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Endocrine disruption by several aniline derivatives and related mechanisms in a human adrenal H295R cell line and adult male zebrafish

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ABSTRACT

Aniline and aniline derivatives have been widely used in the production of pesticides, pharmaceuticals, cosmetic, dyes, rubber, and adhesives products. These chemicals can easily be released into the environment through industrial and municipal discharges or as degradation byproducts. Several studies have suggested that aniline and some of its derivatives could cause reproductive toxicity in aquatic organisms. However, knowledge on the endocrine disruption potentials of these chemicals is limited only to aniline and associated mechanisms are rarely investigated. The objective of this study was to investigate the potential of major aniline derivatives, i.e., 3,4-dichloroaniline (3,4-DCA), 1-naphthylamine (1-NPA), and 4,4′-methylenedianiline (4,4′-MDA), to disrupt sex steroid production and other biological processes. For this purpose, the human adrenal H295R cell line and adult male zebrafish (*Danio rerio*) were used. In the H295R cell line, all tested aniline derivatives decreased testosterone (T) levels. Regulatory changes of several steroidogenic genes, i.e., down-regulation of *StAR* or *CYP17* genes, and up-regulation of *CYP19A*, observed in the H295R cells could explain the sex hormone disruption. In male zebrafish, generally similar directions of changes, i.e., decreases in T levels and increased E2/T ratios, were observed. Again, down-regulation of key steroidogenic genes such as *cyp17* or *3β-hsd*, but slight up-regulation of *cyp19a* gene observed in the fish could explain the sex hormone changes. The results of our study demonstrate that all tested aniline derivatives could influence steroidogenesis and disrupt sex hormone balance toward reduced androgenicity. Consequences of anti-androgenicity following long-term exposure warrant further investigation.

1. Introduction

Aniline, a parent molecule of the aromatic amines family, is one of the important building blocks of numerous chemicals. Aniline and its derivatives, e.g., 3,4-dichloroaniline (3,4-DCA), 4,4′-methylenedianiline (4,4′-MDA), and 1-naphthylamine (1-NPA), have been widely used as precursors or intermediates for many pesticides, pharmaceuticals, cosmetics, dyes, rubber, and adhesives products (Lewis, 2007; Mattarozzi et al., 2013; Saleh et al., 2016; Wang et al., 2016). Several anilines are identified as high volume production chemicals in many countries including the USA and EU member states (Di Girolamo et al., 2009; European Commission, 2006; 2012; Sihtmäe et al., 2010; US EPA, 2009). In 2016, aniline production worldwide was more than 5.6 million tons (Wang et al., 2016).

As precursors or intermediates of the production of many dyes and personal care products, aniline and its derivatives can be released from various products. For example, 1-NPA can be released from hair colour, henna, tattoo, and cosmetics (Akyuz and Ata, 2008). 1-NPA and 4,4′-MDA can be released from rubber products under a certain temperature, and 4,4′-MDA can be formed from residual isocyanates remaining in polyurethane adhesive (Pezo et al., 2012). Pharmaceuticals can be another source of the anilines in the environment because aniline is used for preparation of antipyretics, analgesics, anti-allergics, and vitamins (Kawakami et al., 2010). Moreover, these compounds can be released into the environment as breakdown products, and these examples are 1-NPA from the dyes, herbicides, and pesticides (Babcock et al., 1993; Guzman Mar et al., 2006), 3,4-DCA from the herbicides, e.g., diuron, linuron, and propanil (Marlatt and Martyniuk, 2017; Saleh et al., 2016), and 4,4′-MDA from polyurethane and rubber products (Campanella et al., 2015; Rubio et al., 2014).

While the information on the environmental levels of many anilines is relatively limited, a few anilines have been often detected in river

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water and sediments around the world (Boulahlib et al., 2016; Jurado-Sanchez et al., 2012; Saleh et al., 2016; Zhao et al., 2001). In Scope Creek and Chattahoochee River water in the USA, 3,4-DCA was detected up to 68.2 ng/L (US Geological Survey, 2012). In the sediment of a river in Zonguldak industrial area of Turkey, 1-NPA was detected up to a seasonal mean concentration of 186.45 ng/kg (Akyuz and Ata, 2006). The parent compound of 3,4-DCA, i.e., diuron, was detected up to 230 ng/L in the major harbours of Korea and up to 1360 ng/L in a fishing port of Jinhae Bay of Korea (Kim et al., 2014). Because of the wide range of source and detection in the environment, some anilines have been investigated for toxicological effects.

Several experimental and epidemiological studies suggest that aniline may cause adverse effects on the endocrine system and reproduction. Following exposure to aniline, fetal male rats showed reduced anogenital distance suggesting anti-androgenic effects (Holm et al., 2015). In the human adrenal carcinoma cell line (H295R), exposure to aniline led to increased testosterone levels (Holm et al., 2015). In *Daphnia*, a freshwater crustacean, aniline influenced reproductive parameters following 21 days of exposure (Trubetskova and Lampert, 2002). Similar toxic effects have been reported in aquatic organisms for other anilines. In Nile tilapia (*Oreochromis niloticus*), exposure to diuron metabolite, i.e., 3,4-DCA led to increasing 17β-estradiol in female, and decreasing testosterone levels in male fish (Pereira et al., 2015, 2016). Following exposure to 1-NPA in embryo larvae of zebrafish, toxic effects on survival and development were observed (Horie et al., 2017). In rare minnow (*Gobiocypris rarus*) larvae, a 72 h exposure to 3,4-DCA caused a dose-dependent decrease of growth and increase of malformation. While evidence indicating endocrine disruption potentials of aniline and its derivatives is accumulating, knowledge on underlying mechanisms is still limited. Moreover, only very few aniline derivatives have been studied, and therefore there is a significant information gap on the toxicity of other commonly used and detected aniline derivatives.

The objective of this study was to evaluate endocrine disrupting potentials of major aniline derivatives and to identify possible underlying mechanisms, using both in vitro and in vivo experiments. For this purpose, the human adrenal H295R cell line and adult male zebrafish were employed. H295R cells have been widely used for the screening of the endocrine disruption potentials of various chemicals specially focusing on changes in steroidogenesis (Hecker et al., 2011; Hecker and Giesy, 2008; Hilscherova et al., 2004; Jo et al., 2014; Kraugerud et al., 2011; Sanderson et al., 2002; Sanderson, 2006). In addition, zebrafish have been widely used to investigate the effects of environmental chemicals on sex hormone disruption and to characterize the underlying mechanisms at an organism level (Ji et al., 2013; Liu et al., 2012; Ma et al., 2012; Segner, 2009; Segner et al., 2003; Sohn et al., 2016; Wang et al., 2015). The results of this study will help identify aniline derivatives of potential toxicological concern, and stimulate further studies on endocrine disruption consequences of aniline derivatives.

2. Materials and methods

2.1. Chemicals

3,4-DCA (CAS No. 95-76-1, purity: ≥98%), 1-NPA (CAS No. 134-32-7, purity: ≥99%) and 4,4′-MDA (CAS No. 101-77-9, purity: ≥97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as a solvent for both in vitro and in vivo studies. The final concentration of the solvent in the exposure media was 0.005% (v/v) for zebrafish exposure and 0.1% (v/v) for H295R cell assays.

2.2. Zebrafish culture and exposure

About six months old adult male zebrafish (*Danio rerio*, wild type) were obtained from a commercial vendor (Green Fish, Seoul, Korea). The fish were acclimated in the laboratory in an in-house culture environment at least for seven days before the experiment. Fish were exposed to six concentrations of each test chemical, i.e. 3,4-DCA, 1-NPA, or 4,4′-MDA, including a water and a solvent control. For each concentration or control, four replicates (2 L beaker each) with 4 male fish were used. The exposure concentrations for each chemical were determined at sub-lethal levels, i.e., 0.024, 0.12, 0.6, or 3.0 mg/L for 3,4-DCA; 0.04, 0.2, 1.0, or 5.0 mg/L for 1-NPA; and 0.2, 1.0, 5.0, or 25 mg/L for 4,4′-MDA, which were confirmed based on preliminary range finding tests. The study was carried out for 14 days according to the OECD test guideline No. 204 (OECD, 1984). During the exposure, the fish were maintained at 26 ± 1 °C under 14:10 h light: dark photoperiod, and fed with freshly hatched *Artemia naupli* twice a day. The exposure media (> 90%) were replaced daily with newly prepared test media. During renewal, both new and old exposure media were saved for measurement of water quality parameters including temperature, pH, dissolved oxygen, and conductivity every other day. After the 14 day exposure, the fish were sacrificed, and blood samples were collected from caudal vein using capillary tube. To obtain sufficient volume for hormone measurement, the blood samples from four fishes were pooled, and plasma was separated by centrifugation (8000 rpm for 10 min). The plasma was stored at −80 °C until hormone analysis. Moreover, testes was collected and stored at −80 °C until analysis by quantitative real-time PCR (qRT-PCR) analysis for major steroidogenic genes.

2.3. H295R cell culture and exposure

Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma-Aldrich) were used for the H295R cell line. The cells were cultured at 37 °C in a 5% CO_2 atmosphere (Hilscherova et al., 2004), and the media were renewed every other day. The exposure concentration range was determined at 1.0, 10.0, and 100.0 mg/L for all three test compounds. All test concentrations showed > 80% cell viability compared to that of the control based on the results of the WST-1 cell proliferation assay (Roche Applied Science, Mannheim, Germany) (supplement Fig. S1). For each exposure concentration, control and solvent control, three replicates were prepared. The 24-well plate was used for exposure. The cells were seeded at a density of 3.0×10^5 cells/mL, and after 24 h of incubation, the cells were exposed to the chemical for another 48 h. After the exposure, the culture medium was collected for sex steroid hormone measurement, and the cells were collected for quantification of the gene expression. Each experiment was conducted for three times independently for each compound, and the average value from three independent tests was used for statistical analysis.

2.4. Chemical analysis

The concentrations of 1-NPA, 3,4-DCA, and 4,4'-MDA in the fish exposure media were measured at the beginning and after 24 h of exposure. Exposure media were collected on three different days with 4 technical replicates for each concentration. Briefly, 1 mL of the water sample was filtered with 0.2 μm syringe filter. After that, the sample was diluted with water to ensure the concentration fell within the calibration curve range. All three chemicals were analyzed using an ultra-high pressure liquid chromatography (UHPLC) system Nexera (Shimadzu Corporation, Kyoto, Japan) coupled with API 4500 Triple Quadrupole Mass Spectrometry System (AB SCIEX, Ontario, Canada). Chemicals were separated using ACQUITY UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) column. The injection volume was 5 μL, and the flow rate was 0.3 mL/min. Water and methanol were used as mobile phase with 20:80 ratios in isocratic mode. Total run time was 3 min. Analytical conditions using UHPLC-MS/MS are shown in Supplement Table S1 and MS/MS parameters are shown in supplement Table S2. The limit of detection was 0.13 ng/mL for 1-NPA, 0.22 ng/mL for 3,4-

Table 1
Primer sequences used for the qRT-PCR analysis for H295R cell and zebrafish samples.

Assay	Gene name	Accession No.	Description	Sequence (5′-3′)
in vitro H295R	*β-actin*	NM_001101	Forward	CAC TCT TCC AGC CTT CCT TCC
			Reverse	AGG TCT TTG CGG ATG TCC AC
	StAR	NM_000349	Forward	GTCCCACCCTGCCTCTGAAG
			Reverse	CATACTCTAAACACGAACCCCACC
	3βHSD	NM_000198	Forward	TGC CAG TCT TCA TCT ACA CCA G
			Reverse	TTC CAG AGG CTC TTC GTG
	CYP17	NM_000102	Forward	AGC CGC ACA CCA ACT ATC AG
			Reverse	TCA CCG ATG CTG GAG TCA AC
	CYP19A	NM_000103	Forward	AGG TGC TAT TGG TCA TCT GCT C
			Reverse	TGG TGG AAT CGG GTC TTT ATG G
in vivo zebrafish	*β-actin*	NM_131031	Forward	TGCTGTTTTCCCCTCCATTG
			Reverse	TCCCATGCCAACCATCACT
	star	NM_131663	Reverse	GGTCTGAGGAAGAATGCAATGAT
			Reverse	CCAGGTCCGGAGAGCTTGT
	3βhsd	AY279108	Forward	AGGCACGCAGGAGCACTACT
			Reverse	CCAATCGTCTTTCAGCTGGTAA
	cyp17	AY281362	Forward	TCTTTGACCCAGGACGCTTT
			Reverse	CCGACGGGCAGCACAA
	cyp19a	AF226620	Forward	GCTGACGGATGCTCAAGGA
			Reverse	CCACGATGCACCGCAGTA

DCA, and 0.15 ng/mL for 4,4'-MDA.

2.5. Measurement of sex hormones and gene expression

In both H295R cell medium and zebrafish, sex hormones of 17β-estradiol (E2) and testosterone were measured using enzyme-linked immunosorbent assay (ELISA) with commercial kits (Cayman Chemical; 17β-estradiol [Cat No. 582251] and testosterone [Cat No. 582701]). Hormones were extracted from 500 µL of H295R cell medium or 10 µL of fish plasma. The samples were diluted with 400 µL of ultrapure water. The diluted samples were centrifuged at 2000 g for 10 min after adding 2 mL of diethyl ether, and then the upper layer was collected. The extraction by diethyl ether was done twice. The sample was evaporated under nitrogen flow, and was reconstituted with 300 µL of EIA buffer for the H295R cell line and 120 µL for the zebrafish for hormone measurement using the ELISA kits (Ji et al., 2010). The H295R cell line assay was repeated for three times independently and the results were combined for statistical analysis. For fish, plasma samples from four male fish in each replicate were pooled and measured for hormones. For each treatment or control, four replicates were measured for the hormones.

Measurement of four major steroidogenic genes was carried out by qRT-PCR. Gene expression levels were quantified in the H295R cells for three times independently. For fish, testes were collected from three male zebrafish from each replicate, and pooled for measurement of transcriptional changes. For each treatment or control, a total of four replicates were measured, and used for statistical analysis. For this purpose, H295R cells or fish testes samples were homogenized, and total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA). The complementary DNA (cDNA) was synthesized for all samples using an iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA) from 100 ng/µL of extracted RNA. After the extraction of RNA and synthesis of cDNA, quality and concentration of RNA and cDNA were confirmed by Epoch (BioTek, Winooski, VT, USA). The qRT-PCR sample was prepared by adding 18 µL of premix and 2 µL of cDNA sample in a 96 well plate. The premix contained 10 µL of Light Cycler DNA Master SYBR Green I mix (Roche Diagnostics Ltd., Lewes, UK), 1.8 µL of forward and reverse primer (10 pM), and 4.4 µL of nuclease-free water. The qRT-PCR was carried out using Light Cycler 480 (Roche Applied Science, Indianapolis, IN, USA). Thermal cycling was 95 °C for 10 min followed by denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s with a total 40 PCR cycles. After the final amplification, melting curve analyses were carried out to identify the desired PCR products from the primer dimers or contaminants. The relative expression level of mRNA of each targeted gene was normalized with that of the reference gene (*β-actin* in both H295R and zebrafish) and calculated by threshold cycle (C*t*) number using ΔΔCt method (Livak and Schmittgen, 2001). The primer sequences of targeted genes in H295R and zebrafish are listed in Table 1.

2.6. Statistical analysis

The normality of distribution and homogeneity of variances were assessed by Shapiro-Wilk's test and Levene's test, respectively. When the

Table 2
Measured concentrations (mg/L) for nominal experimental concentrations of 3,4-DCA, 1-NPA, or 4,4′-MDA in the fish exposure media at 0 and 24 h of exposure.

3,4-DCA				1-NPA				4,4′-MDA			
Nominal	Measured			Nominal	Measured			Nominal	Measured		
	0 h	24 h	Average		0 h	24 h	Average		0 h	24 h	Average
0.00 (C)	ND	ND	ND	0.00 (C)	ND	ND	ND	0.00 (C)	ND	ND	ND
0.00 (SC)	ND	ND	ND	0.00 (SC)	ND	ND	ND	0.00 (SC)	ND	ND	ND
0.024	0.030	0.041	0.035	0.040	0.014	0.015	0.015	0.20	0.091	0.056	0.074
0.12	0.11	0.16	0.13	0.20	0.15	0.13	0.14	1.0	0.67	0.50	0.58
0.60	0.44	0.33	0.38	1.0	1.2	0.50	0.83	5.0	5.4	3.9	4.6
3.0	2.1	1.8	1.9	5.0	5.6	1.6	3.6	25	25	20	22

C: Control; SC: Solvent control; ND: Not detected. Average: average of the measured concentrations for the fresh (0 h) and the 24 h old media. Each measured concentrations are an average of 12 independent samples collected throughout the exposure (N = 12).

data did not follow normal distribution, log transformation was performed to make them normally distributed. To compare for differences among the treatments, one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was performed using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). All results are presented as mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Concentrations of chemicals in fish exposure media

Measured concentrations of 3,4-DCA, 1-NPA, or 4,4′-MDA for nominal experimental concentrations are shown in Table 2. The average of the measured concentrations for the fresh (0 h) and old (24 h) media of the highest experimental concentrations of 3,4-DCA, 1-NPA, or 4,4′-MDA were about 63%, 72%, or 88% of the nominal concentrations, respectively (Table 2). In the lowest experimental concentration, measured concentrations for 1-NPA and 4,4′-MDA were less than 50%, while those for 3,4-DCA were greater than 100% of the nominal concentration. Because of relatively huge differences between nominal and measured concentrations, the measured concentrations, i.e., averages of the measured concentrations for the fresh and the 24 h old media, were used for presentation and interpretation of the results throught the manuscript.

3.2. Responses in H295R cells by aniline derivatives

3.2.1. Concentration of sex hormones

In the H295R cell line, all three aniline derivatives showed a significant dose-dependent decrease of T concentrations along with an increase in E2/T ratio (Fig. 1). E2 levels were not changed after 3,4-DCA and 1-NPA exposure, but significantly decreased after 4,4′-MDA exposure (Fig. 1). Forskolin, a positive control chemical used in the H295R cell line assay showed a significant increase of both T and E2 concentrations (supplement Fig. S2).

3.2.2. mRNA expression level of steroidogenic genes

The *CYP17* gene was significantly down-regulated and the *CYP19A* gene was significantly up-regulated by both 1-NPA and 3,4-DCA in the H295R cells (Fig. 2). After exposure to 4,4′-MDA, *StAR*, *CYP17* and *CYP19A* were significantly down-regulated (Fig. 2). However, the *3βHSD* gene was not altered by any test chemicals. All four targeted genes showed significant up-regulations by the positive control chemical, i.e., forskolin (supplement Fig. S3).

3.3. Responses in adult male zebrafish by aniline derivatives

3.3.1. Plasma sex hormones

All three tested chemicals influenced the sex hormone balances of the adult male zebrafish leading to higher E2/T ratio. Significant decreases of both T and E2 were observed by 3,4-DCA and 4,4′-MDA, and the extent of decrease was much greater for T (Fig. 3). While the hormonal changes by 1-NPA were not consistent, E2/T ratio showed similar pattern of increase.

3.3.2. Gonadal steroidogenic genes

Among the measured gonadal steroidogenic genes, both *star* and *cyp17* genes were significantly down-regulated after exposure to 3,4-DCA or 4,4′-MDA (Fig. 4). For 1-NPA, *3β-hsd* and *cyp17* genes were down-regulated with a significant up-regulation of *star* gene at the lowest experimental concentration (0.015 mg/L) (Fig. 4). The transcription of *cyp19a* gene was not influenced by the exposure to any test anilines, except 4.6 mg/L 4,4′-MDA which showed a significant up-regulation (Fig. 4).

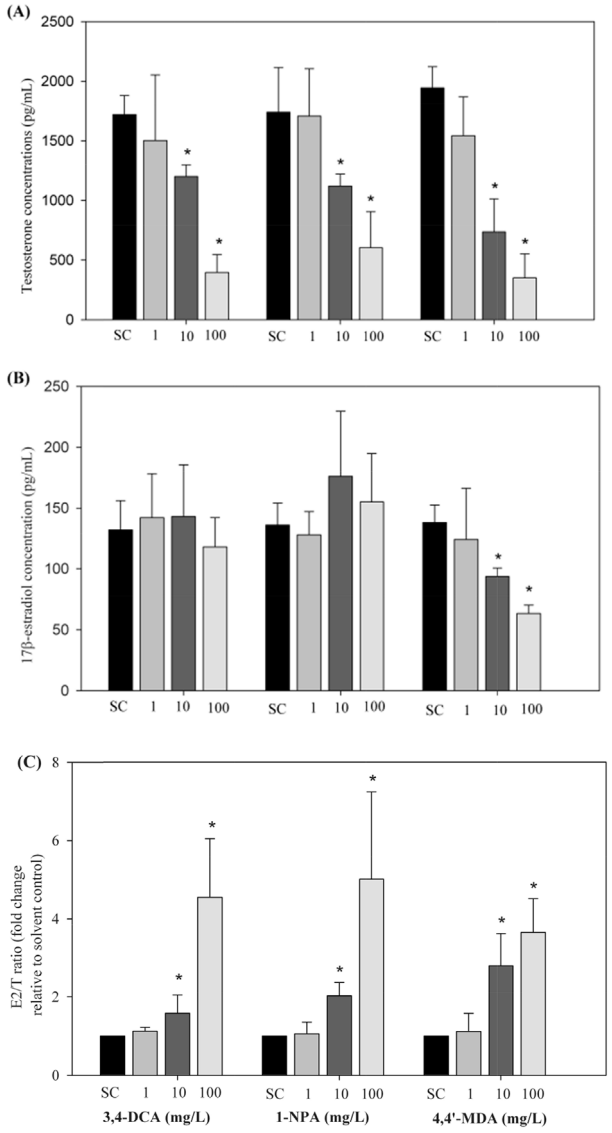

Fig. 1. (A) Testosterone (T), (B) 17β-estradiol (E2), and (C) E2/T ratio measured in the cultured medium of H295R cells after 48 h exposure to 3,4-DCA, 1-NPA and 4,4′-MDA. Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

4. Discussion

A significant dose-dependent decrease of T concentrations along with the increase of E2/T ratio by all three tested aniline derivatives which were observed in the H295R cells (Fig. 1) indicates endocrine disruption potential of the anilines toward greater E2 to T ratio. The increased E2/T ratio was mostly due to significant reduction of T levels by exposure to the tested anilines (Fig. 1A). Significant down-regulation of major steroidogenic genes such as *StAR* and *CYP17* suggests that the decreased T levels may be due to inhibition of upstream steroidogenesis. Down-regulation of *StAR* and *CYP17* genes (Fig. 2) may decrease cholesterol uptake and androstenedione synthesis, respectively, which may lead to reduced production of steroid hormones. The *StAR* gene plays an important role in the transport of cholesterol into the mitochondrial membrane, which is the first step in the steroid synthesis process (Clewell et al., 2010). The enzyme encoded by *CYP17* gene is responsible for the conversion of progesterone to androstenedione.

Up-regulation of *CYP19A* gene in the H295R cells observed following exposure to 3,4-DCA and 1-NPA could be interpreted as

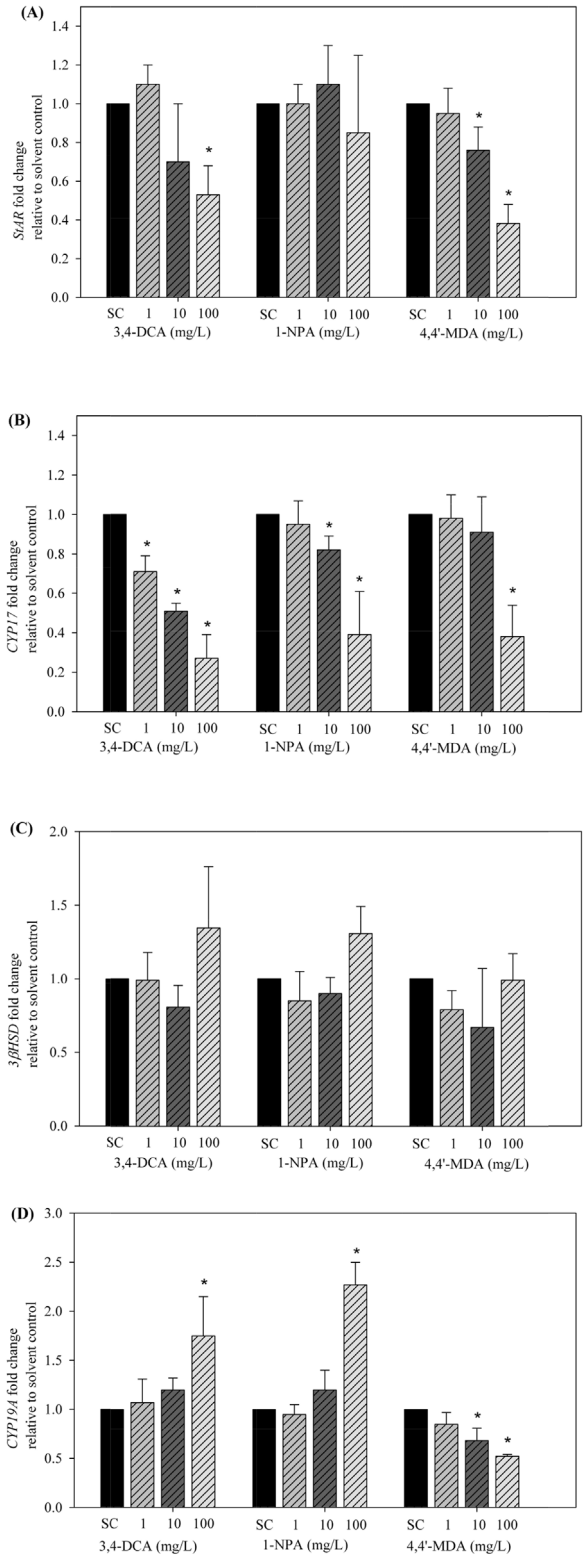

Fig. 2. mRNA expression level of (A) *StAR*, (B) *CYP17*, (C) *3βHSD* and (D) *CYP19A* gene in H295R cell after 48 h exposure to 3,4-DCA, 1-NPA and 4,4′-MDA. Results are shown as mean ± SD of three independent experiments. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

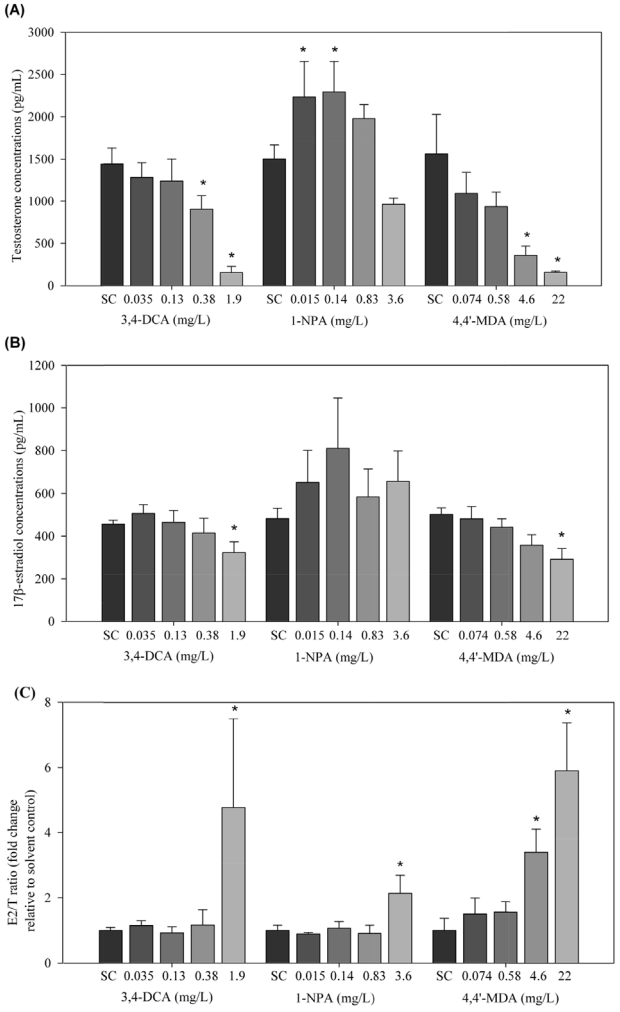

Fig. 3. Plasma concentration changes of (A) Testosterone (T), (B) 17β-estradiol (E2), and (C) E2/T ratio measured in male zebrafish after 14 d of exposure to 3,4-DCA, 1-NPA and 4,4′-MDA. Results are shown as mean ± SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

increased conversion of T to E2 (Fig. 2D). Aromatase, the product of the transcription of the *CYP19A* gene, catalyzes the conversion of T to E2 (Hilscherova et al., 2004; Trant et al., 2001). Therefore, the up-regulation of *CYP19A* may lead to enhancement of the conversion of T to E2, which in turn can decrease T levels. Significant up-regulation of the *CYP19A* gene may explain the E2 levels which were not affected, even though several genes in the up-stream of the steroidogenic pathway were down-regulated. Decreased E2 levels following exposure to 4,4′-MDA (Fig. 1B) can be also explained, in this context, by down-regulation of *CYP19A* gene (Fig. 2D). The observations in H295R cells indicate that the three aniline derivatives could alter the hormonal balances toward decreasing T levels, through modulating the steroidogenic pathway.

To date, endocrine disruption potential of aniline derivatives has seldom been reported, except for aniline. Holm et al. (2015) reported that aniline could increase T synthesis without change of E2 level in H295R cells. In the same study, authors also showed that paracetamol, one of the major metabolites of aniline, could decrease T levels and increase E2 levels in the cells through alteration of the steroidogenic pathway, e.g., down-regulation of *CYP17* gene and up-regulation of *CYP19A* gene. For other anilines, however, sex hormone disruption potentials and steroidogenic alteration have never been investigated in the cells.

The observations in H295R cells were supported by the observations in the zebrafish (Figs. 3 and 4). Employing adult male zebrafish, we found that sex hormones were affected in a consistent way. Among

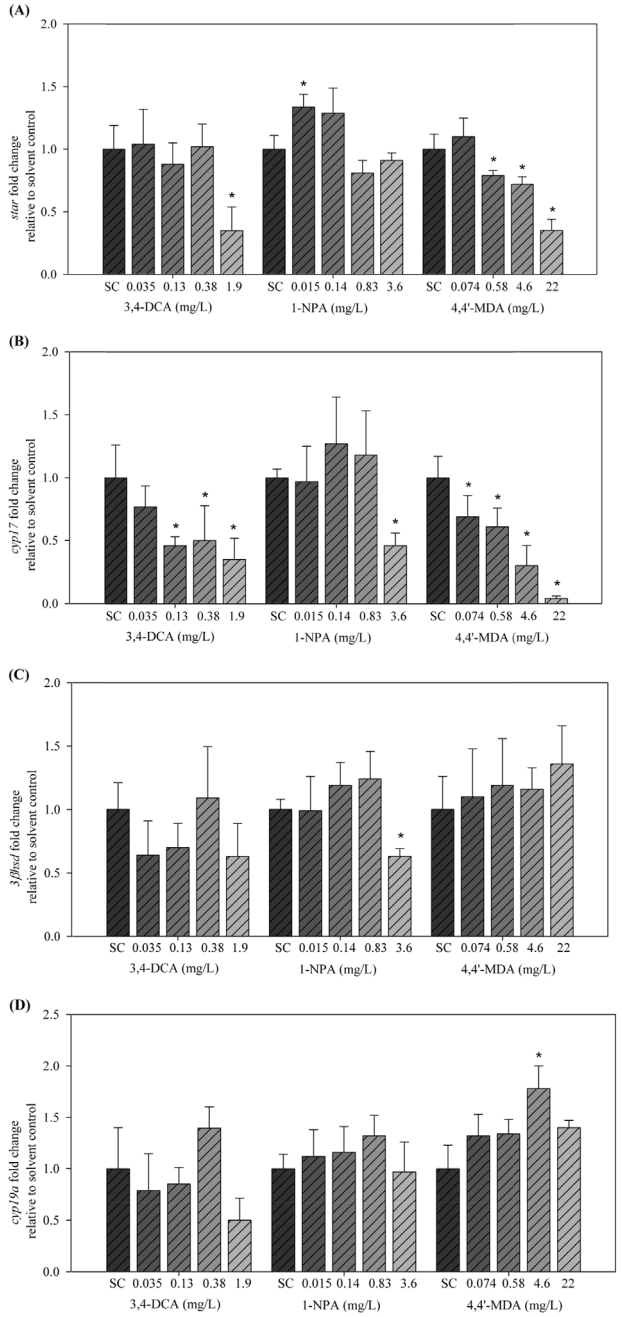

Fig. 4. mRNA expression level of (A) *star*, (B) *cyp17*, (C) *3βhsd*, and (D) *cyp19a* gene in male zebrafish gonad after 14 d exposure to 3,4-DCA, 1-NPA, and 4,4′-MDA. Results are shown as mean ± SD of four replicates for each concentration. Asterisks represent a significant difference ($p < 0.05$) from solvent control (SC, treated with 0.1% DMSO).

three tested anilines, 3,4-DCA and 4,4′-MDA caused significant decreases of both T and E2 levels (Fig. 3A and B). A significant increase of E2/T ratio was also observed, and this may be either because the extent of T decrease was much greater than that of E2 decrease, or because basal level of E2 was lower than that of T in the male fish and therefore did not react as dramatically as T did. Because E2/T ratio has been considered as a sensitive biomarker of sex hormone alteration and an indicator of endocrine disruption (Ji et al., 2013; Orlando et al., 2004), however, greater E2/T ratio in zebrafish upon exposure to 3,4-DCA, 1-NPA, and 4,4′-MDA might be linked to adverse effects on gametogenesis and reproduction of fish (Shang et al., 2006).

Decreases of both sex hormones in the adult male fish, which are similar to the case of H295R cells, could be explained by significant down-regulation of key steroidogenic genes, e.g., *star* and *cyp17* following exposure to 3,4-DCA and 4,4′-MDA (Fig. 4A and B). Unlike H295R cells, adult male zebrafish exposure did not show notable up-regulation of *cyp19a* gene (Fig. 4D), and therefore E2 levels decreased slightly (Fig. 3B). In the literature, to date, only 3,4-DCA has been reported to disrupt sex hormone balance in fish (Pereira et al., 2015, 2016). Following exposure to 3,4-DCA, T was decreased in male tilapia and E2 was increased in female tilapia fish. These observations in the male tilapia fish are the same as those observed in the present study in the male zebrafish, even thoughregulatory changes of the related steroidogenic genes were not evaluated in the tilapia fish study.

The hormonal imbalance observed in the adult male zebrafish was in line with the observations from the H295R cells, except for an inconsistent T level by the lower experimental level exposure to 1-NPA. Exposure to 0.015 or 0.14 mg/L 1-NPA caused up-regulation of *star* gene (Fig. 4A), and increase of T (Fig. 3A). However, at the highest experimental concentration, i.e., 3.6 mg/L, *3β-hsd* and *cyp17* genes were down-regulated. The product of *3β-hsd* is essential for the conversion of 17α–OH–pregnenolone to 17α–OH–progesterone which in turn converts to androstenedione by *cyp17*. Therefore, the down-regulation of *3β-hsd* and *cyp17* genes possibly leads to reduced sex hormone production. The up-regulation of *star* gene expression at the low experimental level can explain the T level increase by 1-NPA. Even though the extent of change was the least among the three tested aniline derivatives, 1-NPA exposure also led to increased E2/T ratio (Fig. 3C). While it is clear that 1-NPA disrupts sex hormone balance in both cell line and the fish, the mechanisms of action seem to be different between the human adrenal cells and the adult male zebrafish. The aromatase gene, *CYP19A*, was significantly up-regulated in the H295R cell while no changes were observed in the male zebrafish, upon exposure to 1-NPA. The regulation of steroidogenic enzymes in the cell lines can be different from that in the fish, therefore leading to different responses (Baker, 2001; Villeneuve et al., 2007). In addition, sex related difference between the male fish and the adrenal cell line originated from a female human, i.e., H295R cell, may in part explain the observed difference in steroidogenic enzyme regulation.

Our observations showed that several anilines such as 3,4-DCA, 1-NPA, and 4,4′-MDA can disrupt hormone balances in male zebrafish, possibly through alteration of steroidogenic pathway. However, this study has several limitations. First, only T was measured in the fish plasma. While T is an important precursor of active androgen in fish, 11-ketotestosterone (11-KT) is considered as a better indicator of male sex hormone in fish (Schulz et al., 2010). Secondly, we tested only male zebrafish in the present study, therefore, potential effects on female fish are remained to be investigated in the future. In addition, we measured only several major genes in the steroidogenic pathway, therefore other modes of action that may lead to sex hormone disruption could not be tested. Finally, the experimental concentrations which are orders of magnitude higher than those detected in the environment should be noted. Therefore, further studies employing environmentally relevant concentrations with long-term exposure design are warranted to understand ecological consequences of aniline compounds in the water.

In conclusion, our observations in both H295R cells and adult male zebrafish clearly demonstrate that the three tested aniline derivatives have a potential to disrupt sex hormone balance, i.e., decrease of T, possibly through alteration of some steroidogenic genes. Long-term consequences of this endocrine disruption, including reproduction, warrant further investigations.

Conflicts of interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.05.003.

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