Early life Exposure to Triclosan Impacted Thyroid Follicular Structure and Decreased Thyroid Hormone Levels in Zebrafish

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Research

Keywords: Triclosan, thyroid hormone, histology, thyroid follicles, zebrafish

Posted Date: March 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-17788/v1>

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Abstract

Background: Triclosan (TCS) is an antimicrobial chemical widely used in personal care products. Most of TCS is discharged into aquatic ecosystem after usage. While TCS has potential thyroid-disrupting effect, it is unknown whether early exposure to TCS affects thyroid gland and thyroid hormone levels. We used zebrafish model to examine this topic.

Methods: The fertilized zebrafish eggs were exposed to TCS. We inspected hatching and mortality of larvae. The total triiodothyronine (TT₃), total thyroxine (TT₄), free triiodothyronine (FT₃), and free thyroxine (FT₄) levels were measured at 7, 14 and 120 days post fertilization (dpf). Thyroid histopathological analysis was conducted at 120 dpf of zebrafish.

Results: The hatching rate of embryos decreased from 95.8% in control group to 18.3% in TCS highest exposure in 3 days. In 900 ng/mL TCS exposure all larvae died within one day. With increasing concentration of TCS exposure, TT₃ and FT₃ level decreased after 14 days. FT₄ decreased, and the height and nuclear area of thyroid follicular cell increased with TCS exposure after 120 days.

Conclusions: High TCS exposure decreased the hatching rate of zebrafish embryos, and also decreased thyroid hormone levels owing to structural change of thyroid follicles. Our study raised the concern that TCS exposure in early life may profoundly affect neurodevelopment.

Background

Triclosan (TCS) is an antimicrobial chemical widely used in personal care products including soaps, toothpastes, shampoos, and cosmetics for more than 40 years^[1]. Most of TCS is discharged into aquatic ecosystem after usage^[2]. Due to the extensive use of TCS in personal care products, TCS has been detected in more than half of the rivers and lakes^[3] and in drinking water^[4]. TCS concentrations reached up to 86.2 ng/mL in some rivers^[3, 5]. Furthermore, TCS can be bio-accumulated in fish^[6]. So far, we and others have detected TCS in urine samples of pregnant women and cord blood of newborns^[7, 8]. Importantly, these studies in pre-birth cohort have shown that maternal urinary TCS concentration was associated with decreased maternal and neonatal thyroid hormone levels (maternal serum FT₄ and cord blood FT₃)^[9, 10]. Besides, a study of adult zebrafish showed that TCS exposure resulted in hyperplasia of thyroid follicles^[11]. There were only two related studies on potential thyroid-disrupting effect of TCS in fish^[11, 12], it is unknown whether early life exposure to TCS will affect offspring thyroid gland and thyroid hormone (TH) levels. In this study, we aimed to use zebrafish to examine this topic.

Zebrafish is a proved model to study thyroid gland and hormones because the development of its thyroid system is generally comparable to human^[13]. While thyroid hormones are critical for fetal neuro-development, up to our knowledge, no study has examined early exposure to TCS (from fertilized eggs, embryo to adult stage) and zebrafish thyroid hormone levels. In this study, we used zebrafish model to examine the impact of early exposure to TCS at environmentally relevant levels and the TCS levels in

human bio-samples on the hatching rate, mortality, and thyroid hormones levels, as well as the histopathology of thyroid follicles.

Materials And Methods

TCS

TCS (Irgasan, 5-chloro-2-(2,4-dichlorophenoxy) phenol, $\geq 97.0\%$ purity (HPLC)) was purchased from Sigma-Aldrich (United States).

Zebrafish strains and maintenance

In this study, we used the zebrafish strains of AB wild-type line. First, 5-month-old male and female zebrafish were acclimated in tanks containing dechlorinated tap water for 4 weeks and under a photoperiod of 14:10 hour light/dark cycle. The fish were fed with brine shrimp twice per day. The remaining food and feces were removed at thirty minutes after feeding.

Next, zebrafish fertilized eggs were obtained/collected within 30 min after natural mating, rinsed in water, and the unfertilized eggs were discarded. Zebrafish larvae were fed with paramecium twice per day from 5 days post fertilization (dpf) till 14 dpf. After that, brine shrimp was fed from 15 dpf. The pH (maintained at 7.5 ± 0.5), conductivity (maintained at $550 \pm 50 \mu\text{S}$), temperature (maintained at $28 \pm 0.5^\circ\text{C}$), and dissolved oxygen of the exposure media were monitored.

TCS exposure

We randomly assigned and exposed fertilized zebrafish eggs (600 eggs per TCS group) to 6 levels TCS exposures from 0 (Dimethyl Sulphoxide, DMSO as blank control), 3, 30, 100, 300 to 900 ng/mL up to 7 and 14 days, and we put 200 fertilized zebrafish in each culture dish of 100 ml medium. We changed half of the medium each time for twice per day. The fertilized zebrafish eggs were cultured at $28.0 \pm 0.5^\circ\text{C}$ under 14:10 light/dark photoperiod cycle in a climate chamber during the duration of the experiment.

After TCS treatment for 7 and 14 days post fertilization (dpf) respectively, larvae TH levels were measured. Specifically, at 7 dpf, fish larvae were collected and put into three groups for each TCS treatment level as three replicates (50 larvae each replicate) for THs measurement. Then, the larvae left were continuingly exposed to TCS/or control until 14 dpf, fish larvae were collected and put into three replicates per each TCS treatment level (100 larvae in each replicate) for THs measures.

In addition, we exposed fertilized zebrafish eggs to 6 TCS exposures levels (30 eggs per each exposure level) for 120 days. At 120 dpf, the fish were deceased by immersing in ice-cold water. The heads were used for thyroid histological study and the bodies of fish were collected for thyroid hormone measurement. All fish samples (larvae at 7 and 14 dpf, heads and bodies of fish at 120 dpf) were immediately frozen and stored at -80°C for measurement of thyroid hormones.

These exposure doses of TCS were set based on the exposure levels of human and wildlife^[9, 14]. 3-100 ng/mL was close to the levels of urinary TCS concentrations in Chinese pregnant women^[9] as well as TCS levels in some rivers/lakes^[3]. We used DMSO as a solvent to enhance its solubility because TCS has low water solubility^[15], and the concentration of DMSO in both the experimental and control groups was 0.01%. Zebrafish embryos are tolerant to low concentrations (0.01%) of DMSO^[16].

Hatching rate

The hatching of fertilized eggs was inspected and recorded twice per day at the first 3 days (i.e. 72 hours). Hatching rate was calculated as the number of larvae hatched during the first 3 days divided by the total number of fertilized eggs in each TCS exposure level. The death of larvae was also inspected and recorded twice per day, and dead larvae were immediately removed from dishes/tanks. Mortality was calculated as the number of larvae death divided by the total number of hatched larvae.

Thyroid hormone measurements

We measured thyroid hormone levels of zebrafish at 7, 14 and 120 dpf. The samples of larvae at 7 and 14 dpf, and the body (without head, viscera or gut) of fish at 120 dpf stored at -80°C were taken out from freezer and immediately sonicated in 0.01 M PBS (0.01 M) at 1mg/5 μL (w/v, w: wet weight of 50 or 100 larvae or every fish body, v: volume of 0.01 M PBS, mg/ μL ; pH 7.2) and homogenized at 4°C . Next, to disrupt the structures, we put samples on intermittent sonic oscillation for 5 minutes on ice, and then vortexed vigorously for 10 min. Then, all the samples were centrifuged at $12,000 \times g$ for 5 min at 4°C . The supernatant was collected for thyroid hormones and total protein concentration measurement. Total protein concentration was measured with the Pierce[®] BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) to normalize thyroid hormone concentration^[17]. Free triiodothyronine (FT₃), free thyroxine (FT₄), total triiodothyronine (TT₃) and total thyroxine (TT₄) levels were measured with ELISA (enzyme-linked immunosorbent assay) (Labor Diagnostika Nord commercial kit, Germany). The limit of detection is 0.1 ng/mL for TT₃, 8 nmol/L for TT₄, 0.3 pg/mL for FT₃ and 1pg/mL for FT₄. Values below the limit of detection (LOD) were replaced with values equal to the LOD divided 2.

Hematoxylin and eosin (H&E) staining

The heads of zebrafish at 120 dpf frozen at -80°C were taken out from freezer and fixed in 10% neutral formalin (Zhongshan Beijing Biotechnology Co., Ltd., Beijing, China) at temperature 25°C for at least 12 h, and transferred to 70% ethanol. Each zebrafish head was placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in wax blocks. Serial transverse cross-sections were cut using a microtome 5 μm and dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. The sections were then stained with hematoxylin for 4-5 min and with eosin for 1-2 min at 25°C (Zhongshan Beijing Biotechnology Co., Ltd.). Stained thyroid follicle sections were observed and photographed under light microscopy (BX53-p; Olympus Corporation, Tokyo, Japan). We

located thyroid follicles according to their being dispersed among the afferent branchial arterioles (such as ventral aorta) in the throat region ^[18].

The nuclear size and height of follicular cells

Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantitatively analyze. The nuclear size of the thyroid follicular cells was assessed quantitatively by measuring the long and short diameters of the cell nuclei. The size of at least 50 thyroid follicular cell nuclei from each fish at a total magnification of $\times 1000$, at least three different follicles or from three separate areas of the section in cases where follicular structure was unclear or absent. Photograph of each follicle was taken at the largest follicle diameter (determined by observing serial sections) with an Olympus digital camera (BX53-p; Olympus Corporation, Tokyo, Japan). The nuclear size was calculated based on the formula for an ellipse (long diameter $\times$ short diameter $\times \pi/4$) ^[18]. The height of follicular cell was calculated by five measurements along the follicle perimeter at regular intervals, 40-80 follicular cell heights for each fish ^[19].

Statistical analysis

The ANOVA F-test was used to compare the differences in hormone levels, the height and the nuclear size of the thyroid follicle cells, the chi square test was used to compare the difference in hatching rate and mortality of larvae among TCS exposure groups. The level of significance was two-sided p value < 0.05 . All analyses were performed using the SAS 9.3 software (SAS Institute, Inc., Cary, NC, USA).

Results

The effect of TCS exposure on hatching rate

In this study, the hatching rate of zebrafish embryos decreased with increasing TCS exposure at 2 dpf. In control group, hatching rate was 42% in the first 48 hours and 95.8% at 72 hpf, and the larvae presented a well-developed head, body and tail. Following the higher concentrations of TCS exposure, hatching rate decreased especially in the first 48 hours. The lowest was 18.3% in the highest (900 ng/mL) TCS exposure group ($p < 0.01$) (**Table 1**) at 72 hpf.

The effect of TCS exposure on mortality of zebrafish larvae

In TCS exposure level of 900 ng/mL, larvae died shortly after its hatching. In larvae exposed to the highest level (900 ng/mL) of TCS, the mortality reached 100% at 3 dpf. In the other TCS exposure levels, the mortalities were similar at 7 dpf (**Table 2**).

After 7 days exposure, we randomly collected fish larvae (50 larvae each for triplicate) from each TCS exposure group to measure thyroid hormone levels. We then continued to observe mortality in the remaining larvae during 8 and 14 dpf, and the mortality was similar among TCS exposure levels from 0 to 300 ng/mL ($p=0.16$, **Table 2**).

The thyroid hormone levels in Zebrafish larvae after 7 days of TCS exposure

The TT_3 , TT_4 , FT_3 and FT_4 levels did not differ significant among 5 TCS exposure groups from 0 to 300 ng/mL (**Figure 1** and **Table S1**). For the TCS exposure level of 900 ng/mL, since the larvae all died by the day three soon after they were hatched, thyroid hormone levels were not measured in this group.

The thyroid hormone levels in larvae after 14 days of TCS exposure

TT_3 and FT_3 levels in TCS exposure groups were all lower than the control group after 14 days of exposure. Following exposure to TCS from 3 ng/mL to 300 ng/mL for 14 days, TT_3 were on average 0.188 to 0.194 ng/mg lower than that of control group (all $p < 0.01$, **Figure 1** and **Table S2**). Also, FT_3 levels were 9.49 - 10.74 ng/mg lower ($p < 0.01$) in larvae exposed to TCS level at 30, 100, 300 ng/mL than the control group. For FT_4 level, overall it tended to decrease with higher level of TCS exposure (p trend < 0.05 , **Table S2**). Similarly, TT_4 levels were lower in larvae exposed to TCS than that in control group, but the differences were not statistically significant.

The thyroid hormone levels in zebrafish after 120 days of TCS exposure

The effects of TCS exposure for 120 days on thyroid hormone levels are presented in **Figure 2** and **Table S3**. Following exposure to TCS at 30, 100, and 300 ng/mL for 120 days, FT_4 levels were on average 0.06, 0.08, 0.09 pg/mg lower than that of control group (all $p < 0.05$) (**Figure 2** and **Table S3**). The TT_3 , TT_4 , and FT_3 levels were similar among 5 TCS groups.

Thyroid Histopathology in zebrafish after 120 days of TCS exposure

With the increasing exposure levels to TCS, the thyroid follicular cell of zebrafish at 120 dpf became hypertrophy (enlarged follicular cell size and nuclear size) and hyperplasia (increased number of follicular cell), and the nuclear area and the epithelial cell height of thyroid follicles was significant increased (**Figures 3** and **4**, **Table S4**). The oval thyroid follicles of zebrafish in control group consisted of an outer thyroid epithelial layer which surrounded into an inner lumen; the inner lumen of thyroid follicles was filled with colloid (**Figure 4**, **Panel A**). After exposure to TCS for 120 days, colloid in follicle decreased obviously (**Panel B, C, D, E and F** in **Figure 4** and **Figure 5**), the number of follicular cells increased (**C, D, E, and F** in **Figure 4**; **B, C, D, E and F** in **Figure 5**), with angiogenesis (**B, C, D, E and F** in **Figure 4**; **F** in **Figure 5**), focal hyperplasia (**C, D, E and F** in **Figure 4**; **B, C, D and E** in **Figure 5**), and diffuse hyperplasia (**F** in **Figure 4**) in thyroid follicles. Colloid depletion of follicles was observed in all zebrafish exposed to TCS (**B, C, D, E and F** in **Figure 4**). In the zebrafish exposed to high TCS levels at 100 ng/mL and 300 ng/mL, the thyroid follicles had obvious morphological alterations, for example, the epithelial cells became edema, thicker and increased significantly, follicular interstitial hyperplasia and inflammatory cells increased (**D, E and F** in **Figure 4**).

Discussion

In this study, we examined the effects of early TCS exposure on the thyroid hormone (TH) levels at two growth phases (larvae and adult) and found thyroid morphological change after 120 days of TCS exposure. High TCS exposure decreased the hatching rate of zebrafish embryos. Furthermore, TCS exposure also decreased TT_3 , FT_3 and FT_4 levels after 14 days and decreased FT_4 level after 120 days owing to histopathological change of thyroid follicular cell. With increasing concentration of TCS exposure, the height and nuclear size (area) of thyroid follicular cell increased with TCS exposure after 120 days. Considering the importance of thyroid hormone to nervous system development, our study implicates that TCS exposure in early life may profoundly affect neurodevelopment.

Zebrafish is a useful animal model for the study on thyroid hormones and the chemical pollutions in water, and its high (71%) genetic is similar to human^[20]. We found that with the increase of TCS exposure concentration zebrafish hatching rate decreased and the mortality rate increased of zebrafish larvae. This result was consistent with previous finding in zebrafish^[14], after being exposed to 500 ng/mL of TCS for 6 days, a significantly delayed hatchability and increased mortality were observed in zebrafish. After larvae stage, zebrafish step into sexual mature phase at 120 dpf^[21]. We observed adverse effect of early exposure to TCS (from fertilized eggs, embryo to adult stage) on thyroid hormone levels, and this might be due to the cumulative effect of TCS exposure on the physiological characteristics of the development of zebrafish thyroid glands. The first follicular thyroid of zebrafish larvae emerges at about 55 hpf and weak signals of T_4 can be detected in submandibular about 72 hpf^[22]. There are about 6-7 thyroid follicles at 7 dpf^[13]. The thyroid hormone level of zebrafish became stable after 7 days^[23]. In our experiments, TT_4 , FT_4 and FT_3 levels in TCS exposed groups tended to decrease, although not significant, after 7 days of exposure. The thyroid disruption effect of TCS was more obvious after 14 days of exposure. FT_4 level decreased significantly with the increasing TCS exposure concentration both at 14 and 120 dpf. The impact of TCS exposure on thyroid hormone levels (TT_3 , FT_3 and FT_4) in zebrafish larvae at 14 dpf provides causal support to our previous findings in population study^[9]. Our previous study observed an inverse association between maternal urinary TCS and cord blood FT_3 level in Chinese newborns^[9].

THs are secreted and released by the thyroid gland. FT_3 is the most active form of TH, and it comes from FT_4 deiodination. FT_3 and FT_4 , TT_3 and TT_4 can transform into each other and maintain dynamic balance in blood. Most TT_4 and few TT_3 are synthesized by thyroid follicular epithelial cells directly and then are transported to blood and be function^[24, 25]. TT_4 and TT_3 are usually used as an indicator of the reserve capacity of the thyroid gland^[26]. Once the thyroid function is disrupted, the FT_3 and FT_4 levels are the first to be affected, lead to TT_3 and TT_4 level and histopathological change of thyroid^[27]. In general, T_4 is more sensitive compared with the changes of T_3 ^[26, 28, 29], however, in this study, with higher TCS exposure levels, TT_3 and FT_3 concentrations decreased statistically in larvae at 14 dpf. Exposure to 120 dpf, TT_4 , FT_3 and FT_4 levels were all lower than that at 14 dpf, and the height and nuclear area of thyroid follicular cell at 120 dpf were obviously increased with the increasing TCS exposure concentration. The

histopathology results are consistent with the changed levels of thyroid hormone in this study. The changes in the epithelial cell height and follicle nuclear size are likely the result of negative feedback of decreased thyroid hormone.

To study the impact of environmental chemicals exposure on health effects, if zebrafish larvae were used as models, generally the whole fish is used as biological samples^[12, 30], and in our study, we measured THs levels in whole body of larvae at 7 and 14 dpf. However, if adult zebrafish were used as models, plasma or body are generally used as biological samples^[31], but we measured TH levels in body (without head, viscera or gut) of adult fish at 120 dpf. In zebra fish, THs both expressed in hepatic and muscle^[32]. THs levels in muscle can reflect the growth and development of the body, and in our study, we found that TCS exposure level was related to TH levels in the muscle and therefore presumably affects the body growth of the zebrafish.

TCS was found in wastewater treatment plants worldwide^[3]. One study found that TCS has been detected in more than half of the rivers and lakes in Savannah, Georgia, USA, with a median level of 0.14 ng/mL and the maximum level of 2.3 ng/mL^[3]. In some stream, the concentration even reached up to 86.2 ng/mL^[3]. This study selected the TCS exposure levels of 3, 30, 100, 300 and 900 ng/mL based on our population exposure level, wildlife zebrafish exposure level and the TCS level in environmental data. The lowest concentration (3 ng/mL) was selected based on the median urinary TCS concentration (2.52 ng/mL), and 100 ng/mL was close to the highest urinary TCS concentrations from population of pregnant women in China^[9]. In fish, the LC50 for TCS ranges from 180 to 602 µg/L^[33-35]. In zebrafish, TCS LC50 values was 420 µg/L (95% confidence interval, 400 ng/mL- 470 ng/mL) for embryos in exposure to TCS for 96 hours (starting from fertilized eggs), and 340 µg/L for zebrafish adults also with an exposure of 96 hours^[14]. TCS exposure level of 100 ng/mL in this study was 25% of the concentration for LC50. We also use relatively higher concentrations of TCS (300 and 900 ng/mL) to study possible toxicity during short-term laboratory exposures. The exposure concentrations 900 ng/mL of TCS was a 10-fold of the highest urinary TCS concentration in our previous population study in China. While the toxic effects of TCS were well recognized on bacterial resistance, reproductive toxicity and cytotoxicity in aquatic organisms^[36-38], the results on thyroid hormone disruption were inconsistent^[39, 40]. Previous studies found that exposure of TCS decreased thyroid hormone levels and inhibited metamorphosis^[39, 41], or did not change the thyroid hormone level nor alter the metamorphosis in anuran^[40]. Most animal studies mainly focused on rodents^[42, 43]. In adult rat, TCS treatment reduced the levels of blood TT₄ and FT₄, while no change was found in the level of blood TSH^[44, 45]^[46, 47]. Another study in female mice found that exposed to TCS (300 mg/kg/day) during pregnancy decreased approximately 30% of TT₄ in dams at the postpartum day 22^[47]. Only two recent studies have examined the influence of TCS exposure on thyroid disruption in fish^[11, 12]. One study found that TCS exposure caused hatching delay of 6-13 hours in medaka fish^[12]. The other study found that sub-chronic (21 days) exposure to 100 µg/g TCS caused hyperplasia of thyroid follicle in adult zebrafish^[11]. A recent study^[48] found that offspring of

zebrafish that exposed to TCS decreased survival rate and delayed maturation. This can be explained by decreased thyroid hormone resulting from TCS exposure.

We speculate that TCS may inhibit thyroid hormones secretion by acting as a disruptor of the hypothalamic-pituitary-thyroid axis or due to the increased thyroid hormones clearance. Mechanisms have been studied but there is controversial. TCS exposure may promote^[11] or inhibit^[49] the sodium-iodide symporter (NIS)-mediated iodide uptake in animals. TCS increased TSH gene transcription and decrease thyroid hormones in circulation of zebrafish^[11]. In rat, TCS inhibited the activity of thyroid peroxidase (TPO) at concentration of 50 ng/mL to inhibit thyroid hormone synthesis^[49]. In addition, evidence suggests that TCS inhibited liver phase I and II enzyme mRNA expression in zebrafish liver cells^[50]. TCS may interfere with thyroid function by increasing the hepatic enzymes activity of glucuronyltransferase and pentoxyresorufin-O-deethylase (PROD) in livers^[51, 52] or by activating of the nuclear receptor, pregnane X receptor (PXR)^[43], as a result, the metabolism of T₄ causing decrease in T₄ bioavailability^[42]. In 2018, it was also found that TCS caused the hypothyroidism in thyroid cells of rats through p38/TRHr-dependent pathway^[37]. Still, the mechanism that TCS inhibit the thyroid hormones need further investigation.

Our study found that zebrafish exposed with TCS for 120 days (from embryo to adult phase) caused thyroid histopathology change and disturbed thyroid hormone levels. We are the first study to explore the thyroid disturbed effect of long-time TCS exposure on zebrafish from fertilized eggs, larvae to adult phases, and to focus on effect of TCS exposure on thyroid hormones of zebrafish from early life. We did not measure TSH level in zebrafish, although TSH receptor is responsible for thyroid gland differentiation in zebrafish^[53], Alt et al.^[54] reported that in zebrafish the formation, growth and differentiation of thyroid follicles appears to be independent of TSH. The limitation of our study is that we did not measure the concentration of TH levels in whole body of adult zebrafish, the trend of TH levels at 120 dpf were not fully consistent with that at 14 dpf.

Conclusions

In summary, we found that exposure to TCS delayed the hatching of zebrafish embryos and decreased thyroid hormone (FT₃, FT₄ and TT₃) levels in zebrafish larvae, also decreased FT₄ level in zebrafish adult. Furthermore, TCS exposure also affect the structure of thyroid follicles by increasing nuclear area and height of zebrafish thyroid follicle cell. Considering that the thyroid hormones are crucial for neurodevelopment in early life, our study raises the concern that early TCS exposure (from conception) may profoundly influence child neurobehavioral development at such highly sensitive window.

Declarations

Funding Information

This study was supported by grants from the National Natural Science Foundation of China [81673178, 81961128023] and Shanghai Municipal Education Commission—Gaofeng Clinical Medicine Grant [grant number 20152518].

Competing financial interests

The authors declare they have no actual or potential competing financial interests.

Ethics approval and consent to participate

All animal in this study underwent ethics approval by Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding authors on reasonable request.

Author contributions

Conceptualization, Fengxiu Ouyang; Methodology, Fengxiu Ouyang, Ning Tang, Weiye Wang, Xiaogang Yu and Xu Wang; Formal analysis, Ning Tang and Pianpian Fan; Investigation, Fengxiu Ouyang and Ning Tang; Resources, Fengxiu Ouyang and Weiye Wang; Data curation, Ning Tang and Pianpian Fan; Writing—original draft preparation, Ning Tang and Fengxiu Ouyang; Writing—review and editing, Fengxiu Ouyang, Pianpian Fan, Xiaogang Yu, Xu Wang and Weiye Wang; Supervision, Fengxiu Ouyang; Project administration, Fengxiu Ouyang and Ning Tang; Funding acquisition, Fengxiu Ouyang.

Acknowledgements

Not applicable.

Consent for publication

Not applicable

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Tables

Table 1. The influence of TCS exposure on the hatching rate of zebrafish embryos

Treatment	fertilized eggs n	number of larvae hatched	
		up to 48 hpf n (%)	up to 72 hpf n (%)
TCS exposure levels (ng/mL)			
0 (control)	600	252 (42.0%)	575 (95.8%)
3	600	287 (47.8%)	580 (96.7%)
30	600	207 (34.5%)	563 (93.8%)
100	600	141 (23.5%)	561 (93.5%)
300	600	169 (28.2%)	521 (86.8%)
900	600	26 (4.3%)	110 (18.3%)
<i>p</i>		<0.01*	<0.01*

χ^2 test was used to test the difference; hpf: hours post fertilization

Figures

Table 2. The influence of TCS exposure on the larvae mortality in 14 days post fertilization

Treatment	Larvae hatched in 3 dpf n	Larvae death in 7 dpf n (%)	Number of live larvae at the beginning of 8 dpf [#] n	Larvae death from 8 to 14 dpf n (%)
TCS exposure levels (ng/mL)				
0 (control)	575	36 (6.3%)	389	16 (4.1%)
3	580	41 (7.1%)	389	18 (4.6%)
30	563	50 (8.9%)	363	18 (5.0%)
100	561	72 (12.8%)	339	9 (2.7%)
300	521	45 (8.6%)	326	9 (2.8%)
900	110	110 (100%)	—	—
<i>p</i>		<0.01*		0.16

dpf: days post fertilization

* χ^2 test was used.

[#] number of larvae at the beginning of 8 dpf= total hatched larvae in 72 hpf - number of larvae death up to 7 dpf - 150 larvae used for measuring thyroid hormones at the 7th day (i.e. 50 larvae/ measure $\times$ 3 measures = 150 larvae for each exposure group).

Figure 1

Thyroid hormone values in zebrafish larva exposed to control and TCS for 7 and 14 days after fertilizing. A-D: TT₃, TT₄, FT₃, FT₄ level at 7 dpf; E-H: TT₃, TT₄, FT₃, FT₄ level at 14 dpf. At 14 dpf, p trend for TT₃ = 0.04, p trend for FT₃ = 0.001, p trend for FT₄ = 0.04. Thyroid hormones concentrations in larvae were measured in three measures for each exposure group, with each measure homogenized from 50 fish at 7dpf, and 100 fish at 14 dpf. *p < 0.05, ** p < 0.01.

120 dpf

Figure 2

Thyroid hormone values in zebrafish exposed to control and TCS for 120 days after fertilizing. Thyroid hormones concentrations in fish at 120 dpf were measured for each exposure group, with each sample homogenized from every single fish at 120 dpf. p trend for FT₄ = 0.01. * p < 0.05, ** p < 0.01.

Figure 3

Effects of TCS exposure at different concentration on nuclear area (A) and the height (B) of thyroid follicular cells. p trend < 0.0001 both for nuclear size and epithelial cell height of thyroid. **: p < 0.01 in TCS exposure group v.s. control group.

Figure 4

Structure of thyroid follicles with different TCS exposure concentration. (A) Control zebrafish follicle squamous to cuboidal follicular epithelium, with colloid in follicle. (B) Hypertrophy of follicles with little colloid in the lumen and angiogenesis in 3 ng/mL TCS exposure. (C) Hypertrophy of follicles, focal hyperplasia and angiogenesis in 30 ng/mL TCS exposure. (D) Hypertrophy of follicles, edema of epithelial cells, hyperplasia and angiogenesis in 100 ng/mL TCS exposure. (E, F) Hypertrophy of follicles,

edema of epithelial cells, diffuse hyperplasia and angiogenesis in 300 ng/mL TCS exposure. (va = ventral aorta; c = colloid; f = thyroid follicle; ag = angiogenesis.)

Figure 5

Representative photomicrographs of single follicle structure and thyroid follicle cells with different TCS exposure concentration. (A) Control zebrafish follicle squamous to cuboidal follicular epithelium. (B) Hypertrophy and mild hyperplasia of follicle cells in 3 ng/mL TCS exposure. (C) Hypertrophy, hyperplasia

and edema of follicle cells 30 ng/mL TCS exposure. (D) Focal hyperplasia with increased number of epithelial cells, depletion of the colloid at the edges in 30 ng/mL TCS exposure. (E) Hypertrophy and hyperplasia of follicles and obviously increased cell height in 100 ng/mL TCS exposure. (F) Severe hypertrophy and edema of thyroid follicular epithelial cells in 300 ng/mL TCS exposure. (fc = thyroid follicular cell; black arrows = hypertrophy; white arrows = hyperplasia)

Supplementary Files

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