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Impairment of reproduction of adult zebrafish (*Danio rerio*) by binary mixtures of environmentally relevant concentrations of triclocarban and inorganic mercury

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ABSTRACT

Effects of chemical mixtures at environmentally relevant concentrations on endocrine systems of aquatic organisms are of concern. Triclocarban (TCC) and inorganic mercury (Hg^{2+}) are ubiquitous in aquatic environments, and are known to interfere with endocrine pathways via different mechanisms of toxic action. However, effects of mixtures of the two pollutants on aquatic organisms and associated molecular mechanisms were unknown. This study examined effects of binary mixtures of TCC and Hg^{2+} on histopathological and biochemical alteration of reproductive organs in zebrafish (*Danio rerio*) after 21 d exposure. The results showed that: 1) At concentrations studied, TCC alone caused little effect on hepatic tissues, but it aggravated lesions in liver caused by Hg^{2+} via indirect mechanisms of disturbing homeostasis and altering concentrations of hormones; 2) Histological lesions were more severe in gonads of individuals, especially males, exposed to the binary mixture. Exposure to TCC alone (2.5 or 5 μg/L) (measured concentration 140 or 310 ng/L) or Hg^{2+} alone (5 μg/L or 10 μg/L (measured concentration 367 or 557 ng/L) slightly retarded development of oocytes, whereas co-exposure to nominal concentrations of 5 μg/L TCC and 10 μg /L Hg^{2+} promoted maturation of oocytes. In males, maturation of sperm was slightly delayed by exposure to either TCC or Hg^{2+}, while their combinations caused testes to be smaller and sperm to be fewer compared with fish exposed to either of the contaminants individually; 3) Lesions observed in fish exposed to binary mixtures might be due to altered transcription of genes involved in steroidogenesis, such as cyp19a, 3beta-HSD, cyp17, 17beta-HSD and modulated concentrations of testosterone and estradiol in blood plasma. The observed results further support the complexity of toxic responses of fish exposed to lesser concentrations of binary chemical mixtures. Since it is impossible to collect empirical information in controlled studies of all possible combinations of toxicants, the application of omics methods might improve the predictive capabilities of results of single classes of chemicals.

1. Introduction

Mixtures of pollutants at environmentally relevant concentrations are of concern due to their potential to adversely affect humans and wildlife. While most information on chemicals is derived from controlled laboratory studies with individual chemicals, wildlife and human are exposed to mixtures in various compartments of the environment or in food or water. Obtaining realistic information on effects of chronic exposures to small concentrations of mixtures is currently the newest frontier in environmental toxicology, especially for chemicals classified as health and beauty or personal care products that are expected to be released into the environment in combinations (Brausch and Rand, 2011; Brack, 2015). Pollutants from consumer products are pseudo-persistent in the environment because of their continual release, and wildlife or humans are exposed to fairly constant concentrations for long periods of time. Exposure can be via direct contact or indirect exposure. For example exposure to pollution through the diet or for infants via the umbilical cord or milk. Combinations of small concentrations of micropollutants might cause significant toxicity, even when exposure to each chemical alone causes no observable adverse effects. Among mixtures of

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micropollutants, those that are endocrine disruptors (EDs) are important because the neuro-endocrine systems of wildlife and humans maintain homeostasis, which is what maintains integrity of the organism to survive in its ambient environment or to facilitate growth and / or reproduction. Results of some studies have shown that mixtures of chemicals with the same molecular mechanism, such as endocrine disrupting chemicals that act through the same nuclear receptors (e.g., estrogenic, anti-androgenic agents), can be predicted by use of the dose (concentration) addition approach (Kortenkamp, 2007). While theoretically it is possible to predict effects of mixtures composed of chemicals with different modes of action by use of the independent action model, this model is not appropriate if there are interactions between the two toxicants such that their effects are not entirely independent (Chung et al., 2011). Recently, approaches based on molecular biology have revolutionized what is possible in biology. While this has brought about a paradigm shift in environmental toxicology, it is still difficult to predict effects on apical endpoints such as survival, growth and reproduction (Brack, 2015). Once chemicals are assigned to a particular adverse outcome pathway, based on their critical mechanism of action, it might be possible, by understanding the various molecular systems involved in each pathway to determine where they might be overlaps or "cross-talk" between the various pathways (Nie, 2001; Liu et al., 2011).

Triclocarban (TCC) and Hg^{2+} are often found together in personal care products, through their extensive use in skin lightening soaps, creams and other cosmetics (Bocca et al., 2014). With widespread application of these personal care products they are ubiquitous in tissues of people and the environment. For instance, mean concentrations of Hg in blood and urine of 286 people using some of these personal care products were 45.2 and 17.1 μg/L, respectively (Sin and Tsang, 2003). The mean concentration of Hg was 3.08 μg/L in blood of residents of Korea, United States (0.94 μg/L), Germany (0.58 μg/L), and Canada (0.69 μg/L) (Kim et al., 2015). TCC and its metabolites were also detected in urine and blood (Pycke et al., 2014; Schebb et al., 2011a, 2011b). As environmentally pervasive chemicals, TCC and Hg^{2+} have occurred simultaneously in aquatic environments at concentrations as great as 6.8 μg /L (Halden and Paull, 2004, 2005; Zhao et al., 2011), such as the Yangtze River, China (Liu et al., 2015; Zhu et al., 2015) and Savannah River, Georgia, USA (Hinck et al., 2008; Kumar et al., 2010). Inorganic mercury is a siderophilic (soft electron acceptor) metal found ubiquitously in sediment and water (Crump and Trudeau, 2009). Concentrations of Hg in water of 20 reservoirs in Maryland ranged from 1 to 50 ng/L (Sveinsdottir and Mason, 2005). Even in global oceans, concentrations of dissolved Hg are in the range of 0.02–0.5 ng L^{-1} (Douglas et al., 2012). TCC and inorganic Hg^{2+} are both known to interfere with various endocrine pathways via different mechanisms of action, and Hg^{2+} has been shown to impair reproductive function and induce lesions in reproductive organs of zebrafish (Crump and Trudeau, 2009; Baldigo et al., 2006; Beckvar et al., 2005.).

Although TCC itself exhibited little or no potency as an agonist for the androgen receptor (AR) or estrogen receptor (ER), it could amplify the action of estradiol and testosterone in vitro or in vivo (Christen et al., 2010; Chung et al., 2011; Duleba et al., 2011; Huang et al., 2014; Tarnow et al., 2013). Although co-exposure to TCC and Hg^{2+} was likely for human and aquatic organisms, their joint effects on the endocrine system were ambiguous. To predict effects on functions of the endocrine system this study investigated whether exposure to environmentally relevant concentrations (μg/L) of TCC in combination with Hg^{2+} would cause histopathological effects on gonads and altered somatic indices for gonads (GSI) and livers (HSI)) as well as molecular responses in zebrafish. Steroidogenic genes including 3beta-HSD, 17beta-HSD, and CYP17 enzymes which participate in synthesis of testosterone, and aromatase CYP19a as a critical enzyme that converts testosterone into estradiol along the HPG axis and liver vitellogenin (vtg1) as well as concentrations of estradiol (E_2) and testosterone (T) were chosen to examine the molecular mechanism of toxicity caused by joint exposure of TCC and Hg^{2+}.

2. Materials and methods

2.1. Test organisms and exposure experiment design

Sexually mature zebrafish (AB-type, 3–4 months old) were obtained from the Institute of Hydrobiology, Chinese academy of sciences (Wuhan) and were acclimated in dechlorinated tap water with a temperature of 25 ± 1 °C, pH ranging from 7.4 to 8.1, 16:8 h light/dark cycle for 2 weeks in laboratory before tests (OECD, 2009).

To avoid the normal reproduction of female with male which could interfere the gonad histopathology (spermatogenesis and growth of follicles), 10 male of mean, wet mass (wm) 0.21 g, or female (0.58 g, wm) zebrafish were exposed separately in 3 L dechlorinated tap water (temperature of 25 ± 1 °C, pH ranging from 7.4 to 8.1, 16:8 h light/dark cycle) containing various concentrations of Hg^{2+} (Sigma-Fluka, Hg^{2+} standard stock solution for ICP, 1000 ± 2 mg/L, 12% HNO_3) and/or TCC (Sigma-Aldrich, > 99.0%) for 21 d in glass jars with three replicates, specific exposure concentrations are shown (Table 1.) Dimethyl sulfoxide (DMSO) was used as solvent for TCC with a final concentration of 0.5‰ v/v in all the treatment groups and vehicle control. During exposure, fish were fed commercial pellet food (INCH-GOLD, China) at 3% of body mass once daily. Jars were aerated to saturation and 50% solutions were renewed daily. pH was maintained at 7.4 ± 0.3 during the period of exposure. Before termination of the exposure, fish were fasted for 24 h and then euthanized by keeping them on ice. Body mass and length (snout to vent) were measured. Briefly, blood was collected from the caudal vein in a glass capillary tube treated with sodium heparin. Gonads were separated from liver, mass determined and subsequently stored in −80 °C until further analysis. Body mass and length were used to evaluate phenotypic effects by calculating the condition factor (k) (Eq. (1)). No mortality occurred during the exposure. Gonad-somatic (GSI) and hepatic-somatic (HSI) indices were calculated (Eqs. (2) and (3)).

$$k = 100 \times \left(\text{body weight}/\text{length}^3\right), \quad (1)$$

gonad-somatic index (GSI) (Eq. (2)).

$$\text{GSI} = 100 \times (\text{gonad weight}/\text{body weight}), \quad (2)$$

and hepatic (liver)-somatic index (HIS) (Eq. (3)).

$$\text{HSI} = 100 \times (\text{liver mass}/\text{body mass}) \quad (3)$$

2.2. Exposure concentration monitoring

Once a week through the exposure period, concentrations of Hg^{2+} and TCC in each exposure group were measured by use of a DMA-80 direct Hg analyzer (Milestone, Italy). The method detection limit (MDL) for Hg is 0.005 ng) (QC:95–105%) (Ma et al., 2015) when the SPE-IE-HPLC-MS method was used (see Section 2) immediately before half-renewal of exposure solutions.

2.3. Histological analysis

After 21 d exposure, 6 female and 6 male zebrafish were randomly collected. After quickly collecting blood from the caudal vein, gonads and liver were carefully isolated, and then quickly

Table 1
Mean concentrations of inorganic mercury (Hg^{2+}) and/or triclocarban (TCC) in solution during exposures.

Test chemical	ID	Nominal concentration (μg / L)	Measured concentration (μg / L)		geometric mean concentration[a] (μg / L)	
			Male	Female	Male	Female
TCC	CK	Control	ND	ND	ND	ND
	2.5TCC	2.5	0.14 ± 0.00	0.088 ± 0.001	0.43	0.34
	2.5TCC+5H	2.5	0.14 ± 0.01	0.097 ± 0.012	0.44	0.36
	5T	5.0	0.32 ± 0.01	0.17 ± 0.04	0.92	0.66
	5TCC+10H	5.0	0.31 ± 0.01	0.16 ± 0.05	0.91	0.65
Hg^{2+}	CK	Control	ND	ND	ND	ND
	5H	5.0	0.37 ± 0.02	0.39 ± 0.01	0.99	1.0
	2.5TCC+5H	5.0	0.34 ± 0.01	0.39 ± 0.01	0.95	1.0
	10H	10	0.56 ± 0.02	0.61 ± 0.02	1.7	1.8
	5TCC+10H	10	0.56 ± 0.03	0.60 ± 0.02	1.7	1.8

Measured concentrations: concentrations before renewal of the water once a week. Mean concentrations were calculated for three groups of water samples (every group contained three parallels) collected during the adult fish exposure experiment (Once a week).
ND: not detected. Values are mean ± standard deviation of the 6 samples (three replicate samples collected from every week)
Note: CK, 2.5TCC, 5TCC, 5H, 10H, 2.5TCC+5H, 5TCC+10H to replace vehicle control, 2.5 μg / L, 5 μg / L TCC; 5 μg / L, 10 μg / L Hg^{2+}; 2.5 μg / L Hg^{2+} co-exposed with 5 μg / L TCC and 5 μg / L Hg^{2+} co-exposed with 10 μg / L TCC in order to avoid complex expression.
[a] geometric mean concentration: geometric mean calculated from the monitored concentration both before and after the renewal.

fixed with 10% formalin for more than 24 h. Subsequently samples were embedded in paraffin, sectioned (4 μm thick) and stained with hematoxylin-eosin. Histologic lesions were observed by optical microscope and digital pictures taken through the microscope. Stages of maturity of oocytes were classified as: (1) previtellogenic oocytes, (2) vitellogenic oocytes, or (3) post-ovulatory follicles. Numbers of normal and affected oocytes (e.g. atretic follicles) were enumerated. Quantitative staging of ovaries was based on relative percentages of oocytes/follicles at different stages of development by counting their numbers based on previously described methods (Wang et al., 2015).

2.4. Quantification of hormones

Samples of blood (three replicates, one replicate contained 10 male or female fish) from the caudal vein were immediately centrifuged at 3000*g* for 10 min, supernatant (plasma) was collected and stored at −80 °C for use in subsequent quantification of hormones. Plasma was diluted 10–50 fold with ELISA buffer, and an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to determine concentrations of estradiol (E_2) (Cat No. 582251) and testosterone (T) (Cat No. 582701) according to the manufacturer's recommendations.

2.5. RNA extraction and Real-time quantitative PCR

RNA was extracted from liver and gonad. Total RNA of each liver or gonad from 8 female or 8 male zebrafish was collected by use of the trizol method of Overturf et al. (2014). Quantity and quality of RNA were measured by use of a Nanodrop 1000 Spectrophotometer. The ratio between the absorbances at 260 nm and 280 nm was used to evaluate purity of RNA, which was between 1.8 and 2.0. Complementary DNAs (cDNAs) were synthesized from RNA by use of the RevertAid First Strand cDNA Synthesis Kit, according to the manual (Thermo scientific, America). Subsequently, RT-qPCR assay was performed in triplicate with selected gene-specific primers (Table S1) and Maxima SYBR Green/ROX qPCR Master Mix (Company info of qPCR kit). Forty amplification cycles were completed by use of a real-time PCR instrument (ABI, USA) with the following temperatures and durations: 95 °C for 15 s, 58 °C for 30 s, 72 °C for 45 s. Specificities of products of PCR were verified by dissociation curves and the Ct value of each gene obtained through analyses of amplification curves. Expression of each gene was analyzed with $2^{-\triangle\triangle ct}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analyses

Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levene's test conducted with the R language (P-value >0.05). Differences between groups were evaluated with one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test (95% confidence interval) by SPSS® (version 18.0; SPSS Inc., Chicago, IL). Differences between groups were considered as statistically significant if $p<0.05$.

3. Results

3.1. Concentrations in exposure media

Measured concentrations of TCC and Hg^{2+} alone or in combination were always less than nominal concentrations, but there was no significant difference in actual concentrations between single and co-exposures. For exposures of males, mean measured concentrations of TCC were 1.4×10^2 and 3.2×10^2 ng/L (single exposures), and 1.4×10^2 and 3.1×10^2 ng/L (mixtures), respectively. Mean concentrations of Hg^{2+} were 3.7×10^2 and 5.6×10^2 ng/L (single exposures), $3.4\times10^2$and 5.6×10^2 ng/L (mixtures), respectively (Table 1). Measured concentrations of TCC and Hg^{2+} for either single or joint exposures accounted for approximately 5–6% of nominal concentrations. Corresponding Geometric mean concentrations of TCC and Hg^{2+} before and after renewals were 4.3×10^2 and 9.2×10^2 ng/L (TCC, individual chemical exposures), and 4.4×10^2 and 9.1×10^2 ng/L (TCC, mixtures), 9.9×10^2 and 1.7×10^3 ng/L (Hg^{2+}, individual chemical exposures), 9.5×10^2 and 1.7×10^3 ng/L (Hg^{2+}, mixtures). (Table 1) For females, measured concentrations of Hg^{2+} decreased similarly to what was observed for males. Mean concentrations of TCC were 88 and 1.7×10^2 ng/L (single exposures), and 97 and 1.6×10^2 ng/L (mixtures), respectively. Mean concentrations of Hg^{2+} were 3.9×10^2 and 6.1×10^2 ng/L (single exposures), 3.9×10^2 and 6.0×10^2 ng/L (mixtures), respectively (Table 1). Geometric mean concentrations of TCC in females were 3.4×10^2 and 6.6×10^2 ng/L (individual chemical exposures), 3.6×10^2 and 6.5×10^2 ng/L (mixtures). Geometric mean concentrations of Hg^{2+} were 1.0 and

Table 2
Morpho-anatomical indices after exposure of zebrafish to inorganic mercury (Hg^{2+}) and/or triclocarban (TCC).

Zebrafish	Male						
	CK	2.5TCC	5H	2.5TCC+5H	5TCC	10H	5TCC+10H
K-factor (%)	1.2 ± 0.2	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
HSI (%)	0.21^{a} ± 0.02	0.18^{a} ± 0.04	0.16^{a} ± 0.06	0.21^{a} ± 0.05	0.23^{ab} ± 0.03	0.17^{a} ± 0.02	0.29^{b} ± 0.04
GSI (%)	0.54^{a} ± 0.15	0.44^{a} ± 0.10	0.36^{a} ± 0.06	0.28^{b} ± 0.02	0.31^{a} ± 0.07	0.29^{a} ± 0.06	0.13^{c} ± 0.02
	Female						
	CK	2.5TCC	5H	2.5TCC+5H	5TCC	10H	5TCC+10H
K-factor (%)	1.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.7 ± 0.3	1.5 ± 0.3	1.6 ± 0.2
HSI (%)	2.7^{ab} ± 1.1	2.2^{a} ± 0.2	1.7^{b} ± 0.0	1.5^{b} ± 0.2	2.4^{a} ± 0.2	1.3^{b} ± 0.3	1.8^{ab} ± 0.3
GSI (%)	14^{ab} ± 1	9.1^{ab} ± 3.1	13^{ab} ± 3	14^{ab} ± 4	8.0^{b} ± 0.7	10^{ab} ± 3	15^{a} ± 1

Data are expressed as mean ± SEM of three replicate samples (each replicate contained 8 fish); Superscript letters represented significant difference.

1.8 ng/L (individual exposures) 1.0 and 1.8 ng/L (mixtures) (Table 1).

3.2. Health index of experimental animals and hepatic toxicity

At the end of 21 d exposure, phenotypic changes (*k*-factor) were not significantly different between controls and any of the treatments for either male or female zebrafish (Table 2). There was a small decrease in HSI of zebrafish exposed to TCC or/and Hg^{2+} with no statistically significant differences in measures of adverse effects on liver (Table 2, Fig. 1) except 5TCC+10H group which caused a significant increase of HSI of male fish compared with that of the control and 10H group (Table 2).

Severe lesions were observed in both male and female fish exposed to Hg^{2+}. These effects were on cell cavitation, nuclear pyknosis, and loss of cytoplasmic inclusions (Fig. 1. C, E–G). The most serious lesion observed was enlarged cells and cavitation caused by exposure to 10 μg Hg^{2+}/L. Exposure to TCC did not cause changes on tissues of males or females (Fig. 1. B, D). Similar damage to liver was observed in fish exposed to the binary mixture or Hg^{2+} alone.

3.3. Histopathology of gonads

In the vehicle control, lumens of seminiferous tubules were populated with mature spermatozoa (Fig. 1.H). In males exposed to TCC or Hg^{2+} only, slightly retarded spermatogenesis was observed with few sperm being present. Larger numbers of spermatocytes were observed under environmentally-relevant concentrations (Fig. 1. I, J, L, M). Severe lesions of testes with enlarged areas of cavities in seminiferous tubules accompanied with apoptosis of germ cells were observed in fish exposed to binary mixtures, especially in fish exposed to the greatest mixture group (Fig. 1. K, N). Not only apoptosis of germ cells but significantly lesser GSI was observed after joint exposure to Hg^{2+} and TCC (Table 2), especially in fish exposed to 5TCC+10H. However, the binary mixture promoted maturation of oocytes instead of delayed development as was caused in females by exposure to Hg^{2+} or TCC alone. Ovaries of the solvent controls were filled with oocytes at all stages of maturation, including previtellogenic oocytes, vitellogenic oocytes, postvitellogenic oocytes, oogonia (Fig. 2. A). Minor lesions such as atretic oocytes, cytoplasm and membrane separation and deformation of membranes were observed in zebrafish exposed to 2.5TCC+5H, 5TCC, 10H or 5TCC+10H (Fig. 2. D, F, G). Growth of follicles was significantly affected in individuals exposed to 5 μg/L TCC or 10 μg/L Hg^{2+} or their combined treatment. Significantly greater percentages of pre-vitellogenic oocytes were observed in individuals exposed to 5TCC and 10H treatment with decreased post-vitellogenic oocytes percentages of the same groups (Fig. 2. H). In contrast, exposure to 5TCC+10H shifted pre-vitellogenic oocytes towards vitellogenic and post-vitellogenic oocytes. The different percentages of stages of oocytes could affect masses of gonads, but there were no statistically significant differences in GSI of females, inverse relationships between concentrations of both declining trend was still observed except a slight increase in individuals exposed to 5TCC+10H group, which might have been caused by development of follicles.

3.4. Concentrations of hormones

Concentrations of T in blood plasma were 1.5- to 2-fold less in males except those exposed to 5TCC+10H (Fig. 3). Meanwhile, a similar tendency of lesser concentrations of E_2 in male fish was observed compared to vehicle control except the greater concentration binary mixture group which resulted in greater concentrations (Fig. 3). In females, concentrations of T in 2.5 TCC, 5H, 2.5 TCC+5H groups were significantly less than those in control individuals. After exposure to 5 μg Hg^{2+}/L, 5 μg TCC/L or 10 μg / L Hg^{2+} treatment, concentrations of E_2 in blood plasma were 2.28-fold, 2.27-fold and 1.82-fold, less than that of control, female fish, respectively (Fig.4). Significantly greater concentrations of E_2 were observed in fish exposed to the greater concentration, binary mixture.

3.5. Expression of mRNA of genes

Expressions of mRNA of 3β-HSD, cyp17, 17-β-HSD and cyp19a in testis of fish exposed to either Hg^{2+} or TCC were less compared to those of controls. Down-regulation was more significant in individuals exposed to greater concentrations. Expressions of mRNA of cyp17 in individuals exposed to 5H, 2.5 TCC+5H, 5 TCC or 10H. Expression of mRNA of Cyp19a in males exposed to 2.5 TCC+5H, 5 TCC or 10H were down-regulated by more than 5-fold compared to expressions of these genes in unexposed male (Fig. 3. A) However, exposure to the greater concentration binary mixture (5TCC+10H) resulted in slight upregulation of mRNA of cyp17 and cyp19a in male fish. (Fig. 3. A) Also there were recovery of mRNAs of 3β-HSD, 17-β-HSD of individuals exposed to 5TCC+10H compared with the corresponding single exposure. Expression of mRNA for Vtg1 was not altered in males exposed to 10H, but slightly up-regulated by 1.70-fold in livers of males exposed to 5TCC. However, expression of Vtg1 was 8.4-fold greater in livers of

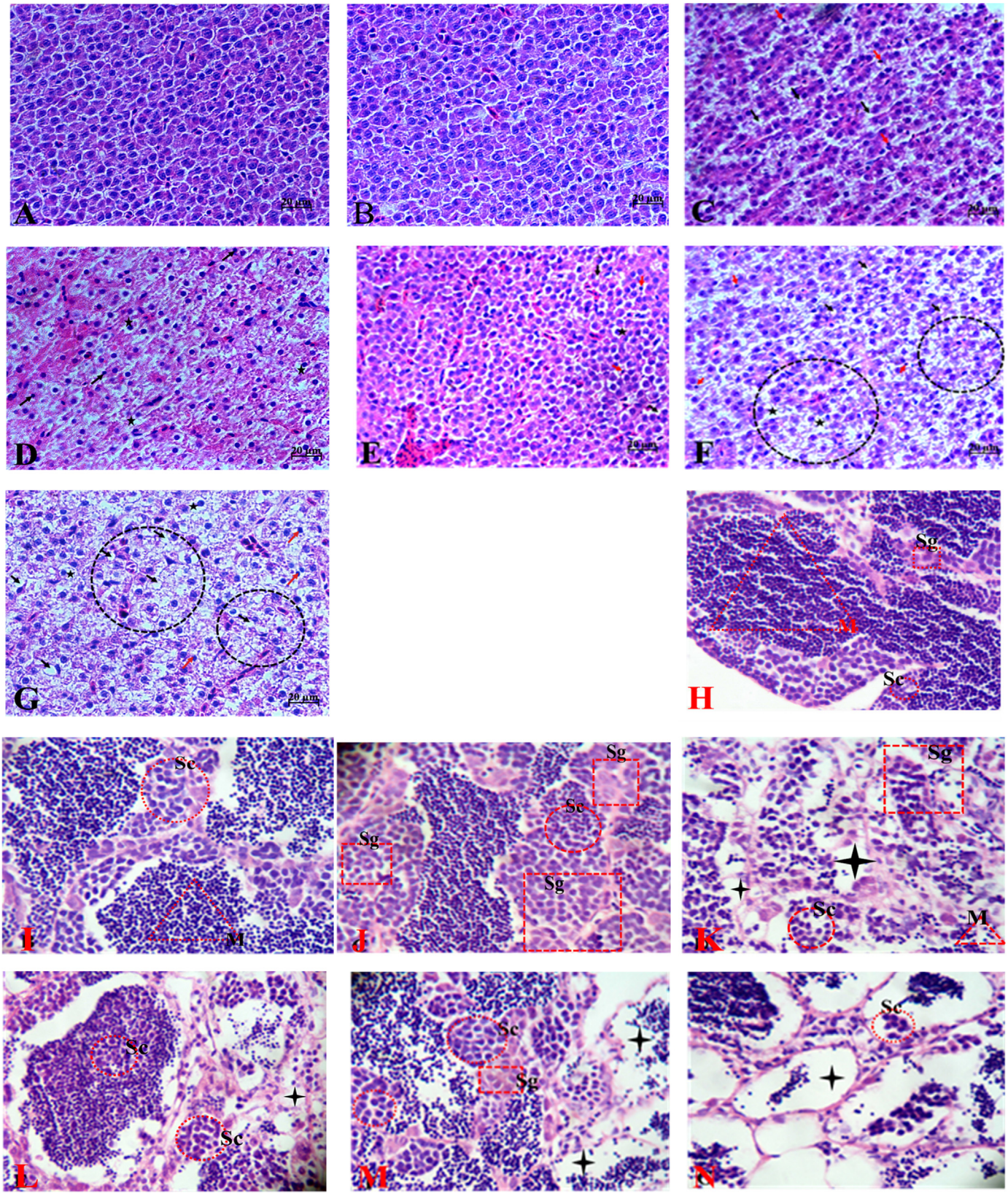

Fig. 1. Representative photomicrographs demonstrating lesions in livers (A–G) and testis of male zebrafish (H–N). A, H: control; B, I: 2.5 μg/L TCC group; C, J: 5 μg/L Hg^{2+}; D, K: 2.5 μg/L TCC +5 μg/L Hg^{2+}; E, L: 5 μg/L TCC; F, M: 10 μg/L Hg^{2+}; G, N: 5 μg/L TCC +10 μg/L Hg^{2+}. M: mature sperms, Sc: spermatocyte, Sg: spermatogonia (✦) seminiferous tubule cavity. Lesions in livers (A–G): formation of cavaties (➘), pycnosis of nucleus ➘, loss of cytoplasmic inclusions (★).

males exposed to 5TCC+10H (Fig. 3. A) than that of unexposed individuals.

In females exposure to lesser concentrations of Hg^{2+} or TCC or their binary mixtures, resulted in down-regulation of expression of mRNA for 17-β-HSD. There was no significant difference in expression of mRNA for 17-β-HSD between individuals exposed

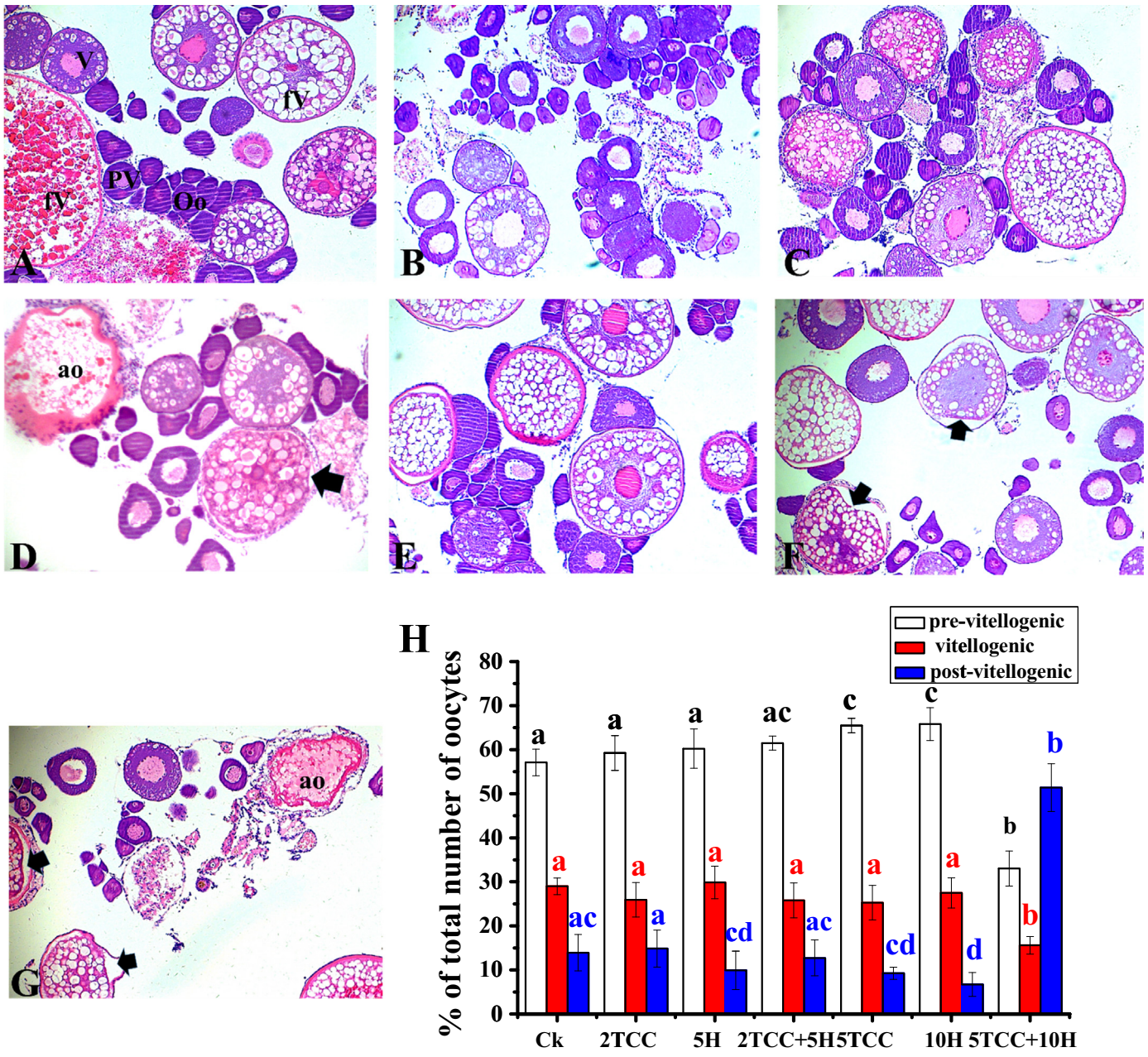

Fig. 2. Photomicrographs of H&E stained sections of zebrafish ovaries (A–G, 10 ×). A: control; B: 2.5 μg/L TCC; C: 5 μg/L Hg^{2+}; D: 2.5 μg/L TCC combined 5 μg/L Hg^{2+}; E: 5 μg/L TCC; F: 10 μg/L Hg^{2+}; G: 5 μg/L TCC co-exposed 10 μg/L Hg^{2+}. PV: pre-vitellogenic oocytes, V: vitellogenic oocytes, fv: post-vitellogenic oocytes, Oo: oogonia; ao: atretic oocytes, Cytoplasm and membrane separation and membrane deformation (➡); H: Proportion of oocytes in different stage after 21 d exposure. (n=6, Different superscript letter indicates statistically significant difference).

singly to 5 μg/L TCC or 10 μg/L Hg^{2+} or their binary mixtures. Significant down-regulation of cyp17 was observed in individuals exposed to all treatments with Hg^{2+} or/and TCC, especially in the 2.5TCC, 5H, 2.5TCC+5H, 5TCC groups. (Fig. 4. A) Up-regulations of cyp19a mRNA were only observed in individuals exposed to 5TCC+10H. Expression of mRNA for Vtg1 was down-regulated by 2.4 and 2.2-fold, respectively, in livers of individuals exposed to 5H or 10H, while 2.2-fold up-regulation was observed in livers of individuals exposed to 5TCC+10H (Fig. 4. A).

4. Discussion

The cause for measured concentrations of TCC and Hg^{2+} in individual or joint exposures being less during the exposures than nominal concentrations was likely due to adsorption by surfaces of devices, especially of Hg^{2+}. Lesser concentrations of TCC in solution were attributed to both adsorption to surfaces of devices (approximately 35%) and uptake and adsorption by zebrafish (Supporting information, Fig. S1) (Schebb et al., 2011a, 2011b). Measured concentrations of TCC that were less in water in which females were exposed than those in solutions in which males were exposed could be explained by the difference between mean masses of males (0.207 ± 0.023 g) and females (0.584 ± 0.03 g). These results highlighted the importance to measure the actual exposure concentration during toxicity test because the actual concentration of chemical is important for assessing its risk with its corresponding toxicological responses.

The larger area of cavities in hepatic tissue after exposure to binary mixtures was mainly attributed to effects of Hg^{2+}, which is

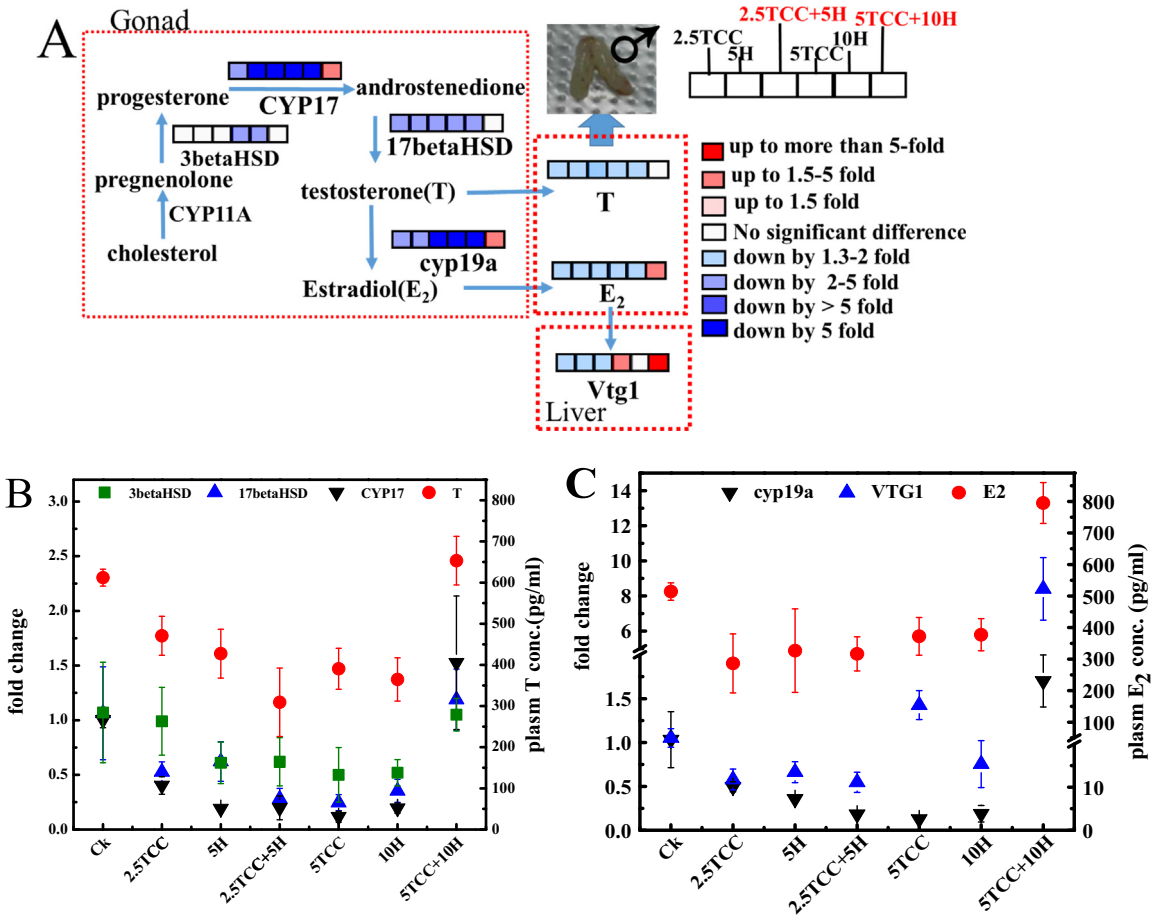

Fig. 3. Altered HPG axis by binary chemical exposure in male zebrafish. (A) Steroidogenic pathway in male zebrafish. Differential gene expression and plasma steroid concentration are shown in different colors. Colors correspond to significance of fold changes. The group with no significant difference compared with control is shown in white. (B) Fold changes in concentrations of testosterone in blood plasma and relevant expressions of mRNA; (C) Fold changes in expression of mRNA for VTG1 and CYP19A and concentrations of 17β-estradiol in blood plasma. Values are presented as mean ± SD.

consistent with results of previous studies (De Oliveira Ribeiro et al., 2002; Ung, 2010; Giari et al., 2008). Similar degree of hepatic tissue damage between Hg^{2+} and binary mixture groups suggested that larger areas of cavities in hepatic tissue after binary mixture treatment was mainly attributed to the Hg^{2+} exposure for zebrafish. Although lesions in liver were caused by exposure to Hg^{2+} and the binary mixture, greater HSI was observed in the 5TCC+10H group compared to the 10H group might be attributed to greater concentrations of estradiol in the 5TCC+10H group which might stimulate the transcript and protein expression of vitellogenin leading to an enlargement of the liver consequently (Fig. 3) (Mandiki et al., 2004). All suggested that TCC could potentiate the liver lesions caused by Hg^{2+} via indirect mechanism of disturbing hormone homeostasis. Given the central role of liver in chemical bio-transforming and cycling, as well as in blood constituent biosynthesis, the binary mixture of TCC and Hg^{2+} could affect whole body physiology of fish.

The delayed spermatogenesis and follicular development retarded by Hg^{2+} or/and TCC might be hormone-dependent. And the alteration of GSI might be related to the changes of hormone level and histopathology of the treated fish. Apparently, retarded phenomenon was more severe along with low T levels. It was postulated that decreased T level might be partly responsible for degraded spermatogenesis in the present study. This hypothesis was also verified by previous research. Dziewulska and Domagala (2003) and Gomez et al. (1999), reported that spermatogenesis in which stem cells divided into mature spermatozoa were hormone-dependent (androgen, follicle-Stimulating hormone, luteinizing hormone) processes. Any removal of them can induce germ cell apoptosis (Sofikitis et al., 2008). Lesser numbers of sperm cells males exposed to 5TCC+10H might have been attributable to significant enhancement of E_2 (Fig. 3) which even at lesser concentrations ($10^{(-9)}$ and $10^{(-10)}$ mol/L) could induce apoptosis of all germ cells through mimicking a gradual withdrawal of gonadotrophin (BlancoRodriguez and MartinezGarcia, 1996; Pentikainen et al., 2000). In brief, Hg^{2+} or/and TCC disturbed steroid homeostasis and further triggered apoptosis of germ cells, spermatogenesis delay, and lesser GSI. In females, changes in lesions in ovaries between single exposure of high concentration TCC and Hg^{2+} than that for the mixture seemed consistent with the patterns of greater concentrations of E_2 and upregulation of expression of VTG mRNA, which might provide partial interpretation. VTG was essential for vitellogenesis, maturation of oocytes and biosynthesis of yolk (Cardenas et al., 2003; Nagahama and Yamashita, 2008). These results indicated that development of follicles was affected by exposure to TCC or Hg^{2+} individually or their binary mixture through disturbing E_2 and VTG synthesis. Although exposure of to greater concentrations of binary mixtures increased concentrations of E_2 in blood plasma of both females and males, the greater concentration of E_2 stimulated maturation of oocytes, but also resulted in apoptosis of germ cells.

Apoptosis of germ cells following exposure to the greater concentration binary mixture could cause adverse effects on reproductive fitness of fish. Defects in development of follicles might result in lesser production of eggs as well as rates of fertilization and hatching (Zhao et al., 2015a, 2015b). Because concentrations

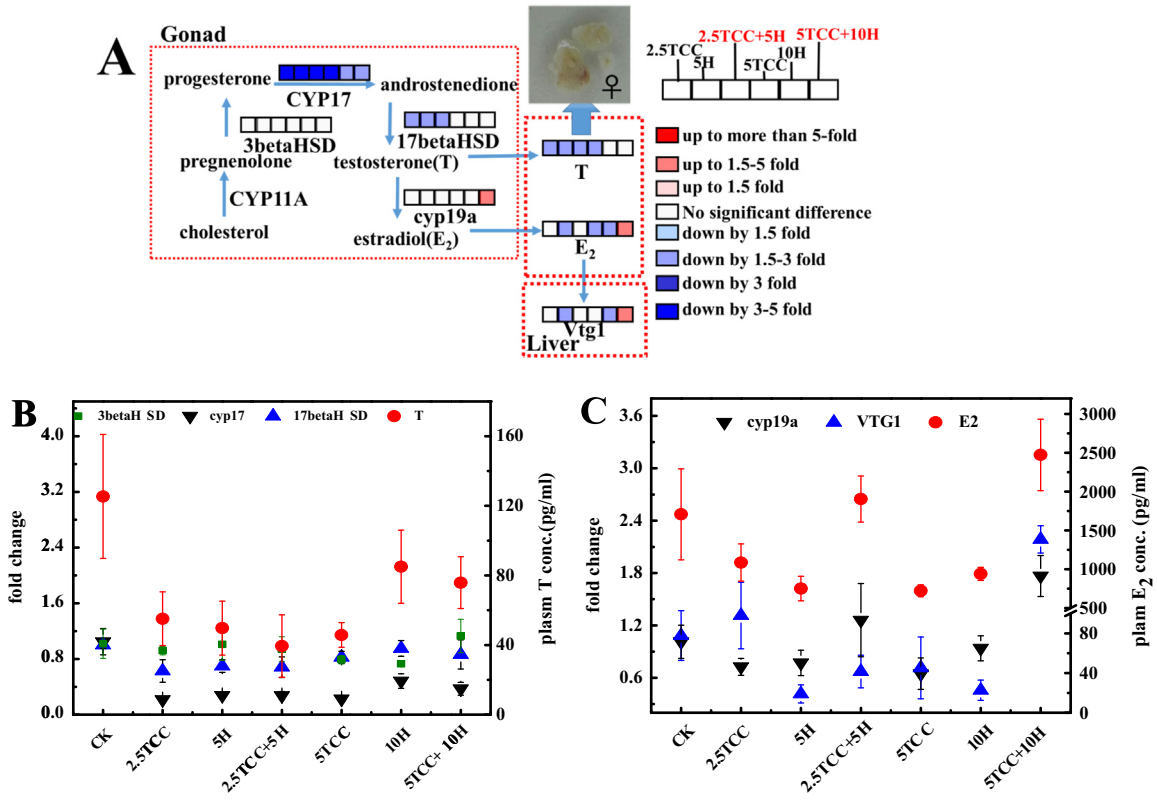

Fig. 4. Altered HPG axis by binary chemical exposure in female fish. (A) Transcriptional expression of steroidogenic genes and steroid concentration in female fish. Different color-filled squares mean significant difference among exposure groups. The filled white color means no significant difference with control; (B) Fold changes of plasma testosterone concentration and the relevant mRNA expressions; (C) Fold changes of VTG1 and CYP19A mRNA expression, and concentrations of 17β-estradiol in blood plasma. Values were presented as mean ± SD.

used in the current study are environmentally relevant, observed lesions of gonads in both males and females caused by exposure to the binary mixture might affect reproduction (egg production, fertilization) and could be used as an indicator of possible TCC and/or Hg^{2+} exposure.

The potential mechanism impairment of reproduction by TCC, Hg^{2+} and their binary mixture might be through modulation of expressions of steroidogenic genes and disturbance of synthesis of steroids, which further effected development of follicles. In fish gonads, along the HPG axis (Fig. 3 A, Fig. 4A), transformation of pregnenolone to testosterone is catalyzed by the enzyme, 3beta-HSD, cyp17, 17beta-HSD. Aromatase (cyp19a) is a critical enzyme which converts Testosterone to 17beta-estradiol of fish. The similar trends of steroid and gene transcription change that Positive correlation among testosterone levels and the expressions of transcription of hormone regulating genes and between E_2 and cyp19a and the tendency of Vtg1 expression was almost consistent with estradiol content trend except for the TCC treatment suggested that TCC/Hg^{2+} single and binary mixture might disrupt the balance of steroid hormones by disturbing the steroidogenesis relevant mRNA expression in gonad.

5. Conclusions

Results of the study reported here demonstrated that exposure to the binary mixture of 310 ng/L (male) or 161 ng/L (female) TCC with 580 ng/L Hg^{2+} caused frank lesions in gonad tissues, particularly in testes, which were smaller and contained fewer spermatozoa. These serious lesions might be attributable to disturbances of endocrine hormones and transcription of steroidogenesis-related genes. The proposed molecular mechanism of for effects of the mixture of Hg^{2+} and TCC on reproductive performance of zebrafish provided a deeper understanding the joint mode of action of the Hg^{2+} and TCC mixture.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.08.026.

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Support information

Impairment of Reproduction of Adult zebrafish (*Danio rerio*) by Binary Mixtures of Environmentally Relevant Concentrations of Triclocarban and Inorganic Mercury

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Materials and Methods

Measured concentrations

Once a week through the exposure period, 1 L exposure solution was collected from each treatment group for TCC detection, then passed through a solid-phase extraction LC-C18 (500 mg, 6 ml, Agilent) and eluted with 4 ml methanol (HPLC reagent). Eluates were dried, reconstituted with 1 ml methanol (HPLC reagent) and analyzed by API4000 (ABSCIEX, American). The column used was the Eclipse Plus C18 (150 mm ×2.1 mm×5.0 μm), and the mobile phase was water (A), and MeOH (B). Start 88% B, hold 6.5 min. The flow rate was 200 μL/min, and a constant temperature of 25 ^{o}C and 5 μL was injected. The mass spectrometer was used for detection of TCC, with the following optimized parameters: ESI negative, nitrogen drying gas, collision gas (5 units), curtain gas (20 units), ion source gas 1 (45 units), ion source gas 2 (55 units), turbo ionspray voltage (-4500 V), turbo probe temperature (450 ^{o}C), dwell time (100 ms), entrance potential (-10 V) and collision cell exit potential (-15 V). The system was controlled by Analyst® (version 1.4.2) software. Two multi-reaction monitoring (MRM) transitions 313.1 Q1/ 159.8 Q3 declustering potential -126.7V and Colision energy -19.04 eV and 313.1 Q1/ 125.9 Q3declustering potential -129.8 V and collision energy -31.45 eV were optimized. Recovery of this method was 90%-110%. 100 ml exposure solution was collected and filtered through 0.22 μM Sterile Syringe Filter (ANPEL Scientific instrument, shanghai), finally stored containing 2% HNO_3 (Guaranteed reagent) for further example Hg^{2+} content analysis.

Added proof test

To monitor fates of added chemicals during exposures，actual concentrations before/after addition of fish and food were measured (Fig.S2). 10 L dechlorinated tap water containing 10 ug Hg^{2+}/L or 5 ug TCC/L aerated with zeolite and 50% solutions were renewed daily. Fishes used in the test had fasted for 24 h before exposure. Two jars each contained 30 fish which were fed with commercial pelleted food (INCH-GOLD, China) at a rate of 3% body mass, which was designated the “fish +food” group. Another two jars (“fish” group) also contained 30 fish without feeding food during the exposure. There weren’t fish or food in the two jars (“water” group). After 1, 3 and 5 d of exposure before solutions were partially-renewed, concentrations of TCC or Hg^{2+} were measured by use of the methods described above.

Results

Measured concentrations of TCC in the “water” group were approximately 70% of the nominal concentration after 1, 3 or, 5 d exposure (Fig.S1.A). Measured concentrations of Jars containing fish or the “fish + food” group were decreased to similarly low concentration. These results suggested that TCC was absorbed by zebrafish from water. Alternatively, there were minor differences among measured concentrations of Hg^{2+} among the groups “fish”, “water”, “fish + water”(Fig.S1.B), which suggested that adsorption to the walls of the jars was the main reason for losses of Hg during the study and that lesser proportions were accumulated by fish.

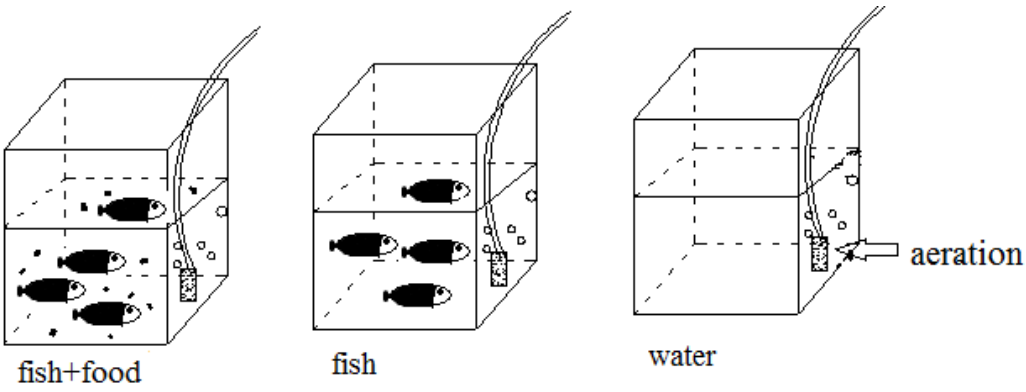

Fig.S2. Schematic diagram of study to determine changes in concentrations during exposures.

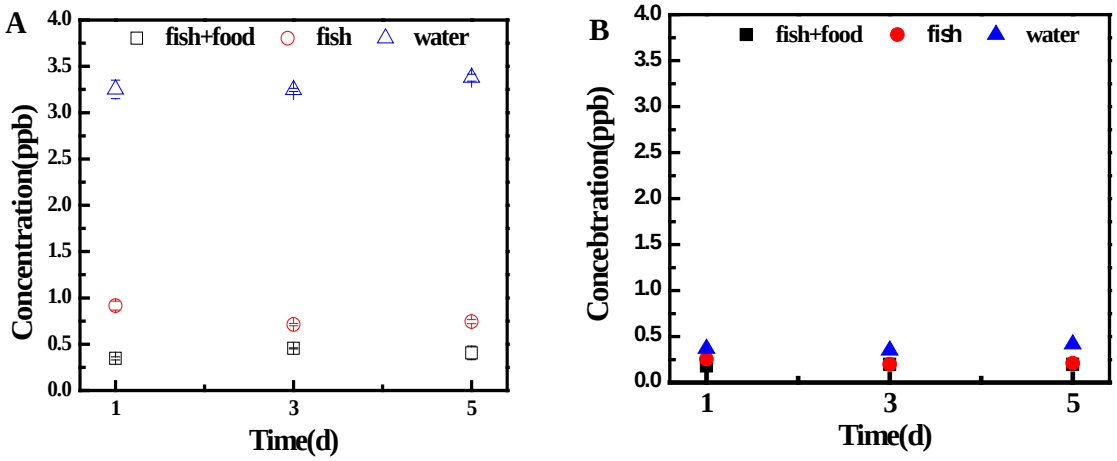

Fig.S1.Measured concentrations (μg TCC/L) (A) and Hg^{2+} (B) of different treatment groups after different durations of exposure (1, 3 or, 5 d).“□” represents the treatment group including fishes, normal food feeding, “○”represent the groups only contained fishes without food;“△”represent the same exposure conditions without fishes, food. Measured concentration were detected from three replicate samples.

Table S1. Primers parameter of RT-qPCR.

Gene symbol	Sense primer(5’-3’)	Antisense primer(3’-5’)	Tm(◦C)
β-actin(NM_131031.1)	**AGATGGGAACCGCTGCCTCTT**	**GTGGTCTCGTGGATACCGCAA**	**58**
3betaHSD(AY279108)	**AACTCTGGTTTTCCACACTGCGTC**	**CAGCAGGAGCCGTGTAGCTTTAA**	**58**
17betaHSD(NM_205584)	**GTGTGTAATGCCGGTGTGGGT**	**AAGGTCTGTATGGTGCGGATGGT**	**58**
cyp17(AY281362)	**AAAGTCTCCTGTCGGCTCGCTA**	**TGCTGGCACAAATCCATTCATCT**	**58**
cyp19a(AF226620)	**TCTCTACGTTTTCACCCGGTC**	**CATTCAGTATGATGTTTGTTCCTTT**	**58**
vtg1(NM_001044897)	**AACGAACAGCGAGAAAGAGATTG**	**TGATGGGAACAGCGACAGGAA**	**58**