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Effect of long-term exposure to bezafibrate on steroidogenesis and reproduction of Japanese medaka (*Oryzias latipes*)

고지혈증 치료제 bezafibrate의 만성노출에 따른 수서생물의 생식영향과 기전

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ABSTRACT

Effect of long-term exposure to bezafibrate on steroidogenesis and reproduction of Japanese medaka (*Oryzias latipes*)

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Fibrates are commonly used lipid regulator to control hypercholesterolemia in humans, and have been frequently detected in the freshwater environment. Bezafibrate is one of lipid regulators and has been detected in streams at up to 202.7 ng/L. While lipid regulation may lead to altered steroidogenesis, endocrine disruption potential of bezafibrate in aquatic non-target organisms has not been well documented. Present study aimed to understand ecological risks of this lipid regulator, and to identify potential endocrine disruption and related mechanisms. The PNEC is the concentration below which exposure to a chemical is not expected to cause an adverse effects. The MEC is the detecion levels found in environmental sample. The PNEC value of bezafibrate was calculated at 2.3 μg/L and hazard quotient (HQ) based on PNEC and MEC values was estimated at 0.09. Following long-term (135

day) exposure of Japanese medaka (*Oryzias latipes*) to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L), reproduction performance of the fish, e.g., number of eggs, hatchability, and hatching time, was affected. Such changes were accompanied by sex hormone alterations, i.e., significant E2 increase in female and decreasing T in male fish, at 165 days post-hatching (dph). Changes in sex hormone concentrations were supported by regulational changes of several steroidogenic genes. In gonads, steroidogenic genes such as *star*, *3β-hsd, cyp11b*, *and cyp19a* showed up-regulation following the exposure. Bezafibrate appears to regulate lipid metabolism, as suggested by up-regulation of *ppara1*, *ppara2* gene expression were observed in both male and female fish. However, plasma cholesterol levels were only reduced in male fish following the exposure. Increased steroidogenesis following long-term bezafibrate exposure in fish is different from the observations of the 30 day early life stage exposure and other previous reports. Our observations clearly show that long-term exposure to this lipid regulator can stimulate steroidogenesis among aquatic non-target species and increase estrogenicity that could lead to changes in reproduction.

Keywords : Fibrate, multi-generation, lipid regulator, steroidogenesis, reproduction, ecological risk assessment, Japanese medaka.

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1. Introduction

Pharmaceuticals are widely used to treatment or livestock of human and animal disease (Boxall et al., 2012). These physiologically active anthropogenic chemicals have been detected in the aquatic environment at concentrations ranging from μg/L in the effluents of wastewater treatment plants to ng/L in surface waters (Fent et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003a; Metcalfe et al., 2003b; Mimeault et al., 2005). Pharmaceuticals are designed to alter biological responses in humans and animals, and therefore could influence normal physiology of non-target species such as water fleas and fish. However, little is known about their effects to aquatic organisms (Skolness et al., 2012; Weston et al., 2009). Due to the biological activities and often persistent natures of pharmaceutical residues (Monteriro and Boxall, 2009), their potential effects on aquatic organisms are of growing concern (Boxall et al., 2012).

Based on National Health Insurance service (NHIS) in Korea, the number of hyperlipidemia patients has increased by 11.5% for the last several years (NHIS, 2013). The prevalence of hyperlipidemia was among the highest in WHO region of Europe (54% for both sexes) according to Global Health Observatory (GHO) data. Such increase in the disease prevalence has led to the increased in worldwide use of cholesterol-lowering medications. Mantel-Teeuwisse et al. (2002) reported the prevalence of lipid lowering

drug use increased from 0.5% to 2.3% in women and from 0.6% to 2.9% in men from 1991 to 1998 in the Netherland (Mantel-Teeuwisse et al., 2002). During the 25-year study period use of cholesterol-lowering medication increased linearly from less than 1% of the participants in 1992 to 6.5% in 2010 in Northern Sweden (Johansson, 2012). The use of cholesterol-lowering medication in the United States grew from 5% in 1988-1994 to 17% in 2001-2004, and reached 23% in 2007-2010 (Kuklina et al., 2013). Health insurance reimbursement data showed the use of lipid-lowering medication increased rapidly among people with diabetes in the early 2000s in Finland (Vehko et al., 2013). During the 2003-2012, prescription of cholesterol-lowering medication in the United States increased with age, from 17% of adults aged 40-59 to 48% of adults aged 75 and over (Kit et al., 2014).

Fibrates are commonly used pharmaceuticals to control lipidemic diseases such as hypercholesterolemia in humans. On that basis, fibrates are consistently released from aquatic environment. In addition, aquatic organisms was continuously exposed to in this environment, eventually aquatic organisms could be affected by the fibrates. Bezafibrate is one of the frequently used fibrates. This drug is rapidly and almost completely absorbed in the human body. A peak plasma concentration of about 10 mg/L is reached after about 2 hours(h) following an immediate-acting formulation of bezafibrate (300 mg) in healthy volunteers. With 400 mg bezafibrate retard, a peak concentration of about 6 mg/L is reached after 3-4 h.

94−96% of bezafibrate is bound to protein in human serum, and the apparent volume of distribution is about 17 L. Elimination is also rapid, with almost exclusive renal excretion. After a 300mg dose 94% of drug was recovered in the urine within 24 h and 3% in the feces within 48 h. The elimination half−life of bezafibrate is about 1−2 h (Actavis New Zealand, 2013; Kajosaari et al., 2004; Miller and Spence, 1998; Monk and Todd, 1987).

Bezafibrate has been detected in aquatic environment, at up 25.5 ng/L and 4.6 μg/L in surface water of Spain and sewage treatment plant (STP) effluents of Germany (Silva et al., 2011; Ternes, 1998a). However, ecotoxicological information about bezafibrate is mostly confined to short−term exposure, limiting our understanding on its potential risks in ambient environment.

Fibrates reduce the level of plasma cholesterol by activating peroxisome proliferator−activated receptors (PPARs). All steroid hormones derived from cholesterol, and normal adrenal steroidogenesis depends on cholesterol delivery from plasma (Evans, 1988; Staels et al., 1998; Velasco−Santamaria et al., 2011). Reduced plasma cholesterol level has been linked to adverse effects on spermatogenesis (Velasco−Santamaria et al., 2011). However detailed mechanisms of the processes remain unclear. A few short−term exposure studies have reported in fish that the lipid regulator could affect sex hormone balance. However little is known for consequences of long−term exposure to bezafibrate in fish. This knowledge gap is important because in order to understand potential

effect of endocrine disruption and the modes of action that lead to reproductive performance, short-term exposure gives only limited snapshots and longer term exposure tests are necessary (Nash et al., 2004).

The present study was conducted with two objectives. Firstly, acute and chronic ecotoxicological information was collected for bezafibrate using standard experimental organisms such as water fleas and fish. This information can be used to derive predicted no effect concentrations (PNECs) and to estimate ecological risks for aquatic environment. Secondly, endocrine and reproduction related effects of bezafibrae in fish were investigated following long-term (165 day) exposure. Related mechanisms of action were examined at hormones and gene transcription levels. The results of this study will provide valuable information on adverse endocrine effects of bezafibrate on non-target aquatic organisms, and on its potential ecological risk.

2. Materials and Methods

2.1 Test chemicals

Bezafibrate (CAS No. 41859-67-0) was purchased from Sigma-Aldrich (St. Lousie, Mo, USA; C4522). Dimethyl sulfoxide (DMSO) was used as a solvent with the final concentrations in the exposure media being less than 0.1%(v/v). Test solutions for each pharmaceutical were prepared immediately before the experiments.

2.2 Maintenance of test organisms

2.2.1 Crustaceans – *Daphnia magna* (*D. magna*) and *Moina macrocopa* (*M. macrocopa*)

The two crustaceans (*D. magna* and *M. macrocopa*) were cultured in Elendt M4 media and maintained in Environmental Toxicology Laboratory of Seoul National University since 2003, following the protocols by US Environmental Protection Agency (US EPA) guidelines, Oh and Choi (2012) (EPA, 2002; Oh and Choi, 2012). Both freshwater crustaceans were fed with chlorella twice a day. Water quality parameters, including temperature, dissolved oxygen, pH, and conductivity were regularly measured.

2.2.2 Fish – Japanese medaka (*Oryzias latipes*)

The Japanese medaka; *Oryzias latipes*) were cultured in conditioned water, which was manufactured with sodium bicarbonate, calcium

sulfate and sea salt in distilled water, at 25 ± 1℃ under a 16:8 h light-dark photoperiod. *O. latipes* were fed with *Artemia nauplii* (< 24 h after hatching) twice a day. Measuring water quality parameters was the same as that of measured in crustaceans.

2.3 Acute and chronic toxicity test with two crustaceans (*D. magna* and *M. macrocopa*)

The 48 h acute toxicity test with *D. magna* and *M. macrocopa* was carried out US EPA guidelines (2012), OECD test guidelines 202 (OECD, 2004). Test concentrations were prepared by dissolving bezafibrate in Elendt M4 media with 3-fold dilution, i.e., 0, 3.7, 11.11, 33.33, 100, or 300 mg/L of *D. magna*, and 0, 18.75, 37.5, 75, 150, or 300 mg/L of *M. macrocopa*. Four replicates with five neonates (<24 h old) were exposed for 48 h. The immobilized crustaceans were counted after 24 h and 48 h exposure. Test crustaceans were not fed during the test. The result of acute toxicity test was used for range of test concentrations in the chronic toxicity test.

The 21 day *D. magna* and 7 day *M. macrocopa* chronic toxicity test were assessed following the protocols by OECD test guidelines 211 (OECD, 2012) and Oh and Choi (2012) (OECD, 2012; Oh and Choi, 2012). Exposure concentrations of 0, 3.7, 11.11, 33.33, 100, or 300 mg/L were chosen based on a preliminary range finding test. The test solutions were renewed 3 times per week and offspring of

parent crustaceans were counted and removed daily. Mortality of parent crustaceans and the number of survival offspring were counted every day. Also, survival, time to first reproduction, number of young per adult, and number of young per brood were measured. The population growth rate (PGR, r) was indicated using the Euler–Lotka equation (Lotka, 1913). During the test two crustaceans were fed with *chlorella* 300 μL daily per each organism. Water quality parameters, including temperature, dissolved oxygen, pH and conductivity were measured during acute and chronic toxicity test.

2.4 Ecological risk assessment

Ecological risk assessments were performed to characterize the degree of contamination and to evaluate the adverse effects of bezafibrate in real aquatic environment (Norton et al., 1992). The hazard quotient (HQ) of bezafibrate was calculated by dividing the measured environmental concentration (MEC) reported previous studies by the PNEC value of bezafibrate (Table 4). PNEC for bezafibrate was derived from the most sensitive toxicity test data and employed a appropriate assessment factor of 10, as recommended by European Chemicals Bureau (2003). Only toxicity data that measured ecologically relevant endpoint, e.g., mortality, immobilization, reproduction and growth inhibition were used. A HQ value is calculated to be less than 1, then no adverse effects are expected as a result of exposure.

2.5 *Oryzias latipes* long-term exposure toxicity test

A long-term exposure toxicity test was initiated with newly fertilized eggs for 165 days (Fig. 1). This test was designed to assess the effects on development and reproduction performance During the test, *O. latipes* were fed with freshly *Artemia nauplii* twice a day. Water quality parameters, including temperature, dissolved oxygen, pH and conductivity was measured regularly.

2.5.1 Waterborne exposure for early-life stage (ELS) test

Early life stage (ELS) test was initiated with newly fertilized eggs (<24 h after spawning). The test was carried out the OECD test guideline 210 early-life stage toxicity test (OECD, 2013) and measured to assess the effects on development, e.g., hatchability, time to hatch and survival. Fertilized eggs were exposed to 0, 0.01, 0.1, 1, 10, or 100 mg/L of bezafibrate. DMSO was used as a solvent at concentrations of less than 0.1%(v/v) in the test. Each treatment was composed of four replicates with 15 eggs each in 50 mL beakers. Test solutions were renewed three times a week.

2.5.2 Larvae and juvenile stage

Hatched fry were transferred to other beaker (250 mL) and were recorded hatchability, time to hatch and survival until all embryos were hatched. After 30 day exposure, five juvenile *O. latipes* in

each treatment were randomly chose and measured for body length and dry weight. Another five juvenile *O. latipes* were randomly chose for whole body gene transcription and were immediately stored in RNA later RNA Stabilization Reagent (QIAGEN, Korea Ltd., Seoul, Korea). The other juvenile *O. latipes* were moved into 1 L beaker and maintained until 165 days post-hatching (dph).

2.5.3 Adult stage – reproduction performance and screening endocrine disruption

After 135 day exposure, six pairs of fish were randomly choose from each treatment for mating. Test solutions were same exposure condition for 14 days. The two mating pairs spawned for the first 3 days were removed. Since then, eggs spawned for 11days collected and counted every day. The collected eggs transferred to clean conditioned water, and hatchability and time to hatch were recorded.

Five adult *O. latipes* in each treatment at 165 dph were randomly chose and anesthetized in ice. The tail of adult *O. latipes* was cut, and blood from caudal vein was collected using a heparinized glass capillary tube. Plasma was collected after blood centrifugation (8,000 rpm for 10min at 4℃) and then the supernatant was collected and stored at −80℃ until sex hormones, such as 17β-estradiol (E2), testosterone (T) and cholesterol analysis. After dissection, liver and gonads in each *O. latipes* were immediately stored in RNA later RNA Stabilization Reagent (QIAGEN, Korea

Ltd., Seoul, Korea) for RNA extraction.

2.6 Sex hormones and cholesterol measurement

Plasma was used for sex hormones and cholesterol measurement. Sex hormones, such as E2 and T, were measured by competitive enzyme-linked immunosorbent assay (ELISA) using commercially available kits (17β-estradiol (Cat No.582251), Testosterone (Cat No. 582701, Cayman Chemical Company, Ann Arbor, MI, USA). For cholesterol measurement, the extracted plasma samples were diluted 1:300 with cholesterol assay buffer and froze at -80℃ before the measurement. Cholesterol were measured by ELISA using commercially available kits (Cholesterol (Cat. No. 10007640), Cayman Chemical Company, Ann Arbor, MI, USA).

2.7 Gene transcription by Quantitative real-time polymerase chain reaction (PCR)

The total RNA of the sample were extracted from whole body, liver and gonad using a RNeasy minikit (QIAGEN, Valencia, CA, USA). The complementary DNA was synthesized from the purified RNA samples using an iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR reaction mixture contained 1.8 μL of forward and reverse primers, 3.0 μL of cDNA template, 3.4 μL of RNase-free

water, 10 μL of SYBR Green Master Mix (Applied biosystems). Thermal cycling was 50℃ for 2 min in pre-incubation, 95 ℃ for 10 min in activation, 40 cycles at 95 ℃ for 15 sec in denaturation and 60℃ for 1min in anneal/extend. The amount of PCR product was quantified using the threshold cycle *C*t number. For each selected gene, quantification was performed with five replicate samples. The mRNA expression level of each target gene was normalized to the mRNA content of its reference gene (*β-actin*) using the $^{\Delta\Delta}C$t method (Livak and Schmittgen, 2001). Primer information that was used for *O. latipes* is listed in Table S1.

2.8 Statistical analysis

The median effective concentration (EC_{50}) and 95% confidence intervals in 48 h acute toxicity test of *D. magna* and *M. macrocopa* were calculated by probit analysis using ToxStat version 3.5 (West Inc., Cheyenne, WY, USA). No observed effect concentrations (NOECs) and Lowest observed effect concentration (LOECs) were calculated by Fisher's exact test in ToxStat. For endpoints in fish toxicity test data, one-way analysis of variance (ANOVA) with Dunnett's test as well as linear regression analysis were performed to examine trends in the data, using SPSS 21.0 for Windows® (SPSS Inc., Chicago, IL, USA) to test for differences from control, dose-response pattern. *p*-values less than 0.05 considered to be statistically significant.

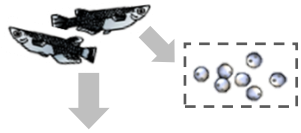
Duration		Endpoints
0 dpf	Eggs 	Japanese medaka eggs exposed to bezafibrate
7 dpf (0 dph)	Larvae 	hatchability, time to hatch
9 dph	Fry 	fry survival
30 dph	Juvenile 	juvenile survival, Length, Weight mRNA expression
135 dph	Adult 	F_1 (the number of eggs, the number brood, hatchability, time to hatch)
165 dph		Blood (steroid hormone, cholesterol) Organs (mRNA expression)

Fig. 1. Experimental procedures for *O. latipes* long-term (165 dph) exposure toxicity test. The 7th day after the fertilization of the egg was assigned as the first day post-hatch (dph). At 135 dph, six mating pairs from the control or each treatment were selected for the examination of effects on reproduction performance.

3. Results

3.1 Acute and chronic toxicity of bezafibrate on *D. magna* and *M. macrocopa*

After 48 h exposure test, EC_{50} was estimated to be 234.64 mg/L (95% confidence interval (CI) 192.49 – 276.80 mg/L) in *D. magna* and 247.07 mg/L (95% CI 203.50 – 290.65 mg/L) in *M. macrocopa* (Table 1). The effect of 21 day *D. magna and* 7 day *M. macrocopa* chronic toxicity test were shown in Table 2. After 21-day chronic exposure of *D. magna*, the survival NOEC for bezafibrate was 33.33 mg/L. The first day of reproduction was significantly delayed at ≤33.33 mg/L. The number of young per adult in *M. macrocopa* significantly decreased at 300 mg/L. For *M. macoropa*, the number of young per female and the number of young per brood were significantly decreased at 300 mg/L. The population growth rate were decreased (β = −0.779 and β = −0.871) by exposure to bezafibrate in a concentration dependent manner (Table 2).

Table 1. Results of 48 h acute toxicity test with *D. magna* and *M. macrocopa*.

Test organism	Exposure duration (h)	EC_{50} (95% CI) (mg/L)
D. magna	48	234.64 (192.49 – 276.80)
M. macrocopa	48	247.07 (203.50 – 290.65)

Abbreviation: EC_{50}: the median effective concentration; CI: confidence interval.

Table 2. Results of chronic toxicity test with *D. magna* (21 day) and *M. macrocopa* (7 day).[a]

Test organisms	Concentration (mg/L)	Survival (%)	First day of reproduction (d)	No. of young per female	No. of young per brood	Growth (mm)	Population growth rate
D. magna	Control	100	7.8±0.9	110.1±6.7	23.1±2.4	3.8±0.1	0.427
	3.70	100	8.5±0.7	109.2±11.0	23.3±1.0	4.0±0.2	0.387
	11.11	100	8.8±0.8	108.4±16.7	23.6±2.7	3.9±0.2	0.374
	33.33	100	9.0±0.7*	103.9±13.9	23.2±2.9	3.8±0.3	0.365
	100	90	9.1±0.6*	98.9±10.9	20.2±1.8*	3.6±0.3	0.346
	300	0*	NA[b]	NA	NA	NA	NA

[a] Values represent mean ± standard deviation of each bezafibrate concentration.

[b] NA: not applicable due to significant mortality.

* Asterisk denotes a significant difference from the control (p<0.05).

Table 2. Continued.

Test organisms	Concentration (mg/L)	Survival (%)	First day of reproduction (d)	No. of young per female	No. of young per brood	Growth (mm)	Population growth rate
M. macrocopa	Control	90	3.0±0.0	58.7±11.3	20.2±2.4	–	0.894
	18.75	90	3.0±0.0	55.1±12.6	20.9±3.8	–	0.875
	37.50	90	3.0±0.0	57.3±7.4	22.9±3.1	–	0.871
	75	90	3.1±0.3	62.7±10.1	22.8±3.0	–	0.905
	150	100	3.2±0.4	57.5±8.0	21.6±2.7	–	0.880
	300	60	3.5±0.6	32.3±9.2*	14.6±3.4*	–	0.683

[a] Values represent mean ± standard deviation of each bezafibrate concentration.

[b] NA: not applicable due to significant mortality.

* Asterisk denotes a significant difference from the control (p<0.05).

3.2 Sex endocrine and reproduction related effects following long-term exposure of *Oryzias latipes*

3.2.1 Effects on early-life stage (ELS) test - 30 day post-hatch (dph)

The results of ELS test were shown in Table 3. The ELS test were observed to significantly effects on hatchability, fry survival and juvenile survival at 100 mg/L. There were no significant effect but concentration-dependent delayed in time to hatch was observed.

Table 3. Results of early life stage (ELS) test in *O. latipes* exposed to bezafibrate.

Concentration (mg/L)	Hatchability (%)	Time to hatch (d)	Fry survival (7 d)	Juvenile (30 dph)		
				Survival (%)	Dry weight (mg)	Length (cm)
Control	96.7±3.8	8.5±1.6	96.4±4.1	86.3±5.3	1.18±0.11	0.98±0.11
0.01	93.3±5.4	8.5±1.4	92.6±6.3	83.9±3.6	1.38±0.66	0.95±0.11
0.1	90.0±3.8	8.7±1.7	88.7±4.8	77.9±5.4	1.36±0.38	0.98±0.08
1	93.3±5.4	8.3±1.7	92.6±6.3	73.1±4.0	1.54±0.29	1.03±0.10
10	91.7±3.3	8.7±1.5	90.8±4.1	74.6±3.7	1.02±0.54	0.97±0.12
100	85.0±6.4*	9.2±1.5	81.9±8.6*	68.2±8.3*	1.32±0.56	0.93±0.07

*Asterisk denotes a significant difference from the control (p<0.05).

Table 4. Derivation of predicted no effect concentration (PNEC) and hazard quotient (HQ) of bezafibrate.

NOEC (mg/L)[C]	AF[C,d]	PNEC (ng/L)	MEC (ng/L)[C]	HQ
0.023 [a]	10	2300	202.7 [b]	0.09

[a] Based on the lowest Crustaceans (*Ceriodaphnia dubia*) 7 d reproduction NOEC in previous study.

[b] Based on the maximum detected concentration in Italy water (previous study).

[C] Based on long-term NOECs from species representing three trophic levels (fish, Daphnia and algae).

[d]Abbreviation: NOEC (no observed effective concentration), AF (assessment factor), MEC (measured environmental concentration)

Table 5. Studies of acute and chronic toxicity test of bezafibrate on aquatic organisms.

Exposure type	Organisms	Test species	Duration	Endpoints	Concentration (95% CI) (mg/L)	Reference
Acute	Bacterium	*Vibrio fischerif*	15min	luminescence EC_{50}	178.73 (162.06−197.12)	Rosal et al., 2010
			30min	luminescence EC_{50}	172.73 (155.52−191.85)	Rosal et al., 2010
	Crustacean	*Ceriodaphnia dubia*	48 h	immobilization EC_{50}	75.79 (60.13−81.01)	Rosal et al., 2010
		Thamnocephalus platyurus	24 h	mortality LC_{50}	39.69 (24.93−63.17)	Isidori et al., 2007
		D. magna	24 h	immobilization EC_{50}	100.08 (80.02−120.54)	Rosal et al., 2010
			48 h	immobilization EC_{50}	240.40 (230.12−250.68)	Rosal et al., 2010
			48 h	immobilization EC_{50}	>200	Hernando et al., 2004
			48 h	immobilization EC_{50}	30.3	Han et al., 2006
			48 h	immobilization EC_{50}	25	Sanderson, 2003
			48 h	immobilization EC_{50}	234.64 (192.49−276.80)	This study
		M. macrocopa	48 h	immobilization EC_{50}	247.07 (203.50−290.65)	This study

Table 5. Continued.

Exposure type	Organisms	Test species	Duration	Endpoints	Concentration (95% CI) (mg/L)	Reference
Chronic	Algae	*Pesudokirchneriella subcapitata*	72 h	growth EC_{50}	>60	Isidori et al., 2007
	Crustacean	*Ceriodaphnia dubia*	7 d	reproduction NOEC	0.023	Isidori et al., 2007
			7 d	reproduction LOEC	0.047	Isidori et al., 2007
		D. magna	21 d	reproduction NOEC	11.11	This study
			21 d	survival NOEC	100	This study
		M. macrocopa	7 d	reproduction NOEC	>300	This study
			7 d	survival NOEC	>300	This study
	Fish	*O. latipes*	30 dph	survival NOEC	10	This study

Abbreviation: EC_{50}: median effective concentration, LC_{50}: lethal concentration, NOEC: no observed effect concentration, min: minutes, h: hours, d: days, dph: day post-hatch.

Table 6. Concentrations of bezafibrate measured at each sampling sites in previous studies.

Country (city)	Sampling site	Concentration (ng/L)				Reference
		Min.	Max.	Median	Mean	
Germany	STP effluent	–	4600	2200	–	Ternes, 1998b
Germany (Montreal)	WWTP effluent	–	–	–	35	Garcia-Ac et al., 2009
Germany (Montreal)	South raw sewage				50	Garcia-Ac et al., 2009
Germany (Montreal)	North raw sewage				53	Garcia-Ac et al., 2009
Italy (Milan)	River	134.3	202.7	168.5	168.5	Zuccato et al., 2000
Italy (Pizacenza, Cremona)	River	15.1	22.4	18.75	18.75	Zuccato et al., 2000
Italy (Sondrio)	River	1	6	3.5	3.5	Zuccato et al., 2000
Canada (Detroit)	River	–	200	52	–	Metcalfe et al., 2003c
Italy (Chivasso)	River	–	–	–	1.58	Calamari et al., 2003
Italy (Mezzano)	River	–	–	–	1.09	Calamari et al., 2003
Italy (Boscone)	River	–	–	–	0.79	Calamari et al., 2003
Italy (Piacenza)	River	–	–	–	2.75	Calamari et al., 2003
Italy (Cremona)	River	–	–	–	2.66	Calamari et al., 2003
Italy (Casalmaggiore)	River	–	–	–	1.92	Calamari et al., 2003

Abbreviation: Min.: minimum, Max.: maximum

Table 6. Continued.

Country (city)	Sampling site	Concentration (ng/L)				Reference
		Min.	Max.	Median	Mean	
Italy (Pieve saliceto)	River	–	–	–	2.3	Calamari et al., 2003
Italy (Parco Lambro)	River	–	–	–	57.15	Calamari et al., 2003
Germany (Montreal)	River	–	–	–	8	Garcia-Ac et al., 2009
UK (South Wales)	Taff river	5	66	33.18	35.5	Kasprzyk-Hordern et al., 2008
UK (South Wales)	Ely river	3	76	39.5	39.5	Kasprzyk-Hordern et al., 2008
Spain (Catalonia)	Llobregat River	0.03	15.06	7.55	1.02	Pal et al., 2010
Germany (Berlin)	Lake Wannsee	–	–	–	170	Heberer et al., 2011

Abbreviation: Min.: minimum, Max.: maximum

3.2.2 Effects on reproduction performance

The results of reproduction performance were shown in Fig. 2. After 135 day exposure, a variety of parameters, e.g., hatchability and time to hatch, related to reproduction were measured. The total number of eggs for 11days in control or each treatment were significantly reduced at 100 mg/L (Fig. 2B). Hatchability was significantly reduced at 100 mg/L and time to hatch was significantly delayed at ≤1 mg/L (Fig. 2C and 2D).

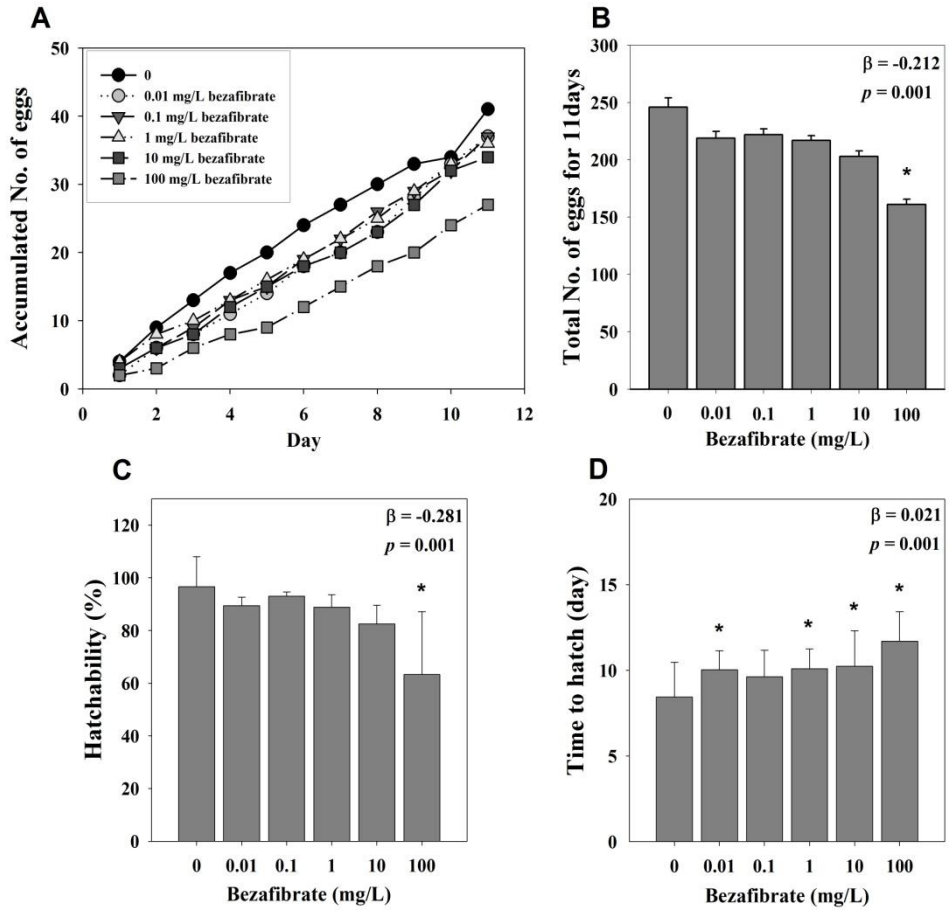


Fig. 2. Effects on reproduction performance of adult *O. latipes* pairs at 135 dph. (A) Accumulated number of eggs per mating pair, (B) Total number of eggs for 11days in control or each treatment, (C) Hatchability (%) and (D) Time to hatch (day) were determined. Values represent the mean ± standard deviation of six mating pairs. Data are expressed as fold− change relative to the control. Asterisk (∗) indicates a significant difference from control (p<0.05). The β (slope) and p values for trends were determined based on linear regression.

3.2.3 Effects on sex hormones levels

The results of plasma E2 and T were shown in Fig. 3. In male fish, the plasma T levels were decreased, but statistical significance was not observed (β = −0.461) (Fig. 3B). In female fish, the plasma E2 levels were significantly increased at 100 mg/L (Fig. 3A). However E2 levels in male fish and T levels in female fish showed no significant difference compared with control (Fig. 3A and 3B).

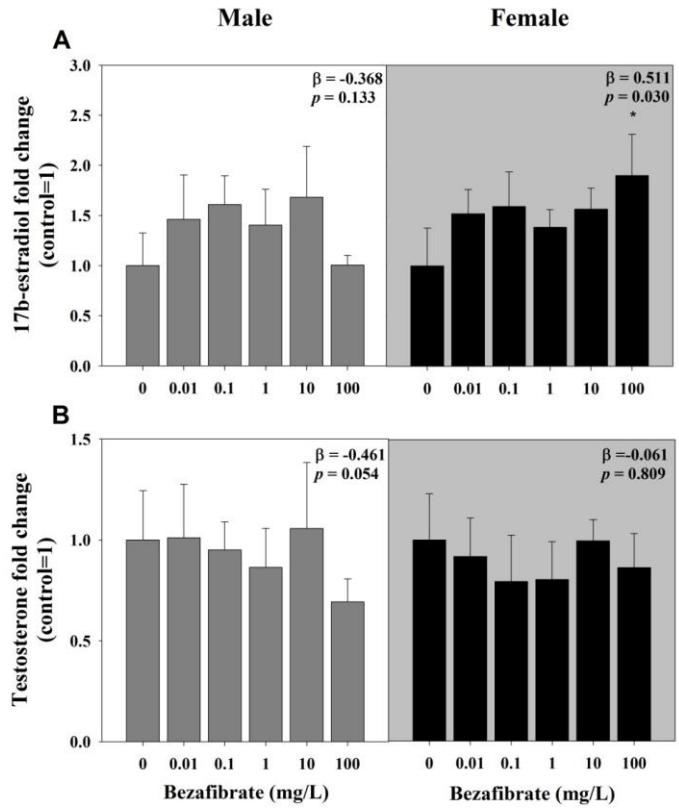


Fig. 3. Effects of bezafibrate on adult *O. latipes* plasma sex hormone levels. (A) 17β−estradiol (E2) and (B) testosterone (T) measured by ELISA in adult male and female *O. latipes* after exposure to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L) for 165 dph. Values represent the mean ± standard deviation of six mating pairs. Data are expressed as fold− change relative to the control. Asterisk (*) indicates a significant difference from control (p<0.05). The β (slope) and p values for trends were determined based on linear regression.

3.2.4 Effects on transcription of endocrine related genes in adult stage

The results of transcription of related genes were shown in Fig. 4, 5, 6A, and 6B. After 165 day exposure, Transcription of *vtg1*and *vtg2* genes were significantly increased at 100 mg/L in the liver of both male and female (Fig. 4C and 4D). In male, the significantly increased of *era* gene was observed at 100 mg/L. However, the decreased of *erβ* gene was observed by exposure to bezafibrat in a concentration dependent manner ($\beta = -0.411$) (Fig 4A and 4B). In female fish, the significantly increased of *erβ* gene was observed at 100 mg/L (Fig. 4B). In gonad, transcription of *erα* gene was significantly increased at <0.1 mg/L in both male and female (Fig. 5A). In female fish, the significantly increased of *cyp17* gene was observed at 100 mg/L and significantly increased of *cyp19a* gene was observed at <10mg/L (Fig. 5E and 5H). Some steroidogenic genes, such as *3β-hsd*, *cyp11b* and *cyp19a* showed increasing trend in male fish (Fig. 5D, 5G and 5H).

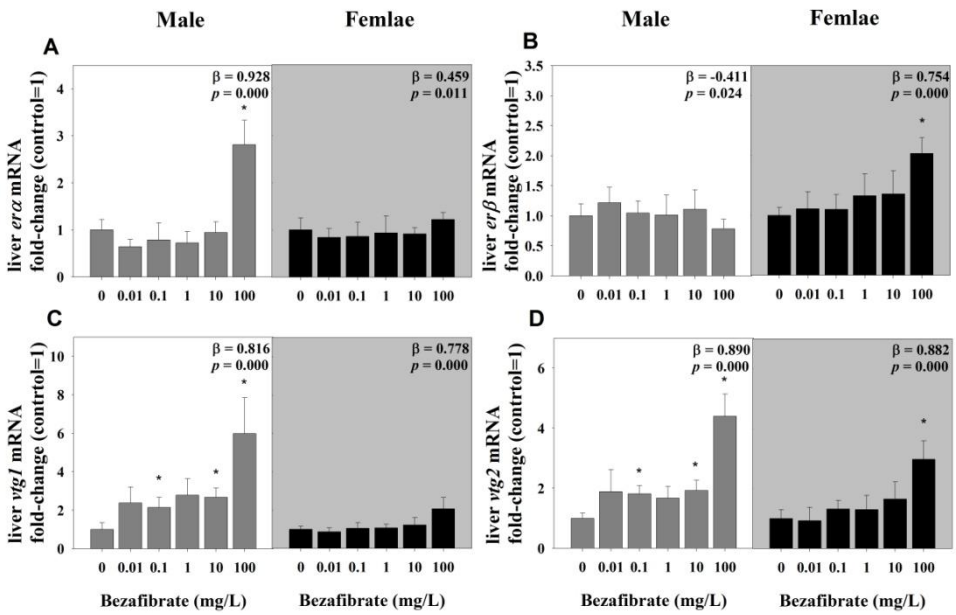


Fig. 4. Effects on (A) *erα*, (B) *erβ*, (C) *vitellogenin1* (*vtg1*) and (D) *vitellogenin2* (*vtg2*) gene transcription measured by real-time PCR in adult male and female *O. latipes* liver after exposure to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L) for 165 dph. Values represent the mean ± standard deviation of six mating pairs. Data are expressed as fold- change relative to the control. Asterisk (*) indicates a significant difference from control ($p<0.05$). The β (slope) and p values for trends were determined based on linear regression.

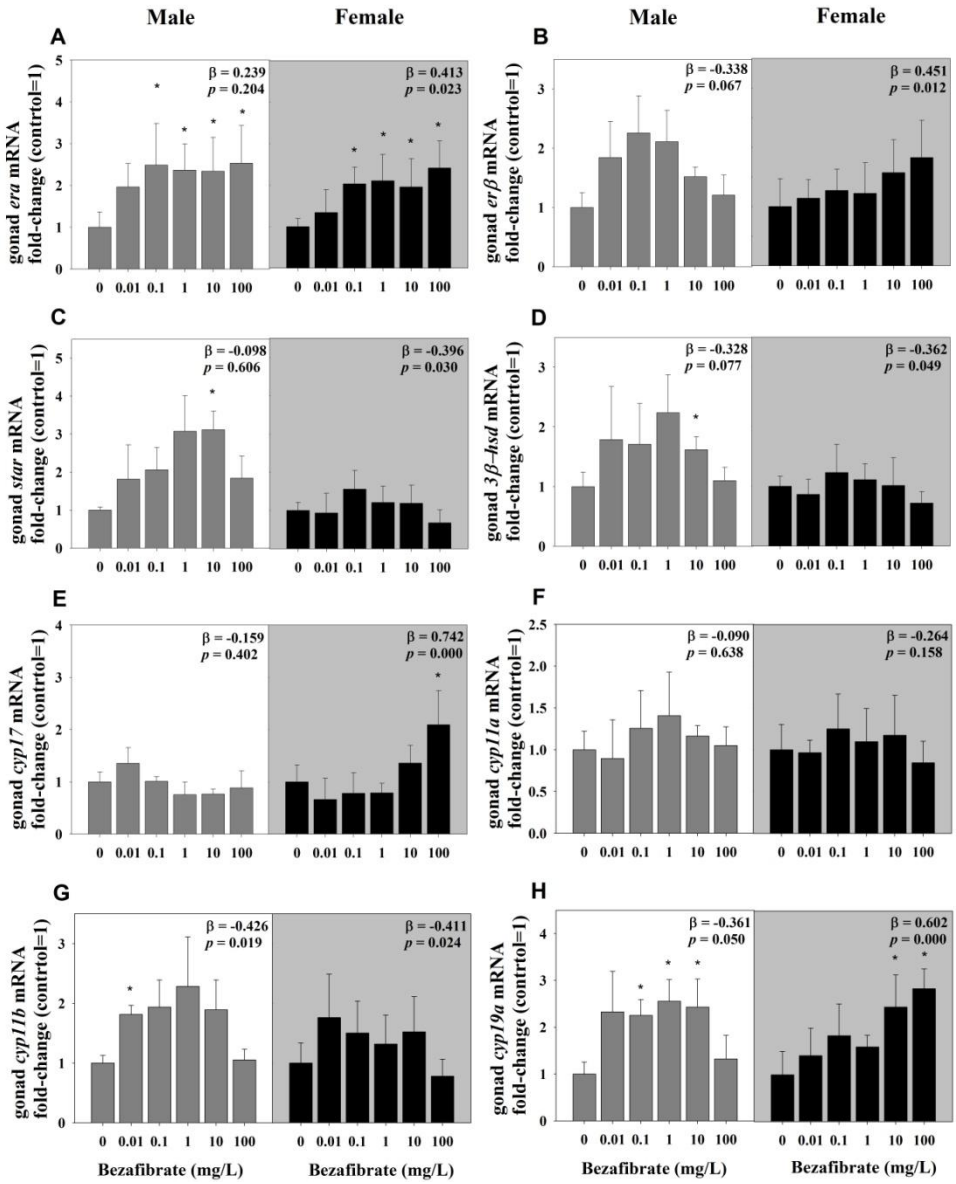


Fig. 5. Effect on (A) *erα*, (B) *erβ*, (C) *star*, (D) *3β-hsd*, (E) *cyp17*, (F) *cyp11a*, (G) *cyp11b* and (H) *cyp19a* gene transcription measured by real-time PCR in adult male and female *O. latipes* gonad after exposure to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L) for 165 dph. Values represent the mean ± standard deviation of six mating pairs. Data are expressed as fold- change relative to the

control. Asterisk (*) indicates a significant difference from control (p<0.05). The β (slope) and p values for trends were determined based on linear regression.

3.2.5 Effects on cholesterol level and related to lipid regulation genes

The results of plasma cholesterol level and transcription of related to lipid regulation genes were shown in Fig. 6. Transcriptions of *ppara1* and *ppar α2* genes were significantly increased at 100 mg/L in the liver of both male and female fish. The levels of cholesterol in male fish were decreased in a concentration dependent manner (β = −0.636) (Fig. 6C). However the level of cholesterol in female fish showed no significant difference compared with control (β = 0.256) (Fig. 6C).

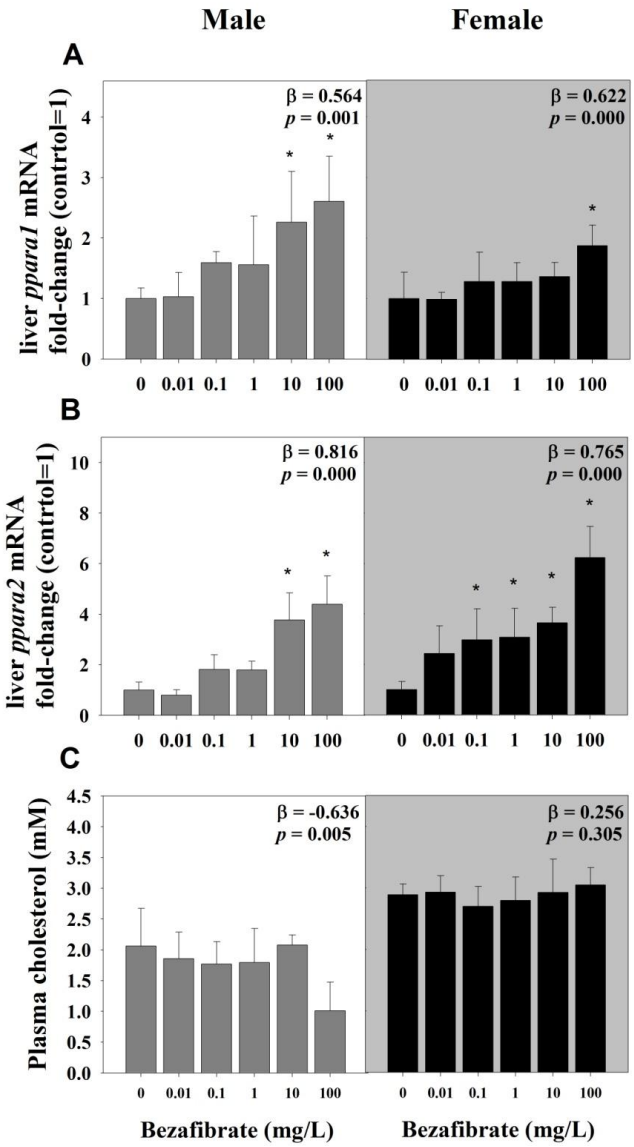


Fig. 6. Effects on (A) *ppar α 1*, (B) *ppar α 2* gene transcription and (C) plasma cholesterol levels. (A) *ppara1*, (B) *ppara2* genes transcription measured by real-time PCR in adult male and female *O. latipes* liver and (C) plasma cholesterol measured by ELISA in plasma of adult *O. latipes* after exposure to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L) for 165 dph. Values represent the mean ±

standard deviation of six mating pairs. Data are expressed as fold-change relative to the control. Asterisk (*) indicates a significant difference from control ($p<0.05$). The β (slope) and p values for trends were determined based on linear regression.

3.2.6 Effects on transcription of endocrine related genes in juveniles stage

The results of 30 dph juvenile fish endocrine related gene transcription such as *erα*, *erβ*, *cyp17*, *cyp19*, *vtg1*, *vtg2* were shown in Fig. 7. In the whole body of juvenile fish at 30 dph, most endocrine related genes such as *erα*, *erβ*, *cyp17*, *cyp19* and *vtg1* were significantly decreased at 100 mg/L. In addition, concentration dependent decreases in *vtg2* genes was observed (β = −0.325, p = 0.080).

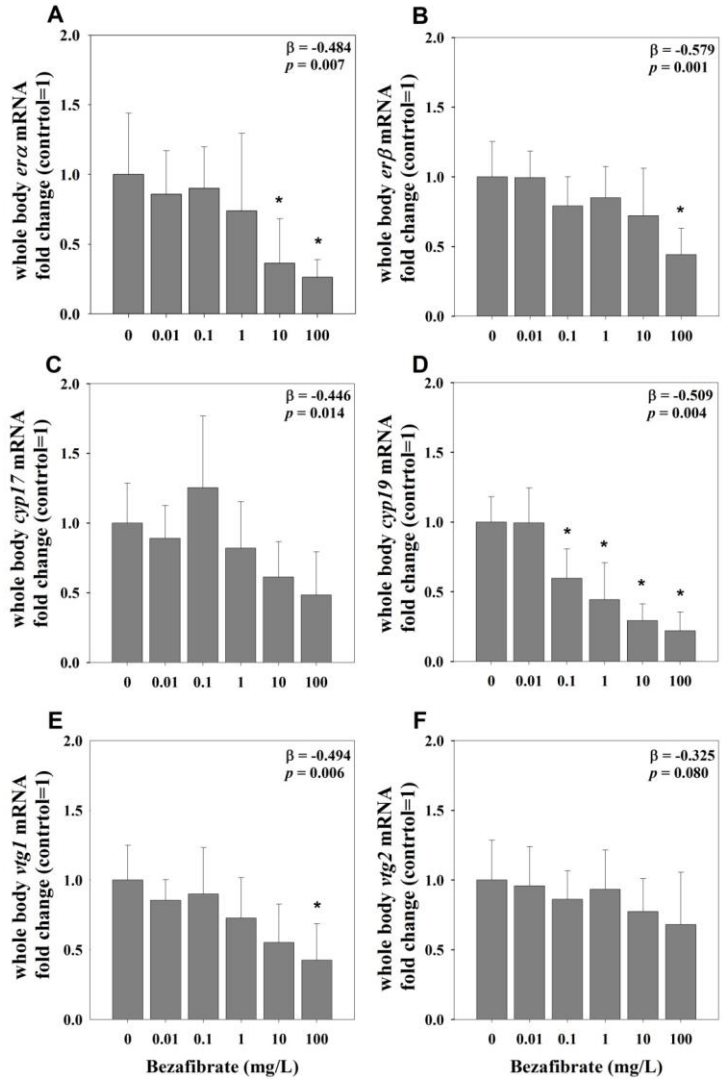


Fig. 7. Effects on (A) *erα*, (B) *erβ*, (C) *cyp17*, (D) *cyp19a*, (E) *vtg1,* (F) *vtg2* gene transcription measured by real-time PCR in whole body *O. latipes* after exposure to bezafibrate (0.01, 0.1, 1, 10, or 100 mg/L) for 30 dph. Values represent the mean ± standard deviation. Data are expressed as fold-change relative to the control. Asterisk (*) indicates a significant difference from control ($p<0.05$). The β (slope) and p values for trends were determined based on linear regression.

4. Discussion

4.1 Acute and chronic ecotoxicity of bezafibrate and risks in ambient water

Acute and chronic toxicity of bezafibrate on aquatic organisms have been reported at mg/L (Table 5). The observed 48 h *D. magna* EC_{50} of 234.64 mg/L (95% CI : 192.49 – 276.80 mg/L) in the present study is similar to 240.40 mg/L (95% CI : 230.12 –250.68 mg/L) reported in previous study (Rosal et al., 2010). *D. magna* (48 h EC_{50} : 234.64 mg/L) showed similar sensitivity to bezafibrate compared to *M. macrocopa* (48 h EC_{50} : 247.07 mg/L) in terms of acute lethal toxicity results. However, after chronic exposure, *D. magna* showed much sensitive reproduction damages– reproduction NOECs were observed at 11.11 and >300 mg/L in 21–day exposure of *D. magna* and 7 day exposure of *M. macrocopa*, respectively. Such difference might be due to difference in exposure duration, e.g., 21 vs. 7 days. For *O. latipes*, 30 dph survival NOEC was observed at 10 mg/L, the second highest concentration.

Employing the present and previous reports on ecotoxicity information of bezafibrate including the most sensitive toxicity value of *Ceriodaphnia dubia* 7 day reproduction NOEC, i.e., 0.023mg/L. The PNEC value was derived at 2.3 μg/L using an assessment factor of 10 following European Chemicals Bureau (2003). Comparing with occurrence information of bezafibrate worldwide (Table 6), even the maximum detected concentration reported in

Italy water, which is 202.7 ng/L in Lambro river (Zuccato et al., 2000), is an order of magnitude lower than the proposed PNEC of 2.3 μg/L. However, the levels of this lipid regulator in STP effluents could reach the PNEC (Table 6), hence, the area number significant STP influence could be potentially affected.

4.2 Endocrine related effects on fish following long-term exposure

Long-term exposure to bezafibrate damages the reproduction capacity of the fish, e.g., decreased hatchability, delay in time to hatch and reduced number of eggs (Fig. 2). Our observation is comparable to several other studies that have reported similar reproduction damages of fibrates in fish. Bezaifbrate led to a high incidence of germ cell syncytia in the tubular lumen and an increased number of cysts containing spermatocytes, which indicate testicular degeneration in male zebrafish (*Denio rario*) after 21 day dietary exposure (Velasco-Santamaria et al., 2011). Clofibric acid, similar fibrate lipid regulator, affected the reproductive axis of fathead minnows (*Pimephales promelas*) after 21 days exposure at 1 mg/L (Runnalls et al., 2007).

Observed adverse reproduction effects may be in part explained by changes in sex hormone levels. In the present study, the plasma E2 levels in female fish were significantly increased at 100 mg/L (Fig. 3A). Schulz et al. (2010) showed that the levels of androgens

like T and 11-KT increase during the initial stage of spermatogenesis and increase to a greater extent around spawning time in fish (Schulz et al., 2010). In male fish, the plasma E2 levels showed no significant difference compared with control but it showed an increasing pattern except for highest concentration (Fig. 3A). Alternations of sex hormone levels have been reported in fish previously, but the exposure duration was generally no longer than 21 days. Fish plasma 11-KT levels significantly reduced after 21 day dietary exposure to bezafibrate (70mg bezafibrate/g food) in zebrafish (Velasco-Santamaria et al., 2011). Following 96 h or 14 days waterborne exposure to gemfibrozil at 1.5 and 1500 μg/L, a reduced concentration of plasma T was observed in goldfish (*Carassius auratus*) (Mimeault et al., 2005). The decreased plasma T levels observed in fathead minnow after 21 day exposure to 0.01 and 1 mg/L clofibric acid (Runnalls et al., 2007). Sex hormones play important roles in fish spermatogenesis. Hence measurement of sex hormones has been suggested to be one of the most integrative and functional endpoints for reproduction in fish (Ma et al., 2012).

The sex hormonal change could be explained by transcriptional changes of steroidogenic and other related genes in fish (Fig. 3,4, and 5). Increased of plasma E2 level in female fish (Fig. 3) and up-regulation of *vtg1*, *vtg2* genes and *er*α, *er*β genes in liver (Fig. 4) showed increased estrogenicity of fish following exposure to bezafibrate. *VTG* is an egg yolk protein precursor and is one of the most frequently used biomarkers of exposure to estrogenic

endocrine disruptive chemicals (EDCs) in the environment (Navas and Segner, 2006; Scott et al., 2006). *VTG* is generally synthesized in the female during oocyte maturation, whereas *VTG* is absent or present at very low concentrations in male or juvenile fish (Benson, 1998; Filby et al., 2007; Kidd et al., 2007; Tyler et al., 1996). *VTG* can be induced by estrogenic chemicals, and has been used as a valuable biomarker of exposure to endocrine disrupting chemicals.

In addition, up-regulation of steroidogenic acute regulatory protein (*star*) gene was observed in male gonad following 165 days exposure to 10 mg/L of bezafibrate (Fig. 5C). *star* is one of the first rate-limiting steps in steroidogenensis and is responsible for delivery of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Black et al., 1994; Stocco, 2001; Velasco-Santamaria et al., 2011; Young et al., 2005). Our observation of up-regulation *star* gene suggests the increased mobilization of cholesterol to increase steridogenesis (Velasco-Santamaria et al., 2011) following long-term exposure to lipid regulator which is expected to reduce plasma cholesterol. Another steroidogenic genes, such as *3β-hsd*, *cyp11b*, and *cyp19a*, generally showed increased transcription following the exposure to bezafibrate (Fig. 5) toward enhanced steroidogenesis.

Bezafibrates, a lipid regulator, reduces the level of plasma cholesterol through the activation of PPARs, particularly *PPARα*. *PPARa* is essential transcription factor to lipid metabolism (Kota et al., 2005; Staels et al., 1998). In this study, significant up-

regulation of *ppar α 1*, and *ppar α 2* genes was observed in both adult male and female fish (Fig 6A and 6B). In addition, the decreasing pattern of plasma cholesterol levels were observed in male fish (Fig. 6C). Our observation shows that this lipid regulator may exert the same pharmacological function in the fish. Similarly, Prindiville et al. (2011) showed that a decrease in plasma lipoprotein, including cholesterol, in rainbow trout (*Oncorhynchus mykiss*) following the injection of 100 mg gemfibrozil/kg.

Because all steroid hormones derived from cholesterol, and normal adrenal steroidogenesis depends on cholesterol delivery from plasma (Evans, 1988; Staels et al., 1998; Velasco-Santamaria et al., 2011), an alteration of cholesterol level could affect the steroid balance. Velasco-Santamaria et al. (2011) reported the reduced plasma cholesterol level has been linked to adverse effects on spermatogenesis in zebrafish following exposure to bezafibrate. Also, a time dependent monotonic decrease in the plasma cholesterol concentration and significantly decrease in the plasma 11-KT were observed after 21 day exposure at 70 mg bezafibrate/g food in fish (Velasco-Santamaria et al., 2011). Similarly, in the present study, we found down-regulation of whole body genes including two important steroidogenic genes such as *cyp17* and *cyp19*, two *vitellogenin* genes such as *vtg1* and *vtg2*, and two estrogen receptor genes (Fig. 7). These transcriptional changes clearly indicate that early life stage (ELS) exposure to bezafibrate for relatively short duration of time could lead to

decreased estorgenicity in young fish. This observations are quite different from those observed from adult fish following >5 months exposure in the present study. Effects of bezafibrate on steroidogenic gene regulation appear to be influenced by duration of exposure. For instance, while down-regulation of *3β-hsd* and *cyp19a* genes was observed after 48 h, no changes were observed after 21 day exposure in fish (Velasco-Santamaria et al., 2011). Among humans, 4 weeks of bezafibate therapy to patients resulted in significantly reduced serum lipids, but no difference in serum lipid was observed at a 1-year treatment (Eriksson and Angelin, 1987).

Most of previous studies on endocrine toxicity of bezafibrate in fish have been based on short-term exposure. The present study clearly shows that longer term exposure to bezafibrate can cause increased estrogenicity in adult Japanese medaka fish, and may eventually cause adverse reproduction consequences. Reduced estrogen during early life stage of fish also warrants further investigation, because sex hormone balance in early stage of life plays crucial roles in normal development of the fish (Crain et al., 2008; Jin et al., 2009; Rasier et al., 2006).

5. Conclusion

Bezafibrate is one of the frequently used lipid regulators that have been used to control lipidemic diseases like hypercholesterolemia in human. It has been detected in ambient water at high levels worldwide. Compared with ambient levels currently reported, bezafibrate is not expected to cause direct risks in most water bodies, except for hotspots near STP outfall. The PNEC value of bezafibrate was calculated at 2.3 μg/L and HQ was estimated at 0.09. Following long-term exposure, bezafibate can cause adverse reproduction consequences, and such reprodcution damage was associated with altered sex hormone balances and steroidogenesis. Interestingly, bezafibrate increased estrogenicity in adult fish following long-term exposure, while the directions of change were opposite in juvenile fish after 30 day exposure. Details of mechanisms related to different responses from long-term and short-term exposure warrant further investigatons.

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Supporting information

Table S1. Real-time PCR primer sequences for the genes used in this study.

Gene name	Accession number	Primer sequence (5' – 3')[a]
β-Actin	NM_001101	ATCACCATTGGCAATGAGAGGTT
		GCAGGACTCCATACCAAGGAA
ERα	D28954.1	GACGGAGATCTTCGACATGCT
		GCAGACGAATTCCTCAGGTTTGA
ERβ	AB428449	GCAGTCCAAATCCACCTGTTG
		GGCCCAGCATCAGGATCT
PPARa1	AB469411.1	ACCAAACCCTGACAAGCTAACTG
		ACTGCTGGAGTTAGTTTTTCTTTCCT
PPARa2	AB469412.1	GCTTTATCACACGCGAGTTCCT
		GCTCCATCATGTCGCTAAACG
VTG I	AB064320	ACTCTGCTGCTGTGGCTGTAG
		AAGGCGTGGGAGAGGAAAGTC
VTG II	NM_001104840	CTATACAAACTTGGATTGGGTCTTCCA
		CTTTCAGGATAGGCCTCCAACT
StAR	DQ988930.1	GGAATCCCAATGTGAAAGAGGTCAA
		GCAGACACCTCATGGGTAATCAT
CYP11A	EF537029.1	ACACTCCTATGGACTTTGTATGAATTAGC
		GCCACCTCCAACCTCAGTTC
CYP11B	AB105880.1	GGCGTACCAGCGTCTGT
		CCCCAGAACTCCACCGTAGA
3β-HSD	NM_001137565	CCGACTGGCCACATGCT
		ACCAAGCAGGAAGCGACATC

[a] Upper and lower sequences represent forward and reverse primers, respectively

Table S1. Continued.

CYP17	NM_001105094	AGCCACCATCAGGGAGGT
		TGTCACTGAGGGCCACATG
CYP19A	D82968.1	ACAACATCAACTTTACTGCAGAGCTT
		CGCACTGCCTCACGTTCT

[a] Upper and lower sequences represent forward and reverse primers, respectively

Table S2. Measured concentrations of bezafibrate for given nominal concentrations in test media before and after the exposure.

Nominal Concentration (mg/L)	Measured bezafibrate concentration (mg/L)		
	Before exposure (0 h)	After exposure (48 h)	Average concentration (mg/L)
Control	ND [a]	ND [a]	ND [a]
0.01	0.005	0.031	0.018
0.1	0.06	0.18	0.12
1	0.81	0.85	0.83
10	6.05	4.85	5.45
100	75.6	53.1	64.35

[a] ND: Not detected.

국문초록

고지혈증 치료제 bezafibrate의 만성노출에 따른 수서생물의 생식영향과 기전

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Fibrate계 의약물질은 고콜레스테롤혈증 치료에 일반적으로 사용되어지는 지질 저하제이며, 이는 수계에서 자주 검출되어진다. 지질저하제 중 하나인 bezafibrate는 하천에서 최대 202.7 ng/L수준까지 검출 되어졌다. 지질 저하로 인해 스테로이드 합성이 변화되어 잠재적인 내분비계교란 가능성이 있지만, 수생 생물에서의 bezafibrate에 대해 관련 연구는 많지 않다. 본 연구에서는 지질저하제인 bezafibrate의 생태학적 위해성을 알아 내고, 잠재적인 내분비계 교란과 이와 관련된 기전을 확인하고자 하였다. Bezafibrate의 예측 무영향 농도값 (PNEC)은 2.3 μg/L으로 산출되어졌고, 예측 무영향 농도 (PNEC)와 실측환경농도 (MEC)값을 토대로 유해지수 (HQ)는 0.09로 추정되었다. Bezafibrate(0.01, 0.1, 1, 10, 100

mg/L)를 일본산 송사리에 만성(135일)노출 시키면, 물고기의 알의 갯수, 부화율, 그리고 부화시간같은 생식능력에 영향을 미쳤다. 이런한 변화는 부화 후 165일의 암컷 성어에서 에스트로겐이 유의하게 증가하고, 수컷 성어에서의 테스토스테론 감소와 같은 성호르몬의 변화를 동반하였다. 성호르몬의 변화는 여러 스테로이드 관련 유전자의 조절의 변화에 의해 뒷받침 되어진다. 생식소에서 *star*, *3β-hsd*, *cyp11b*, 그리고 *cyp19a*와 같은 스테로이드 관련 유전자는 bezafibrate 노출 후에 증가됨을 보여준다. 지질 조절과 관련된 유전자인 *ppara1*, *ppara2* 유전자 발현의 증가가 암컷과 수컷 성어 모두에서 관찰되어진 것으로 보아, bezafibrate는 지질 대사를 조절하는 것으로 보인다. 하지만 혈장 중 콜레스테롤의 수준은 만성(165일)노출 후 오직 수컷 성어에서 감소하였다. Bezafibrate의 물고기 만성노출에 따른 스테로이드 합성의 증가는 30일 초기생장단계 독성평가의 결과와 이전의 다른 연구 결과들과는 달랐다. 본 연구 결과 지질저하제인 bezafibrate의 만성(165일)노출은 수생 생물종들의 스테로이드 합성을 활성화시키며, 또 이는 생식변화로 이어질 수도 있는 estrogenicity가 증가되었음을 보여준다.

주요어 : Fibrate, multi-generation, lipid regulator, steroidogenesis, reprodcution, ecological risk assessment, Japanese medaka

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