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Endocrine Disruption Induced by Some Sulfa Drugs and Tetracyclines on *Oryzias latipes*

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Abstact: There has been increasing concern in recent years about the occurrence, fate and toxicity of pharmaceutical products in aquatic environment. Although these compounds have been detected in a wide variety of environmental samples including sewage effluent, surface waters, ground water and drinking water, their concentrations generally range from the low ppt to ppb levels. It is therefore often thought to be unlikely that pharmaceuticals will have a detrimental effect on the environment. This study was conducted to determine the endocrine disruption effects of the several pharmaceutical residues in water using adult Japanese medaka (*Oryzias latipes*). The common antibiotics were used sulfa durgs (sulfamethoxazole and sulfamethazine) and tetracycline drugs (oxytetracycline and tetracycline). Positive control that was induced Vtg (vitellogenin) in male fish was used 17β-estradiol. Vtg was qualified and quantified through Western blotting and ELISA. After SDS gel electrophoresis, the dominant protein band was identified to molecular weight approximately 205 kDa in whole body samples of vitellogenic female. In female medaka exposed to 17β-estradiol, there was no significant difference in total protein induction. In contrast, three to five day exposure of male fish to 17β-estradiol resulted in more than 60.0‰ increase of total protein compared to that of control males ($p<0.01$). In case of antibiotics, female fish didn't show significant difference, but male fish was showed significant difference. In addition, Vtg induction in male fish was observed with all the test chemicals. On concentrations greater than 0.1 ppm of sulfamethoxazole, 1 ppm of sulfamethazine, 1 ppm of oxytetracycline and 20 ppm of tetracycline, Vtg induction was increased in a dose response manner. This study is one of the early reports suggesting potential endocrine disruption mechanism of antibiotic pharmaceutical products in aquatic ecosystem. Although the effect concentrations obtained from this study were high as unrealistically as in environments, it is endocrine disruption that we should be considered as one of the important consequences of pharmaceutical contamination at water environment, and warrants due attention in future researches.

Keywords: pharmaceuticals, endocrine disruption, sulfamethoxazole, sulfamethazine, oxytetracycline, tetracycline, *Oryzias latipes*

Introduction

Endocrine disruptors may have adverse effects on wild life and humans. There are many endocrine disrupting chemicals (EDCs) that may adversely affect reproduction in animal. (Kim *et al.* 2003, Hwang and Kim 2002). These endocrine-active compounds have been reported to have both adverse effects on animal reproductive systems (Leopold; Santell and Awoniyi 1976) and some beneficial effects on human health, including protective effects on cancer, cardiovascular disease, brain function, alcohol abuse, osteoporosis and menopausal symptoms (Bingham *et al.* 1998).

A great concern about the occurrence, fate and toxicity of pharmaceutical products in the aquatic environment have been showed in recent years (Jones *et al.* 2001). These compounds have been detected in a wide variety of environmental samples such as sewage effluent, surface waters, and ground waters. It is therefore often thought to be possible that pharmaceutics will have some detrimental effect to environment.

Potential concerns from the environmental presence of these pharmaceuticals include abnormal physiological processes and reproductive impairment, increased incidences of cancer (Davis *et al.* 1995), the development of antibiotic-resistant bacteria (Sumpter *et al.* 1995), and the potential increased toxicity of chemical mixtures (Daughton *et al.* 1999). For many substances, the potential effects

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on humans and aquatic ecosystems are not understood clearly.

A number of chemicals in the environment have been shown, or are suspected, to have endocrine-disrupting potential that might have caused a decline in reproductivity of wildlife species. To examine this, various studies have been performed *in vivo* and *in vitro* using pharmaceuticals has caused synthesis in high concentrations of plasma vitellogenin, which is an egg yolk precursor, in male fish (Jobling *et al.* 1996).

In the present study, four pharmaceutical products, i.e., sulfamethoxazole, sulfamethazine, oxytetracycline, and tetracycline based on the reports of their frequent occurrences in the environment, and evaluate endocrine disruption using a fish, *Oryzias latipes*. The aim of this study was to determine the effects of pharmaceuticals on several aspects of endocrine function at fish (*Oryzias latipes*), specifically vitellogenin production when males were exposed to pharmaceutical residues. The understanding of the nature and magnitude of the response to antibiotics will act as a baseline for further investigations, which will focus on the estrogenic potential of human health.

Materials and Method

Fish Maintenance

The commercial orange-red variety of adult medakas (body length 2.5-3.5 cm) were generously provided by Dr. Sung K. Lee, Korea Institute of Toxicology. These reproductively active fish were acclimated for 7 days before exposure by placing each group (40 adults per group, 20 of each sex) into an aerated holding container of 30 *l*. All new fish were checked daily for a week for signs of illness and maturity. Healthy fish were kept in dechlorinated tap water at 25 ± 1°C under a 14:10 hour-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 and Drug Exposure

We used the fish groups where more than 90% individuals had plasma Vtg levels below the detection limit by ELISA. 17β-estradiol (E_2 : Sigma E-8875) was used as a positive control. E_2 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* E_2 for 3~5 days. After exposure of male fish to E_2 (10 ng/m*l*) for 3~5 days, whole body samples were collected. The Vtg of these samples was quantified using the ELISA. Groups of two male and two female adult fish kept in glass beakers (250 m*l*), with a 200 m*l* test-solution volume. To evaluate the estrogenic potency of the chemicals, the exposure were as above. Test solutions were exchanged every 2 days. Oxytetracycline (Sigma O-5750) and sulfamethazine (Sigma S-6256) was dissolved in ethanol (10 mg/10 m*l*) and diluted to 1, 5 and 10 ppm in rearing water just before use. Male and Female fish were exposed to 1, 5 and 10 ppm for 3~5 days. By the similar method, Male and Female fish were exposed to 0.1, 0.5, 1, 5 and 10 ppm for sulfamethoxazole (Sigma A-7507); 1, 5, 10 and 20ppm for tetracycline (Fluka 87128).

Whole Body Sampling and Bradford Assay

Fish were kept on ice for 1~2 minute, and whole body was minced 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 minute, 4°C), the supernatant were kept frozen at −70°C until use.

The supernatant was injected into tubes for 5~20 μ*l* using pipet. It was added PBS (Sigma T-9039) to make a total volume of 100 μ*l* and 1 m*l* for Bradford buffer (Coomassie brilliant blue 100 mg BIORAD 161-0406, 95% ETOH 50 m*l*, H_2PO_4 100 m*l*, D.W 200 m*l*). Absorbances were determined at 595 nm with spectrophotometer after 2 minute but before 30 minute. The standard curve for the Bradford assay was remained linear only from about 5 μg to 20 μg of BSA (Sigma B4287). The absorbances of unknown protein samples were fell outside of this range, the margin of error was

became very high. It was also possible to express the amount of BSA was measured along the x-axis as a concentration.

SDS-PAGE and Western Blot Analysis

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sample and Vtg standard(Biosense V01013301-001) loaded with sample buffer. Based on the protein concentrations determined, each sample was resolved using SDS-PAGE. A standard protein to measured molecular weight has been high molecular weight range marker (Sigma M-3788). The proteins were separated for 1hour at constant voltage (100V). SDS-PAGE samples were electroblotted using Western Blot System(KS-8460, Marysol) to a transfer membrane (nitocellulose membrane, Millipore ITVH-0010). The membrane was blocked for 1.5 hours in blocking buffer while shaking, and incubated while shaking with primary antibody (1:1000; mouse anti-striped bass Vtg monoclonal antibody; Biosense CK-4B3) for 1 h. After washing, the membrane was incubated shaking with secondary antibody (1:2000; Peroxidase-Labeled Affinity Purified Antibody to Mouse IgG KPL 04-18-18) for 1h. DAB substrate reagent (InnoGenex A-0401) was used for staining protein bands of the antigen.

Monoclonal Antibody-coated Microtiter Plates and Quantification of Vtg

A sandwich ELISA was developed to determine Vtg levels using 96-well microtiter plates (EnbioTec IBTM-3500, Japan). The wells were coated with 50 μl (10 μg/ml) of monoclonal antibody in TBS and incubated at 4°C overnight. The microtiter plate was set up with sufficient wells for running all blanks (zero standard), standards and samples as required. 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 hour at 37°C. After washing three with TBST, 50 μl of the HRP-labeled at a 1:2000 dilution and incubated for 1 hour at 37°C. The wells were washed three times with TBST and injected 50 μl for 20 min at 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. Absorbances were determined at 450 nm with spectrophotometer.

Assay Validation and Statistics

Vtg Con.[1)](‰) = C/D × 1,000

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(‰)

One-way analysis of variance (ANOVA) was performed to detect differences between treatment groups. Pairwise differences were determined using Tukey-Krammer post-hoc test.

Results

Protein and Vitellogenin Standard Curve

The assay using BSA for total protein quantification in fish (*Oryzias latipes*) was follows. Fig. 1 showed the typical standard curve for quantification of fish total protein. The assay range of the standard curve was between 0 and 22.5 μg/ml. As a result, protein standard curve was fitted by quantification sensitivity, R_2 value was 0.9851.

Vtg standard was assayed by using purified Vtg. Fig. 2 showed the typical standard curve for quantification of fish Vtg. The assay range of the standard curve was between 0 and 100 ng/ml. For a result, Vtg standard curve was measured by sensitivity, R_2 value was 0.9958.

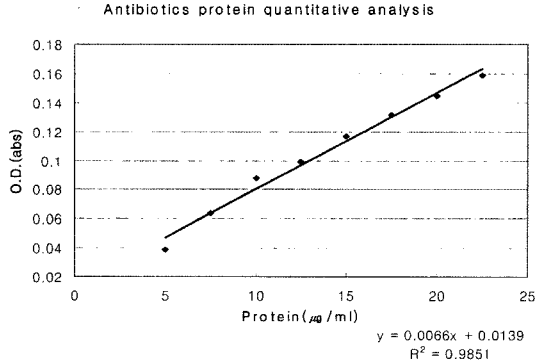


Fig. 1. Total protein quantification standard curve fitting.

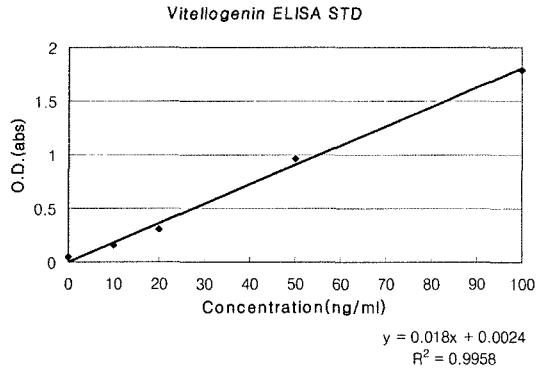
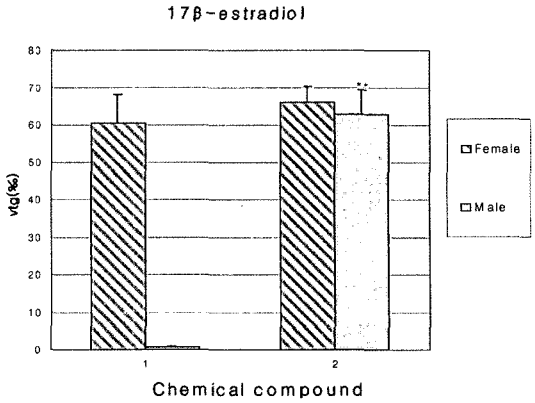


Fig. 2. Vitellogenin standard curve fitting.



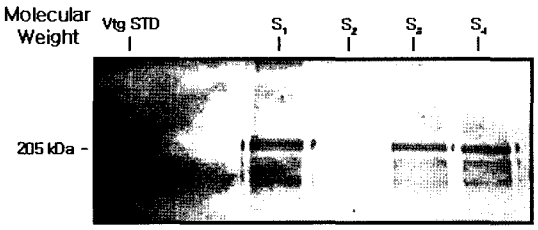
1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/ml (Female & Male)
: Significant difference by Negative Control T-TEST (: $p<0.01$)

Fig. 4. Vitellogenine induction by 17β-estradiol in fish.

17β-estradiol(E_2) Using the Positive Control

17β-estradiol(E_2) was used as a positive control that was induced Vtg in male fish. In this study, reasons for using 10 ng/ml as the concentration E_2 exposure were as follows : (a) the concentration was considered close to 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 Western blot of protein separated on SDS-PAGE. After native electrophoresis, the immunogloblins recognized a single protein band in samples of vitellogenic female fish, whereas no binding was observed in samples of male fish. 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. Molecular weight of 200 kDa for a major protein subunit of fish vitellogenin under denaturing conditions and these authors determined a molecular weight of 420 kDa for the native form of vitellogenin. Male fish used control, male and female fish exposed 17β-estradiol(E_2) showed deep band, but female fish used as control showed faint band (Fig. 3).



S_1: Negative Control (Female)
S_2: Negative Control (Male)
S_3: Positive Control E_2 10 ng/ml (Female)
S_4: Positive Control E_2 10 ng/ml (Male)

Fig. 3. Result of Western blot for female & male fish were exposed to 17β-estradiol 10 ng/ml for 3~5 days.

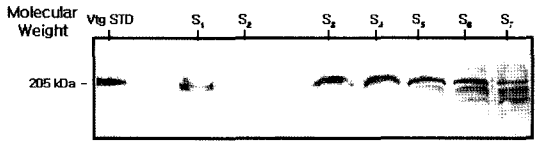
Result of total protein qantification & ELISA for female & fale fish were exposed to 17β-estradiol 10 ng/ml for 3~5 days. Vtg mean value was calculated by Vtg concentration by ELISA/total protein by protein qantification. The value was expressed by permil (‰). As a result, female fish didn't showed significant difference but 3~5 exposure of male fish to 17β-estradiol produced concentrations of 63.07‰, value was elevated than control male fish (0.76‰) and showed a significant difference, $p<0.01$.

Antibiotics

In this study, antibiotics were used sulfa drugs (sulfamethoxazole and sulfamethazine) and tetracyclines (oxytetracycline and tetracycline).

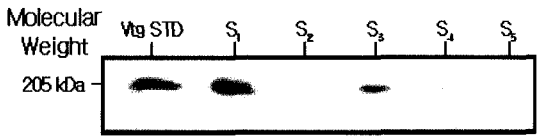
Western blot results showed approximately 205 kDa, that is similar to myosin at High Molecular Weight Range Sigma Marker. Vtg band was gradually darkened to 0.1~10 ppm for sulfamethoxazole (Fig. 5), 1~10 ppm for sulfamethazine (Fig. 6), 1~10 ppm for oxytetracyclinen (Fig. 7) and 1~20 ppm for tetracycline (Fig. 8), according to exposed concentration of each chemicals.

Vtg concentration of each chemicals was quantified by total protein qantification & ELISA. Male fish



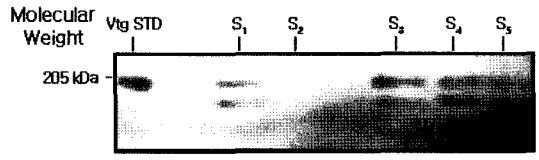
S_1: Negative Control (Female)
S_2: Negative Control (Male)
S_3: Sulfamethoxazole 10 ppm (Male)
S_4: Sulfamethoxazole 5 ppm (Male)
S_5: Sulfamethoxazole 1 ppm (Male)
S_6: Sulfamethoxazole 0.5 ppm (Male)
S_7: Sulfamethoxazole 0.1 ppm (Male)

Fig. 5. Result of Western blot for female & male fish were exposed to sulfamethoxazole 0.1, 0.5, 1, 5 & 10 ppm for 3~5 days.



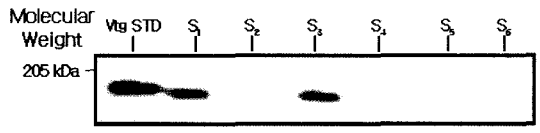
S_1: Negative Control (Female)
S_2: Negative Control (Male)
S_3: Sulfamethoxazole 10 ppm (Male)
S_4: Sulfamethoxazole 5 ppm (Male)
S_5: Sulfamethoxazole 1 ppm (Male)

Fig. 6. Result of Western blot for female & male fish were exposed to sulfamethazine 1, 5 & 10 ppm for 3~5 days.



S_1: Negative Control (Female)
S_2: Negative Control (Male)
S_3: Oxytetracycline 10 ppm (Male)
S_4: Oxytetracycline 5 ppm (Male)
S_5: Oxytetracycline 1 ppm (Male)

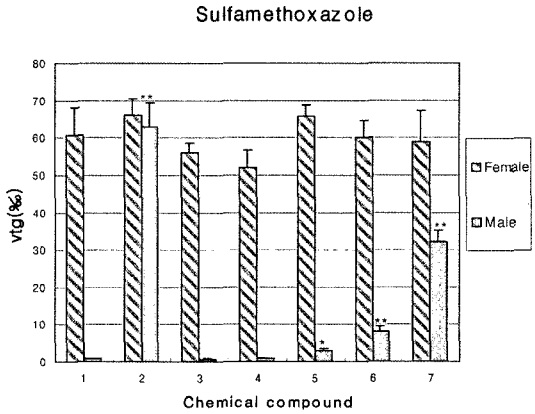
Fig. 7. Result of Western blot for female & male fish were exposed to oxytetracycline 1, 5 & 10 ppm for 3~5 days.



S_1: Negative Control (Female)
S_2: Negative Control (Male)
S_3: Tetracycline 20 ppm (Male)
S_4: Tetracycline 10 ppm (Male)
S_5: Tetracycline 5 ppm (Male)
S_6: Tetracycline 1 ppm (Male)

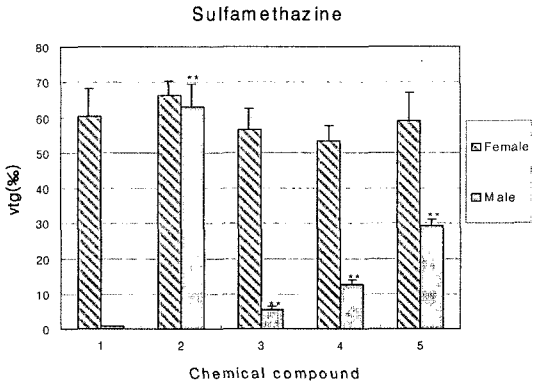
Fig. 8. Result of Western blot for female & male fish were exposed to tetracycline 1, 5, 10 & 20 ppm for 3~5 days.

exposed to sulfamethoxazole respectively appeared 0.71, 0.78, 2.79, 8.06 & 32.15‰ of 0.1, 0.5, 1, 5 & 10 ppm respectively, value was elevated than control male fish (0.76‰). Male fish exposed 0.1 & 0.5 ppm to sulfamethoxazole was made Vtg band but didn't show significant difference. Male fish exposed to 1, 5 & 10 ppm of sulfamethoxazole showed significant difference. Male fish exposed



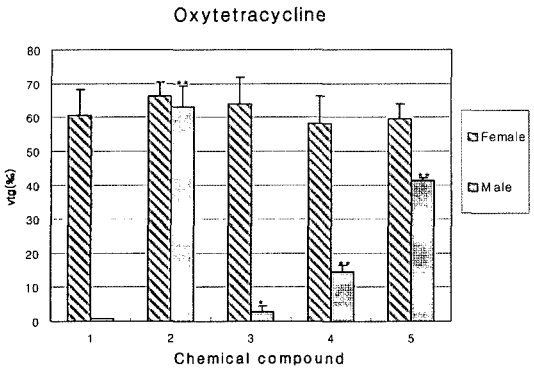
1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10 ng/ml (Female & Male)
3: Female & Male fish were exposed to Sulfamethoxazole 0.1ppm for 3~5days
4: Female & Male fish were exposed to Sulfamethoxazole 0.5ppm for 3~5days
5: Female & Male fish were exposed to Sulfamethoxazole 1ppm for 3~5days
6: Female & Male fish were exposed to Sulfamethoxazole 5ppm for 3~5days
7: Female & Male fish were exposed to Sulfamethoxazole 10ppm for 3~5days
*,**: Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.01)

Fig. 9. Vitellogenine induction by Sulfamethoxazole in fish.



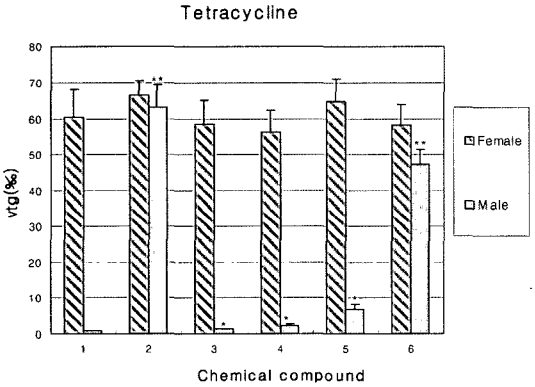
1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10ng/ml (Female & Male)
3: Female & Male fish were exposed to Sulfamethazine 1ppm for 3~5days
4: Female & Male fish were exposed to Sulfamethazine 5ppm for 3~5days
5: Female & Male fish were exposed to Sulfamethazine 10ppm for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

Fig. 10. Vitellogenine induction by sulfamethazine in fish.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10ng/ml (Female & Male)
3: Female & Male fish were exposed to Oxytetracycline 1ppm for 3~5days
4: Female & Male fish were exposed to Oxytetracycline 5ppm for 3~5days
5: Female & Male fish were exposed to Oxytetracycline 10ppm for 3~5days
*,** : Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.001)

Fig. 11. Vitellogenine induction by oxytetracycline in fish.



1: Negative control (Female & Male)
2: Positive control 17β-estradiol(E_2) 10ng/ml (Female & Male)
3: Female & Male fish were exposed to Tetracycline 1ppm for 3~5days
4: Female & Male fish were exposed to Tetracycline 5ppm for 3~5days
5: Female & Male fish were exposed to Tetracycline 10ppm for 3~5days
6: Female & Male fish were exposed to Tetracycline 20ppm for 3~5days
*,** : Significant difference by Negative Control T-TEST (*:p<0.05, **:p<0.01)

Fig. 12. Vitellogenine induction by Tetracycline in fish.

to sulfamethazine respectively appeared 5.42, 12.71 & 29.26‰ of 1, 5 & 10 ppm respectifvely, value was elevated than control male fish (0.76‰). Male fish exposed to 1, 5 & 10 ppm of sulfamethazine showed significant difference.

The teracycline drug also was estimated, female fish didn't show significant difference. The value was estimated at 2.67, 14.61 & 41.35‰ to oxytetracycline (1, 5 & 10 ppm) and 1.25, 2.26, 6.79 & 47.33‰ of tetracycline (1, 5, 10 & 20 ppm). It was appeared to significantly different from control male fish.

Compared to other *in vivo* endpoints such as induction of testis-ova (Gray and Metcalfe 1997), synthesis of plasma vitellogenin and inhibition of gonadal growth (Jobling *et al.* 1996), reproductive toxity was examined the same level at pharmaceuticals in this study.

As a result, female fish exposed to four antibiotics didn't show estrogen effect equal pharmaceuticals. By way of comparision, endocrine disruptor effect was detected in male fish. This values were high as estimated Vtg level at pharmaceutically induced.

Discussion

Accordingly the aim of this study was to determine the effects of pharmaceuticals on several aspects of endocrine function in fish (*Oryzias latipes*), specifically vitellogenin production when males are exposed to sulfamethoxazole, sulfamethazine, oxytetracycline and tetracycline.

A red dye, prontosil, synthesized in Germany by Klarer and Mietzsch in 1932, was tested but found to be ineffective against bacteria *in vitro*. However, Domagk reported in 1935 that it was strikingly active *in vivo* against hemolytic *Streptococcal* and other infections. This was due to the conversion in the body of prontosil to sulfanilamide, the active drug. Since then the sulfonamide molecule has been chemically altered by the attachment of many different radicals, and there has been a proliferation of active compounds. In spite of the advent of the antibiotic drugs, the sulfonamides are among the most widely used antibacterial agents in the world today, chiefly because of the low cost and their relative efficacy in some common baterial diseases (Lullmann *et al.* 2000). The synergistic action of sulfonamide with trimethoprim has brought about an enormous resurgence in sulfonamide use everywhere during the last decade. Sulfonamides are structural analogues of p-amino benzoic acid (PABA) The action of sulfonamides is bacteriostatic and is reversible by removal of the drug or in the presence of an excess of PABA. Different sulfonamides may show quantitative but not necessarily qualitative differences in activity. Sulfonamides can inhibit both gram-positive and gram-negative bacteria, *Norcadia*,

Chlamydia trachomatis and some protozoa. Some enteric bacteria are inhibited but not *Pseudominas*, *Serratia*, *Proteus*, and other multi-resistant organisms. The tetracycline are a large group of drugs with a common basic structure and activity. Chlorotetracycline, isolated from *Streptomyces aureofacien*, was introduced in 1948. Oxytetracycline, derived from *Streptomyces rimosus*, was introduced in 1950. Tetracycline, obtained by catalystic dehalogenation of chlorotetracycline, has been available since 1953. The most recently developed tetracyclines have emphasized good absorption combined with prolonged blood levels (Lullmann *et al.* 2000). Tetracyclines are the prototype of 'broad-spectrum' drugs. They are bacteriostatic for many gram-positive and gram-negative bacteria, including some anaerobes : for rickettsiae, clamydiae, mycoplasmas, and L forms ; and for some protozoa, eg, amebas. Equal amounts of tetracyclines in body fluids or tissues have approximately equal antimicrobial activity. Susceptible microbial populations obtain small numbers of organism resistnat to tetracyclines. These lack an active transport mechanism across cell membranes and thus do not concentrate tetracycline in their cells. Alternatively, resistant bacteria may lack passive permeability to tetracyclines.

In case of antibiotics, female fish didn't show significant difference, while male fish was showed significant difference. In addition, Vtg concentration of antibiotics was increased 50% rate to upward of 10 ppm exposure concentration range, in comparative with control male fish. When exposed to antibiotic residues in water environment, endocrine disrupting potentials that might have caused a decreasing in reproductive activity of aqua-life species. In addition, it was suggested that physico-chemical alterations, such as an induction of plasma vitellogenin level, were caused by much lower concentration (even at below ppm) than those at which a down regulated in reproductivity was induced actually (Tompson *et al.* 2000). The consequential fate of antibiotics residues in water will be better defined only after the appropriate occurrence data becoming available. Based on the limited reference available, however, levels of detection of these antibiotics in water environments were far lower than the effective doses (Kolpin *et al.* 2002). However it should be noted that the antibiotics levels which induced elevated Vtg in male fish were in general much lower than those causing acute toxicity, which are often used to calculate predicted no effect concentrations (PNECs) in ecological risk assessment. We need information to conclude the effects of antibiotics in water environment. 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 antibiotics contamination in water.

This study is one of the early report that demonstrates estrogenic potency of commonly used antibiotics to Japanese medaka in Korea. The potential mechanism of the estrogenicity of these medicinal products needs to be investigated in depth.

Acknowledgement

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