1] : Vitellogenin concentration (‰)

The data obtained for samples were compared by analysis of variance. Significant differences among means were determined by Duncan's multiple-range test. Differences were considered significant at $P<0.05$ or $P<0.01$.

Results

Vitellogenin Standard Curve Fitting

We assayed using BSA for total protein quantification in fish (*Oryzias latipes*). The range of the standard curve was between 0 and 22.5 μg/ml. Then a protein quantification standard was measured and a calibration curve was developed; the r^2 value of 0.9851 was obtained.

Medaka Vtg standard was assayed by using purified Vtg. Fig. 1 shows the typical standard curve for quantification of Medaka Vtg. The assay range of the standard curve was between 0 and 100 ng/ml. Vtg standards resulted in r^2 value of 0.9958.

17β-estradiol(E_2) using the Positive Control

17β-estradiol(E2) was used positive control induced Vtg in male Medaka. Thompson *et al.* (2000) reported the EC50 of adult fish exposed to E_2 was 200 ng/l In our observation, the plasma Vtg level in fish depended on the E_2 concentration (data not shown). In this study, the reasons for using 10 ng/ml as the concentration E_2 exposure were as follow: (a) the concentration was considered close to

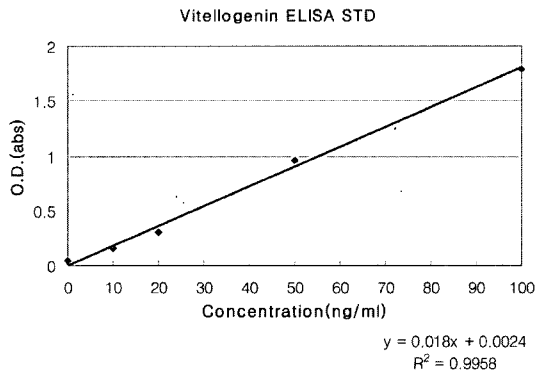


Fig. 1. Vitellogenin standard curve fitting.

the threshold for Vtg elevation; (b) minor changes of the Vtg level in the samples would be easy to follow by the E_2 concentration.

Induced Vtg was confirmed by observation of a band by Western blot of protein separated on SDS-PAGE. After native electrophoresis, the immunoglobulins recognized a single protein band in samples of vitellogenic female medaka, whereas no binding was observed in samples of male medaka (Scholz *et al.*, 1999).

Following SDS gel electrophoresis, the antibodies bound to a dominant protein band with a molecular weight of approximately 205 kDa in whole body samples of vitellogenic female.

Control male and, male and female medaka exposed to 17β-estradiol (E2) showed deep band, but control female medaka showed faint band (Fig. 2).

With Male and female medaka exposed to 17β-estradiol 10 ng/ml for 3~5 d, Vtg mean value was estimated by ELISA/Total protein by Protein quantification.

In this result, female medaka did not show significant difference. However 3-5 exposure of male medaka to 17β-estradiol produced elevated concentration of 63.07‰, which was significantly greater than that of the control male (0.76‰) ($p<0.01$).

Exposure to test pharmaceuticals

Commonly used pharmaceuticals i.e., caffeine, ketoconazole, acetaminophen and diltiazem were evaluated for their Vtg induction capacity in fish. Western blot results showed approximately 205

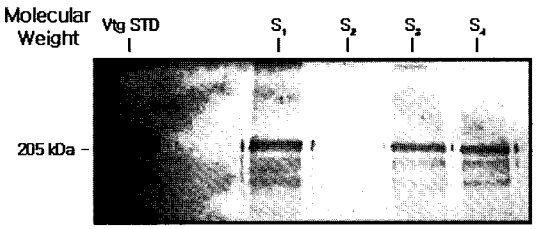
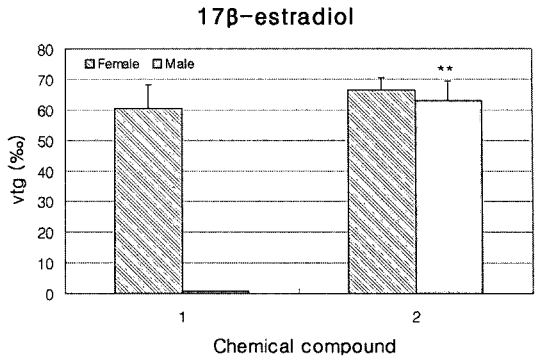


Fig. 2. Result of Western blot for female & male medaka were exposed to 17β-estradiol 10 ng/m*l* for 3-5 days.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/m*l* (Female & Male)
*: Significant difference by Negative Control T-TEST (**:$p< 0.01$)

Fig. 3. Vitellogenin induction by 17β-estradiol in *Oryzias latipes.*

kDa, that is similar to myosin at high molecular weight range Sigma branded marker. Vtg band was gradually fainted to 2-8 ppm for caffein (Fig. 4), 1-100 ppm for ketoconazole (Fig. 5), 4-16 ppm for acetaminophen (Fig. 6) and 0.5-2 ppm for diltiazem (Fig. 7), according to exposed concentration of each chemicals. Although Vtg band wasn't showed to 0.5 ppm for diltiazem.

Vtg concentration of each chemicals was quantified by total protein qantification & ELISA. Female and male medaka were exposed to 2-8 ppm for caffeine, 1-100 ppm for ketoconazole, 4-16 ppm for acetaminophen and 0.5-2 for diltiazem. Vtg meanvalue was estimated Vtg concentration by ELISA/total protein.

While caffeine treated female medaka did not shown significant difference, male medaka exposed to 2, 4 & 8 ppm of caffeine produced concentrations of 3.35‰, 17.33‰ & 20.36‰, respectively. Even

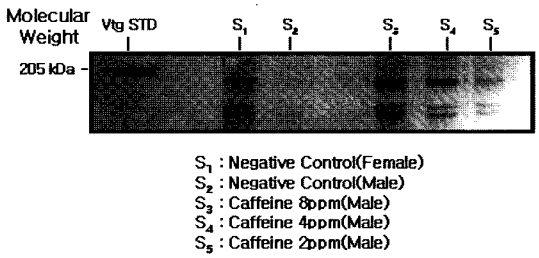


Fig. 4. Result of Western blot for female & male medaka were exposed to caffeine 2, 4 & 8 ppm for 3-5 days.

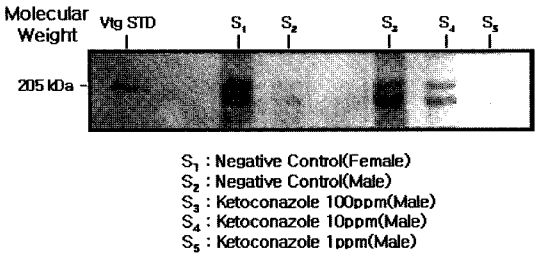


Fig. 5. Result of Western blot for female & male Medaka were exposed to Ketoconazole 1, 10 & 100 ppm for 3-5 days.

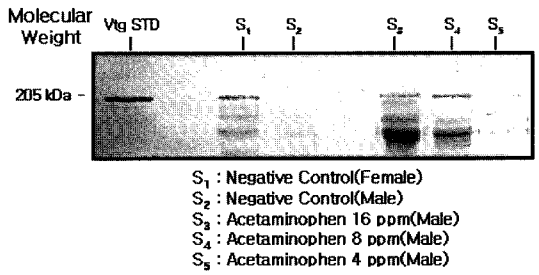


Fig. 6. Result of Western blot for female & male Medaka were exposed to acetaminophen 4, 8 & 16 ppm for 3-5 days.

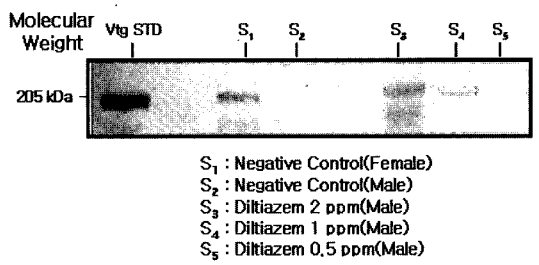
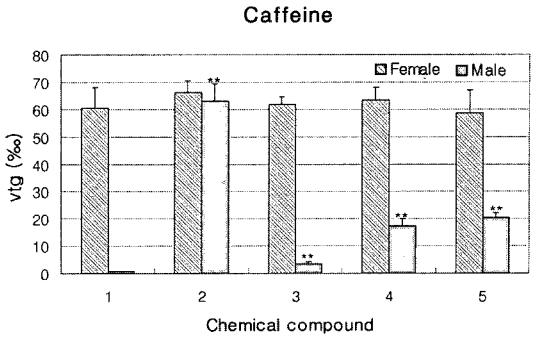
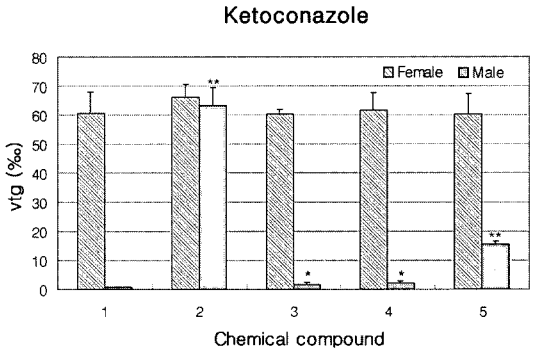


Fig. 7. Result of Western blot for female & male Medaka were exposed to diltiazem 0.5, 1 & 2 ppm for 3-5 days.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/ml (Female & Male)
3: Female & Male Medaka were exposed to Caffeine 2 ppm for 3-5 days
4: Female & Male Medaka were exposed to Caffeine 4 ppm for 3-5 days
5: Female & Male Medaka were exposed to Caffeine 8 ppm for 3-5 days
: Significant difference by Negative Control T-TEST (:p<0.01)

Fig. 8. Vitellogenine induction by Caffeine in Japanese Medaka.



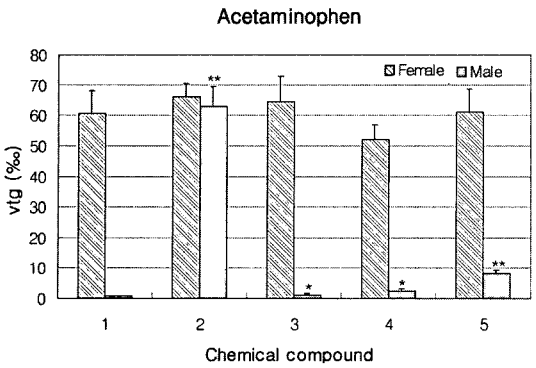
1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/ml (Female & Male)
3: Female & Male Medaka were exposed to Ketoconazole 1 ppm for 3-5 days
4: Female & Male Medaka were exposed to Ketoconazole 10 ppm for 3-5 days
5: Female & Male Medaka were exposed to Ketoconazole 100 ppm for 3-5 days
*,**: Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.01)

Fig. 9. Vitellogenin induction by ketoconazole in Japanese Medaka.

the male medaka exposed to the lowest dose, i.e., 2 ppm of caffeine resulted in Vtg significantly different from the control male (0.76‰). With ketoconazole, male medaka showed significant increases of Vtg, i.e., 1.55‰, 2.18‰ & 15.4‰, after exposure to 1, 10, and 100 ppm for 3‰~5 d ($p<0.05$).

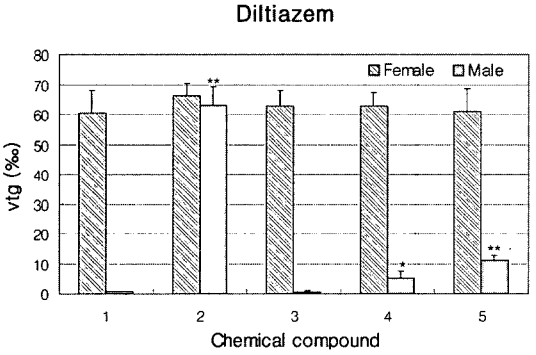
Acetaminophen and diltiazem treated female medaka did not show significant differences in Vtg induction. However, exposure of the male fish to acetaminophen of 4, 8, and 16 ppm produced Vtg concentrations of 1‰, 2.27‰ & 8.23‰, respectively, and diltiazem exposure of the male fish to 0.5, 1, and 2 ppm produced Vtg concentrations of 0.58‰, 5.18‰, and 11.11‰. Except the 0.5 ppm of diltiazem exposure, all the concentrations of acetaminophen and diltiazem resulted in significant increases of Vtg in male medaka ($p<0.05$).

Based on the current study, the test pharmaceuticals do not affect estrogenicity of the female medaka. However, with male medaka, all the test pharmaceuticals caused significant induction of Vtg.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/m*l* (Female & Male)
3: Female & Male Medaka were exposed to Acetaminophen 4 ppm for 3-5 days
4: Female & Male Medaka were exposed to Acetaminophen 8 ppm for 3-5 days
5: Female & Male Medaka were exposed to Acetaminophen 16 ppm for 3-5 days
*,** : Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.01)

Fig. 10. Vitellogenin induction by acetaminophen in Japanese Medaka.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/m*l* (Female & Male)
3: Female & Male Medaka were exposed to Diltiazem 0.5 ppm for 3-5 days
4: Female & Male Medaka were exposed to Diltiazem 1 ppm for 3-5 days
5: Female & Male Medaka were exposed to Diltiazem 2 ppm for 3-5 days
*,** : Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.01)

Fig. 11. Vitellogenin induction by diltiazem in Japanese Medaka.

Discussion

Many man-made chemicals including endocrine disruptors, widespread in the environment are in connection with human life, and may have serious consequences on the health and reproduction of people or wildlife (Kazuto *et al.*, 2002, Kim and Hwang, 2002). The objective of this study was to determine the effects of pharmaceuticals i.e., caffeine, ketoconazole, acetaminophen, diltiazem. as endocrine disruptors in fish (*Oryzias latipes*).

Vtg was quantified by Western blot and ELISA. Total protein was analyzed by using BSA. Vtg induction was confirmed by Western blot of protein separated on SDS-PAGE. After native electrophoresis, the immunoglobulins recognized a single protein band in vitellogenic female samples, whereas no binding was observed in samples of male medaka (Scholz *et al.*, 1999), which was again confirmed in the present study.

Following SDS gel electrophoresis, the antibodies bound to a dominant protein band with a molecular weight of approximately 205 kDa in whole body samples of vitellogenic female. When exposed to 17β-estradiol, female medaka did not exhibit significant differences in Vtg induction, however 3~5 exposure of male fish to 17β-estradiol produced Vtg concentrations of 63.07‰, which was significantly different from that of the control male (0.76‰, $p<0.01$). With all test pharmaceuticals, female fish did not show significant differences in Vtg induction, while male fish did show significant increase of Vtg induction.

The potential consequences of pharmaceutical residues in water will be better understood only after the appropriate occurrence data becoming available. Based on the limited information available, however, levels of detection of these compounds in streams (ppt to low ppb levels) are far lower than the effective doses (Kolpin *et al.*, 2002). However it should be noted that the pharmaceutical levels which induced elevated Vtg in male fish were in general much lower than those causing acute mortality, which are often used to derive predicted no effect concentrations (PNECs) in ecological risk assessment. Ecological significance of Vtg induction in male fish should be further investigated: That is, other organizational level endpoints, such as measurement of sex steroid hormone titers, histopathological observations, and reproduction success should be looked into in the future studies to gain better understanding of potential ecological consequences of pharmaceutical contamination in water.

This study is one of the first that demonstrates estrogenic potency of commonly used pharmaceuticals in Japanese medaka. The potential mechanism of the estrogenicity of these medicinal products needs to be investigated.

Acknowledgement

This work was supported by Korea Research Foundation Grant (KRF-2004-042-D00112).

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