Endocrine Disrupting Effects of Several Pharmaceuticals to Oryzias Latipes

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Abstract

Endocrine disrupting effects of four pharmaceutical products were evaluated with fish. The test pharmaceuticals, i.e., sulfamethoxazole, sulfamethazine, oxytetracycline and tetracycline have been often detected in aquatic environment, but their ecological hazard on receptors of various trophic levels has seldom been evaluated. In the present study, we conducted acute toxicity assays with a fish, Japanese medake (*Oryzias latipes*).

The vitellogenin induced in female fish normally, but a endocrine disrupting chemical could give effects even male fish. We have tried 4 pharmaceutic chemicals to find out the endocrine disrupting effects. Sulfamethoxazole 1, 0.5 ppm induced vitellogenin even at male Japanese medaka. Sulfamethazine 10, 5, 1 ppm could induced vitellogenin at male fish. Oxytetracycline 10, 5, 1 ppm could induced vitellogenin With the fish. Tetracycline 10, 5 ppm could induced vitellogenin at male fish.

Some pharmaceuticals such as sulfamethoxazole, sulfamethazine, oxytetracycline and tetracycline could give effects to male *Oryzias latipes*. They could induced vitellogenin under exposure range 0.5 ~ 10 ppm of chemicals at male *Oryzias latipes*.

Introduction

Pharmaceutically active compounds are complex molecules with different functionalities, physiochemical and biological properties. They are developed and used because of their ionic nature. Their molecular weights range typically from 300 to 1000. Under environmental conditions molucules can be neutral, cationic, anionic, or zwitterionic. They also often have basic or acidic funtionalities. Pharmaceuticals can be classified according to their effects, but also crosswise according to their chemicals structure. Normally, pharmaceuticals and disinfectants are classified according to their therapeutical purpose (e.g. antibiotics, analgesics, antineoplastics, anti-inflammatory substances, antibiotics, antihistaminic agents, contrast media, etc). Many pharmaceuticals are biotrasformed in the body. Biodegradation modifies the chemical structure of their active molecules, which in turn often results in a change in their physicochemical and pharmaceutial properties. Recently numerous reports have been published indicating the

occurrence of many pharmaceuticals in various environmental media, however, ecological consequences of the discharge of these pharmaceutical residues have seldom been thoroughly investigated.

In the present study, we chose four human pharmaceutical products, i.e., sulfamethoxazole, sulfamethazine, oxytetracycline and tetracycline based on the reports of their frequent occurrences in the environment, and evaluated their endocrine disrupting effects to Japanese medaka (*Oryzias latipes*). We aimed at estimation of their potential endocrine disrupting effect to fish. The information gleaned from this study will be useful for formulation of appropriate risk management decisions for protection of aquatic ecosystem from these pharmaceutical residues.

Materials and Method

Fish maintenance conditions

A commercial orange-red variety of adult medaka (body length 2.5-3.5 cm) was supplied by Korea Institute of Toxicology. 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℃ under a 14:10h-light/dark photo-period and fed a commercial food(Tetramin) twice a week. Water was renewed once a week.

Experimental induction of vitellogenin synthesis by 17β-estradiol treatment

We used the fish groups where more than 90% individuals had plasma Vtg levels below the detection limit by ELISA. E2 was dissolved in ethanol(1mg/ml) and diluted to 10ng/ml in rearing water just before use. Male and Female medaka were exposed to 10ng/ml E2 for 3~5days. At 3~5days after exposure was begun, hall body samples were collected. The Vtg of these samples was quantified using the ELISA.

Chemical compound exposures

Sulfamethoxazole was dissolved in ethanol(1mg/ml) and diluted to 1, 0.5 and 0.1 ppm in rearing water. Oxytetracycline, Sulfamethazine and tetra cycline were dissolved in ethanol (10mg/10ml) and diluted to 1, 5 and 10ppm in rearing water just before use. Male and Female medaka were exposed for 3~5days.

Whole body sampling

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

Preparation of monoclonal antibody-coated microtiter plates.

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

Quantification of Vtg by ELISA

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㎕ of standards(purified-Vtg) or samples were added and incubated for 1h at 37℃. After washing three with TBST, 50㎕ of the HRP-labeled at a 1:2000 dilution and incubated for 1h at 37℃. The wells were then washed three times and pipette 50㎕ for 20min of room temperature(20~28℃) equilibrated substrate TMB(100mM sidium phosphate, 50mM sodium citrate, 0.05% H_2O_2; pH5.0) into all wells. The wells were added 50㎕ of stop solution. Absorbances were determined at 450nm with spectrophotometer.

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Results and Discussion

Vitellogenin standard and protein quantification

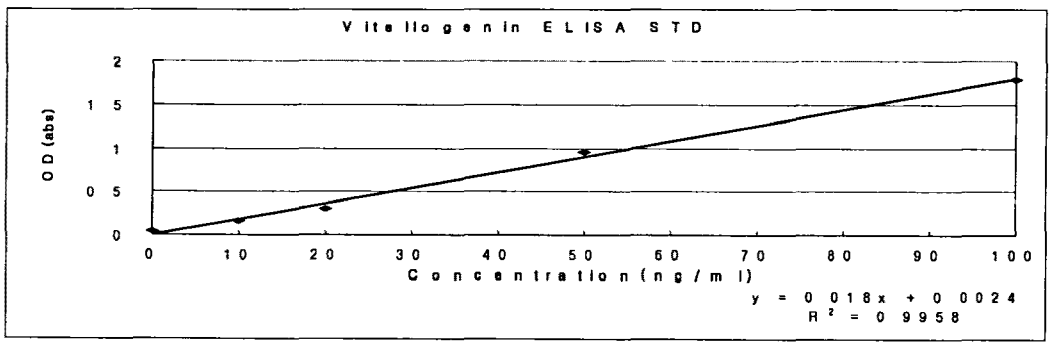


Fig. 1. Vitellogenine standard curve fitting.

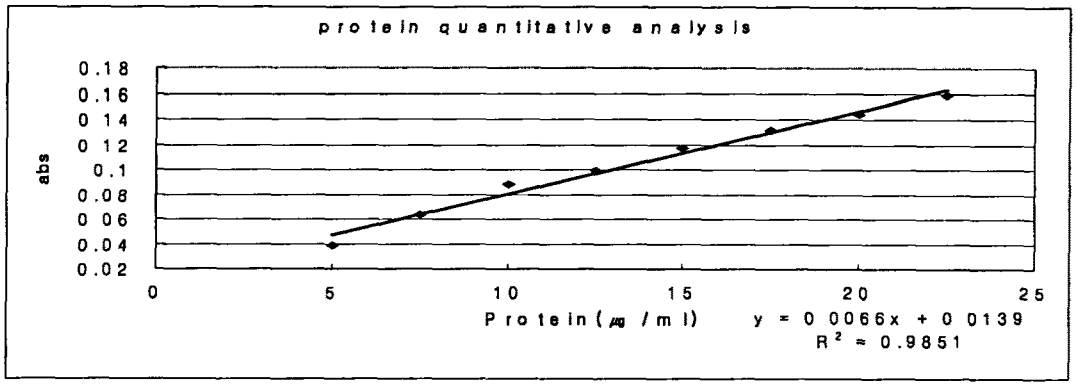


Fig. 2. Protein quantification standard curve fitting.

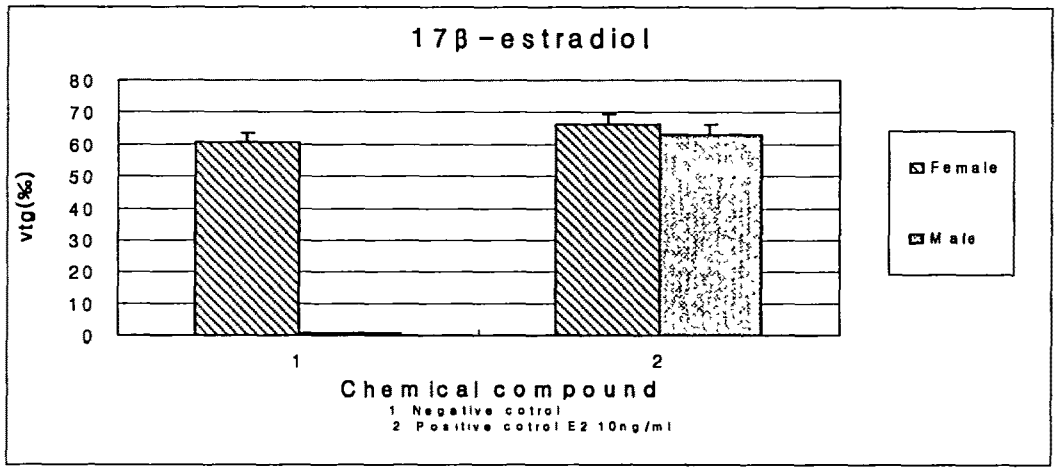


Fig. 3. Vitellogenine induction by 17β-estradiol in Japanese medaka.

Vitellogenin induction by sulfa drugs

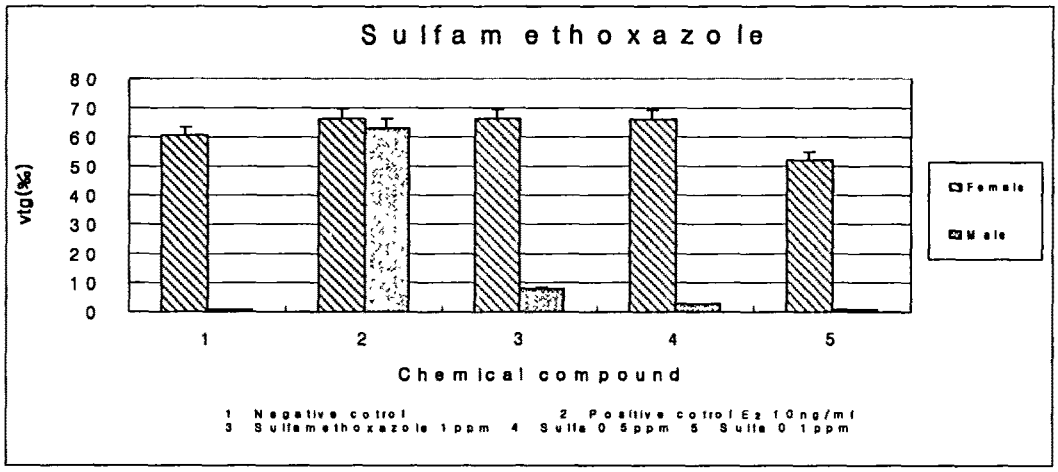


Fig. 4. Vitellogenine induction by suflfamethoxazole exposure at Japanese medaka.

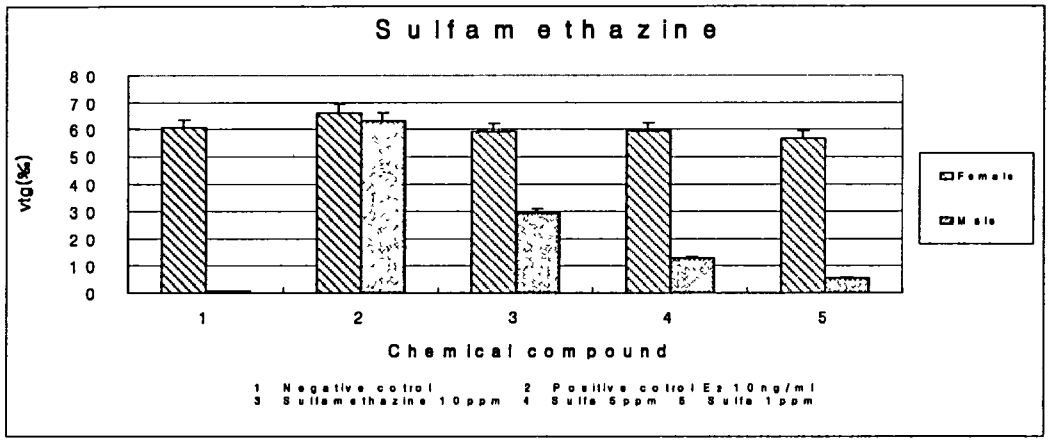


Fig. 5. Vitellogenin induction by sulfamethazine exposure at Japanese medaka.

Vitellogenin induction by tetracyclines

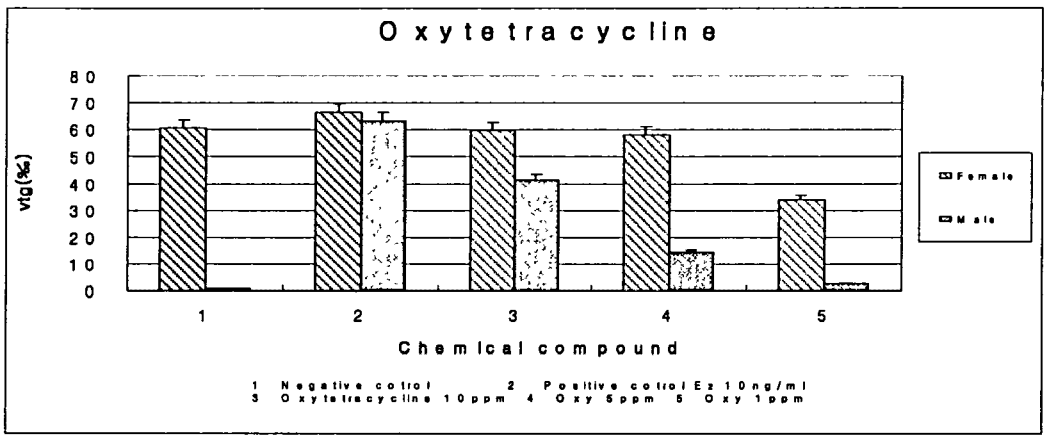


Fig. 6. Vitellogenin indution by oxytetracycline exposure at Japannese medaka.

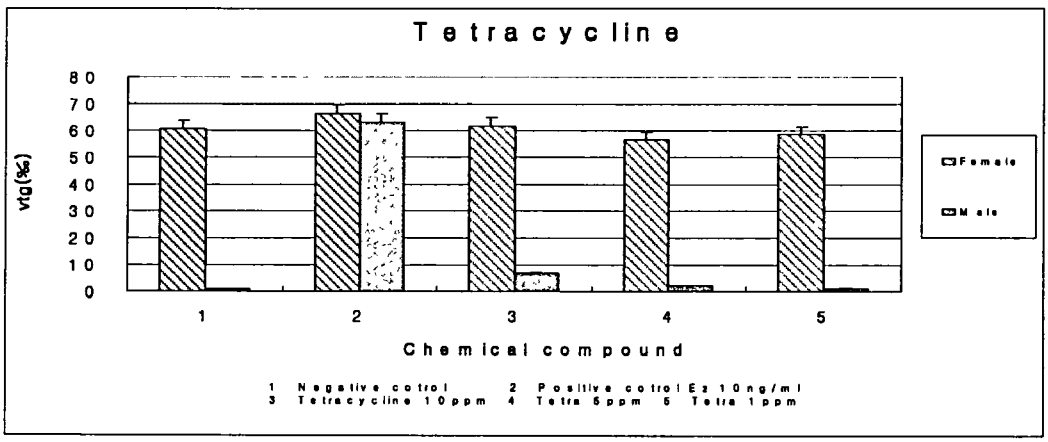
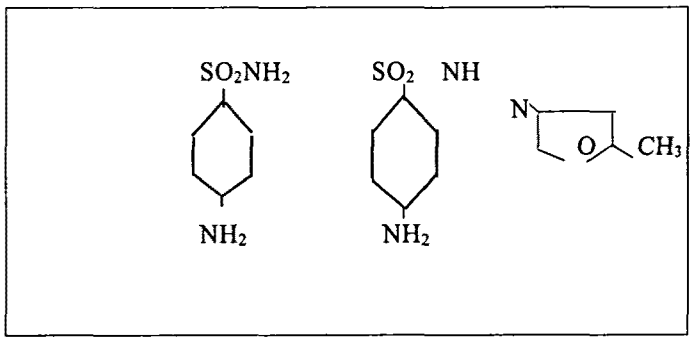


Fig. 7. Vitellogenin indution by tetracycline exposure at Japannese medaka.

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. 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. Chloro-tetracycline, 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. All of the tetracycline have the basic structure shown below.

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.

Conclusions

The sulfa drugs (sulfamethoxazole and sulfamethazine) and tetracyclines (oxytetracycline and tetracycline) were induced vitellogenine at male Japanese medaka.

References

1) Ethan D. Clotfelter. Val Nolan Jr and Ellen D. Ketterson, The Effects of Experimentally Elevated Testosterone and Food Deprivation on Food Consumption and Prey Size Preferences in Male Dark-Eyed Juncos. Ethology. 107, 439-449. (2001).
2) Arias P., Pinochet L. F., and A. Disi. Study of the Nervous Tissue Development in Rainbow Trout Embryo Treated with Oxytetracycline. Anat. Histo. Embryol. 31, 144-147, (2002).
3) Toften H. and M. Jobling, Development of Spinal Deformities in Atlantic Salmon and Arctic Charr Fed Diets Supplemented with Oxytetracycline. Journal of Fish Biology, (1996) 49, 668-677.
4) Toften H, Jobling M. Feed intake and growth of Atlantic salmon, Salmo salar L., fed diets supplemented with oxytetracycline and squid extract, Aquaculture Nutrition, 1997 3; 145-151.
5) Sangungruang K, Endo M, Ueno R, Development of a method for forced oral administration of xenobiotics in shrimp. Fisheries Science, 2004 ; 70: 463-466.
6) Bertram G, Katzung, MD, Phd, Basic and Clinical Pharmacology, a LANGE medical book, 565-591, 1989.