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Exposure to monocrotophos pesticide during sexual development causes the feminization/demasculinization of the reproductive traits and a reduction in the reproductive success of male guppies (*Poecilia reticulata*)

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ABSTRACT

Monocrotophos is a highly toxic organophosphorus pesticide that has been confirmed to be an endocrine-disrupting chemical. To evaluate the influence of this pollutant on the reproductive system of male fish, we studied the sex steroid levels, reproductive traits, sex ratio, and reproductive success in male guppies (*Poecilia reticulata*) exposed to 40% monocrotophos pesticide at the nominal concentrations of 0.01, 0.10, and 1.00 mg/L for 90 days from birth to adulthood in a semi-static exposure system. Radioimmunoassay and western blot analyses demonstrated that the long-term exposure to monocrotophos pesticide during the sexual development of male guppies caused a significant increase in 17β-estradiol levels and consequently induced vitellogenin synthesis, suggesting the feminization of the males. Monocrotophos pesticide also caused a significant decrease in testosterone levels, which consequently inhibited testis growth and reduced the sperm count and the area and intensity of their sexually attractive orange spots, which collectively indicated the significant demasculinization of the male sexual characteristics. Furthermore, these changes in the sexual characteristics at the cellular and organ levels translated into ecologically important effects on the reproductive success at the individual level, as measured by a decrease in offspring production and survival rate. The present study provides the first evidence that monocrotophos pesticide can cause severe reproductive abnormalities in fish due to its endocrine-disrupting action.

Introduction

Monocrotophos (MCP, CAS number 6923-22-4) is listed as a United Nations Environment Program Prior Informed Consent chemical, and it is still extensively used in agriculture in developing countries, such as China, Pakistan, and Kuwait. High levels of pesticide residue have led to excessive MCP levels in the environment and several incidents of contamination. Waste effluent was collected from a factory manufacturing the MCP pesticide near Pune, India, and the MCP content ranged from 0 to 125 mg/L (Bhadbhade et al., 2002). Tariq et al. (2004) reported the presence of MCP in the shallow groundwater of four intensive cotton–growing districts in Pakistan, with the highest detected concentration of 8.3 μg/L. Additionally, 4 μg/L and 0.165 μg/L MCP were detected in rain water in India and in source water in China, respectively (Kang and Zhang, 2000; Kumari et al., 2007). MCP residue concentrations were 0.4 mg/kg and 0.06 mg/kg in vegetables from India and Ghana, respectively (Arora, 2009; Darko and Akoto, 2008; Kumari et al., 2004), and MCP contamination was also detected in food from Kuwait and China (Pan et al., 2008; Sawaya et al., 1999). MCP was reported to be responsible for deaths of Swainson's hawks (*Buteo swainsoni*) in Argentina (Goldstein et al., 1999). MCP is an organophosphorus pesticide with high cytotoxicity (Bing et al., 2002) and genotoxicity (Saleha Banu et al., 2001). In our previous study, it was demonstrated that both vitellogenin (VTG) mRNA expression and secretion were significantly induced in male goldfish exposed to an MCP-based pesticide, suggesting that MCP pesticide has significant estrogenic properties and is therefore a potential endocrine-disrupting chemical (EDC) (Tian et al., 2009). We further proved that MCP pesticide exerts its estrogenic effects via interfering with the reproductive axis at multiple sites and consequently causing an increase in 17β-estradiol (E_2) plasma levels and a decrease in plasma testosterone (T) concentrations (Tian et al., 2010).

Because sex steroids play a main role in the regulation of sex differentiation, sexual characteristics, reproductive behavior, and reproductive capacity, the EDCs that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones can cause a range of reproductive abnormalities in males, such as the induction of VTG synthesis (Sumpter and Jobling, 1995), a reduction in testis size (Cardinali et al., 2004; Gimeno et al., 1996;

Abbreviations: CI, Coloration index; EDC, endocrine-disrupting chemical; E_2, 17β-estradiol; HSI, hepatosomatic index; GSI, gonadosomatic index; GPI, gonopodial index; MCP, monocrotophos; T, testosterone; VTG, vitellogenin.

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Kinnberg et al., 2000), morphologically altered testis (Kinnberg et al., 2000), diminished secondary sex characteristics (Baatrup and Junge, 2001), delayed sexual maturity (McMaster et al., 1991), and, with further severity, sex reversal (Bayley et al., 2002; Nielsen and Baatrup, 2006; Toft and Baatrup, 2003). Considering the significant effects of MCP pesticide on the estrogen and androgen levels in male fish, attention should be paid to the influence of this pesticide on the male reproductive system.

Therefore, we conducted a systematic study on the reproductive toxicology of MCP pesticide in male guppies with the aim of evaluating the adverse effects and intrinsic toxicological properties of MCP pesticide using reproductive traits (including VTG synthesis and sexual characteristics), sex ratio, and reproductive success as apical endpoints. The guppy was chosen to be the experimental model for several reasons. Guppies are live-bearing fish with a short reproductive period whose sperm count, body coloration, gonad size, sexual behavior, and reproductive rate are independent of season (Houde, 1997). Gonad differentiation and the development of secondary sexual characteristics occur during the juvenile period, and the fish sexually mature in approximately three months. The adult male has bright coloration and performs distinct courtship behaviors, his anal fin is developed into a copulatory organ (the gonopodium) for internal fertilization, and the sperm ejaculates can be isolated for sperm counting without harming the fish (Matthews et al., 1997). Hence, the male guppies are suitable for studying the effects of MCP pesticide on the reproductive system.

Guppies were treated with a long-term exposure of MCP pesticide during the entire vulnerable juvenile period of gonad development to investigate if MCP pesticide would induce an imbalance of the androgen and estrogen levels, thereby disturbing the normal development of the male reproductive traits and influencing the sex differentiation and reproductive success in the adult male guppies. First, the response of testicular E_2 and T to MCP pesticide in the male guppies was quantified to determine if the same endocrine-disrupting effects of MCP pesticide on male goldfish that were seen in a previous study also exist in male guppies. Second, the hepatic synthesis of VTG in the male guppies in response to the MCP pesticide exposure was determined. Third, a series of biomarkers was selected to determine the influences of MCP pesticide on the primary and secondary sexual characteristics, including the number of ejaculated sperm cells at the cellular level, testis size (gonadosomatic index, GSI), length of the gonopodium (gonopodial index, GPI), and body coloration (coloration index, CI) at the organ level. Finally, the effects of MCP pesticide on the sex ratio, offspring production, and survival rate were analyzed to determine if the abovementioned changes in the sexual characteristics at the cellular and organ levels translated into a disturbance of the sex differentiation and/or reproductive success of the individuals.

Materials and methods

Fish exposure and sample protocol. An MCP-based pesticide (3-hydroxyl-N-methyl-*cis*-crotonamide dimethyl phosphate, 40% water-soluble preparation) was purchased from the Qingdao pesticide factory in China. The concentration given on the label was 40%, which was consistent with the actual concentration determined by gas chromatography in our previous study (40 ± 0.1%) (Ru et al., 2003). The half-life of MCP is approximately 66 days at pH 7.0 and 20 °C (Wang and Zhang, 1989).

To obtain newborn experimental fish, adult Red Albino guppies (*Poecilia reticulata*) from a local dealer in Qingdao, China were used as a broodstock. The breeders were maintained in 50-L aquaria, and cylindrical net enclosures were placed in the maternity aquaria as juvenile refuges to minimize the risk of the adult cannibalism of the newborn guppies. The newborn guppies were removed daily from the breeding aquaria and were randomly distributed to four groups over a three-day period until each group contained 48 young fish. Three groups were exposed to MCP pesticide at nominal concentrations of 0.01, 0.10, and 1.00 mg/L, which are 1/10,000, 1/1000, and 1/100 of the 96-h LC_{50} (about 100 mg/L; our unpublished results), respectively. A control group (dechlorinated tap water) completed the exposure design. Each group of fish ($n = 48$) was kept in four 10-L aquaria (12 fish/tank) containing 6 L dechlorinated tap water and the appropriate MCP dose using a semi-static toxicity test (3 L water was renewed daily to keep the MCP concentration constant). The water temperature was maintained at 25 ± 2 °C, the dissolved oxygen content was 7.0 ± 0.1 mg/L, the pH was 7.6 ± 0.2, and the photoperiod was 14 h light and 10 h dark. During the experiment, the guppies were fed twice daily with freshly hatched *Artemia nauplii* and commercial fish feed. The remaining food and feces were removed daily. From the ninth week of the exposure to MCP pesticide, the aquaria were monitored three times daily for newly hatched offspring.

After 90 days of exposure, the guppies were anesthetized in MS–222 (Sigma-Aldrich, St. Louis, MO, USA). The gender was identified for the calculation of sex ratio. Twenty males from each group were randomly selected for the subsequent experiments. The body length and body weight were recorded, and the condition factor was calculated as follows: condition factor = 100 × body weight (g)/body length $(cm)^3$. After the fish were imaged for the subsequent determination of the GPI and CI, the sperm cells were collected to determine the sperm count. The skin, testis, and liver were each dissected. The skin was sampled to determine the carotenoid content. The testis was weighed to determine the GSI and then immediately frozen in liquid nitrogen and stored at −80 °C for the measurement of the sex steroids. The liver tissue was weighed to calculate the hepatosomatic index (HSI) and was immediately frozen in liquid nitrogen and was stored at −80 °C for the detection of VTG. The experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Ocean University of China.

The detection of the testicular sex steroid levels by radioimmunoassay. The testes from five fish were homogenized together in 5 μL Tris–HCl buffer (0.1 M, pH 7.4) per 1 mg testis tissue. After centrifugation at 600 ×*g* for 30 min at 4 °C, the supernatant was collected, extracted with diethyl ether, concentrated under a flow of nitrogen to remove the diethyl ether, and re-dissolved in Tris–HCl buffer for the measurement of the sex steroids.

The E_2 and T levels were detected by radioimmunoassay using commercial kits from the Tianjin Nine Tripods Medical and Bioengineering Co., Ltd, China. The hormone levels were measured according to the manufacturer's instructions. The assay detection limits were 0.50 pg/mL for E_2 and 0.10 ng/mL for T. For both E_2 and T, the inter- and intra-assay coefficients of variation were <8%. The cross-reactivities of the antibody for E_2 to estriol, progesterone, estrone, T, and cholesterol were 9.0×10^{-2}%, 1.0×10^{-2}%, 7.0×10^{-1}%, 1.0×10^{-2}%, and 1.0×10^{-3}%, respectively. The cross-reactivities of the antibody for T to dihydrotestosterone, estriol, E_2, androstenedione, and progesterone were 1.6×10^{-2}%, 5.2×10^{-17}%, 5.0×10^{-2}%, 2.1×10^{-4}%, and 5.0×10^{-12}%, respectively.

The determination of the hepatic VTG levels by western blot analysis. The body weight and the wet weight of the isolated liver were measured. The HSI was calculated as the percentage of the liver weight to the body weight. The presence of VTG in the liver samples of the male guppies was verified by western blot analysis using a polyclonal antiserum against goldfish VTG prepared by our laboratory, which can bind to VTG produced by other teleosts, such as zebrafish (*Danio rerio*), red drum (*Sciaenops ocellatus*), and red sea bream (*Pagrosomus major*) (Li, 2006; Shi, 2007). The livers of the untreated females were used as a positive control. Six livers from each group were homogenized in 4 μL homogenization buffer (50 mM Tris–HCl, 0.02% aprotinin, and 1 mM phenylmethanesulfonyl fluoride) per