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Environmentally relevant concentrations of mercury exposure alter thyroid hormone levels and gene expression in the hypothalamic–pituitary–thyroid axis of zebrafish larvae

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Abstract Mercury (Hg) is one of the most toxic heavy metals that can cause severe damage to fish. Studies have demonstrated that Hg has a specific affinity for the endocrine system, but little is known about the effects of Hg on thyroid endocrine system in fish. In this study, zebrafish embryos were exposed to environmentally relevant concentrations of 1, 4, and 16 μg/L Hg^{2+} (added as $HgCl_2$) from 2 h post-fertilization (hpf) to 168 hpf. Thyroid hormone (TH) levels and mRNA expression levels of genes involved in the hypothalamus–pituitary–thyroid (HPT) axis were determined. The results showed that exposure to 16 μg/L Hg^{2+} increased the whole-body thyroxine (T4) and triiodothyronine (T3) levels. The transcription levels of corticotrophin releasing hormone (*crh*) and thyroid stimulating hormone (*tshβ*) were up-regulated by Hg^{2+} exposure. Analysis of the mRNA levels of genes related to thyroid development (*hhex*, *nkx2.1*, and *pax8*) and THs synthesis (*nis* and *tg*) revealed that exposure to higher Hg^{2+} concentrations markedly up-regulated *hhex*, *nkx2.1*, *nis*, and *tg* expression, while had no significant effect on the transcripts of *pax8*. For the transcription of two types of deiodinases (*deio1* and *deio2*), *deio1* showed no significant changes in all the treatments, whereas *deio2* was significantly up-regulated in the 16 μg/L Hg^{2+} group. In addition, Hg^{2+} exposure up-regulated thyroid hormone receptor β (*trβ*) mRNA level, while the transcription of *trα* was not changed. Overall, our study indicated that environmentally relevant concentrations of Hg^{2+} exposure could alter TH levels and the transcription of related HPT-axis genes, disturbing the normal processes of TH metabolism.

Keywords Mercury exposure · Thyroid endocrine system · Thyroid hormones · Hypothalamic–pituitary–thyroid axis

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Introduction

Mercury (Hg) is classified as a priority pollutant by many countries such as China, USA, and EU countries due to its low biodegradability, bio-accumulation, and high toxicity (Larras et al. 2013; Zhang et al. 2016a). This toxicant is released to the environment from natural and anthropogenic sources. During the last several decades, human activities (such as coal combustion and mining) have resulted in a pronounced increase in anthropogenic Hg emission, which discharge into the aquatic environment directly or indirectly through atmospheric deposition, and consequently has become a potential threat to aquatic organisms and human beings (Larras et al. 2013; Zhang et al. 2016b). Previous studies have documented that Hg exposure can cause a variety of adverse effects on fish, such as behavioral changes (Berntssen et al. 2003), growth inhibition (Friedmann et al. 1996), and developmental damage (Zhang et al. 2016b). In recent years, growing amounts of data have shown that Hg has a specific affinity for the endocrine

system, and thus the potential endocrine-disrupting effects of Hg have received some attention. For example, Hg can alter the level of sex hormones (such as 17β-estradiol and testosterone) and gene expression in the hypothalamic–pituitary–gonadal axis, interfering with the process of oogenesis and spermatogenesis (Hedayati and Hosseini 2013; Zhang et al. 2016a). However, very few studies have looked at the toxic effects of Hg on thyroid endocrine system, an important endocrine system in fish.

Thyroid hormones (THs) are one of the most important hormones required by fish for growth, development, metamorphosis, and metabolism (Nelson and Habibi 2009). As in mammals, the synthesis of THs in fish is regulated by the hypothalamus–pituitary–thyroid (HPT) axis. In teleosts, hypothalamus controls the synthesis and release of corticotrophin-releasing hormone (CRH) (like thyrotropin-releasing hormone in mammals) which acts on the pituitary, stimulating the release of thyroid-stimulating hormone (TSH), which in turn acts on the thyroid to regulate the synthesis of THs (in particular thyroxine, T4) (De Groef et al. 2006). Meanwhile, plasma TH levels have a negative feedback effect on TSH release. To date, many studies have shown that contaminant exposure can disrupt thyroid endocrine system by interfering with the HPT axis. For example, subacute microcystin-LR exposure could disturb TH homeostasis by disrupting the synthesis and conversion of THs in juvenile zebrafish (Liu et al. 2015). Chinese rare minnow larvae exposed to Cd^{2+} led to a significant reduction in TH levels and alterations of gene expression in the HPT axis (Li et al. 2014a). Furthermore, studies on fish from Hg-contaminated areas found that Hg could interfere with thyroid function and transcription of thyroid-related genes in juvenile or adult fish (Mulder et al. 2012; Fu et al. 2017). Unfortunately, no information is available on the influence of environmentally relevant concentrations of Hg^{2+} exposure on TH levels and gene transcription in the HPT axis of fish at embryonic-larval stages.

The zebrafish embryo/larva is an ideal model for toxicological researches, which has been widely used to investigate endocrine disruption by chemicals (Yu et al. 2013). The purpose of this study is to evaluate effects of environmentally realistic concentrations of Hg exposure on thyroid endocrine system in the early development stage of fish. To achieve this, zebrafish embryos were exposed to different concentrations of Hg^{2+} (0, 1, 4, and 16 μg/L; added as $HgCl_2$) for 7 days (from 2 h post-fertilization (hpf) to 168 hpf), and thyroxine (T4) and triiodothyronine (T3) levels in the whole body were measured. Moreover, the mRNA expression of genes involved in the HPT axis was analyzed, including corticotrophin releasing hormone (*crh*), thyroid stimulating hormone (*tshβ*), hematopoietically expressed homeobox protein (*hhex*), NK2 homeobox 1 (*nkx2.1*), paired box 8 (*pax8*), sodium/iodide symporter (*nis*), thyroglobulin (*tg*), deiodinase type 1 and 2 (*deio1* and *deio2*), and thyroid hormone receptor α and β (*trα* and *trβ*). Our findings will facilitate understanding of the endocrine toxicity mechanisms of Hg exposure in fish.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals and was approved by the Committee of Laboratory Animal Experimentation of Chongqing Normal University.

Chemicals

$HgCl_2$ (purity ≥ 99.5%) was purchased from Shanghai Sinopharm Group Corporation (Shanghai, China) and dissolved in pure water for stock solution. 3-Aminobenzoic acidethyl ester methanesulfonate (MS-222) was obtained from Sigma (St. Louis, MO, USA). Other chemicals used in this study were of analytical grade.

Zebrafish maintenance and embryo exposure

The experimental procedures have been described in our recent study (Zhang et al. 2016b). Briefly, adult zebrafish were cultured in a flow-through system (28 °C) and fed with freshly hatched *Artemia nauplii* and commercial flake diet. Normal adult zebrafish were placed in tanks with a ratio of 2:1 (male:female) overnight. Spawning was triggered in the morning when the light was turned on and completed within 30 min. Embryos were collected 2 hpf and examined under a stereomicroscope. Approximately 6000 embryos were randomly distributed into 12 glass aquariums (500 embryos per aquarium) containing 2 L of $HgCl_2$ solution (0, 1, 4, and 16 μg/L Hg^{2+}) until 168 hpf by which time could

maintain relatively high levels of thyroid hormones (Chang et al. 2012). The selected exposure concentrations were ascertained for two reasons: (1) these doses were close to environmentally realistic concentrations in Hg-polluted waters (Gray et al. 2000; Berzas Nevado et al. 2003); (2) by a range-finding study, mortality in the highest concentration was not more than 50% and the other two doses led to lower mortality to obtain sufficient samples for analysis. During the experiment, half of the water was renewed daily and the Hg concentrations were monitored by using cold vapor atomic fluorescence spectrometry, and the measured values for four treatments were 0.07 ± 0.02, 1.27 ± 0.13, 3.76 ± 0.46, and 15.12 ± 0.78 μg/L (mean ± SD, $n = 4$), respectively. Zebrafish larvae were maintained in the environment with 28 °C and 14:10 (light:dark) qualification. The controlled conditions for the exposure were as follows: temperature, 27.5–28.2 °C; pH, 7.7–7.8; dissolved oxygen, 6.3–6.7 mg/L; hardness, 140.3–145.6 mg/L as $CaCO_3$.

After 7 days (168 hpf) of exposure, the larvae were washed with UltraPure water and anesthetized with MS-222, and then immediately frozen in liquid nitrogen and stored at − 80 °C for determination of gene expression and hormone levels.

TH extraction and measurement

Thyroxine (T4) and triiodothyronine (T3) levels were measured as described by Yu et al. (2011) using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Uscnlife, Wuhan, China). About 200 larvae from each aquarium were homogenized in 0.4 mL ELISA buffer, and then samples were disrupted by intermittent sonic oscillation for 5 min and vortexed vigorously for 10 min. Next, the samples were centrifuged at 5000×*g* at 4 °C for 10 min. The supernatants were collected and immediately used for T3 and T4 measurement in accordance with the manufacturer's instructions. The detection limit of T3 and T4 was 0.12 and 3.7 ng/mL, respectively. No significant cross-reactivity or interference was observed for each kit.

Gene expression analysis

Gene expression levels were measured by quantitative real-time PCR (qPCR) method following MIQE guidelines (Bustin et al. 2009) as described in Chen et al. (2016). Total RNA was isolated from 30 larvae using RNAiso Plus (TaKaRa, Dalian, China), and the quality was assessed by agarose gel electrophoresis and by the 260/280 absorbance ratio. Afterwards, total RNA was treated with DNase I (TaKaRa, Dalian, China) to eliminate traces of DNA and then reverse-transcribed to cDNA with oligo-dT primers and cDNA Synthesis Kit (TaKaRa, Dalian, China). qPCR assays were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) with a 20-μL reaction volume containing 10 μL of 2 × SYBR Premix Ex Taq™ (TaKaRa, Dalian, China), 0.4 μL of 10 mM each of forward and reverse primers, 1 μL of diluted cDNA template (10-fold), and 8.2 μL of RNase-free water. Primers are given in Table 1. Thermal cycling was done at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30s. All reactions were performed in duplicates, and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. A non-template control and dissociation curve were performed to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Elongation factor 1-alpha (*ef1α*) expression was stable under the experimental conditions and was used as a reference gene. The relative expression levels of specific genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Results were presented as mean ± SD. Prior to statistical analysis, all data were tested for the normality and homogeneity using the Kolmogornov–Smirnov and Levene's tests, respectively. If necessary, data were log-transformed to improve normality and homogeneity of variance. Then, data were subjected to one-way ANOVA and Tukey's multiple range test. Analysis was performed using SPSS 17.0, and the minimum significant level was set at 0.05.

Results

Whole-body T3 and T4 contents

Effects of waterborne Hg^{2+} exposure on the whole-body T4 and T3 contents in zebrafish larvae are shown in Fig. 1. Compared with the control group, both T3 and T4 contents were significantly increased in the 16 μg/L Hg^{2+} group, but were not affected in 1 and 4 μg/L Hg^{2+} groups.

Table 1 Primers used for qPCR analysis

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size	Accession no.
crh	CCTTTCCACCGCCGTATG	GGTTTCTGTTGCCGAGCC	141	NM_001007379
tshβ	CATCCTCATACACTGCCACC	CCCCTCTGAACAATAAAACG	233	AY135147
hhex	GCTCACGCCGATACACCC	CACAGCAGAGGCTTACCCA	120	NM-130934
nkx2.1	AGCCCCAAACACTCAACG	GGTGCTATGCGAGAACTGC	207	NM_131776.1
pax8	GCACATCAGGGCTACGCT	AGACAGGTCCAGGAAGGCT	138	AF072549
nis	GGCATGGTGACAGAAGAACTC	CCCAGCCAGAGCCTAAAAG	107	NM_001089391
tg	GCTGCCGTGGAATAGGAT	GCCGAAAGGATAGAGTTGACTT	170	XM_001335283
deio1	ACCCTGCTCAAAGAAGACCC	CCGATGCCTCCCTGATAGA	137	BC076008
deio2	GCATAGGCAGTCGCTCATTT	TGTGGTCTCTCATCCAACCA	103	NM_212789
trα	CTATGAACAGCACATCCGACAAGAG	CACACCACACACGGCTCATC	85	NM_131396
trβ	CGAGCAGCAGTGCGTTAT	CAGAGGAAAGCAGAATCACG	199	NM_131340
ef1α	GATCACTGGTACTTCTCAGGCTGA	GGTGAAAGCCAGGAGGGC	118	FJ915061

mRNA expression levels of genes involved in the HPT axis

Compared with the control, *crh* transcripts were significantly elevated in 4 and 16 μg/L Hg^{2+} groups (Fig. 2a). The expression of *tshβ* was significantly increased in the highest Hg^{2+} concentration, but lower concentrations (1 and 4 μg/L Hg^{2+}) exposure caused no significant changes (Fig. 2b).

For the expression of genes involved in thyroid development, *hhex* was significantly up-regulated in the 16 μg/L Hg^{2+} group (Fig. 3a), while a significant induction of *nkx2.1* expression was found in 4 and 16 μg/L Hg^{2+}groups (Fig. 3b). However, the expression of *pax8* showed no significant differences among the treatments (Fig. 3c).

The mRNA levels of *nis* and *tg*, two key genes related to THs synthesis, were significantly up-regulated after exposure to 16 μg/L Hg^{2+}, whereas treatment with lower concentrations did not cause such effects (Fig. 4).

For the expression of thyroid hormone receptor isoforms, *trα* had no significant differences (Fig. 5a), while *trβ* was increased significantly after exposure to 16 μg/L Hg^{2+} (Fig. 5b).

For the gene transcription of two isoforms of deiodinases, *deio1* showed no significant changes in all the treatments (Fig. 6a), but *deio2* was significantly up-regulated in the 16 μg/L Hg^{2+} group (Fig. 6b).

Discussion

In the present study, exposure of zebrafish embryos to $HgCl_2$ (16 μg/L Hg^{2+}) for 7 days significantly increased both T3 and T4 contents in larvae, indicating that Hg has the ability to act as an endocrine disruptor which could

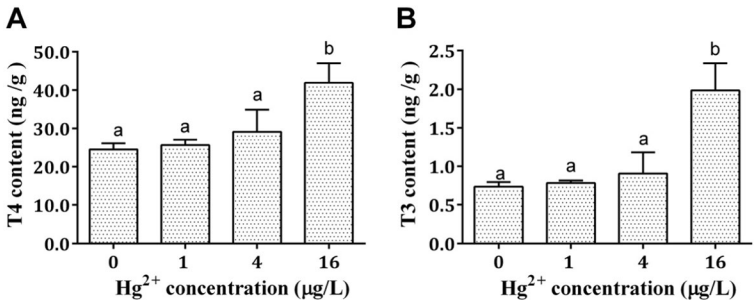

Fig. 1 Effects of waterborne Hg^{2+} exposure on the whole-body contents of thyroxine (T4) and triiodothyronine (T3) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included approximately 200 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

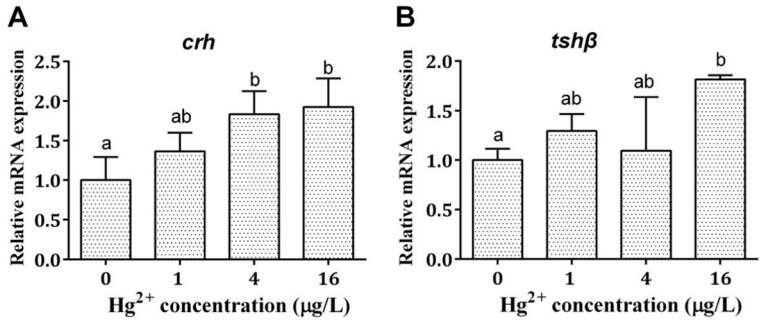

Fig. 2 Effects of waterborne Hg^{2+} exposure on the mRNA expression level of *crh* (**a**) and *tshβ* (**b**) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included 30 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

induce thyroid disruption. Similarly, plasma T4 and T3 levels increased after juvenile rainbow trout exposure to $HgCl_2$ (28 and 112 μg/L Hg^{2+}) for 4 h, but remained unchanged after 72 h of exposure (Bleau et al. 1996). Moreover, Li et al. (2014b) reported that the whole-body T4 level of Chinese rare minnow (*Gobiocypris rarus*) increased after exposure of larvae to 100 and 300 μg/L Hg^{2+} for 4 days, while T3 content was not significantly affected although an increasing trend was found. The apparently contradictory findings may be due to differences in species and developmental stage of fish, and/or differences in dose and duration of Hg^{2+} administration. In fish, THs play an indispensable role in the growth and development, and abnormal TH levels (deficiency or excess) can cause growth retardation (Power et al. 2001). Our parallel paper (Zhang et al. 2016b) showed that $HgCl_2$ exposure caused a decrease in body length of zebrafish larvae. Combined with the correlation analysis results that both T4 and T3 levels were negatively correlated with the body length (data not shown), we suggested that Hg^{2+} induced developmental toxicity might be related to the alteration of TH levels.

To further explore the molecular mechanisms of Hg^{2+}-induced thyroid endocrine toxicity, we examined the transcriptional levels of thyroid-related genes. CRH and TSH secretions act as common regulators of the HPT axis during larvae development in fish, and their gene transcription can be used to evaluate whether environmental chemicals disturb thyroid function (Zoeller et al. 2007; Yu et al. 2011). In the present study, an increase in *crh* and *tshβ* expression levels of Hg^{2+}-treated embryos/larvae was observed, implying that Hg^{2+} activated the HPT axis and disturbed thyroid function. Given the fact that TSH could initiate THs synthesis and release in the thyroid gland, it can be inferred that

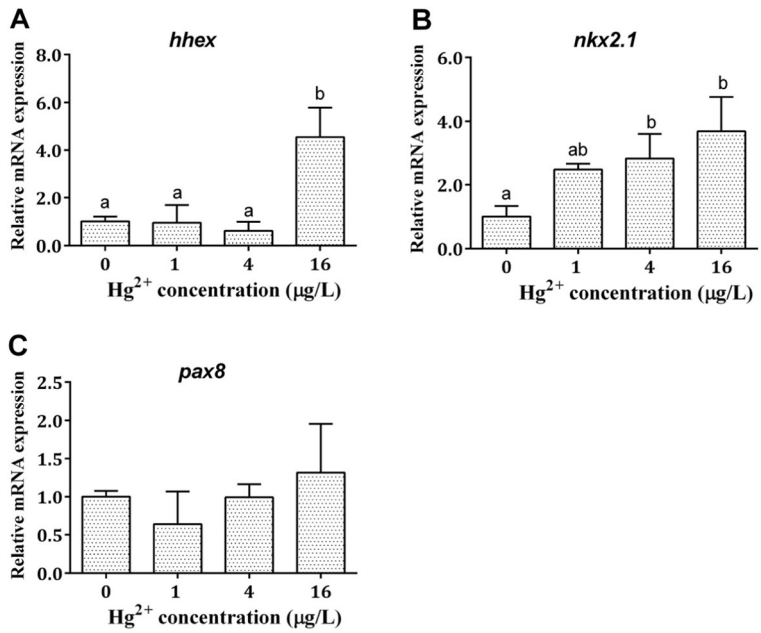

Fig. 3 Effects of waterborne Hg^{2+} exposure on the mRNA expression level of *hhex* (**a**), *nkx2.1* (**b**), and *pax8* (**c**) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included 30 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

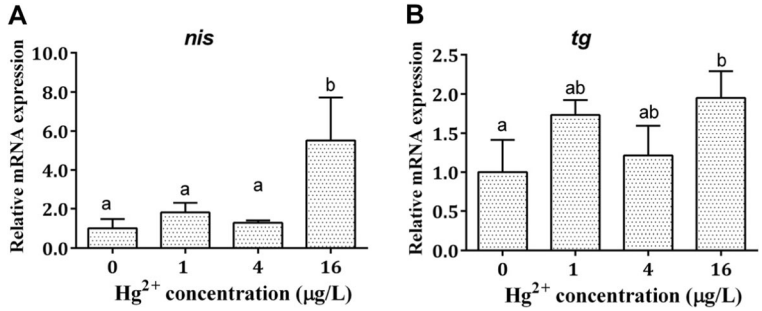

Fig. 4 Effects of waterborne Hg^{2+} exposure on the mRNA expression level of *nis* (**a**) and *tg* (**b**) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included 30 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

the elevation of whole-body T4 and T3 levels in the zebrafish larvae was, at least in part, attributed to the up-regulation of *crh* and *tshβ* expressions.

Thyroid gland development and differentiation during embryogenesis are regulated by several key genes such as *hhex*, *nkx2.1*, and *pax8* (Porazzi et al. 2009; Zucchi et al. 2011). Hhex and Pax8 proteins are essential for the differentiation and formation of thyroid follicles, while the transcription factor Nkx2.1 plays an important role in thyroid development of fish (Wendl et al. 2002; Elsalini et al. 2003). In this study, a significant up-regulation of *hhex* and *nkx2.1* expression and a slight elevation in *pax8* mRNA were found in higher Hg^{2+} concentrations (4 and/or 16 μg/L), probably indicating an induction of thyroid primordium growth and thyroid gland development at embryonic-larval stages of zebrafish under Hg stress (Yu et al. 2010; Zucchi et al. 2011).

It is reported that Nis and Tg are associated with TH synthesis, and the transcriptional level of *nis* and *tg* genes can be used as convenient and sensitive markers for monitoring thyroid activity during development of fish (Manchado et al. 2008; Xie et al. 2015). Nis, a transmembrane glycoprotein, can transport sodium and iodide across the basolateral plasma into thyroid follicle cells to synthesize THs (Dohán and Carrasco 2003). Tg, a dimeric glycoprotein, is secreted by the thyrocyte and stored in the lumen of the thyroid follicle and can be used by the thyroid gland to produce THs (Mercken et al. 1985; Yan et al. 2012). In our study, the expression of *nis* and *tg* was significantly up-regulated by 16 μg/L Hg^{2+} exposure, which might be responsible for the increase in TH production. Likewise, an up-regulation of *tg* expression was also observed in Chinese rare minnow larvae after sublethal exposure to Hg^{2+} (Li et al. 2014b).

In fish, three types of deiodinases have been identified (Deio1, Deio2, and Deio3), playing a key role in the regulation of TH metabolism. Among them, both Deio1 and Deio2 can catalyze the conversion of T4 to active T3 by removing iodine from the outer-ring of T4, while Deio3 converts T4 and T3 to inactive metabolites (Orozco, 2005; Liang et al. 2015; Huang et al. 2016). In this study, *deio2* mRNA level was significantly up-regulated in the highest Hg^{2+} concentration, whereas *deio1* expression showed no significant differences among the treatments, suggesting that *dio1* was not as sensitive as *deio2* for Hg^{2+}-induced thyroid dysfunction. Since Deio1 has a

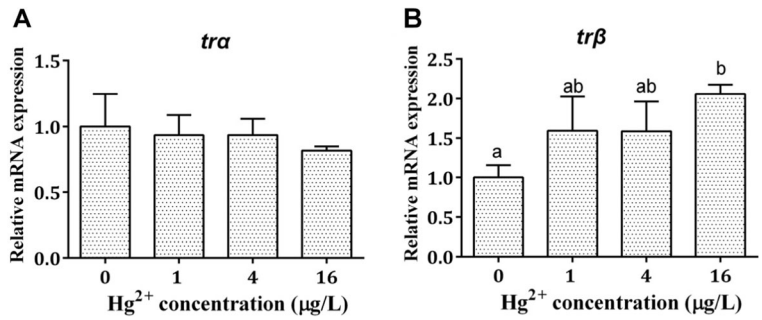

Fig. 5 Effects of waterborne Hg^{2+} exposure on the mRNA expression level of *trα* (**a**) and *trβ* (**b**) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included 30 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

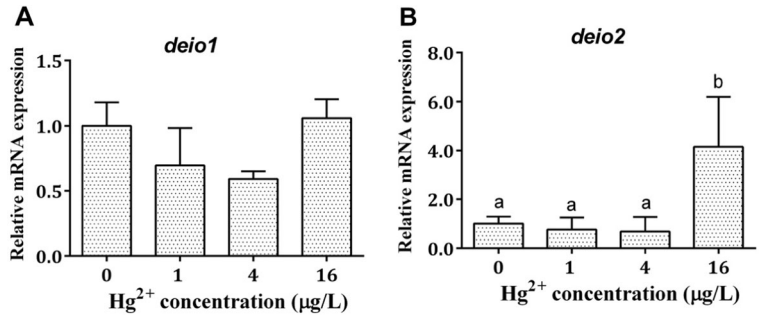

Fig. 6 Effects of waterborne Hg^{2+} exposure on the mRNA expression level of *deio1* (**a**) and *deio2* (**b**) in zebrafish larvae after 168 hpf. Values are mean ± SD of three replicate samples (each sample included 30 larvae). Different letters above bars indicate significant differences among groups ($P < 0.05$)

minimal role in TH homeostasis but is crucial to iodine recovery and TH degradation, and Deio2 plays a vital effect on T3 production at the early embryonic stages for availability of adequate local and systemic T3 (Orozco and Valverde 2005; Van der Geyten et al. 2005; Walpita et al. 2008), it is plausible that the up-regulation of *deio2* and unchanged *deio1* expression might also partially account for the increase of the T3 content.

THs exert their major biological functions by binding to multiple TRs (Power et al. 2001). TRs, members of a large superfamily of nuclear receptors, are encoded by TRα and TRβ genes, playing a crucial role in embryonic and larval development of fish (Liu and Chan 2002). Our study found that Hg^{2+} exposure markedly up-regulated *trβ* mRNA level, but had no significant effect on *trα* expression, which was partly consistent with the study of Li et al. (2014b), where the mRNA transcription of *trα* and *trβ* was both significantly up-regulated in Chinese rare minnow larvae under Hg^{2+} exposure. Here, differential response of expression of *trα* and *trβ* to Hg^{2+} stress suggested that two *tr* subtypes might serve somewhat different functions and *trβ* respond to Hg^{2+}-induced thyroid disruption more efficiently than *trα*. Besides, increased *trβ* expression observed might be attributed to an elevation of T3 content, and abnormal *trβ* transcription could be involved in the disturbance of HPT axis homeostasis (Yu et al. 2013; Li et al. 2014b). Interestingly, TH contents and the expression of the majority of genes showed significant changes only in the highest Hg^{2+} concentration group as compared to the control, which might be related to the mechanism of detoxification and homeostasis of body at low doses. On the other hand, although not significant, the alterations of TH levels and some gene expressions (such as *crh*, *nkx2.1*, and *trβ*) showed an upward tendency with increasing Hg^{2+} concentrations, reflecting the Hg-caused mechanism on the thyroid system.

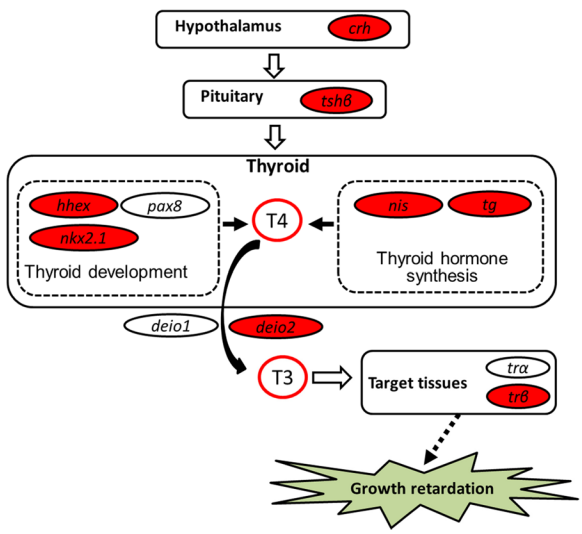

Fig. 7 Schematic diagram of waterborne Hg^{2+} exposure on the thyroid endocrine system in zebrafish larvae after 168 hpf. Genes with red background indicated that the mRNA expression levels of Hg^{2+}-exposed fish were significantly higher than those in the control

In summary, this study demonstrated that exposure of zebrafish embryos/larvae to environmentally relevant concentrations of Hg increased the whole-body TH levels and the transcription levels of genes involved in the HPT axis, indicating that Hg has the ability to act as an endocrine disruptor and could induce thyroid disruption in the early development stage of fish, which might subsequently impair the normal development of zebrafish larvae (Fig. 7). Therefore, the potential risk of thyroid dysfunction induced by Hg is worthy of our vigilance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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