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Developmental toxicity and thyroid hormone-disrupting effects of 2,4-dichloro-6-nitrophenol in Chinese rare minnow (*Gobiocypris rarus*)

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ABSTRACT

In the present study, to evaluate embryonic toxicity and the thyroid-disrupting effects of 2,4-dichloro-6-nitrophenol (DCNP), embryos and adults of Chinese rare minnow (*Gobiocypris rarus*) were exposed to 2, 20, and 200 μg/L DCNP. In the embryo-larval assay, increased percentages of mortality and occurrence of malformations, decreased percentage of hatching, and decreased body length and body weight were observed after DCNP treatment. Moreover, the whole-body T3 levels were significantly increased at 20 and 200 μg/L treatments, whereas the T4 levels were markedly decreased significantly ($p < 0.05$) for all DCNP concentrations. In the adult fish assay, plasma T3 levels were significantly increased whereas plasma T4 levels were significantly reduced in the fish treated with 20 and 200 μg/L ($p < 0.05$). In addition, DCNP exposure significantly changed the transcription levels of thyroid system related genes, including *dio1*, *dio2*, *me*, *nis*, *tr*, and *ttr*. The increased responsiveness of thyroid hormone and mRNA expression levels of thyroid system related genes suggested that DCNP could disrupt the thyroid hormone synthesis and transport pathways. Therefore, our findings provide new insights of DCNP as a thyroid hormone-disrupting chemical.

1. Introduction

The thyroid hormone system plays a crucial role in the development, somatic growth, metabolism, energy provision, and reproduction of vertebrates (Schnitzler et al., 2012). During the early life stages of fish, thyroid hormones (THs) are essential for larval to juvenile metamorphosis (Blanton and Specker, 2007; Porazzi et al., 2009). Exogenous THs were found to accelerate zebrafish development, growth, and differentiation (Brown, 1997). Conversely, exposure to thyroid hormone-disrupting chemicals (THDCs) inhibited the larval metamorphosis (Crofton, 2008; Miller et al., 2009). However, in the embryos, the relationship between THs and embryonic development is not well documented (Blanton and Specker, 2007). The THs may play an important role during embryonic development since high concentrations of maternal THs have been detected in the embryos (Leatherland et al., 1989). Moreover, *dio2* knock-down in zebrafish embryos induced developmental retardation (Walpita et al., 2009). Given the key role of THs in fish development, it is important to identify which THDCs adversely affect thyroid function.

Exposure to THDCs, including polychlorinated biphenyls, dioxins, phthalates, polybrominated diphenyl ethers (PBDEs), and other halogenated organochlorines, has been shown to interfere with the production, transport, and metabolism of THs (Patrick, 2009). In addition, substituted phenols (which have structural similarity to THs) were also considered to be THDCs. Numerous substituted phenols, including 2,4-dichlorophenol (Ghisari and Bonefeld-Jorgensen, 2009), pentachlorophenol (Dallaire et al., 2009; Kawaguchi et al., 2008; Sugiyama et al., 2005), 4-*n*-

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DCNP, 2,4-dichloro-6-nitrophenol; DCP, 2,4-dichlorophenol; Dio, deiodinase; Dpf, days post-fertilization; EDCs, endocrine-disrupting chemicals; ELISA, enzyme-linked immunosorbent assay; GC–MS, gas chromatography/mass spectrometry; Hpf, hours post-fertilization; HPT axis, hypothalamus-pituitary-thyroid axis; LOQ, limit of quantification; Me, malic dehydrogenase; NIS, sodium-iodide symporter; PBDEs, polybrominated diphenyl ethers; PCP, pentachlorophenol; S.E.M., standard error of the mean; T3, 3,5,3′-triiodothyronine; T4, thyroxine; THDCs, thyroid hormone-disrupting chemicals; THs, thyroid hormones; TPO, thyroperoxidase; TR, thyroid hormone receptor; TRHR, thyrotropin-releasing hormone receptor; TTR, transthyretin.

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nonylphenol (Duffy et al., 2014), and 4-*tert*-octylphenol (Croteau et al., 2009), have the potential to disrupt thyroid endocrine activities at several different pre-receptor points of action (Guo and Zhou, 2013). Substituted phenols released into the environment can disrupt the hypothalamus-pituitary-thyroid (HPT) axis by interfering with TH synthesis, cellular uptake and metabolism due to their structural similarity to THs (Boas et al., 2012). Moreover, there is increasing evidence that thyroid hormone receptors (TRs) and transthyretins (TTRs) may be the targets of some substituted phenols (Carr and Patiño, 2011). Recently, pentachlorophenol (Guo and Zhou, 2013) and BPA (Gentilcore et al., 2013) were found to disrupt thyroid functions by binding to TR and TTR, altering HPT axis-related gene expression. However, the thyroid hormone-disrupting effects of DCNP, a substituted phenol and an organochlorine chemical, are not well documented.

2,4-Dichloro-6-nitrophenol (DCNP) is an environmental transformation product of the ubiquitous 2,4-dichlorophenol (DCP) (Vione et al., 2006). Although DCNP is a rarely produced and used chemical, DCP is a key intermediate in the synthesis of chloride-based herbicides; both of these compounds have been detected in the surface water (Chen et al., 2016). DCNP has been detected in the brackish lagoons of southern France at a concentration of 1.32 μg/L, which is comparable to the concentration of the parent herbicide (4.72 μg/L) (Chiron et al., 2007). The photonitration model to produce DCNP has been reported in previous studies: nitrite and nitrate ions may generate reactive species with sunlight and then attack the aromatic ring in the DCP. (Calza et al., 2008; Maddigapu et al., 2010; Vione et al., 2006, 2007). However, there is a lack of data about the toxicity and thyroid hormone disruption activity of DCNP in fish (Tognazzi et al., 2012).

Therefore, in the present study, to further clarify embryo toxicity and thyroid hormone disruption bu DCNP in fish, embryos of and adult rare minnows were exposed to an environmental concentration (2 μg/L) and two higher concentrations (20 and 200 μg/L) of DCNP. The progression of early development, thyroid hormone levels and the mRNA expression levels of thyroid system-related genes were determined. Chinese rare minnow (*Gobiocypris rarus*) has the characteristics of a small size, short life cycle and prolific egg production, all of which contribute to the increase of the sample number, reduction of the deviation and shortening of the required experimental period (Yuan et al., 2016). The advantages of the optical clarity during embryogenesis and the early larval stages facilitate visual in vivo observation of early developmental processes (Zhu et al., 2014). From fertilization to hatching, the embryonic developmental period of Chinese rare minnow lasts for 72 h at 26 °C. The duration of postembryonic developmental period is 30 days, during which time Chinese rare minnows grow uniformly with a growth rate of 0.52 mm per day. Four months after hatch, Chinese rare minnow could attain sexual maturity (Liang and Zha, 2016). In previous studies, Chinese rare minnows were used as a model to evaluate the endocrine disruption effects of environmental pollutants such as 3-amino-1,2,4-triazole (Li et al., 2009) and benzotriazole (Liang et al., 2014).

2. Materials and methods

2.1. Chemicals

DCNP (CAS no. 609-89-2; purity > 98%) was purchased from Sigma-Aldrich (Chemical Co., Missouri, USA). A stock solution of DCNP was prepared by dilution in acetone (purity > 99.9%; Sinopharm, China). All the resultant exposure media contained 0.01% acetone.

2.2. Test fish and culture conditions

The brood stock of Chinese rare minnows has been used by our laboratory to test chemicals for more than 14 years. The brood stock of Chinese rare minnow was raised in dechlorinated tap water (25 °C) using a flow-through system under a photoperiod of 16:8 h (light:dark). Fish were fed a commercial food pellet (Trea, Germany) and newly hatched brine shrimp (*Artemia nauplii*) twice a day. All experimental procedures involving Chinese rare minnows were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of China.

2.3. Measured concentrations of DCNP

The measured concentrations of DCNP were consistent over the renewal interval (24 h). Quantification of DCNP was conducted by gas chromatography/mass spectrometry (GC–MS). The test solutions were analyzed using the selected ion monitoring (SIM) mode and the retention time locking (RTL) method. For the recovery test, the selected optimized elution solvents of DCNP were used. According to the experimental results, the matrix spike recovery for DCNP is 74.56%. The instrument limit of quantification (LOQs) of DCNP is 10 pg. The analyzed concentrations (mean ± standard deviation; % analyzed/nominal) of DCNP in the test solutions during the exposure period were 1.68 ± 0.39 (84%), 16.3 ± 2.63 (82.5%), 158 ± 21.2 (79%) μg/L. For simplicity, nominal concentrations are used when presenting the results in this paper.

2.4. Embryos exposure to DCNP

Fertilized eggs were examined under a dissecting microscope, and those that had reached the blastula stage (2 h post-fertilization, hpf) were selected for experiments. Approximately 1440 embryos were randomly distributed into aseptic 24-well plates containing 1 ml of DCNP solution (0, 2, 20, or 200 μg/L). Forty wells (three healthy embryos per well) were treated as one group, and there were three groups for each treatment. During the experimental period, the exposure solution was replaced daily, and rare minnow larvae were fed cultured live brine shrimp twice daily. When yolk-sac had nearly disappeared, the rare minnow larvae were first fed at 48 h after hatch. The embryos were observed for 120 continuous hours to determine the percentage of mortality, hatching and occurrence of malformations. Lethal effects, including yolk coagulation and gastrula inactivation, and the malformation types, including yolk sac edema, pericardial edema, axial curvature, uninflated swim bladder and caudal fin malformation, were recorded daily. At 14 days post-fertilization (dpf), the larvae were randomly sampled, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis of gene transcription and THs.

2.5. Adult fish exposure to DCNP

Healthy adult rare minnows (seven months old) from the same pair of brood stock were used in this experiment. These adult rare minnows were kept in 16-L glass aquaria and exposed to various concentrations (0, 2, 20, and 200 μg/L) of DCNP. Experiments were carried out in triplicate for each exposure concentration and each glass aquarium contained 30 adult rare minnows. The exposure solutions were completely replaced once per day. Fish were fed twice a day with newly hatched brine shrimp. After 14 and 28 days of exposure, the fish were removed from experimental tanks, anesthetized with MS-222, and immediately sacrificed following the recording of the fishes' length and weight. The tissues were excised, immediately frozen in liquid nitrogen and stored at −80 °C.