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Chlortetracycline Caused Vitellogenin Induction at Male Japanese Medaka (*Oryzias latipes*)

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Abstact: This investigation was intended to find out the estrogenic effect of chlortetracycline (CTC) on vitellogenin induction in adult male Japanese medaka (*Oryzias latipes*). Vitellogenin (Vtg) produced in male fish has been used to as one of a biomarker of endocrine disrupters. The positive control was 17β-estradiol (E2) that induced Vtg in male fish. As a result, male and female fish were exposed to 0.1, 1, 10 and 100 ppm of CTC. Western blot results showed approximately 205 kDa, that is similar to myosin at high molecular weight range Sigma maker. Vtg band was showed fainted to 10 and 100ppm for chlortetracycline. Vtg concentration of CTC was qunatified by total protein quantification and ELISA. Exposure of the male fish to CTC of 0.1, 1, 10 and 100 ppm produced Vtg concentrations of 0.24, 0.12, 7.61 and 40.02‰, respectively, that value was elevated than control male fish (0.14‰). CTC exerted as a Vtg inducer in male fish from 10 ppm, but it was a reducer in female fish from 0.1 ppm level. The results say that vitellogenin induction patterns alter in male medaka treated with CTC, and that CTC may caused endocrine disruption in fish.

Keywords: chlortetracyline, veterinary medicine, endocrine disruption, vitellogenin, Japanese medaka (*Oryzias latipes*), ELISA, Western blot

Introduction

The presence of pharmaceuticals in the environment rapidly emerged as an environmental science issue in the late 1990s. The initial findings of low levels of drugs in aquatic environments triggered a number of scientific publications and conferences, as well as media stories. The potential of these pharmaceuticals to result in effects to wildlife led to substantial speculation with widely divergent predictions. Uncertainty prevailed within the scientific community, particularly with regard to the potential for chronic ecological effects (Williams, 2005; Kang *et al.*, 2005). Some of the effects of various compounds-most notably antihelmintics from veterinary medicine and antibacterial therapeutics-are already known (Daughton and Ternes, 1999; Boxall *et al.*, 2003, 2004; Floate *et al.*, 2005), but there are many other substances that can affect organisms in the environment. This is further complicated by the fact that some pharmaceuticals can cast effects on bacteria and animals well below the concentrations that are usually used in safety and efficacy tests.

Chlortetracycline (CTC) is a broad-spectrum antimicrobial agent used in animal husbandry for both prophylaxis and treatment of respiratory and alimentary tract infections. It is commonly administered for these reasons as an in-feed antibiotic in the pig industry. Currently, there is much debate over the use of antimicrobial agents in livestock production, and their potential to select for antimicrobial resistance. The possibility of resistant organisms of animal origin becoming directly pathogenic to man, or transferring their resistance genes to pathogens of medical importance, is of particular concern. CTC is predominantely excreted in urine and faeces and, due to enterohepatic recirculation, elimination is prolonged. Pig intestinal flora are therefore exposed to fairly low concentrations of CTC over extended periods; conditions that exert a strong selective pressure for the development of resistance. The presence of antibiotics in livestock faeces is also of concern, with regard to their fate and their effect on the environment when they enter the soil. It is therefore important to have specific methods by which to assay these veterinary antibiotics, to assist and support studies investigating both the pressure that these agents apply in selecting resistant isolates, and their impact on the environ-

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ment (Lullmann *et al.*, 2000).

The objective of this study was to scrutinize the effects of CTC on several aspects of endocrine function in Japanese medaka, specifically vitellogenin production from male fishes. An understanding of the nature and magnitude of the response to CTC will act as a baseline for further investigations, which will focus on the estrogenic potential of the environment and human health.

Materials and Methods

We used the fish group where more than 90% individuals had Vtg levels below the detection limit by ELISA. 17β-estradiol (E_2: purity ≥ 98%: Sigma, E-8875) was dissolved in ethanol (1 mg/m*l*) and diluted to 10 ng/m*l* in rearing water just before use. Male and female medaka were exposed E2 concentrations of 10 ng/m*l* in 250 m*l* glass beakers for 3~5 days at 25 ± 1°C. The fish were subjected to expose under the light photoperiod, and were not fed during the exposure periods. At end of the 3~5 days exposure, whole body were sampled, and stored at −80°C until analysis (Kazuto *et al.*, 2002; Akemi *et al.*, 2005). Animals used in this study were d-rR medaka (*Oryzias latipes*), which contain a red pigment color marker on the male Y chromosome. Fish with an XY chromosome have an orange-red phenotype and XX are white. Medaka (d-rR) of about 2 months was kindly provided by the Korea Institute of Toxicology (KIT), in Korea. First-generation medaka eggs obtained from the Laboratory of Freshwater Fish from the Bioscience Center of Nagoya University, in Japan. The fish were placed under a summer photoperiod (16:8-hour light:dark) and fed exclusively with commercial food (Tetramin®) twice a day. Water temperature was maintained at 25 ± 1°C. The test equipment and glass aquarium containing were cleaned at least once a week to prevent any dense bacterial or algal growth. Residual food and feces in the glass aquarium containing were removed daily. Every 7 days all groups of experimental animals were placed into new aquarium with complete renewal of tank water.

Fish were thawed on ice and weighed. Whole body was minced and individually homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.3) with a 1 g : 10 m*l* (weight : volume) ratio of wet mass to buffer volume in a glass homogenizer. The homogenate was the centrifuged at 4°C, 8,000×g and collect the supernatant. The supernatant was withdrawn and immediately frozen at −80°C until use. Protein contents were determined at 595 nm with spectrophotometer by Bradford assay. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sample and medaka Vtg standard (Biosense BATCH-0501) were used at this assay (Kang *et al.*, 2006).

SDS-PAGE samples, whole body sample of male, female and E2-treated male medaka, were electroblotted by use of a Western Blot System (Bio-Rad, Mini Trans-Blot) to a transfer membrane (nitocellulose membrane, Bio-Rad 162-0115). After shaking with primary antibodies (1:500; mouse anti-striped bass Vtg monoclonal antibody; Biosense CK-4B3) and the membrane was incubated shaking with secondary antibody (1:1000; Peroxidase-Labeled Affinity Purified Antibody to Mouse IgG KPL 04-18-18). The membrane was incubated shaking with ABC reagent and DAB substrate reagent (InnoGenex A-0401). (Kang *et al.*, 2005). The microtiter was set up with 96-well microtiter plates (EnbioTec IBTM-3500, Japan). Absorbances were determined at 450 nm with spectrophotometer (Kazuto *et al.*, 2002).

The vitellogenin concentrations were calculated by this equation Vtg Con.(‰) = C/D × 1,000 at 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.

We used 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

Male and female fish were exposed to 0.1, 1, 10 and 100 ppm of CTC. Western blot results showed approximately 205 kDa, that is similar to myosin

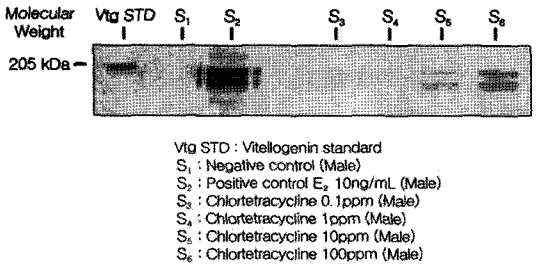

Fig. 1. Result of Western blot for male fish which were exposed to chlortetracycline 0.1, 1, 10, and 100 ppm for 3~5 days.

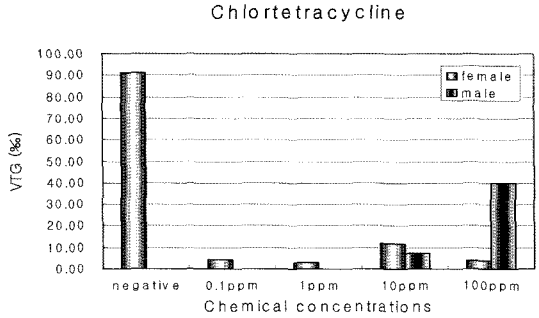

Fig. 2. Vitellogenin induction by chlortetracycline in Japanese medaka.

at high molecular weight range Sigma maker. Vtg band of male fish was showed fainted to 10 and 100 ppm for chlortetracycline as shown in Fig. 1. Vtg concentration of CTC was qunatified by total protein quantification and ELISA. Exposure of the male fish to CTC of 0.1, 1, 10 and 100 ppm produced Vtg concentrations of 0.24, 0.12, 7.61 and 40.02‰, respectively as shown in Fig. 2, that value was elevated than control male fish (0.14‰).

Vtg induction was inhibited by the CTC exposure at female fish. Negative control female fish was expressed Vtg about 90‰ of total protein, but CTC exposed female fish manifested Vtg about 0.12~40.02‰ of total protein, up to the dose of chemical. CTC could exerted to the Vtg protein expression in male fish, but not in female fish. On the contrary CTC acted as a Vtg diminution factor in female fish.

Discussions

The possible sources of human health pharmaceuticals in the environment, excluding nonroutine events such as a potential transportation accident or a waste water treatment plant (WWTP) release of untreated sewage during a storm flow, could theoretically include 1) releases from pharmaceutical manufacturing facilities, 2) disposal of pharmaceutical product from the supply chain (prior to distribution to patient), 3) disposal by patients or health care facilities of unused pharmaceuticals either to wastewater or to solid waste, or 4) patient excretion of an active pharmacuetical ingredient (API) and metaboites to wasetwater. It is generally accepted that the principal source of human pharmaceuticals detected in the aquatic environment is patient excretion. Although there is no definitive mass-balance study substantiating this conclusion, the basis for it is reasonable. The following provides an analysis of each of the 4 possible sources of human pharmaceuticals in the environment. Discharge from pharmaceuticals manufacturing facilities, disposal of pharmaceutical products from the prepatient supply, patient disposal of unused pharmaceutical products, and patient use. But in veterinary pharmaceuticals were possible as these 4 sources and animal husbandary for prophylaxis (Williams RT, 2005). CTC was used as a veterinary medicines by sales amout 2,763 tons in Korea 2005 (Table 1). Also the chemical structure of CTC was shown in Fig. 3.

Vtg concentration of each antibiotics was quantified by total protein quantification and ELISA. Exposure of the male fish to chlortetracycline of 0.1, 1, 10 and 100 ppm produced Vtg concentrations of 0.24, 0.12, 7.61 and 40.02‰, respectively. While

Table 1. Veterinary medicines by sales amount in 2005

Ingredient	CAS No.	Use	Sales amount* (kg)
Chlortetracycline	57-62-5	antibiotics	2,762,951

*: KAHPA 2005 (Korea Animal Health Products Association)

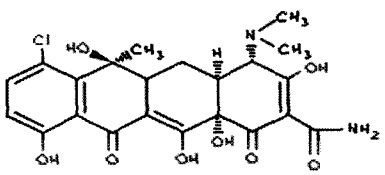

Fig. 3. Structures of chlortetracycline used in this study.

Table 2. Initiation concentrations of chlrotetracycline was inducing endocrine disruption in male medaka

Antibiotics	Sex	Initiation concentrations (ppm)
Chlortetracyline	Female	0.1
	Male	10

male fish was showed significant increase of Vtg induction and the endocrine disruption induced by antibiotics.

However, female medaka did exhibit significant differences in Vtg induction. Exposure of the female fish to chlortetracycline of 0.1, 1, 10 and 100 ppm produced Vtg concentrations of 4.26, 2.78, 12.03 and 3.74‰, respectively. After termination of exposure by chlortetracycline, the Vtg concentration dropped to 1/10~1/30 of in non-exposed fish (90.99‰).

This study demonstrated that the endocrine disruption induced by antibiotics in male and female medaka are affected by treatment with selected estrogenic compounds, and suggests that these results may be useful molecular biomarkers for screening EDCs in the shortest possible time.

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