CHRONIC EXPOSURE TO PENTACHLOROPHENOL ALTERS THYROID HORMONES IN ZEBRAFISH

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Introduction

Pentachlorophenol (PCP) is a broad-spectrum biocide that has been widely used in wood preservatives, pesticides and disinfectants. The annual production of PCP increased to 3,000 t in 2003 following the re-emergence of schistosomiasis in several provinces in China. The continued use of PCP throughout the world means it is still likely to result in environment pollution, with potential risks to human health and the environment. Limited information shows that PCP might disrupt thyroid endocrine functions. Epidemiological studies have reported that exposure to low levels of PCP in the environment is associated with decreased TH levels in human neonates.

THs play a crucial role in the regulation of development, growth, immunity, metabolism, reproduction and behavior in vertebrates. In fish, thyroid homeostasis is controlled primarily by the hypothalamic-pituitary-thyroid (HPT) axis. Recently, Zebrafish (*Danio rerio*) thyroid system has become very popular vertebrate model to screen for thyroid-disrupting chemical pollutants. Several studies have suggested that PCP exposure may affect the expression level of genes associated with TH metabolism and signaling in rats and thypothalamic–pituitary–thyroid (HPT) axis in zebrafish larvae; however, the TH endocrine disruption and environmental risk of lower concentration of PCP for adult fish remains unclear. As such, in the present study we have evaluated the effects of lower concentration of PCP exposure on TH homeostasis in zebrafish, using plasma TH levels and gene expression involved in the HPT axis, to gain an improved understanding of PCP's effect on TH function.

Materials and methods

PCP (purity >99%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). It was dissolved in dimethyl sulfoxide (DMSO) and stored at 4ºC. The TRIzol reagent and PrimeScript Reverse Transcription (RT) Reagent kit were purchased from TaKaRa (TaKaRa, Dalian, China), and the SYBR Real-time PCR Master Mix was purchased from Tiangen (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for T3 and T4 were purchased from EIAab Science Co. Ltd. (Wuhan, China). All other chemicals used in the present study were of analytical grade.

After 70 days of exposure, the adult fish were anesthetized in 0.03% MS-222. The body weight was recorded and blood was collected from the caudal vein of each fish. The blood samples from five fish of the same sex were pooled as one replicate (approximately 40 μL). The pooled blood was then centrifuged at 7,000 ×g for 5 min at 4°C, and the plasma was collected and stored at –80°C until analysis. Plasma total T4 (tT4) and T3 (tT3) levels were measured in adult zebrafish as described previously. In brief, plasma TH levels were measured using ELISA test kits (Uscnlife, Wuhan, China) following the manufacturer's instructions. The detection limits, intra-assay and inter-assay variations reported by the manufacturer are 1.2 ng/mL, 4.3%, and 7.5% for tT4, and 0.1 ng/ml, 4.5%, and 7.2% for tT3, respectively.The liver and brain (including hypothalamus and pituitary) were dissected and preserved in TRIzol reagent (TaKaRa, Dalian, China) for RNA sample preparation. Extraction, purification and quantification of total RNA, first-strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) assays were carried out as previously described. Briefly, total RNA was isolated using TRIzol regent, and digested with RNase-free DNaseI (Promega, Madison, WI, USA), following the manufacturer's instructions. The concentration of total RNA was assayed at 260 and 280 nm using a spectrophotometer (M5, Molecular Devices, CA, USA), and the purity of RNA in each sample was verified by determining the A260/A280 ratio and confirmed by agarose-formaldehyde gel electrophoresis with ethidium bromide staining. The purified RNA was used immediately for RT or stored at –80°C until analysis.

Synthesis of first-strand cDNA was performed using a PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China), following the manufacturer's instructions. The qRT-PCR was performed using a SYBR Green PCR kit (Tiangen, Beijing, China) on an Agilent Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The primer sequences of the selected genes were obtained using the online Primer 3 program (http://frodo.wi.mit.edu/) and are shown in Table 1. Genes responsible/involvement for TH synthesis (tshβ), transport (ttr), binding (trβ), and metabolism (dio1, dio2, ugt1ab and sult1 st5) along HPT axis and live were selected (see table 1 for definition of abbreviations). The thermal cycle was set at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min, and a final cycle of 95 °C for 15 s, 60 °C for 1min and 95 °C for 15 s. Gene expression was measured in quadruplicate and repeated three times. The housekeeping gene ribosomal protein L8 (rpl8) did not vary upon chemical exposure (data not shown) and was used as an internal control. The mRNA expression of each gene was normalized to its corresponding rpl8 mRNA level. The relative mRNA expression was determined by the 2–□□Ct method. On the last day of exposure, one hundred randomly selected eggs from each tank were separately cultured in glass dishes, which contained 250 ml of fresh water without PCP exposure until 4 days post-fertilization (dpf). The number of larvae that exhibited malformations, the hatching rate and the survival rate were determined at 4 dpf.

Results and discussion

Body condition in the F0 Generation

No mortality was observed in the control and PCP-exposed groups, and there were no effects on the hepatic-somatic index and brain-somatic index in either male or female fish. The gonadal-somatic index (GSI) was significantly decreased in females exposed to 27 μg/L PCP, but was not significantly different in males or controls. In males, 27 μg/L PCP exposure significantly inhibited body weight (0.29 ± 0.01 g) compared to control (0.31 ± 0.01 g).

Plasma concentration of TH

Exposure to PCP affected the plasma concentration of tT4 in both female and male zebrafish, while caused effects on tT3 concentrations in males only. In females, the plasma tT4 level showed a significant increase (44.7%) in the highest exposure group. In males, the plasma tT4 level was significantly greater than that of controls by 161%, when exposed to 27 □g/L PCP. In females, tT3 levels was not significantly changed after PCP exposure; however, plasma tT3 levels in male zebrafish were significantly decreased by 34.5% and 38.3% when exposed to 9 and 27 □g/L PCP, respectively, compared with control levels.

mRNA expression profile of selected genes

Several genes involved in the regulation, transport, binding and metabolism of THs were examined. In the brain of female zebrafish, tsh□ gene expression was significantly downregulated by 47.7% in the 27 □g/L PCP-exposed group. Similarly, in the males, a concentration-dependent downregulation of tshβ expression was observed, such that increasing concentrations of PCP was associated with incrementally lower tshβ expression. Furthermore, exposure of males to 27 μg/L PCP also caused a significant downregulation of thyroid hormone receptorβ (trβ) (3.63-fold). In the liver, dio1, dio2, ugt1ab, sult1st5 and ttr expression were examined. In females, dio1 expression was significantly decreased in a concentration-dependent manner, while there was a significant upregulation in dio2 expression that was also dose-dependent. Ttr gene expression was significantly upregulated by 2.2-fold in the 27 □g/L PCP-exposed group, and there was a strong dose-dependent increase in hepatic ugt1ab expression with an approximately 24-fold increase in expression observed in the group exposed to 27□g/L PCP. On the other hand, Sult1 st5 expression was significantly downregulated by PCP exposure at all concentrations studied. In males, the expression of dio1 and dio2 were both significantly downregulated by PCP exposure at concentrations of 1, 9 and 27 □g/L. In contrast to female fish, ttr expression was significantly downregulated by PCP in a concentration-dependent manner in the liver of male fish. The expression of ugt1ab was significantly increased in a concentration-dependent manner, with significant increases observed at doses of 1, 9 and 27 □g/L PCP. Furthermore, treatment with the highest concentration of PCP (27 □g/L) significantly upregulated the expression of sult1 st5.

F1 generation toxicological endpoints

In the offspring of PCP-exposed fish, 76% of the control embryos hatched successfully at 4 dpf, and there was no significant difference in the hatching rate observed in any of the PCP-treated groups. The rate of malformation (spinal curvature) was significantly increased to 1.7% and 1.8% after parental exposure to 9 and 27 □g/L PCP respectively, compared with control, while the recorded survival rates of embryos showed no significant differences after 70 days of parental exposure to PCP.

The results demonstrate that PCP significantly alters plasma TH levels, as well as the expression level of selected genes associated with TH metabolism and signaling in the HPT axis and liver. The concentrations of PCP we used in the present study (0.1, 1, and 9 □g/L) have been reported to be prevalent in the aquatic environment, thus the results suggest that environmental concentrations of PCP might have an adverse effect on fish thyroid systems in an aquatic environment, and could affect the development of both adult fish and their offspring. Although the mortality rate of adult zebrafish was not significantly affected by PCP exposure, a significant decrease in body weight was observed in males exposed to 27 μg/L PCP. These data are in agreement with a previous study, which observed significant effects on growth (total length and body weight) in Japanese medaka (Oryzias latipes) with a 28-day exposure to 200 μg/L PCP. In the study, the inhibition of growth in males might be partially explained by the significant decrease in plasma T3 levels, as T3 plays an essential role in the regulation of development and growth in fish. In females, GSI was significantly reduced in the highest exposure group (27 μg/L PCP). The low GSI might be related to disruption of the endocrine pathways regulating reproduction, generally as the hypothalamic–pituitary–gonadal (HPG) axis signaling. The results indicate that low concentrations of PCP have significant effects on growth of zebrafish, and could be endocrine disrupting chemicals.An increase in plasma T4 levels were observed following PCP exposure in fish. In contrast, most studies have found reduced T4 levels with treatment at higher doses of PCP in fish, rats, ewes, and cell lines. It has been shown that PCP has twice the affinity of T4 to TH serum binding TTR, and also affects thyroid hormone metabolism by competitively inhibiting iodothyronine sulfation in vitro. The increases in circulating T4 levels observed in the study may be due to different mechanisms or reflect toxicity of long-term low dose exposure to PCP. Decreased T3 in males and unaltered T3 in females upon exposure to PCP were observed in the study. Although the reason for the sex difference is unclear, a previous study also reported a similar result, in which significant increased tT3 levels and unaltered tT3 levels was observed in PCP-Na-treated female and male rats, respectively. The significant decrease in T3 observed in the study is consistent with previous studies, and the observed decrease in T3, at least in part, can be attributed to the TH metabolism disrupting properties of PCP. Indeed, this observation corresponds with the PCP-induced downregulation of dio2 and upregulation of sult1st5 observed in the liver of male fish. In fish, TSH secretions function as common regulators of the thyroidal axis as feedback mechanisms triggered by changes in the concentration of circulating THs. Interestingly, previous studies have reported that changes in tshβ mRNA levels may be related to alterations in T4 levels in fish. In addition, downregulation of tshβ gene transcription has been related to increased levels of T4 in fish after exposure to PBDEs. Consistent with these results, the study revealed that the reduction in tshβ expression could be explained as a negative feedback response to increased levels of T4. Physiological actions of TH are usually mediated through interaction with nuclear receptors, and TH receptor β (trβ) is one of the main TH receptor isoforms. In the study, trβ gene transcription was significantly downregulated in the male brain and unaltered in female brain. The downregulation of trβ gene expression in the male brain might result from the PCP-induced decline in plasma T3 levels, as the mRNA transcription of trβ was previously reported to be autoinduced in the fish brain by T3. It has been proposed that TTR is a key TH carrier protein that maintains extra thyroidal stores of TH, regulates the supply of TH to various target tissues, and plays an important role in the thyroid axis in fish. Previous work has demonstrated that phenol compounds such as PCP, halogenated phenols, and tetrabromobisphenol A have strong affinities for TTR. In the current study, ttr gene expression levels were significantly downregulated in the male liver and were upregulated in the female. The results confirmed that PCP strongly influences TTR expression, as previously reported. Interestingly, the effect of PCP on ttr expression was different in female and male livers, which might be associated with the different T3 levels observed in male and female fish. In addition to TTR, albumin and thyroxine-binding globulin can also bind to THs in fish plasma. As such, further studies are needed to better understand the different response to PCP exposure between the sexes.Hepatic deiodinases are important regulators of circulating and peripheral TH levels in vertebrates. In fish, there are three dio genes: dio1, dio2 and dio3. Dio1 has a considerable influence on iodine recovery and TH degradation. Dio2 is responsible for the conversion of T4 to T3, allowing adequate availability

of local and systemic T3, and dio3 is a purely inactivating enzyme. The results demonstrate that dio1 was significantly downregulated in the liver of both females and males, and the expression of dio2 was upregulated in females but downregulated in males. Therefore, we suggest that the downregulation of dio1 is partially responsible for the increased T4 concentrations we observed. Similarly, Yu et al. (2011) demonstrated that exposure to PBDEs downregulated mRNA expression of dio1 and increased plasma T4 concentrations in adult zebrafish. On the other hand, the decrease in dio2 expression in males may, at least partly, be associated with the reduced levels of circulating T3, while the upregulation of dio2 expression in females was associated with an increasing trend in T3 levels. The different expression levels of dio2 induced by PCP in male and female fish is consistent with a previous report, which showed that PCP-Na exposure inhibited the dio2 expression of male rats by 79.2%, but did not affect the dio2 expression of female rats. Additionally, previous studies also provide evidence for sex differences in the magnitude of T3-induced relative mRNA responses for dio3 in liver, as well as dio2 and dio3 in the fish brain. Thus, the cause of the sex differences in dio2 expression may be due to the different levels of T3 in females and males. Interestingly, the significant changes in expression of dio1 and dio2 expression may indicate a regulatory role in response to altered THs levels, and confirm that dio2 is the major contributor to TH activation in fish.It has been suggested that uridinediphosphate glucoronosyltransferases (UGT) and sulfotransferases (SULTs) play important roles in TH homeostasis, via the major pathway for T4 conjugation. UGTs play a role in decreasing circulating THs, and upregulation of ugt gene expression or enzyme activities have generally been observed in rats and zebrafish exposed to different chemicals. In the study, increased ugt1ab expression could possibly be explained as an autoregulatory response to increased T4 levels, by increased biliary elimination of the conjugated hormone within the thyroid axis. Sulfonation has been viewed as a key step in TH metabolism, and may increase the hydrophilicity and the biliary excretion of the hormone. Sulfonation reactions are catalyzed by SULTs. In zebrafish, it has been demonstrated that sult1 st5 appears to be the only known enzyme that displays substrate specificity exclusively for THs and their metabolites. In the present study, we found that sult1 st5 expression was downregulated in female livers and upregulated in male livers, in response to PCP exposure. These results are consistent with a previous study in which the expression of sulfotransferase (SULT) in female and male rare minnow liver were different after PCP treatment. We suggest that downregulation of sult1 st5 in females may be partially responsible for the increased T4 concentrations and unchanged T3 levels, and that the upregulation of sult1 st5 in the male liver might play a role in reducing the circulating levels of T3.

In summary, the results demonstrate that PCP can affect thyroid endocrine system at lower concentrations in fish. The TH disrupting effects of environmental levels of PCP was probably associated with altered TH metabolism, as evidenced by marked alterations in the expression levels of dio1, dio2, sult1 st5 and ugt1ab. Interestingly, sex-specific effects on gene expression and plasma t T3were observed. The causes of these sex-specific differences are unclear and require further study. Nevertheless, the results suggest that analysis of the HPT axis might be suitable for determining thyroid endocrine disruption following PCP exposure.

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