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Fluoride caused thyroid endocrine disruption in male zebrafish (*Danio rerio*)

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ABSTRACT

Excessive fluoride in natural water ecosystem has the potential to detrimentally affect thyroid endocrine system, but little is known of such effects or underlying mechanisms in fish. In the present study, we evaluated the effects of fluoride on growth performance, thyroid histopathology, thyroid hormone levels, and gene expressions in the HPT axis in male zebrafish (*Danio rerio*) exposed to different determined concentrations of 0.1, 0.9, 2.0 and 4.1 M of fluoride to investigate the effects of fluoride on thyroid endocrine system and the potential toxic mechanisms caused by fluoride. The results indicated that the growth of the male zebrafish used in the experiments was significantly inhibited, the thyroid microtrastructure was changed, and the levels of T3 and T4 were disturbed in fluoride-exposed male fish. In addition, the expressional profiles of genes in HPT axis displayed alteration. The expressions of all studied genes were significantly increased in all fluoride-exposed male fish after exposure for 45 days. The transcriptional levels of corticotrophin-releasing hormone (CRH), thyroid-stimulating hormone (TSH), thyroglobulin (TG), sodium iodide symporter (NIS), iodothyronine I (DIO1), and thyroid hormone receptor alpha (TRα) were also elevated in all fluoride-exposed male fish after 90 days of exposure, while the inconsistent expressions were found in the mRNA of iodothyronineII (DIO2), UDP glucuronosyltransferase 1 family a, b (UGT1ab), transthyretin (TTR), and thyroid hormone receptor beta (TRβ). These results demonstrated that fluoride could notably inhibit the growth of zebrafish, and significantly affect thyroid endocrine system by changing the microtrastructure of thyroid, altering thyroid hormone levels and endocrine-related gene expressions in male zebrafish. All above indicated that fluoride could pose a great threat to thyroid endocrine system, thus detrimentally affected the normal function of thyroid of male zebrafish.

1. Introduction

Fluoride is widely distributed in the environment as inorganic or organic compounds as a result of its great reactivity. Fluoride plays an important role in the growth and development of humans and animals (Chachra et al., 2010; Chen et al., 2013; Shim et al., 2011). The fluoride levels in unpolluted fresh surface waters generally range from 0.01 to 0.3 mg/L (Camargo, 2003). However, natural and anthropogenic processes cause accumulation of fluoride compounds in surface waters and groundwater reserves, such as a variety of industrial and domestic emissions of easily dissolved fluoride ions, the improper use of agricultural pesticides and fertilizers, the differentiation of natural fluoride minerals, volcanic eruptions, etc., which are the major sources of fluoride pollution in freshwater ecosystems (Camargo, 2003). The fluoride content of groundwater at fluorosis areas in China was 2.3–8.0 mg/L (Zheng et al., 2006). The fluoride content in boiled water was found to be higher than 5 mg/L, even up to 45 mg/L (Ren and Jiao, 1988). Fluoride concentration in industrial wastewater was determined at 96.8 mg/L (Ding et al., 1998), and in extreme cases, up to 3000–5000 mg/L (Wu et al., 2006).

Fish live in the water and can take up fluoride from the water (Tripathi et al., 2009). Excessive fluoride can lead to detrimental effects in fish, such as abundant accumulation in tissues (Moren et al., 2007; Shi et al., 2009a; Yoshitomi et al., 2007), growth inhibition (Chen et al., 2013; Shi et al., 2009b; Yoshitomi and Nagano, 2012), developmental disorder (Camargo, 2003), metabolic disor-

Abbreviations: CRH, corticotrophin-releasing hormone; THS, thyroid-stimulating hormone; TG, thyroglobulin; NIS, sodium iodide symporter; DIO1, iodothyronine I; DIO2, iodothyronineII; TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta; UGT1ab, UDP glucuronosyltransferase 1 family a, b; TTR, transthyretin.

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der (Bajpai and Tripathi, 2010; Chen et al., 2012) behavioral changes (Camargo, 2003; Tripathi et al., 2004), pathological changes and apoptosis (Bhatnagar et al., 2007; Haque et al., 2012; Cao et al., 2013a,b), chromosome aberration and gene mutation (Tripathi et al., 2009). However, very few studies have looked at the toxic effects of fluoride on thyroid endocrine system in fish, such as thyroid hormone (THs), histopathological changes and endocrine-related gene expressions, and the underlying mechanisms of fluoride on the thyroid system have not been clarified.

Thyroid hormones (THs), secreted and released by the thyroid gland, play an important role in the regulation of growth, development, and metabolism in the vertebrates (Jugan et al., 2010). The thyroid homeostasis is regulated by hypothalamus–pituitary–thyroid axis (HPT), which is responsible for the thyroid hormone dynamics by coordinating their synthesis, secretion, transport and metabolism (Carr and Patiño, 2011). In mammals, the HPT axis functions by stimulating thyrotropin-releasing hormone (TRH) from hypothalamus, which regulates the secretion of thyroid-stimulating hormone (TSH) from pituitary. TSH adjusts the synthesis of THs (T3 and T4). In amphibians and teleosts, corticotrophin-releasing hormone (CRH) stimulates the secretion of TSH (De Groef et al., 2006). THs in plasma of fish are bound to transthyretin (TTR), a specific THs transport protein in teleosts (Power et al., 2000), and only free hormones enter into target cells to launch a response. Previous studies showed that environmental pollutants could interfere with thyroid hormonal homeostasis and HPT axis, such as the hormone levels, enzyme activities, and the alterations of gene transcriptions, which have been used to evaluate the effects on thyroid endocrine disruption (Chen et al., 2012; Yu et al., 2010). Moreover, gene expression levels in HPT axis have been used as endpoints to estimate the detrimental effects of environmental pollutants (Hermsen et al., 2012) and provide further insight for clarifying the mechanisms of environmental pollutants. However, the underlying mechanisms of fluoride on thyroid endocrine disruption in male zebrafish have still not been clarified.

Zebrafish are widely used as a predominant ecological toxicological model in the field of life science research because of its advantages, such as small size, high reproductive performance, rapid organogenesis, morphological and physiological similarities to mammals, high sensitivity to the harmful substances, etc., (Segner, 2009). Previous studies indicated that zebrafish are ideal models for endocrine disruption by chemicals in the laboratory (Chen et al., 2012; Kanungo et al., 2012; Liu et al., 2011; Tu et al., 2013). Therefore, in the present study, to remove sex as a factor/variable, the growth performance, histopathological changes of thyroid gland, the levels of THs (T3 and T4), and the gene mRNA profiles in the HPT axis were examined in male zebrafish exposed to different determined concentrations of 0.1, 0.9, 2.0 and 4.1 M fluoride to investigate the effects of fluoride on thyroid endocrine system of zebrafish and the underlying toxic mechanisms caused by fluoride.

2. Materials and methods

2.1. Test animals

Adult male zebrafish three months old (*Danio rerio*) (AB strain), with mean body weight and body length of 0.19 ± 0.03 g and 2.5 ± 0.3 cm, were obtained from Taiyuan fish hatchery in Shanxi Province, PR China. They were kept in a flow-through system with dechlorinated tap water (pH, 7.0–7.4; water temperature, 28 ± 1 °C; light regime, 14-h light and 10-h dark) for 15 days to acclimate to laboratory conditions before exposure. Fish were fed with commercially available adult zebrafish compound feed.

2.2. Fluoride exposure

Approximately six hundred male zebrafish were randomly divide into four groups and exposed to different concentrations of 0 (control), 20, 40, 80 mg/L fluoride (in the form of NaF) dissolved in 120 L of dechlorinated-tap water in 150 L aquaria for 90 days, respectively. Each concentration had three replicates ($n = 50$ for each replicate). Exposure media were renewed every three days. The selected exposure concentrations were previously ascertained by range-finding study and did not result in any obvious deformation or mortality. The controlled conditions for the exposure were: water temperature, 28 ± 1 °C; light period, 14 h light: 10 h dark; pH, 7.0.–7.4; dissolved oxygen, 7.0 ± 0.1 mg/L; total ammonia nitrogen, 1.05 ± 0.12 mg/L; nitrite nitrogen, 0.062 ± 0.012 mg/L; hardness, 20.0 ± 0.35 mg/L (as $CaCO_3$). Heavy metal concentrations in the exposure media were below detection limits and the chemical components met the water quality standard of fisheries (National Environmental Protection Agency, 1990). Fluoride concentrations were monitored daily during the exposure using the fluoride ion selective electrode method (Inkielewicz et al., 2003). Determined fluoride concentrations in each tank remained relatively constant between the 3-days water renewals. The average measured concentrations of fluoride for 0 (control), 20, 40, 80 mg/L were 0.1 ± 0.02, 0.9 ± 0.26, 2.0 ± 1.90 and 4.1 ± 3.90 M, respectively. The fish were handled according to the National Institute of Health Guidelines for the handling and care of experimental animals. All studies were approved by the Laboratory Animal Care and Use Committee of Shanxi Agricultural University, Shanxi of China in 2014.

After exposure for 45 and 90 days, fish were anesthetized in 80 mg/L of MS-222, and the whole body length and body weight of 30 male zebrafish from each treatment group were measured. Then, eighteen fish from each group were sacrificed and the heads were collected. Considering the possible diurnal fluctuations in hormone levels, the fish were sampled between 8:00 and 10:00 a.m. (Leiner et al., 2000). Blood of nine male fish from each group were collected from the caudal vein with chilled heparinized syringes and was kept on ice. Plasma samples were obtained after centrifugation at $2500 \times g$ for 20 min and were stored at −20 °C for hormone assay. Since thyroid follicles are almost invisible, heads including thyroid follicles, hypothalamus and pituitary were sampled for histopathological analysis and gene expression analysis in HPT axis. Nine heads containing thyroid follicles from each group were fixed in Bouin's solution for histopathological analysis. Nine heads from each group were snap-frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

2.3. Determination of growth parameters

Growth performances were determined as follows: weight gain rate (%) = (final body weight−initial body weight)/initial body weight × 100; length gain rate (%) = (final body length− initial body length)/initial body length × 100; specific growth rate (%/d) = (ln final body weight−ln initial body weight)/d × 100; condition factor (K) (g/cm) = weight/length^3 × 100.

2.4. Histopathological analysis of thyroid

After being fixed for 24 h, the head specimens were dehydrated in graded ethanol, embedded in paraffin blocks, and cut on Rotary microtome (Paraffin machine, Leica RM 2245, Germany) at 5 μm. The sections were stained with Delafield's hematoxylin and alcoholic Eosin, dehydrated in graded alcohol, and then mounted in neutral balsam. Thyroid histopathology endpoints, such as follicle area, colloid area, and the height of epithelial cells from photomicrographs were quantified by Image Pro Plus (version 6.0, Media Cybemetics, USA). Thirty follicles were chosen for analysis in each