보건학석사 학위논문

Assessment of aquatic toxicity and endocrine disruption potential of metformin, an anti-diabetic drug

당뇨병 치료제 metformin의 수생태 독성평가 및 내분비계 교란영향

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환경보건학과 환경보건 전공

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ABSTRACT

Assessment of aquatic toxicity and endocrine disruption potential of metformin, an anti-diabetic drug

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Metformin, one of the most prescribed anti-diabetic pharmaceuticals, has been frequently detected in ambient water. However, knowledge of its effects on aquatic organisms is very limited. In this study, acute and chronic toxicity of metformin was evaluated using two freshwater organisms, a crustacean (*Daphnia magna*) and Japanese medaka fish (*Oryzias latipes*) following Organization for Economic Co-operation and Development (OECD) test guidelines. Endocrine disruption potential of metformin and its underlying mechanisms were also investigated using adult Japanese medaka.

In 21 d chronic *D. magna* test, no observed effect concentration (NOEC) for survival was determined at 40 mg/L of metformin, but significant

changes in reproduction were not observed at this concentration. In early life stage toxicity test with medaka, NOEC for survival was determined at 100 mg/L of metformin. Predicted no effect concentration (PNEC) value of metformin was determined at 4 mg/L, based on *D. magna* 21 d NOEC. Hazard quotient (HQ) was estimated less than 1 suggesting negligible risk.

In adult male Japanese medaka, plasma 17β-estradiol (E2) level and E2 to testosterone (T) ratio were significantly increased following 21 d exposure to metformin. In addition, transcription of steroidogenic genes such as *star, cyp11a, hsd3b, cyp19a, hsd11b2,* and *cyp11b* was significantly up-regulated. In female Japanese medaka, 11-ketotestosterone (11-KT) level as well as *cyp11b* gene transcription level was increased following 21 d exposure to metformin.

Our observation suggests that current level of metformin in ambient water is not likely to be of concern among freshwater organisms. However, its endocrine disruption potential through alteration of steroidogenic pathway warrants long-term exposure studies.

Keywords: anti-diabetic drugs; metformin; ecological risk assessment; endocrine disruption; hypothalamic-pituitary-gonad (HPG) axis
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1. Introduction

Pharmaceuticals can be released into the environment through many pathways, and consequently influencing non-target organisms (Kidd et al., 2007). Metformin is one of the most widely prescribed drugs for Type 2 diabetes mellitus (Marin-Morales et al., 2016). Globally, over 100 million patients are prescribed with metformin per annum (Rena et al., 2013). Metformin was the most produced pharmaceutical in Germany in 2012, with the production amount of 1200 tons/yr (Küster and Adler, 2014). Following therapeutic use, about 38-52 % of metformin is excreted as unmetabolized form through urine (Pentikäinen et al., 1979; Tucker et al., 1981). Therefore, this compound can be released through wastewater treatment plants (WWTPs) to the water environment. While removal efficiency of WWTPs for metformin is high as 68-98.7% (Blair et al., 2013b; Oosterhuis et al., 2013; Scheurer et al., 2009, 2012; Trautwein and Kümmerer, 2011), high mass of metformin can reach aquatic ecosystems (Dong et al., 2013; Scheurer et al., 2012).

Metformin is found in wastewater influents at concentrations between 3.2 to 129 μg/L (Scheurer et al., 2009, 2012; Trautwein and Kümmerer, 2011; van Nuijs et al., 2010), in wastewater effluents at levels between 0.64 and 92 μg/L (Al-Odaini et al., 2011; Blair et al., 2013b; Scheurer et al., 2012; Trautwein and Kümmerer, 2011) and in surface water at levels between 0.029 and 9.2 μg/L (Al-Odaini et al., 2011; Blair et al., 2013a; Kolpin et al., 2002; Scheurer et al., 2009, 2012) worldwide. In Korea, metformin was identified as one of the high-priority pharmaceuticals with potential ecological risks in Korean aquatic ecosystem (Ji et al., 2016). However,

ecotoxicological data are very limited to only few species.

Metformin is also used to treat polycystic ovarian syndrome (PCOS), an endocrine disorder in women showing anovulation with hyperandrogenism, hyperinsulinemia or peripheral insulin resistance. Metformin is suggested to treat PCOS patients by two modes of action (Diamanti-Kandarakis et al., 2010a; 2010b; Lauretta et al., 2016). One mechanism is a direct effect on steroidogenesis which is supported by down-regulation of the steroidogenic enzymes in ovarian cells (Attia et al., 2001; Tosca et al., 2006). The other one is an indirect effect on steroidogenesis by reducing insulin level in ovary (Diamanti-Kandarakis et al., 2006; Nestler and Jakubowicz, 1996).

Therapeutic effect of metformin on PCOS patients suggests its potential endocrine disruption in non-target organisms. Very recently, studies reported metformin could act as an endocrine disruptor in fish. In fathead minnow (*Pimephales promelas*), intersex and reduction of fecundity were observed following one year exposure to metformin (Niemuth and Klaper, 2015). In male and juvenile fathead minnow, upregulation of transcriptions of endocrine related genes (*gnrh3*, *erα*, *cyp3a126*, and *vtg*) were observed following 7-day or 4-week exposure to metformin (Crago et al., 2016; Niemuth et al., 2015). However only one freshwater species was studied and associated mechanisms in fish have not been completely understood.

In the present study, we evaluated acute and chronic toxicity of metformin using two standard freshwater organisms (*Daphnia magna* and Japanese medaka) following Organization for Economic Co-operation and Development (OECD) test guidelines and performed ecological risk assessment. In addition, endocrine disruption effects and its related mechanism were investigated following exposure to metformin using

Japanese medaka. The results of the present study will reduce uncertainty of ecological risk assessment of metformin and help understand the endocrine disruption effects of metformin in aquatic ecosystems.

2. Materials and Methods

2.1. Test Chemical

Metformin hydrochloride (CAS No. 1115-70-4, Purity ≥ 98%) was purchased from Cayman chemical (Ann Arbor, MI, USA).

Concentrations of metformin in the exposure media were measured using Agilent 1100 series High-Performance Liquid Chromatography coupled with Diode-Array Detection (HPLC-DAD). Chromatographic separation was carried out using a Zorbax ODS column (5 μm, 4.6 × 150 mm, Agilent). The mobile phase was 10 mM ammonium acetate buffer (adjusted to pH 3 with HCl)-Acetonitrile solution (3:7; v:v) at a total flow rate of 1.0 mL/min and 20 uL of each sample were injected into the HPLC system (255 nm wavelength).

2.2. Test organisms and maintenance

Test organisms were cultured in Environmental Toxicology Laboratory of Seoul National University (Seoul, Korea). *Daphnia magna* were cultured in-house in moderately hard water manufactured following US Environmental Protection Agency guidelines (2002). *D. magna* were maintained at 21 ± 1 ^{o}C under a 16:8 dark photoperiod in 3 L glass jars. *Daphnia* were fed daily with *Chlorella* (Aquanet, Gyeongnam, Korea). Reference tests using sodium chloride as a reference toxicant were carried out monthly to assure comparable sensitivity among cohorts of test organisms over time.

Adult Japanese medaka (*Oryzias latipes*, about 5-month-old) were obtained from a commercial aquarium (Greenfish, Seoul, Korea). The fish were acclimatized in the laboratory for more than one week before use for

exposure. The dechlorinated fish culture water was prepared by > 24 h of aeration. The fish were maintained at 25 ± 1 ^{o}C under a 14:10 dark photoperiod and fed daily with freshly hatched *Artemia nauplii* (Brine Shrimp Direct, Ogden, UT, USA) twice daily. In both culture water, water quality parameters including hardness, pH, conductivity, temperature, and dissolved oxygen were routinely monitored.

2.3. Acute and chronic toxicity test

2.3.1. *D. magna* toxicity test

The 48 h acute and 21 d chronic *D. magna* toxicity test were conducted following OECD guideline 202 (2004) and OECD guideline 211 (2012), respectively. The test solutions were prepared in culture media.

For acute exposure, four replicates with five neonates each (< 24 h old) were exposed to test concentrations of metformin with 2-fold serial dilution (0, 20, 40, 80, 160, or 320 mg/L). Every 24 h, the number of immobilized organisms was recorded.

For chronic *D. magna* exposure, ten replicates with one neonate each (< 24 h old) were exposed to 0, 2.5, 5, 10, 20, 40, or 80 mg/L of metformin for 21 d. The exposure concentrations were determined based on the results of the acute toxicity test. The exposure medium was renewed at least three times per week and *D. magna* were fed daily with *Chlorella*. The adult survival and the number of living offspring were recorded daily. The neonates produced by each parent were counted and removed daily. First day of reproduction, number of young per adult, number of young per brood,

population growth rate (*r*) and growth (e.g. length) were also determined. The population growth rate (*r*) was calculated using the following equation (Euler-Lotka, 1993):

$$\sum l_x m_x e^{-rx} = 1$$

where l_x is the proportion of individuals surviving to age *x*, m_x is the age-specific fecundity (number of females produced per surviving female at age *x*), *e* is the base of the natural logarithm, and *x* is time in days.

2.3.2. *O. latipes* toxicity test

The 96 h acute medaka toxicity test was conducted following OECD guideline 203 (1992) and US Environmental Protection Agency guidelines (2002). The chronic medaka toxicity test, early-life stage (ELS) toxicity test, was conducted following OECD guideline 210 (2013). The test solutions were prepared in culture media, dechlorinated water.

For acute exposure, 12 days post hatch (dph) larvae were exposed to 0, 20, 40, 80, 160, 320, or 640 mg/L of metformin. Each treatment consists of four replicates with five larvae each in 50 mL beakers. The larvae were fed with *A. nauplii* (< 24 h after hatching) 2 h prior to medium renewal and the exposure medium was renewed at 48 h. The dead larvae were removed as soon as possible and the number of mortality was recorded daily for 96 h.

For fish ELS toxicity test, fertilized eggs (< 24 h after fertilization) were exposed to 0, 3, 10, 30, 100, or 300 mg/L of metformin until 30 dph. Each treatment consists of four replicates with 15 eggs in 50 mL glass beakers. After 4 dph, larvae were fed with *A. nauplii* until 30 dph *ad libitum* twice

daily. At 7 dph, larvae were transferred to 250 mL glass beakers filled in 100 mL exposure medium. Exposure medium was renewed at least three times per week. Dead eggs or fish were removed immediately and the number of mortality was recorded. Larval-juvenile survival and time to hatch were also recorded. At 30 dph, 3 fish per treatment were randomly chosen and measured for body length and weight to calculate condition factor (K = 100 x total weight (g)/total length (cm^3)).

2.3.3. Ecological risk assessment

For ecological risk assessment, toxicity data of metformin obtained from the present study, and those reported elsewhere, e.g., literatures, government documents and toxicity databases (e.g., https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/11) were collected and reviewed. All collected data were evaluated for reliability based on Klimisch categories (Klimisch et al., 1997), and only the data with reliability categories 1 or 2 were chosen.

Predicted no effect concentration (PNEC) was derived from the most sensitive toxicity data divided by the assessment factor (AF) according to the European guidance document for derivation environmental quality standards under the Water Framework Directive (EC, 2011). Hazard quotient (HQ) was calculated by dividing the median or maximum measured environmental concentration (MEC) of surface water and wastewater effluent respectively with PNEC value. The median MEC for HQ was calculated as median value of median concentration of metformin

in each region.

2.4. Endocrine disrupting effects in *O. latipes*

2.4.1. Early-life stage (ELS) exposure

At 30 dph on ELS toxicity test, four to six juvenile medaka per treatment were randomly chosen and anesthetized on ice for analysis of transcriptions of the genes associated with sex hormone regulation.

2.4.2. 21 d adult fish exposure

Adult Japanese medaka (about 5-month old) were exposed to 0, 0.03, 0.3, 3, or 30 mg/L of metformin prepared in dechlorinated water. Exposure medium was renewed at least three times a week by decanting old medium as much as possible and adding newly prepared test medium. The test media were gently aerated during the test. The adult fish were fed with *A. nauplii* (< 24 h after hatching) *ad libitum* twice daily.

Male and female fish were separated in 15 L glass tank filled with 7 L exposure media. Each test tank was divided into four chambers by porous stainless steel wall and four fish of the same sex were allocated per each chamber, i.e., 16 male fish in four replicates of one tank and 16 female fish in another four replicates of the other tank per treatment.

After 21 d of exposure, the fish were anesthetized on ice, and measured for length and weight. Then blood was collected from caudal vein in a glass capillary tube treated with heparin. The blood from two to four fish were pooled, and plasma was separated by centrifugation at 3000 x g for 7 min at

4 ^{o}C. The plasma samples were stored at -80 ^{o}C until hormone analysis. The brain, liver, and gonad were dissected from the sacrificed fish, and organs from two fish were pooled to make each replicate. Gonadosomatic index (GSI = 100 x gonad weight (g)/body weight (g)) and hepatosomatic index (HSI = 100 x liver weight (g)/body weight (g)) were calculated. For gene transcriptions, dissected organs were stored at -80 ^{o}C until analysis.

2.5. Measurement of sex hormones

Plasma sex steroid hormones were measured in both male and female fish by competitive enzyme-linked immunosorbent assay (ELISA) using commercially available kits (17β-estradiol [Cat # 582251], testosterone [Cat # 582701], 11-ketotestosterone [Cat # 582751]; Cayman Chemical, Ann Arbor, MI, USA). Sex hormones were extracted from fish plasma following the method described in Ji et al. (2013). Briefly, 6 μL of fish plasma with 400 μL of ultra-pure water was extracted with 2 mL of diethyl ether at 2100 x g for 10 min. Following evaporation of diethyl ether, the residues were dissolved in 140 μL EIA buffer for ELISA assay.

2.6. Quantitative analysis of the genes related to HPG axis

The transcriptions of several genes related to hypothalamus-pituitary-gonad (HPG) axis were evaluated in whole body of 30 dph juvenile fish and organs (brain, liver, and gonad) of adult fish. Total RNA was extracted from the homogenized sample using RNeasy mini kit (QIAGEN). Complementary DNAs (cDNAs) were synthesized from 500 ng purified RNA samples using iScript^{TM} cDNA Synthesis kits (Bio-Rad, Hercules, CA, USA). Quantitative

real-time polymerase chain reaction (qRT-PCR) was performed with 20 μL of qRT-PCR reaction mix (10 μL of LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics Ltd., Lewes, UK), 4.4 μL of nuclease free water, 1.8 μL of forward and reverse primer, and 2 μL of cDNA sample), and LightCycler® 480 instrument (Roche Applied Science, Indianapolis, IN, USA). The relative expression level of each target gene was calculated with the threshold cycle (Ct) value using $^{2\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Transcriptions of 17 genes as well as one house keeping gene (*rpl 7*) were measured. The Ct values of *rpl-7* were used to standardize the results because the levels of *rpl-7* expression did not change significantly ($p < 0.05$). The primer sequences and PCR efficiencies of selected genes and housekeeping gene are listed in Table S1.

2.7. Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics (version 22.0; SPSS Inc., Chicago, IL, USA). For *D. magna* and fish acute toxicity test, the median effective concentration (EC_{50}), median lethal concentration (LC_{50}), and confidence intervals were calculated by probit analysis.

For *D. magna* chronic toxicity test, normality of data and homogeneity of variances were analyzed by Shapiro-Wilk's test and Levene's test, respectively. No observed effect concentrations (NOECs) and lowest observed effect concentrations (LOECs) were calculated using one-way analysis of variance (ANOVA) followed by Dunnett's test or a non-parametric Kruskal-Wallis test combined with Mann-Whitney *U* test.

Survival of parent *D. magna* was analyzed with chi-square test.

For fish chronic and adult-exposure toxicity test, one-way ANOVA test followed by Dunnett's test was conducted to determine significant differences. In addition, linear regression was applied for trend analysis. In all statistical analyses, *p* value of less than 0.05 was considered to be statistically significant.

Dixon's Q test was performed to identify outliers. It was used to exclude the results of testosterone level in male fish exposed to 30 mg/L of metformin because of the extraction problem.

3. Results

3.1. Measurement of exposure media

Metformin was measured in test media three times respectively, before and after 48 h or 72 h of exposure with *Daphnia magna* and Japanese medaka (*Oryzias latipes*). Most measured concentrations of each sample were more than 80% of the nominal concentration, respectively (Table 1).

Table 1. Measured concentrations of metformin in the exposure media before and after renewal (n=3)

Test	Nominal concentration (mg/L)	Measured concentration[a] (mg/L)			% of nominal concentration
		Before	After[b]	Average	Average
D. magna 48 h acute[c]	Control	ND	ND	ND	NA
	20	20.3±2.9	13.6±5.4	17.0	84.9
	40	38.8±4.3	36.8±11.4	37.8	94.4
	80	78.4±9.4	59.7±22.9	69.1	86.3
	160	133.3±31.5	141.6±48.5	137.4	85.9
	320	311.0±40.7	293.2±111.1	302.1	94.4
D. magna 21 d Chronic[d]	Control	ND	ND	ND	NA
	2.5	2.7±0.3	2.9±0.5	2.8	112.7
	5	4.8±0.4	5.1±0.5	5.0	99.2
	10	8.5±2.2	9.6±0.4	9.1	90.9
	20	15.7±4.5	18.3±0.4	17.0	84.8
	40	30.0±11.5	37.4±3.4	33.7	84.2
	80	59.2±26.2	72.8±8.9	66.0	82.5
O. latipes 96 h acute[e]	Control	ND	ND	ND	NA
	20	16.5±3.2	18.8±1.9	17.6	88.2
	40	36.9±5.1	36.5±3.1	36.7	91.7
	80	68.2±10.0	74.0±4.4	71.1	88.8
	160	152.3±19.4	129.0±43.5	140.6	87.9
	320	228.7±96.9	276.1±51.4	252.4	78.9
	640	556.6±69.8	565.2±91.6	560.9	87.6
O. latipes ELS test[f]	Control	ND	ND	ND	NA
	3	3.3±0.4	3.3±0.8	3.3	109.3
	10	9.5±0.9	9.9±2.3	9.7	97.0
	30	24.4±6.1	24.7±13.0	24.5	81.8
	100	81.1±13.2	67.7±37.5	74.4	74.4
	300	243.7±28.2	242.5±116.2	243.1	81.0

[a] Values represent mean ± standard deviation of each concentration. [b] 48 h, 72 h for *O. latipes* ELS test. ND: below limit of detection. NA: not available, Limit of detection (LOD): [c] 0.74-13 mg/L, [d] 0.2-0.74 mg/L, [e] 0.2-13 mg/L, [f] 0.74-0.76 mg/L

3.2. Acute and chronic toxicity

3.2.1. *D. manga* acute and chronic exposures

In a *D. magna* 48 h acute test, EC_{50} for immobilization was determined at 81.4 mg/L. The effects of 21 d chronic exposure to metformin are shown in Table 2. The *D. magna* NOEC for survival was determined at 40 mg/L of metformin but significant changes in reproduction and growth were not observed.

Table 2. Results of 21 d chronic *D. magna* test with metformin[a]

concentration (mg/L)	Survival (%)	First day of reproduction (day)	No. young per adult	No. young per brood	Growth (mm)	Population Growth rate
Control	90	10.0 ± 0.5	88.2 ± 14.0	21.5 ± 3.3	4.0 ± 0.2	0.337
2.5	100	11.3 ± 0.9	79.9 ± 27.1	20.3 ± 6.3	3.9 ± 0.3	0.298
5	100	11.0 ± 0.5	91.4 ± 22.6	23.0 ± 5.6	4.0 ± 0.1	0.312
10	90	9.6 ± 0.5	108 ± 16.7	25.8 ± 4.8	4.0 ± 0.3	0.357
20	90	10.1 ± 0.8	95.0 ± 21.5	23.8 ± 4.9	4.0 ± 0.1	0.342
40	100	10.2 ± 1.3	107 ± 30.5	26.5 ± 5.3	4.1 ± 0.2	0.355
80	20 *	17 ± 0.0	12.0 ± 8.5	5.5 ± 4.9	3.2 ± 0.1	0.022

[a] Values represent mean ± standard deviation of each concentration. Asterisk (*) denotes a significant difference from the control based on chi-square test ($p < 0.05$).

3.2.2 *O. latipes* acute and chronic exposures

In a 96 h acute test with Japanese medaka, LC_{50} was determined at 383.3 mg/L of metformin. The effects of early-life stage (ELS) exposure to metformin are summerized in Table 3. Survival NOEC was determined at 100 mg/L of metformin. However, other sublethal effects such as time to hatch and growth (length, weight, and condition factor) were not observed at levels up to 100 mg/L of metformin.

Table 3. Effects on survival, hatching, and growth of *O. latipes* following early life stage exposure (30 dph) to metformin[a]

Concentration (mg/L)	Survival (%)		Time to hatch (day)	Juvenile length (mm)	Juvenile dry weight (mg)	Condition factor
	Egg	Juvenile				
Control	93.3 ± 5.44	89.6 ± 11.4	8.71 ± 1.42	12.1 ± 0.739	3.09 ± 0.724	0.174 ± 0.027
3	86.7 ± 9.43	85.9 ± 9.36	8.88 ± 1.90	11.8 ± 0.333	2.92 ± 0.556	0.175 ± 0.018
10	95.0 ± 3.33	91.2 ± 3.66	8.57 ± 1.52	11.9 ± 0.739	2.52 ± 0.431	0.148 ± 0.013
30	98.3 ± 3.33	81.3 ± 11.5	9.66 ± 2.23	12.3 ± 0.739	2.73 ± 0.573	0.147 ± 0.010
100	90.0 ± 3.85	85.3 ± 10.0	9.11 ± 1.82	12.5 ± 1.41	3.08 ± 0.699	0.159 ± 0.020
300	96.7 ± 3.85	46.8 ± 16.6 *	9.36 ± 1.65	10.2 ± 0.994	1.71 ± 0.281	0.162 ± 0.025

[a] Values represent mean ± standard deviation of each concentration (n=4). Asterisk (*) denotes a significant difference from the control ($p < 0.05$) based on Dunnett's test.

3.2.3. Ecological risk assessment of metformin

Ecotoxicity information reported for metformin with reliability categories of 1 or 2 is summarized in Table 4. The most sensitive response was reported from *D. manga* following chronic study, i.e., 40 mg/L of survival NOEC. The assessment factor for deriving PNEC was determined at 10, because chronic toxicity values are available in base set (algae, *Daphnia*, and fish). Therefore PNEC was determined at 4 mg/L.

The environmental concentrations of metformin reported worldwide are summarized in Table 5. The measured environmental concentrations (MECs) in surface water (median: 0.42 μg/L; maximum: 9.2 μg/L) and wastewater effluent (median: 11 μg/L, maximum: 92 μg/L) were selected (Blair et al., 2013a, 2013b; Scheurer et al., 2009), respectively. The concentrations of metformin in waste water effluent were considered as extreme environmental condition.

At the levels of occurrence in ambient water, hazard quotient (HQ) value was determined at less than 1 (Table 6). HQs based on surface water MEC were 0.00011 (median) and 0.0023 (maximum), and HQs based on effluent water MEC were 0.0028 (median) and 0.023 (maximum).

Table 4. Acute and chronic toxicity of metformin reported elsewhere

Taxonomic group	Species	Test duration/endpoint	Parameter	Operator	Effect Conc. (mg/L)	Test type	Reference
Macrophytes	*Lemna minor*	7 d/growth inhibition	EC_{50}	=	110	Acute	Cleuvers, 2003
Algae	*D. subspicatus*	72 h/growth inhibition	EC_{50}	>	320	Acute	ECHA, 2002; Cleuvers, 2003
	Anabaena flos-aquae	6 d/growth inhibition	NOEC	=	800	Chronic	ECHA, 1994a
Crustaceans	*D. magna*	48 h/immobilization	EC_{50}	>	130	Acute	ECHA, 1994b
	D. magna	**48 h/immobilization**	**EC_{50}**	**=**	**81**	**Acute**	**This study**
	D. magna	48 h/immobilization	NOEC	=	78	Acute	ECHA, 1994b
	D. magna	48 h/immobilization	EC_{50}	=	64	Acute	Cleuvers, 2003
	D. magna	**21 d/survival**	**NOEC**	**=**	**40***	**Chronic**	**This study**
	D. magna	**21 d/reproduction, growth**	**NOEC**	**≥**	**40**	**Chronic**	**This study**
	D. magna	21 d/ survival, reproduction	NOEC	≥	32	Chronic	ECHA, 2007
Fish	*L. macrochirus*	96 h/survival	NOEC	≥	982	Acute	ECHA, 1994c
	O. latipes	**96 h/survival**	**LC_{50}**	**=**	**383**	**Acute**	**This study**
	D. rerio	34 d (30 dph)/time to hatch, hatchability, larval development, length, weight, survival	NOEC	≥	12	Chronic	ECHA, 2008
	O. latipes	**30 dph/ survival**	**NOEC**	**=**	**100**	**Chronic**	**This study**
	O. latipes	**30 dph/time to hatch, hatchability, length, weight**	**NOEC**	**≥**	**100**	**Chronic**	**This study**

* The study used for derivation of predicted no effect concentration (PNEC) was indicated in asterisk (*).

This study was indicated in bold fonts.

Table 5. Environmental concentrations of metformin reported in water worldwide

Region	Country	Detection frequency[a]	Concentration (μg/L)		Reference
			Median	Maximum	
Waste water influents	Belgium	22/22	45.1	94.3	van Nuijs et al., 2010
	Germany	1/1	-	56.8	Trautwein and Kümmerer, 2011
		3/3	101	129	Scheurer et al., 2009
		5/5	42.0	105	Scheurer et al., 2012
	USA	6/6	55.0	100	Blair et al., 2013b
Waste water effluents	Malaysia	4/5	2.91	34.2	Al-Odaini et al., 2011
	Germany	3/3	**11.0**	21.0	**Scheurer et al., 2009**
		1/1	-	0.76	Trautwein and Kümmerer, 2011
		5/5	2.1	10.0	Scheurer et al., 2012
	USA	6/6	42.0	**92.0**	**Blair et al., 2013b**
		5/5	27.0	33.0	Blair et al., 2013b
		6/6	0.64	47.0	Blair et al., 2013b
Surface water	Malaysia	5/7	0.074	0.19	Al-Odaini et al., 2011
	Germany	14/14	**0.42**	1.70	**Scheurer et al., 2009**
		18/18	0.8	3.1	Scheurer et al., 2012
	USA	7/7	1.03 (mean of mean)	**9.20**	**Blair et al., 2013a**
		4/84	0.11	0.15	Kolpin et al., 2002

[a] number of metformin detected sites/ total number of sites.

The studies used for measured environmental concentration (MEC) were indicated in bold fonts.

Table 6. Derivation of predicted no effect concentration (PNEC) and hazard quotient (HQ) of metformin

Endpoint	NOEC (mg/L)	AF	PNEC (μg/L)	MEC (μg/L)				$HQ_{based\ on}$			
				Surface water		Wastewater effluent		Surface water		Wastewater effluent	
				MEC_{median}	MEC_{max}	MEC_{median}	MEC_{max}	MEC_{median}	MEC_{max}	MEC_{median}	MEC_{max}
Survival	40[a]	10	4000	0.42[b]	9.2[c]	11.0[d]	92.0[e]	0.00011	0.0023	0.0028	0.023

NOEC: no observed effect concentration, AF: assessment factor, MEC: measured environmental concentration

[a] This study, [b] Scheurer et al., 2009, [c] Blair et al., 2013a, [d] Scheurer et al., 2009, [e] Blair et al., 2013b

3.3. Sex endocrine related effects of metformin on *O. latipes*

3.3.1. Transcriptional changes of sex hormone related genes in fish early life stage

Significant up-regulations of *vtg1* and *vtg2* gene transcription were observed in juvenile Japanese medaka following 30 dph exposures to 30 mg/L of metformin (Fig. 1).

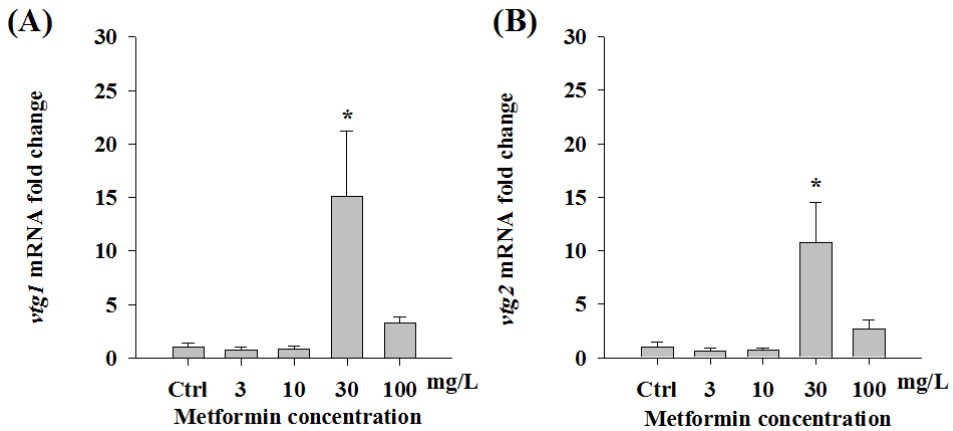

Fig. 1. Effects on (A) *vtg1* and (B) *vtg2* gene transcription in 30 dph Japanese medaka whole body following early-life stage exposure to metformin. The results are shown as mean ± SE of four replicates. It is expressed as fold change relative to control. Asterisk (*) denotes a significant difference from the control (ANOVA, $p < 0.05$).

3.3.2. Effects on plasma sex steroid hormones in adult fish after 21 d exposure

Following 21 d exposure to metformin, the levels of sex hormones were changed in male and female adult fish (Fig. 2). In male fish, level of 17β-estradiol (E2) hormone was significantly increased with 1.4-fold changes at highest concentration (Fig. 2A). Although plasma testosterone (T) and 11-ketotestosterone (11-KT) level were not significantly changed at each concentration, T showed significantly negative trend (β = -0.11, p = 0.033) (Fig. 2B and C). In addition, the level of T or 11-KT were lower in higher concentration groups (0.3, 3, and 30 mg/L), compared to those of lower concentrations (0 and 0.03 mg/L). Therefore, E2/T and E2/11-KT ratio were significantly increased at 30 mg/L and 0.3 mg/L of metformin, respectively in male fish (Fig. 2D and E). In female fish, only the level of 11-KT was significantly increased at 0.3, 3, and 30 mg/L of metformin (Fig. 2C).

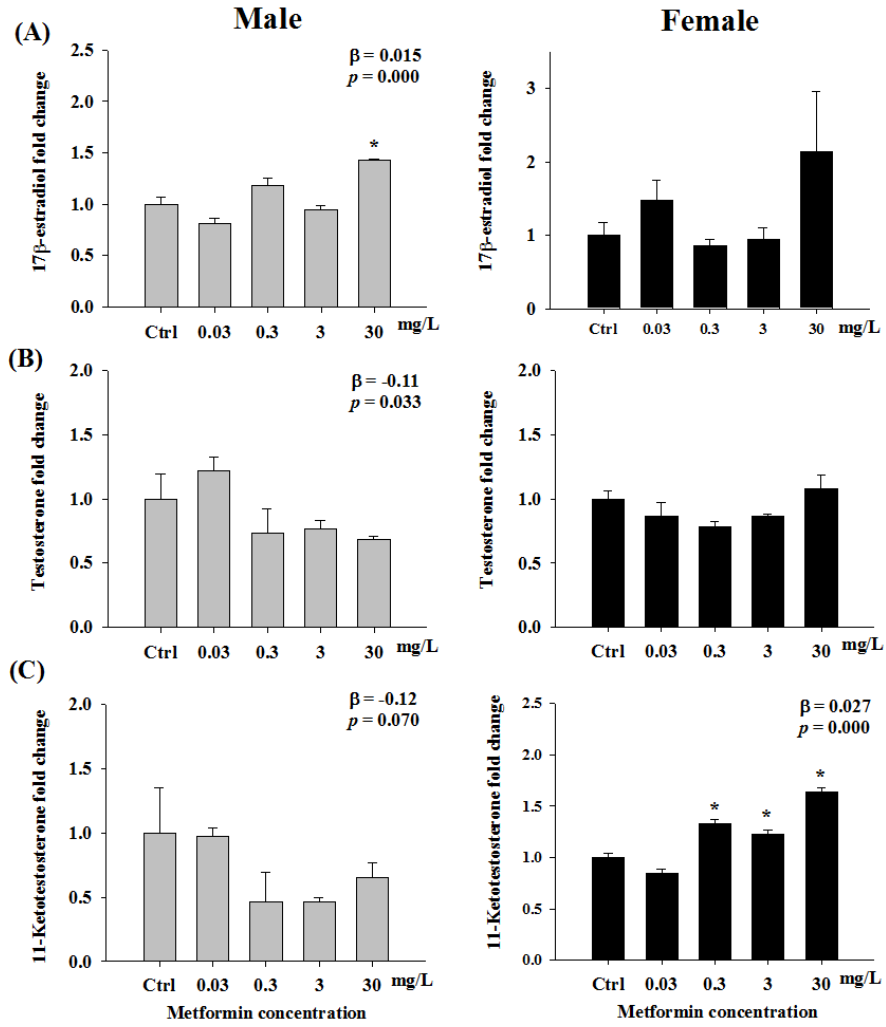

Fig. 2. Effects on (A) 17β-estradiol (E2) hormone level, (B) testosterone (T) level, (C) 11-ketotestosterone (11-KT) level, (D) E2/T ratio, and (E) E2/11-KT ratio of male and female Japanese medaka after 21 d exposure to metformin. The results are shown as mean ± SE of three or four replicates. It is expressed as fold change relative to control. Asterisk (*) denotes a significant difference from the control (ANOVA, $p < 0.05$). Slope and p values of the trend were shown only when the trend was significant.

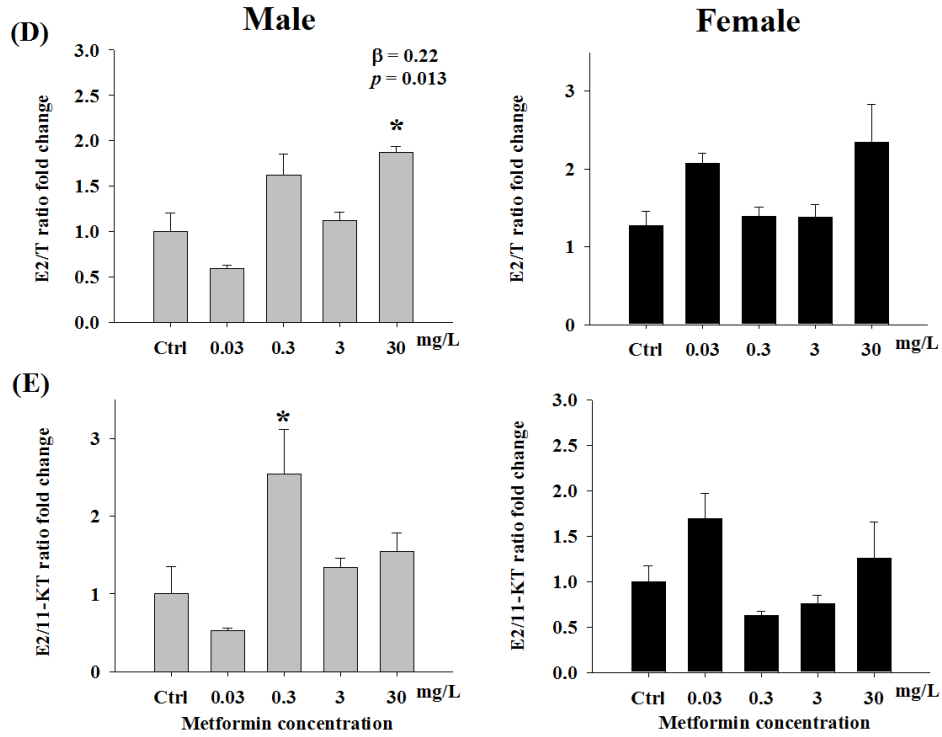

Fig. 2. (Continued).

3.3.3. Transcriptional changes of genes related to HPG-axis in adult fish

Following exposure to metformin, transcription of several genes related to hypothalamus-pituitary-gonad (HPG) axis was changed (Fig. 3-5).

In male brain and liver, transcriptions of *erα*, *erβ* and *vtg1* were significantly up-regulated at 0.3 mg/L of metformin (Fig 3C, 3D, and 4A). Transcription of *vtg2* was significantly up-regulated in concentration dependent manner ($\beta = 0.469$, $p = 0.001$).

In testis, transcriptions of *fshr*, *lhr*, *star*, *cyp11a*, *hsd3b*, *cyp19a*, *hsd11b2*, and *cyp11b* gene were significantly up-regulated at 3 mg/L or 30 mg/L (Fig. 5). In addition, the regulation of these genes showed significantly positive trend by increasing exposure concentration. On the other hands, *cyp17* gene transcription was significantly down-regulated at 0.3 mg/L.

In female fish ovary, transcription level of *hsd11b2* gene was significantly down-regulated (Fig. 5I), while *cyp11b* gene transcription was significantly up-regulated at 30 mg/L, and showed concentration dependent up-regulation ($\beta = 0.072$, $p = 0.03$) (Fig. 5J).

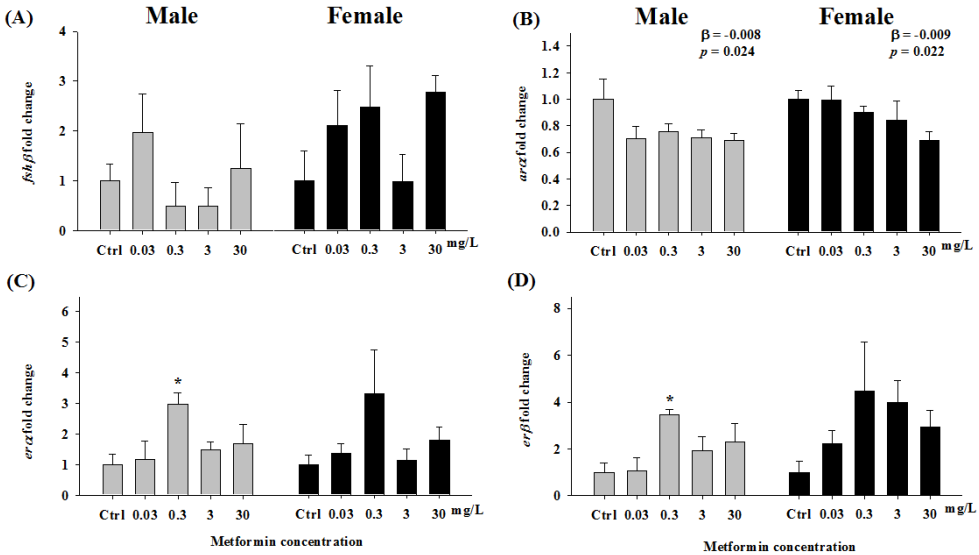

Fig. 3. Effects on (A) *fshβ,* (B) *arα*, (C) *erα*, and (D) *erβ* gene transcription in the brain of male and female Japanese medaka following 21 d exposure to metformin. The results are shown as mean ± SE of three or four replicates. It is expressed as fold change relative to control. Asterisk (*) denotes a significant difference from the control (ANOVA, $p < 0.05$). Slope and p values of the trend were shown only when the trend was significant.

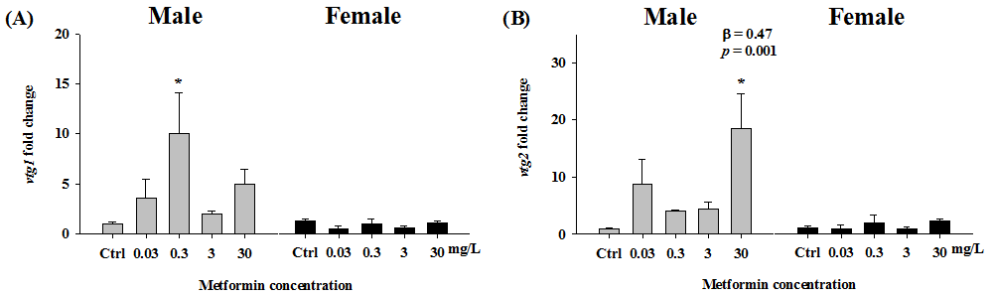

Fig. 4. Effects on (A) *vtg1* and (B) *vtg2* gene transcriptions in the liver of male and female Japanese medaka following 21 d exposure to metformin. The results are shown as mean ± SE of three or four replicates. It is expressed as fold change relative to control. Asterisk (*) denotes a significant difference from the control (ANOVA, $p < 0.05$). Slope and p values of the trend were shown only when the trend was significant.

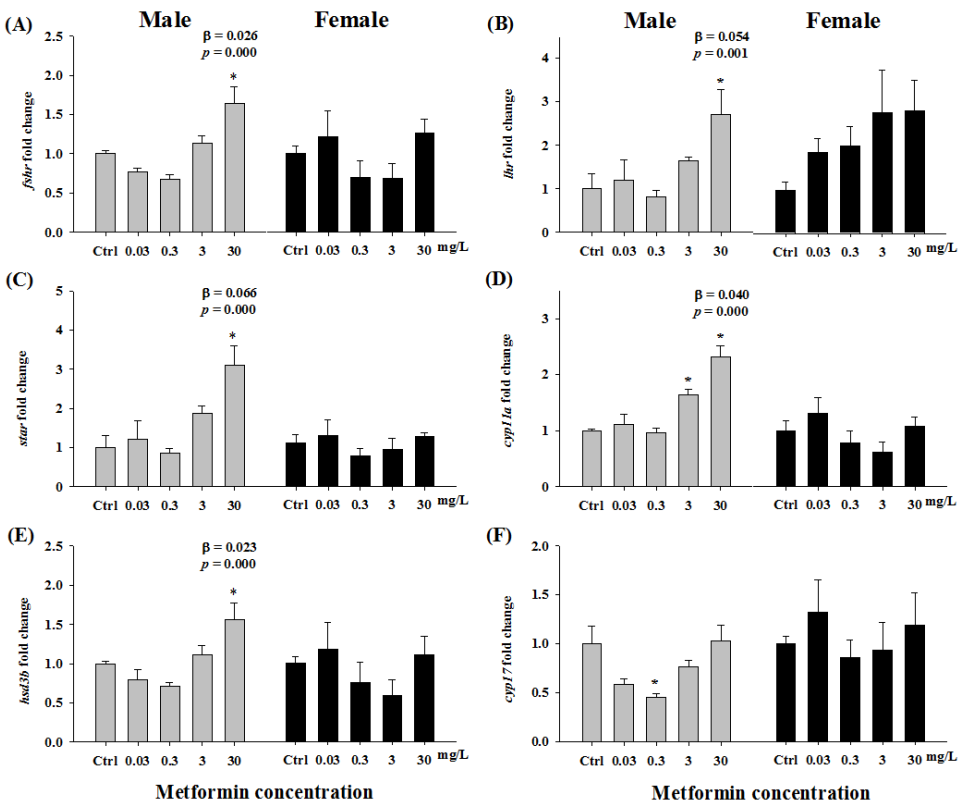

Fig. 5. Effects on (A) *fshr*, (B) *lhr*, (C) *star*, (D) *cyp11a*, (E) *hsd3b*, (F) *cyp17*, (G) *hsd17b3*, (H) *cyp19a*, (I) *hsd11b2*, and (J) *cyp11b* in the gonad of male (testis) and female (ovary) Japanese medaka following 21 d exposure to metformin. The results are shown as mean ± SE of three or four replicates. It is expressed as fold change relative to control. Asterisk (*) denotes a significant difference from the control (ANOVA, $p < 0.05$). Slope and p values of the trend were shown only when the trend was significant.

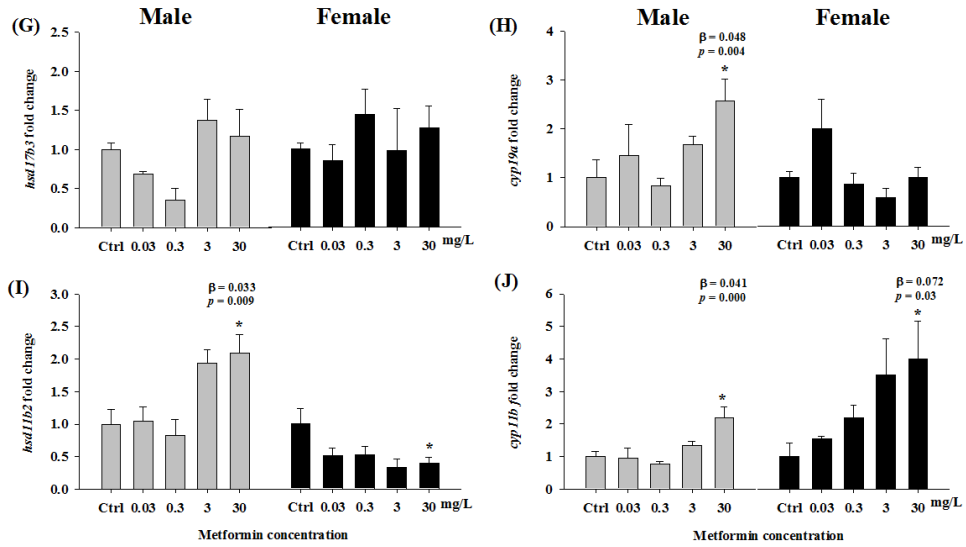

Fig. 5. (Continued).

4. Discussion

The *Daphnia* toxicity of metformin observed in the current study was generally comparable to those reported elsewhere. *D. magna* 48 h EC_{50} of 81.4 mg/L obtained in this study was in the ranges of EC_{50} in other reports ranging from 64 mg/L (Cleuvers, 2003) to 130 mg/L (ECHA, 1994b). The Japanese medaka 96 h LC_{50} estimated in the present study (383 mg/L) was lower than that reported from *L. macrochirus* (NOEC ≥ 982 mg/L) (ECHA, 1994c) (Table 4). In chronic exposures, only the survival was affected, and the effect levels were comparable to those of other previous studies (ECHA, 2007, 2008). In ECHA dossier, the *D. magna* 21 d reproduction NOEC was ≥ 32 mg/L (ECHA, 2007) and the *D. rerio* 30 dph development NOEC was ≥ 12 mg/L (ECHA, 2008) of metformin. However, both reproduction of *D. magna* and growth of Japanese medaka were not affected even at the highest exposure concentration of metformin (Tables 2 and 3). An assessment factor of 10 was therefore applied to 21 d survival NOEC of *D. magna*, 40 mg/L, PNEC was determined at 4 mg/L (Table 4).

Metformin has been frequently detected with detection frequency of 100 % in most cases in many countries (Table 5). Even at the maximum detected level in wastewater effluents (92 μg/L in USA) (Blair et al., 2013b), however, HQ value of metformin was determined below 1 (Table 6), suggesting that its risk is negligible.

Metformin exposure led to changes of sex hormone level in Japanese medaka following 21 d exposure, which may reflect therapeutic mechanisms of this drug on PCOS. In male fish, metformin showed estrogenic effects,

e.g., increases in plasma E2 level and decreases in plasma T and 11-KT levels. Although clear concentration dependence was not observed, E2/11-KT ratio was also increased (Fig. 2). Sex hormone ratio is a sensitive biomarker of abnormal sex hormones in fish (Orlando et al., 2004).

Sex hormone disruptions by metformin have been reported in mammals or *in vitro* studies (Table S3). Similar to our finding, in male chicken and pre-natally exposed mouse, plasma T levels were decreased by metformin. In addition, similar findings were reported in human and mouse testes and H295R cells (Faure et al., 2016; Hirsch et al., 2012; Tartarin et al., 2012). However, different from our observation which showed increase of E2 level in male or 11-KT level in female, in human thecal cells, plasma androstenedione levels decreased (Attia et al., 2001). Decrease of E2 levels was also reported in rat/bovine granulosa cells (Tosca et al., 2006, 2007)

The up-regulation of *vtg* gene transcription observed in 30 dph Japanese medaka (Fig. 1) also supports estrogenic potential of metformin. Vtg, a yolk protein precursor generally produced by female teleost, is a representative biomarker of exposure to estrogenic chemicals in sexually immature fish and male (Jin et al., 2008, 2011; Sun et al., 2014; Yamaguchi et al., 2008). Significant up-regulation of *vtg2* gene transcription in male liver (Fig. 4B) was observed along with up-regulation of E2 level (Fig. 2A) and E2/T ratio (Fig. 2D). Similar to our results, up-regulation of *vtg* gene transcription was reported in juvenile and male fathead minnow following exposure to 10 μg/L and 40 μg/L of metformin but not in female (Crago et al., 2016; Niemuth et al., 2015).

Changes in steroidogenic gene transcriptions explain the biological mechanisms of disrupted hormonal balance in fish (Fig. 5 and Fig. S1). In male fish, transcriptions of many steroidogenesis related genes, steroidogenic acute regulatory protein (*star*), cholesterol side-chain cleavage cytochrome P450 (*cyp11a*), 3ß-hydroxysteroid dehydrogenase (*hsd3b*), cytochrome P450 aromatase (*cyp19a*), hydroxysteroid 11-beta dehydrogenase 2 (*hsd11b2*) and 11 beta-hydroxylase (*cyp11b*) were significantly up-regulated (Fig. 5). *Star*, *cyp11a*, and *hsd3b* genes are in initial step of steroidogenesis pathway. *Cyp19a* is involved in conversion of androgens to estrogens. Both *hsd11b2* and *cyp11b* genes mediate the pathway from T to 11-KT (Fernandino et al., 2012). These changes might increase E2 level (Fig. 2A) and decrease T level (Fig. 2B). Compared to male fish, steroidogenesis gene transcriptions in female fish were less affected by metformin exposure in the present study. Only the transcriptional levels of *hsd11b2* and *cyp11b* were significantly changed at 30 mg/L of metformin with increased 11-KT level. The changes of two genes were in different way but the up-regulation of *cyp11b* was more significant than down-regulation of *hsd11b2*. Therefore, the increase of 11-KT level can be explained by significant up-regulation of *cyp11b* transcription.

Effects of metformin on steroid synthesis and vitellogenin up-regulation in fish have been suggested previously (Crago et al., 2016; Niemuth et al., 2015), but such effects do not appear to be directly mediated by estrogen receptor binding. Niemuth et al. (2015) suggested the effects on insulin

signaling of metformin might up-regulate *vtg* genes, without influencing transcription of major metabolic genes in adult fathead minnow (Niemuth et al., 2015). Crago et al. (2016) suggested the effects of metformin are not through direct estrogen receptor binding but through phase I and II drug-metabolizing enzymes, steroidogenesis, or HPG axis. They observed up-regulation of cytochrome P450 3A4-like isoform (CYP3A126), gonadotrophin releasing hormone 3 (GnRH3), and estrogen receptor α (ERα) genes.

The hormonal changes reported by metformin exposure in mammals or *in vitro* studies can also be explained by alterations in steroidogenesis. Affecting sex hormone balance is one of the important treatment mechanisms of metformin in patients with PCOS (Diamanti-Kandarakis et al., 2010a; Lauretta et al., 2016). The activities of steroidogenic enzymes such as HSD3B2, CYP17A1, CYP11A1, and STAR decreased by metformin in H295R cells, rat or bovine granulosa cells, and human theca cells (Attia et al., 2001; Hirsch et al., 2012; Tosca et al., 2006, 2007). Transcriptional levels such *as star, cyp11a1, cyp17* also decreased (Attia et al., 2001; Hirsch et al., 2012; Tartarin et al., 2012). However, detailed mechanisms of sex hormone disruption in fish warrant further studies including its effects on insulin sensitivity changes, glucose metabolism, and lipid metabolism in the fish.

In this study, we found that metformin exhibited minimal risk on aquatic ecosystem at the current environmental levels. However, changes in hormonal and gene transcriptional levels in Japanese medaka evidence

endocrine disrupting potential of metformin in aquatic organisms. Changes in many steroidogenesis related genes observed in this study suggest metformin can disrupt sex hormone balance via steroidogenesis pathway. For better understanding the consequences of metformin in aquatic ecosystem, long-term exposure studies with low concentration of metformin are required.

5. Reference

Al-Odaini, N.A., Zakaria, M.P., Yaziz, M.I., Surif, S., Abdulghani, M., 2013. The occurrence of human pharmaceuticals in wastewater effluents and surface water of Langat River and its tributaries, Malaysia. Int. J. Environ. Anal. Chem. 93, 245-264.

Attia, G.R., Rainey, W.E., Ph, D., Carr, B.R., 2001. Metformin directly inhibits androgen production in human thecal cells. Fertil. Steril. 76, 517–524.

Blair, B.D., Crago, J.P., Hedman, C.J., Klaper, R.D., 2013a. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. Chemosphere 93, 2116-2123.

Blair, B.D., Crago, J.P., Hedman, C.J., Treguer, R.J.F., Magruder, C., Royer, L.S., Klaper, R.D., 2013b. Evaluation of a model for the removal of pharmaceuticals, personal care products, and hormones from wastewater. Sci. Total Environ. 444, 515–521.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

Crago, J., Bui, C., Grewal, S., Schlenk, D., 2016. Age-dependent effects in fathead minnows from the anti-diabetic drug metformin. Gen. Comp. Endocrinol. 232, 185–190.

Diamanti-kandarakis, E., Christakou, C.D., Kandaraki, E., Economou, F.N., 2010a. Metformin : an old medication of new fashion : evolving new molecular mechanisms and clinical implications in polycystic ovary syndrome. Eur. J. Endocrinol. 162, 193–212.

Diamanti-kandarakis, E., Economou, F., Palimeri, S., 2010b. Metformin in polycystic ovary syndrome. Ann. N. Y. Acad. Sci. 1205, 192–198.

Diamanti-kandarakis, E., Papavassiliou, A.G., 2006. Molecular mechanisms of insulin resistance in polycystic ovary syndrome. Trends Mol. Med. 12, 324-332.

Dong, Z., Senn, D.B., Moran, R.E., Shine, J.P., 2013. Prioritizing environmental risk of prescription pharmaceuticals. Regul. Toxcol. Pharmacol. 65, 60-67.

Euler-Lotka, A.J., 1993. A natural population norm. J. Wash. Academy. Sci. 3, 241–248.

European Chemicals Agency (ECHA), 2008. Database. Metformin hydrochloride-aquatic toxicity. Long-term toxicity to fish (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/6/2/3 (Accessed: Jan. 18, 2017).

European Chemicals Agency (ECHA), 2007. Database. Metformin hydrochloride-aquatic toxicity. Long-term toxicity to aquatic invertebrates (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/6/2/5 (Accessed: Jan. 18, 2017).

European Chemicals Agency (ECHA), 2002. Database. Metformin hydrochloride-aquatic toxicity. Toxicity to aquatic algae and cyanobacteria (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-

/registered-dossier/12522/6/2/6 (Accessed: Jan. 18, 2017).

European Chemicals Agency (ECHA), 1994a. Database. Metformin hydrochloride-aquatic toxicity. Toxicity to microorganisms (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/6/2/8 (Accessed: Jan. 18, 2017).

European Chemicals Agency (ECHA), 1994b. Database. Metformin hydrochloride-aquatic toxicity. Short-term toxicity to aquatic invertebrates (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/6/2/4 (Accessed: Jan. 18, 2017).

European Chemicals Agency (ECHA), 1994c. Database. Metformin hydrochloride-aquatic toxicity. Short-term toxicity to fish (ecotoxicological information) experimental study. Available from URL: https://echa.europa.eu/registration-dossier/-/registered-dossier/12522/6/2/2 (Accessed: Jan. 18, 2017).

European Communities, 2011. Common Implementation Strategy for the Water Framework Directive (2000/60/EC) Guidance Document No. 27. Technical Guidance for Deriving Environmental Quality Standards.

Faure, M., Guibert, E., Alves, S., Pain, B., Ramé, C., Dupont, J. Brillard, J. P., Froment, P., 2016. The insulin sensitiser metformin regulates chicken Sertoli and germ cell populations. Reproduction 151, 527-538.

Fernandino, J. I., Hattori, R. S., Kishii, A., Strüssmann, C. A., Somoza, G. M., 2012. The cortisol and androgen pathways cross talk in high

temperature-induced masculinization: the 11β-hydroxysteroid dehydrogenase as a key enzyme. Endocrinol. 153, 6003-6011.

Hirsch, A., Hahn, D., Kempná, P., Hofer, G., Nuoffer, J. M., Mullis, P. E., Flück, C. E. 2012. Metformin inhibits human androgen production by regulating steroidogenic enzymes HSD3B2 and CYP17A1 and complex I activity of the respiratory chain. Endocrinol. 153, 4354-4366.

Ji, K., Han, E.J., Back, S., Park, J., Ryu, J., Choi, K., 2016. Prioritizing human pharmaceuticals for ecological risks in the freshwater environment of Korea. Environ. Toxicol. Chem. 35, 1028–1036.

Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J.P., Choi, K., 2013. Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish. J. Hazard. Mater. 254-255, 242–251.

Jin, Y., Shu, L., Huang, F., Cao, L., Sun, L., Fu, Z., 2011. Environmental cues influence EDC-mediated endocrine disruption effects in different developmental stages of Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 101, 254-260.

Jin, Y., Wang, W., Sheng, G.D., Liu, W., Fu, Z., 2008. Hepatic and extrahepatic expression of estrogen-responsive genes in male adult zebrafish (*Danio rerio*) as biomarkers of short-term exposure to 17β-estradiol. Environ. Mornit. Assess. 146, 105-111.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901.

Klimisch, H. J., Andreae, M., Tillmann, U., 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regul Toxicol. Pharm. 25, 1-5.

Kolpin, D., Furlong, E., Zaugg, S., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U. S. Streams, 1999-2000 : A National Reconnaissance. J. Environ. Sci. Technol. 36, 1202–1211.

Küster, A., Adler, N., 2014. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Phil. Trans. R. Soc. B, 369, 20130587.

Lauretta, R., Lanzolla, G., Vici, P., Mariani, L., Moretti, C., Appetecchia, M., 2016. Insulin-sensitizers, polycystic ovary syndrome and gynaecological cancer risk. Int. J. Endocrinol. 2016.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25, 402–408.

Marin-Morales, M.A., Campos-Pereira, F.D., Navarro, F.F., de Oliveira, J.A., 2016. Eco-toxicological Impact of Pharmaceuticals for Human Use in Aquatic Systems. Aquat. Toxicol.

Moermond, C.T., Smit, C.E. 2015. Derivation of water quality standards for carbamazepine, metoprolol, and metformin and comparison with monitoring data. Environ. Toxicol. Chem.

Nestler, J.E., Jakubowicz, D.J., 1996. Decreases in ovarian cytochrome P450c17α activity and serum free testosterone after reduction of insulin secretion in polycystic ovary syndrome. N. Engl. J. Med. 335, 617-623.

Niemuth, N.J., Jordan, R., Crago, J., Blanksma, C., Johnson, R., Klaper,

R.D., 2015. Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. Environ. Toxicol. Chem. 34, 291–296.

Niemuth, N.J., Klaper, R.D., 2015. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere 135, 38–45.

Oosterhuis, M., Sacher, F., Thomas, L., 2013. Prediction of concentration levels of metformin and other high consumption pharmaceuticals in wastewater and regional surface water based on sales data. Sci. Total Environ. 442, 380-388.

Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S., Guillette Jr, L.J., 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environ. Health. Perspect. 112, 353.

Oride, A., Kanasaki, H., Purwana, I.N., Miyazaki, K., 2010. Effects of metformin administration on plasma gonadotropin levels in women with infertility, with an *in vitro* study of the direct effects on the pituitary gonadotrophs. Pituitary 13, 236–241.

OECD, 1992. OECD 203 fish acute toxicity test. 203 1-9.

OECD, 2004. OECD 202 Daphnia sp., acute immobilisation test. OECD/OCDE 202 1-12.

OECD, 2004. OECD 210 fish early life stage toxicity test. Oecd/Ocde 220 1–22.

OECD, 2012. OECD 211 Daphnia magna reproduction test. 211 1–25.

Pentikäinen, P.J., Neuvonen, P.J., Penttilä, A., 1979. Pharmacokinetics of metformin after intravenous and oral administration to man. Eur. J. Clin. Pharmacol. 16, 195–202.

Rena, G., Pearson, E.R., Sakamoto, K., 2013. Molecular mechanism of action of metformin: Old or new insights? Diabetologia 56, 1898–1906.

Scheurer, M., Michel, A., Ruck, W., Sacher, F., 2012. Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment. Water Res. 46, 4790-4802.

Scheurer, M., Sacher, F., Brauch, H.J., 2009. Occurrence of the antidiabetic drug metformin in sewage and surface waters in Germany. J. Environ. Monit. 11, 1608–1613.

Sun, L., Lin, X., Jin, R., Peng, T., Gender-specificity, V.Á ., 2014. Toxic effects of bisphenol A on early life stages of Japanese medaka (*Oryzias latipes*). Bull. Environ. Contam. Tox. 93, 222–227.

Tartarin, P., Moison, D., Guibert, E., Dupont, J., Habert, R., Rouiller-Fabre, V., Frydman, N., Pozzi, S., Frydman, R., Lecureuil, C., Froment, P., 2012. Metformin exposure affects human and mouse fetal testicular cells. Hum. Reprod. 27, 3304–3314.

Tosca, L., Solnais, P., Ferré, P., Foufelle, F., Dupont, J., 2006. Metformin-induced stimulation of adenosine 5′ monophosphate-activated protein kinase (PRKA) impairs progesterone secretion in rat granulosa cells. Biol. Reprod. 75, 342-351.

Tosca, L., Chabrolle, C., Uzbekova, S., Dupont, J., 2007. Effects of

metformin on bovine granulosa cells steroidogenesis: possible involvement of adenosine 5’ monophosphate-activated protein kinase (AMPK). Biol. Reprod. 76, 368–378.

Tosca, L., Froment, P., Rame, C., McNeilly, J.R., McNeilly, A.S., Maillard, V., Dupont, J., 2011. Metformin decreases GnRH- and activin-induced gonadotropin secretion in rat pituitary cells: potential involvement of adenosine 5’ monophosphate-activated protein kinase (PRKA). Biol. Reprod. 84, 351–62.

Trautwein, C., Kümmerer, K., 2011. Incomplete aerobic degradation of the antidiabetic drug Metformin and identification of the bacterial dead-end transformation product Guanylurea. Chemosphere. 85, 765-773.

Tucker, G.T., Casey, C., Phillips, P.J., Connor, H., Ward, J.D., Woods, H.F., 1981. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br. J. Clin. Pharmacol. 12, 235-24 6.

U.S. Environmental Protection Agency, 2002. Methods for Measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, EPA 600/4-90/027F. Washington, D.C..

van Nuijs, A.L.N., Tarcomnicu, I., Simons, W., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2010. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. Anal. Bioanal. Chem. 398, 2211–2222.

Yamaguchi, A., Kohra, S., Ishibashi, H., Arizono, K., 2008. In vivo anti-

estrogenic effects of Menadione on hepatic estrogen-responsive gene expression in male medaka (*Oryzias latipes*). J. Health Sci. 54, 596–601.

Supporting information

Table S1. Primer sequences for the quantitative RT-PCR (qRT-PCR) analysis used

Gene	Sequence primer (5'-3')	Antisence primer (5'-3')	%Eff.[a]
rpl 7	cgccagatcttcaacggtgta	aggctcagcaatcctcagcat	116
fshβ	gacggtgctaccatgaggat	ttaacagctcggcatgtctg	103
cyp19b[b]	agtgcgtgttggagatggtga	catgaagaagaggctgatggaga	94.7
erα[b]	gacggagatcttcgacatgct	gcagacgaattcctcaggtttga	101
erβ[b]	gcagtccaaatccacctgttg	ggcccagcatcaggatct	110
arα[b]	gcaaaaggactgccaggtttc	tgacctccatcctaaagcgaac	105
vtg1	ctccagctttgaggccatttac	acagcacggacagtgacaaca	101
vtg2[b]	ctatacaaacttggattgggtcttcca	ctttcaggataggcctccaact	94.4
fshr	gctgcgcctttaaaaacaag	gcaaggacggagataatcca	94.1
lhr	ccgacctggaatttgactgt	gcaaaggccaggttacacat	98.3
star[b]	ggaatcccaatgtgaaagaggtcaa	gcagacacctcatgggtaatcat	117
cyp11a[b]	acactcctatggactttgtatgaattagc	gccacctccaacctcagttc	119
cyp17[b]	agccaccatcagggaggt	tgtcactgagggccacatg	114
hsd3b	gacacgccccatttaagcta	ggaaaaggtgaacgtcgtgt	97.6
hsd17b3b	gtggagcgattttctcaagg	gttccctgctcggaggtact	101
hsd11b2	cagtgttggagctgacagga	acaaacaccagcgttgttca	91.1
cyp11b	ctagacgacgtggcgaaagact	cctctgctcctcttccttctcg	107
cyp19a[b]	acaacatcaactttactgcagagctt	cgcactgcctcacgttct	104

[a] Efficiency of primer (%) was determined based on the standard curve of Ct values obtained from a 4-fold dilution series of cDNA (e.g., 1, 1:4, 1:16) in duplicate. [b] Kim et al. (2014); other primer sequences were designed using Primer 3 online software ver. 4.0.0 (http://primer3.ut.ee/).

Table S2. Somatic indices in male and female Japanese medaka following 21 d exposure to metformin[a]

concentration	Body length (mm)		Body weight (g)		GSI[b]		HSI[c]	
(mg/L)	Male	Female	Male	Female	Male	Female	Male	Female
Control	16.4±0.62	17.4±1.1	0.47±0.03	0.52±0.07	0.53±0.29	3.15±1.4	1.34±0.39	1.93±0.27
0.03	16.5±0.64	19.0±1.1	0.48±0.05	0.61±0.06	0.96±0.24	2.14±0.80	1.35±0.33	1.54±0.30
0.3	16.5±0.92	17.9±0.46	0.44±0.06	0.51±0.08	1.32±0.61	1.79±0.83	1.74±0.23	1.54±0.49
3	17.8±0.68 *	18.9±1.0	0.45±0.04	0.52±0.06	0.75±0.20	1.97±0.58	1.26±0.43	1.64±0.21
30	16.8±0.35	19.4±0.41 *	0.45±0.02	0.56±0.04	0.80±0.24	2.77±1.59	1.40±0.29	1.43±0.36

[a] Values represent mean ± standard deviation of each concentration (n=4). GSI (Gonadosomatic index) = 100 x gonad weight (g)/body weight (g), HSI (Hepatosomatic index) = 100 x liver weight (g)/body weight (g))

* Asterisk (*) denotes a significant difference from the control ($p < 0.05$) based on chi-square test or Dunnett's test.

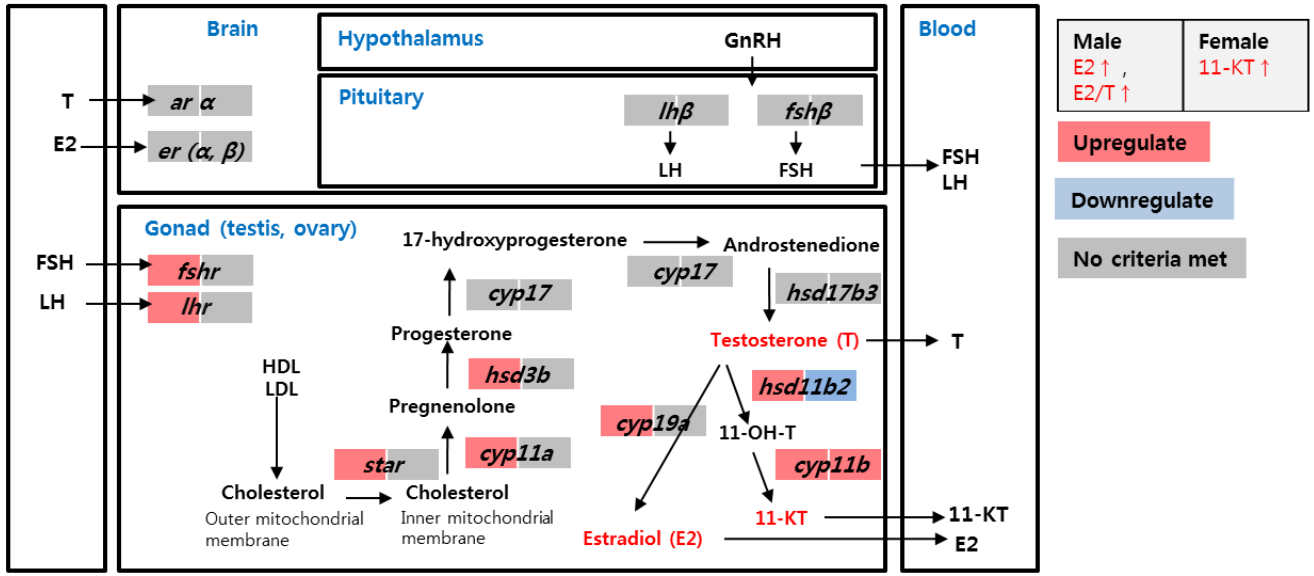

Fig. S1. Overview of sex hormone related effects in male and female Japanese medaka following 21 d exposure to metformin.

Table S3. Endocrine disruption and related mechanisms of metformin reported elsewhere

Test organisms		Results	Reference
Human	PCOS patients	17-OH-progesterone↓, T↓, LH↓	Nestler and Jakubowicz, 1996; Oride et al., 2010
In vivo	Mouse (male fetus), Chicken (male)	T↓	Tartarin et al., 2012; Faure et al., 2016
In vitro	Pituitary (rat)	FSH↓, LH↓; *fshβ* mRNA ↓	Tosca et al., 2011
	Pituitary (mouse)	*fshβ* promoter activity ↑	Oride et al., 2010
	Testis (human)	T↓	Tartarin et al., 2012
	Testis (mouse)	T↓; *star, cyp11a1, cyp17a1, 3βhsd, lhr* mRNA↓	Tartarin et al., 2012
	Ovarian theca, granulosa cell (human, rat, bovine)	Androstenedione↓, E2 ↓, Progesterone↓; 3BHSD, STAR, CYP17, CYP11A , CYP19A1 protein↓; *cyp17* mRNA↓	Attia et al., 2001; Tosca et al., 2006; Tosca et al., 2007
	H295R cell	Androgen↓; CYP17A1, HSD3B2 activity↓ *hsd3b2* mRNA↓	Hirsch et al., 2012
Fish	Fathead minnow (75-85 dpf or male adult)	Intersex in male ↓, reproduction↓; T – , VTG ↑(tendency) *vtg, GnRH3, ERα GCYP3A126* mRNA↑	Niemuth et al., 2015; Niemuth and Klaper, 2015; Crago et al., 2016

T: testosterone, LH: luteinizing hormone, FSH: follicle stimulating hormone, E2: 17β-estradiol

국문초록

당뇨병 치료제 metformin의
수생태 독성평가 및 내분비계 교란영향

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많이 사용되는 당뇨병치료제 중 하나인 메트폴민 (metformin)은 수계에서 빈번하게 검출이 보고되고 있다. 하지만 이 물질의 잠재적인 생태독성에 대한 연구는 부족한 실정이다. 본 연구는 두 담수생물인 물벼룩(*Daphnia magna*)과 어류인 Japanese medaka (*Oryzias latipes*)를 이용하여 OECD Test Guideline에 따라 급성, 만성 독성시험을 수행하였다. 또한 성어 Japanese medaka를 이용하여 메트폴민이 내분비계교란에 미치는 영향 및 구체적인 기전을 확인하고자 하였다.

D. magna 21일 만성독성시험 결과, 생존율 NOEC (No observed effect concentration, 무영향관찰농도)은 40 mg/L로 확인되었으나, 이 농도에서 생식 영향은 관찰되지 않았다. 어류 초기생장단계 시험 (early life stage test) 결과, 생존율에 대한 NOEC은 100 mg/L로 확인되었다.

메트폴민의 PNEC (Predicted no effect concentration, 예측무영향농도) 값은 *D. magna* 21일 NOEC 값인 40 mg/L를 토대로 4 mg/L로 산출되었다. HQ (hazard quotient, 유해지수)는 1 미만으로 나타나, 현재 노출 수준에서 생태위해성은 없는 것으로 나타났다.

성어 Japanese medaka에서 메트폴민 21일 노출 후, 수컷에서는 17β-estradiol (E2) 호르몬 수준과 E2/T (testosterone) 비율이 유의하게 증가하였다. 또한 스테로이드합성(steroidogenesis)관련 유전자인 *star, cyp11a, hsd3b, cyp19a, hsd11b2*, *cyp11b* 발현이 유의하게 증가하였다. 암컷에서는 11-ketotestosterone (11-KT) 호르몬 수준이 *cyp11b* 유전자 발현 수준과 함께 유의하게 증가하였다.

본 연구를 통해, 메트폴민은 현재 수계 농도수준에서는 직접적인 생태독성 영향이 없는 것으로 나타났다. 하지만 스테로이드합성 관련 유전자 교란을 통해 성호르몬 교란 영향이 있는 것으로 나타났다. 따라서 수계에서 저농도의 장기노출영향에 대한 내분비계 교란영향에 대한 추가 연구가 필요할 것으로 사료된다.

주요어: 당뇨병 치료제; 메트폴민; 생태독성평가; 내분비계 교란; 시상하부-뇌하수체-생식선 축

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