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Effect of low-dose malathion on the gonadal development of adult rare minnow *Gobiocypris rarus*

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ABSTRACT

Malathion is an organophosphorus pesticide that extensively used in agriculture and veterinary practices. To investigate the effects of low dose malathion on rare minnow *Gobiocypris rarus* gonadal development, we exposed adult rare minnow to environmentally relevant concentration malathion (2 and 20 μg/L) for 21 days. Gonadal histology, sex hormone levels and mRNA expressions of steroidogenic genes were investigated. Malathion at both 2 and 20 μg/L significantly up-regulated rare minnow testicular weight and promoted the progression of spermatogenesis. Neither ovarian weight nor process of ovary development was markedly changed. In testis, 2 μg/L malathion significantly down-regulated testosterone and 11-ketotestosterone levels, and up-regulated mRNA expression of steroidogenic genes. In ovaries, 2 and 20 μg/L malathion significantly inhibited estradiol17β levels and induced testosterone levels, both in concentration dependent manners; mRNA expressions of almost all the detected ovarian steroidogenic genes were up-regulated. The present result suggested that malathion even at low dose could pose a potential threat to adult rare minnow gonadal development.

1. Introduction

Organophosphorus (OP) pesticides, among the most ubiquitous synthetic chemicals in our environment, have broad household and agricultural applications worldwide (Buckley et al., 2004; Rohlman et al., 2007). Toxic mechanisms of action of malathion have been attributed to inhibition of cholinesterases (ChEs), mainly AChE (Taylor et al., 1995). However, later studies showed that many OP pesticides including malathion could interfere with hormone levels through activating estrogen, androgen and other hormone receptors (McKinlay et al., 2008; Mnif et al., 2011). It has been reported that exposure to malathion could result in fertility decline, miscarriages, genetic damage, and birth defects in humans (Rutledge, 1992; Griffin, 1999; Penna-Videau et al., 2012) Increasing number and amount of industrial, agricultural and commercial chemicals discharged into aquatic environment lead to various deleterious effects on aquatic organisms (McGlashan and Hughies, 2001).

Fish could accumulate pollutants directly from contaminated water or indirectly via the food chain (Sasaki et al., 1997). In catfish *Clarias batrachus*, 4 mg/L malathion down-regulated plasma estrone, estradiol-17β (E2) and testosterone (T) levels (Singh and Singh, 1987). Malathion at 3 and 9 mg/L resulted in decreased sex hormone levels, degenerated testicular lobules and atretic oocytes in eel *Monopterus albus* (Singh, 1993). Exposing the laboratory acclimated fish caught in wild to malathion at 0.123 and 1.23 mg/L decreased the sex hormone levels, body weight and ovarian weight (Lal et al., 2013).

However, up to present, most of the studies focused on the malathion's effect on fish at relatively high levels (mg/L or mg/kg). However, in aquatic environment, malathion is distributed at low concentrations (μg/L) (Newhart, 2006). For example, malathion concentrations in waters were ranging from 39.1 to 50.3 μg/L in Ventura County, America (Ando et al., 1996). Segawa et al., (1991) found an average of 49.4 μg/L malathion in freshwater ponds immediately after application. Malathion was reported at 11.1 and 2.62 μg/L in a freshwater channel in California, America (Ando et al., 1996). In Qazvin, Iran, a maximum concentration of 18.12 μg/L malathion was detected (Karyab et al., 2013). However, only a few studies focused on the effect of low dose malathion on fish reproduction. Prathibha et al. (2014) found that 1 and 10 μg/L malathion affected transcript levels of various reproductive related genes, slowed progression of spermatogenesis in testis and increased oocytes with oil droplets in 50 days post hatch catfish. Therefore, to better understand the effect of low dose malathion on fish reproduction, more studies are still required.

It is a known fact that fish gonadal development is closely

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related to sex steroid hormones such as E2, T and 11-ketotestosterone (11 KT) (Kazeto and Kazeto, 2008). Steroidogenc enzymes such as steroidogenic acute regulatory protein (Star), hydroxy-delta-5-steroid dehydrogenase, 3 beta (Hsd3b), cytochrome P450, subfamily XIA, polypeptide 1 (Cyp11a1), Cyp17a1, and Cyp19a1a all play essential roles in sex steroid hormone biosynthesis (Payne and Hales, 2004). Rare minnow *Gobiocypris rarus*, a Chinese freshwater cyprinid, is distributed in the upstream of the Yangze River, Sichuan Province, China. Due to its small size, rapid embryonic development (3 days at 25 °C), short generation time (about 4 months), high fertilization and hatching rates and sensitivity to chemicals, rare minnow is a promising animal model for aquatic toxicology research (Zhong et al., 2005). In the present study, both adult male and female rare minnow were exposed to 2 and 20 μg/L malathion for 21 days, and gonadal histology, sex hormone levels, and mRNA expression of steroidogenic genes were assayed. We aimed to provide more information for the reproduction disrupting effect of low dose malathion.

2. Materials and methods

2.1. Ethics statement

This study has been carried out in accordance with the regulations on experimental animals of Management methods of Laboratory Animals in Shaanxi Province, China (No. 150, 2011). During the whole experiment, fish were humanly treated. Before sacrificed, all fish were anesthetized using tricaine methane sulphonate (MS-222), and every effort was made to minimize suffering. All experimental procedures were approved by the Animal Ethics Committee of Northwest A&F University.

2.2. Animals and malathion exposure

Rare minnow were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan city, Hubei province, China). They were raised in glass tanks with dechlorinated tap water with a 14 h/10 h light/dark cycle and fed with chironomid larvae twice a day. After 14-days acclimation, 6-months old rare minnow were randomly selected and exposed to 2 and 20 μg L^{-1} malathion (Sigma Chemicals, Inc. St. Louis, MO, USA) or the solvent control with 0.001% DMSO (Sigma Chemicals, Inc. St. Louis, MO, USA) in 30 L glass tanks for 21 days. There were 30 individuals in each treatment group (10 individuals per tank in triplicate at the same time). 90 fish (45 males and 45 females) in total were used in this study. Half of the water in each tank was replaced daily with fresh dechlorinated tap water dosed with the appropriate amount of malathion.

2.3. Morphometry, gonadosomatic index and gonadal histology

Following exposure, fish were euthanized with MS-222 (500 mg L^{-1} buffered with 200 mg L^{-1} $NaHCO_3$), and drained on filter paper and the body weight and length were measured using sensitive balance (accuracy: 0.0001 g) and caliper (accuracy: 0.0001 cm) respectively. Following sampling, the gonads were weighed for calculation of the gonadosomatic index (GSI = 100 × gonad weight/body weight) (Ankley et al., 2001). Half gonadal tissue of each fish was fixed with 4% paraformaldehyde solution at 4 °C (15 fish in total, 5 per replicate). 40 h later, the tissues were dehydrated through ascending grades of alcohol series, cleared in xylene and embedded in paraffin. Six μm-thick paraffin sections were cut with a rotary microtome (Leica RM2235, Leica Microsystems) and stained by haematoxylin eosin (HE) methods. Microscopic examination was carried out using an Olympus CHC binocular microscope and photography was done with a Motic Digital Microscope. Number of germ cell on different development stage was measured using Toupview software.

2.4. RNA extraction and reverse transcription

Half gonadal sample of each fish was homogenized in TRIZOL (Invitrogen) and total RNAs were isolated. RNA integrity was checked by analyzing 28S ribosomal RNA (rRNA) and 18S rRNA ratios with 1% agarose gel eletrophoresis. The concentration and purity of isolated RNA were assessed by the spectrophotometric method with a nanodrop spectrophotometer (Thermo Electron Corporation, USA). Total RNAs were further treated with RNase-free DNase I (Promega, USA) to remove genomic contamination. The cDNAs were synthesized from 3 μg total RNA with M-MLV reverse transcriptase (Invitrogen) and oligo $(dT)_{18}$ primer in a 20 μL final reaction volume as previous description (Liu et al., 2012).

2.5. Detection mRNA expression of target genes following malathion exposure

Quantitative real-time PCR (qRT-PCR) was used to evaluate mRNA expression profiles of rare minnow *star*, *hsd3b*, *cyp11a1*, *cyp17a1*, and *cyp19a1a* in the gonadal tissues. At the same time, mRNA expressions of the commonly used housekeeper gene including beta-actin (*actb*), tubulin (*tub*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were also analyzed. Reference gene expression values were analyzed on the website http://www.leonxie.com/referencegene.php to select the most stable one as the reference gene in the present study. qRT-PCR was performed as our previous description (Zhang et al., 2014). The primers were referenced to our previous study (Zhang et al., 2014) and listed in Table S1. All genes were analyzed on 15 fish (5 fish per tank in triplicate). The relative transcript changes under BPA exposure were calculated using $2^{\Delta\Delta Cq}$ method with the formula $F=2^{\Delta\Delta Cq}$, $\Delta\Delta C_q=(C_q$, target gene – C_q, reference gene) $_{malathion}$ – (Cq, target gene – Cq, reference gene) $_{control}$ (Livak and Schmittgen, 2001).

2.6. Detecting steroid hormone levels

Fifteen gonads (5 fish from each triplicate tank) from each treatment group were homogenized using phosphate buffer solution (pH = 7.4). The homogenates were used to evaluate E2, T and 11 KT by ELISA method. Concentrations of rare minnow E2, T and 11 KT in homogenate samples were normalized to protein concentrations of the corresponding samples, which were assayed using the commercial protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing China).

2.7. Data analysis

All data were expressed as mean ± standard error of the mean (SEM) and significant differences were analyzed by one-way ANOVA. Data were tested for normality of distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) prior to analysis. Data that did not meet assumptions of normality and homoscedasticity were transformed (log) and then analyzed by one-way ANOVA. In the data sets for which there was a significant difference, a Tukey posthoc test was carried out ($P < 0.05$). *P*-values less than 0.05 were considered to be significant. Analyses were performed with SPSS Statistics 17.0.