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Chlorpyrifos is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in zebrafish

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ABSTRACT

The potential interference of endocrine disrupting chemicals (EDCs) on aquatic animals and humans has drawn wide attention in recent years. Reports have shown that some organophosphorus pesticides were a kind of EDCs, but their effects on fish species are still under research. In present study, flow cytometry data of HEC-1B cell line showed that chlorpyrifos (CPF) could increase cell proliferation index like 17β-estradiol (E2), but the effect of CPF was weaker than of E2 in the same concentration. Moreover, CPF altered the expression pattern of estrogen-responsive gene VTG and ERα in zebrafish embryos. When exposed to CPF at various concentrations (0, 0.10, 0.25, 0.50, 0.75 and 1.00 mg/L) for 48 h during the embryo stage, compared with controls, the hatching rate of treated groups significantly increased at the same time and the hatching rate of embryos was proportional to CPF concentration. The mRNA expression levels of c-myc, cyclin D1, Bax and Bcl-2, which are closely related to cell proliferation and cell apoptosis, were disturbed by CPF in zebrafish embryos after exposure treated for 48 h. In addition, acridine orange (AO) staining of zebrafish embryos showed that cell apoptosis was appeared in the 0.75, 1.00 mg/L CPF treated groups. Taken together, the results obtained in the present study indicated that chlorpyrifos is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in zebrafish.

1. Introduction

During the last decade, a vast amount of researches have shown that endocrine disrupting chemicals (EDCs), which have caused increasing concern of academia, are closely related to human reproductive health, abnormal development, metabolic disorders and even some cancers (such as breast cancer, testicular cancer) [1,2]. EDCs are substances in our environment, food, and consumer products that interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction [3]. Reported EDCs include a wide range of compounds, such as pesticides, detergents and surfactants, phthalates, alkylphenols, and natural or synthetic estrogens [4,5]. Thereinto, the serious pollution made by uncontrolled use of organophosphorus (OP) pesticide has been a great public health concern all over the world.

Chlorpyrifos (CPF) is a OP pesticide product widely used in the US, China and other countries to manage insect pests on many agricultural crops including tree fruits and nuts, vines, vegetables, corn and soybeans [6]. It has become one of the most widely studied pesticides from both human health and environmental perspectives since the prohibiting of high toxicity pesticides. The primary target organ for chlorpyrifos toxicity is the central and peripheral nervous system [7], due to the ability of the chlorpyrifos to inhibit the enzyme activity of acetylcholinesterase [8]. In 2007, the Unite States Environmental Protection Agency (US EPA) released a draft initial list of 73 chemicals for Endocrine Disruptor screening which included the classic OP insecticide CPF [9]. The World Health Organization's International Program on Chemical Safety defines: An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (International Program on Chemical Safety. Global Assessment of Endocrine Disrupting Chemicals. Available at: http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/). However, few of reports gave such adverse effects of CPF. Reports showed that CPF was recognized as an endocrine disruptor since it has been demonstrated to possess the ability to interfere with the ERβ mRNA steady state level [10,11]. It was also proved that CPF has antiandrogenic activity and showed a significant decrease in testosterone biosynthesis [12]. These results showed that the CPF worked like EDCs, but it still needs more evidence to prove that CPF is an endocrine disruptor. In this study, human endometrial carcinoma cell line HEC-1B and

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estrogen-response genes of zebrafish were selected for evaluating the effects of chlorpyrifos.

Zebrafish is a small freshwater fish with advantages of the species size, short life cycle, high fecundity and easily observed for experimental bioassays [13]. By now, zebrafish has been well recognized as a model vertebrate species for developmental and ecotoxicology studies [14,15]. For many toxic agents, such as OP pesticides, aquatic environments serve as the major route of distribution [16]. The impact of degradation-resistant pesticides in aquatic environment on wild-animals and humans cannot be ignored. Numerous reports have shown the adverse effects of CPF on the embryonic development and larval growth of aquatic organisms [17–20] because of the lower mobility to avoid contamination early on in the life cycle of these two stages. The exposure of CPF on embryonic zebrafish can result in high rate of malformation and death. However, the molecular mechanisms by which CPF induced their toxicity during zebrafish development remain largely unknown. In present study, the hatching rate of 48 h post-fertilization (hpf) was significantly increased in the CPF treated groups compared to the control. This result indicated that the CPF interrupted normal embryonic development and made the hatching earlier.

The present study was designed to investigate the impact of CPF on zebrafish embryonic development. First of all, we demonstrated that CPF was estrogenic by flow cytometry analysis and estrogen marker genes expression pattern analysis. Then, we found that CPF disturbed the embryonic incubation of zebrafish. Finally, the results of gene expressions and AO staining showed that CPF altered the cell proliferation and apoptosis in zebrafish.

2. Materials and methods

2.1. Chemicals

The organophosphorus pesticide chlorpyrifos (CPF) was obtained from Tiancheng Biotechnology company (Shandong, China), with a purity of $\geqslant$98%. 17β-Estradiol (E2), acridine orange (AO) and propidium iodide (PI) were purchased from sigma–Aldrich. E2 was used for cell cycling analysis. AO and PI were dissolved in PBS for staining analysis. All of the other chemicals utilized in this study were of analytical grade.

2.2. Tumor cells maintenance and disposition

Human endometrial cancer cell line HEC-1B was obtained from Chinese Academy of Medical Sciences tumor cell bank in our research. HEC-1B cells were cultivated in growth medium MEM + 10% FBS + 4% antibiotics at 37 °C, 95% air, 5% CO_2. Cells were then washed in PBS buffer and cultivated in growth medium without phenolsulfonphthalein to exhaust the endogenous estrogen 4 days before the treatment. CPF-1 (0.05 mg/L), CPF-2 (0.01 mg/L), CPF-3 (0.005 mg/L) and E2 (0.05 mg/L) were added to growth medium respectively in triplicate. Cells were collected and fixed 2 days later after exposure treatment.

2.3. HEC-1B cell proliferation analysis

To test the potential effects of CPF, a widely utilized stain, propidium iodide, was used to measure cell proliferation. HEC-1B cells with trypsin enzyme digesting was collected and immobilized by ice-chilled 70% ethanol overnight at 4 °C. Cells were then stained following the manufacturer's protocol (BD Biosciences) and washed twice in PBS to prepare single cell suspension. Flow cytometry data was acquired on a FACSJazz (Becton Dickson, USA) and analyzed using FlowJow 7.6.1. Debris was eliminated by gating based on FSC-H and SSC-H parameters. Gates were defined based on differences in fluorescence levels between treatments and controls.

2.4. Zebrafish care

AB line wild-type zebrafish were purchased from China Zebrafish Resource Center (CZRC, China) and raised under our protocol. Briefly, fish were kept in zebrafish breeding system (ESEN, China) with charcoal-filtered tap water at 28 ± 0.5 °C in a 14:10 h light:dark cycle. The fish were fed thrice daily with *Artemia nauplii*. Spawning adults in groups of 2 males and 1 female in a tank were prepared for collecting fertilized eggs. Normally developed embryos (without malformation) at 0.5–1.0 hpf were selected and randomly distributed among the disposable dishes (50 embryos per dish) containing 40 mL fish buffer.

2.5. Zebrafish embryo toxicity test

In our study, exposure solution of zebrafish embryo was renewed every 12 h for maintaining a stable CPF exposure concentration. 6 gradient concentrations of CPF (0, 0.10, 0.25, 0.50, 0.75 and 1.00 mg/L) were added in disposable dishes containing 50 normal embryos. Each concentration was exposed in triplicate. Because of the poor water solubility of CPF, organic solvent acetone was used as solubilizing assistant. The maximum concentration of acetone in exposure was 0.1% and our previous study (unpublished) has shown that acetone concentration under 0.1% did not sway the results.

2.6. Zebrafish embryo morphological observation

Anatomical structures to determine morphological developments were recorded as previously described by [21] with modifications. The zebrafish embryos were observed through a microscope (Olympus, Japan) every 12 h after CPF exposure. The embryos were put on double concave slide containing 3% cellulose and the hatching rate, deformation rate and death rates were recorded for statistical analysis.

2.7. Gene expression

Embryos were collected and pre-served in TRIzol reagent (Invitrogen, NJ, USA) at −80 °C after exposure to CPF in this study. Each concentration was measured in triplicate with a composite of 30 embryos. Using TRIzol regent to isolate total RNA, and concentrations of total RNA were estimated at 260 nm and the quality was verified by measuring the 260/280 nm ratio. To further verify the quality of total RNA, 0.8% gel electrophoresis with ethidium bromide staining was also used. First-strand cDNA synthesis was performed using commercial kits (PrimeScript™ RT reagent Kit with gDNA Eraser, Takara, Japan) following the manufacturer's instructions. IQ5 Multicolor Real-Time PCR Detection System (Bio-rad, USA) was used to analyze mRNA expression level. Because the *β-actin* expression levels were not significantly different between control and CPF treated groups, *β-actin* was chosen as an internal control to normalize the data in this study. Primer sequences were designed using Primer Premier 5 software (Table 1). The thermal cycle was set at 95 °C for 5 min, followed by 35–45 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min. Transcript expression of target genes were obtained by $2^{-\Delta\Delta Ct}$ method and measured in triplicate.