Fish & Shellfish Immunology 43 (2015) 405–414

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

The toxicity of chlorpyrifos on the early life stage of zebrafish: A survey on the endpoints at development, locomotor behavior, oxidative stress and immunotoxicity

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ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form
10 January 2015
Accepted 19 January 2015
Available online 26 January 2015

Keywords:
Chlorpyrifos
Neurotoxicity
Oxidative stress
Immunotoxicity
Zebrafish

ABSTRACT

Chlorpyrifos (CPF) is one of the most toxic pesticides in aquatic ecosystem, but its toxicity mechanisms to fish are still not fully understood. This study examined the toxicity targets of CPF in early life stage of zebrafish on the endpoints at developmental toxicity, neurotoxicity, oxidative stress and immunotoxicity. Firstly, CPF exposure decreased the body length, inhibited the hatchability and heart rate, and resulted in a number of morphological abnormalities, primarily spinal deformities (SD) and pericardial edema (PE), in larval zebrafish. Secondly, the free swimming activities and the swimming behaviors of the larvae in response to the stimulation of light-to-dark photoperiod transition were significantly influenced by the exposure to 100 and 300 μg/L CPF. In addition, the activity of acetylcholinesterase (AChE) and the transcription of some genes related to neurotoxicity were also influenced by CPF exposure. Thirdly, CPF exposure induced oxidative stress in the larval zebrafish. The malondialdehyde (MDA) levels increased and the glutathione (GSH) contents decreased significantly in a concentration-dependent manner after the exposure to CPF for 96 hours post fertilization (hpf). CPF affected not only the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST), but also the transcriptional levels of their respective genes. Finally, the mRNA levels of the main cytokines including *tumor necrosis factor* α *(Tnf*α*)*, *interferon (Ifn)*, *interleukin-1 beta (Il-1*β*)*, *interleukin 6 (Il6)*, *complement factor 4* (C4) in the larvae increased significantly after the exposure to 100 or 300 μg/L CPF for 96 hpf, suggesting that the innate immune system disturbed by CPF in larvae. Taken together, our results suggested that CPF had the potential to cause developmental toxicity, behavior alterations, oxidative stress and immunotoxicity in the larval zebrafish.

1. Introduction

In the last decades, organophosphate pesticides are among the most widely used classes of pesticides. China, India and other developing countries have substantially increased production of organophosphate pesticides even in recent years [1]. As a well-known organophosphate pesticide, chlorpyrifos (CPF) is frequently and extensively used for controlling agriculture and house hold pests all over the world [1]. As reported, about 18,000 tones of CPF were consumed annually in China [2]. Runoff, erosion, leaching are the major routes of CPF entry into surface waters [3–5]. Thus, CPF is commonly monitored in groundwater and surface water [6–8]. Like other organophosphate pesticides, CPF also exerts its pharmacological activity primarily through the binding of the enzyme acetylcholinesterase (AChE) by phosphorylation, leading to the inhibition of this enzyme activity [9,10]. As a result of widespread use of CPF, there is no doubt that the misuses of CPF and other organophosphate pesticides can have adverse effects on non-target organisms including aquatic vertebrates.

The toxicity of organophosphate pesticides have been linked to nausea, dizziness, confusion, increased heart rate, respiratory failure, and even death. A number of previous studies have indicated that CPF exposures are associated with a wide range of toxic effects including nephrotoxicity, oxidative stress, genotoxic and mutagenic effects, alterations in swimming performance, as well as effects on development, in different aquatic organisms [11–14]. Recently, the toxicities of CPF have been reported on zebrafish, but they are

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http://dx.doi.org/10.1016/j.fsi.2015.01.010
1050-4648/

mainly focused on the inhibition of the activity of AChE and their unpredictable consequences. For example, Levin et al. [15] reported that the 100 μg/L CPF administered to zebrafish embryos on Days 1–5 postfertilization caused significant slowing of swimming activity on Days 6 and 9 dpf and had persisting effects of impairing spatial discrimination and decreasing response latency in adulthood. Yen et al. [1] found that the AChE activity was significantly inhibited in larval zebrafish after exposure to 300 nM CPF for 5 dpf. However, the effects on oxidative stress and immunotoxicity and other toxicological endpoints of zebrafish in response to the CPF have received limited concern.

Zebrafish is an established powerful laboratory fish model with a well characterized genome allowing for the application of sophisticated molecular approaches to investigate mechanisms of toxicity [16–21]. Recently, the expression of some genes was adopted as a powerful tool to analyze the neurotoxicity and immunotoxicity in zebrafish induced by different environmental chemicals. For example, in a recent study, Fan et al. [22] observed that the expression profiles of genes such as *glial fibrillary acidic protein (Gfap)*, *myelin basic protein (Mbp)*, *Elval3*, *neurogenin 1 (Ngnl)*, *sonic hedgehog a (Shha)* may be useful biomarkers for rapid evaluation of the developmental neurotoxicity potential of chemicals in zebrafish. As reported, the cytokines, such as tumor necrosis factor (TNF), interleukins (IL), etc., have an important role in initiating responses once a pathogen penetrates the host [23]. And the transcriptional levels of these genes are also considered as effective biomarkers induced by environmental chemicals [24].

Although there are many papers on the biochemical and physiological influences of CPF on aquatic organisms, a survey of many different CPF toxicity endpoints including the locomotor behavior in response to light change stimulus, the transcription of several key genes related to neurotoxicity, innate immune system and oxidative stress have not been evaluated, to our knowledge, in a single study dealing with early life stage of zebrafish. Such an inclusive work would help to understand the mechanisms of CPF toxicity in fish comprehensively. In the present study, we thus attempted to compare CPF toxicity effects measured in the model of the early life stage of zebrafish at multiple endpoints on development, locomotor behavior, oxidative stress and immunotoxicity. Our results bring some insights into the toxicity mechanisms of CPF in the aquatic ecosystem.

2. Materials and methods

2.1. Chemicals

CPF (Purity > 99%) was purchased from Sigma–Aldrich. The chemical was used as received. Stock solutions of CPF were prepared by dissolving it in DMSO. The commercial kits for determining malondialdehyde (MDA), glutathione (GSH) contents and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), AChE activities were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein concentrations were determined by the bicinchoninic acid (BCA) protein kit provided by Sangon Biotech (Shanghai, China). TRIzol reagent for isolating RNA was purchased from Takara Biochemicals (Dalian, China). The reverse transcriptase kit and SYBR green PCR kit were purchased from Toyobo (Tokyo, Japan).

2.2. Maintenance of zebrafish

Healthy five-month-old adult fish were selected and acclimatized separately in glass tanks at ambient temperature (27 ± 1 °C) with 14-h light/10-h dark cycles. The fish were fed two times a day with brine shrimp. A total of fifty male and fifty female fish were selected and reared separately for spawning. Then, a total of ten male and ten female fish were selected randomly and maintained together for spawning each time. Embryos were collected and staged using standard procedures as outlined by Westerfield [25]. The eggs were mixed gently before exposing to CPF to eliminate the difference between the different breeder groups.

In all experiments, water was dechlorinated and filtered through activated carbon prior to use. The eggs were transferred to various exposure chambers containing CPF with known concentrations at least 60 min after the initial spawning. Non-fertilized eggs were separated from the fertilized ones using a pipette. Control embryos were exposed to water only containing same volume of DMSO. In all experiments, the exposure solutions were changed daily. Incubation was carried out at ambient temperature (28 ± 1 °C) with 14 h light/10 h dark cycles in a constant temperature-light incubator (Laifu, Ningbo, China).

2.3. Embryo toxicity test

To determine the hatchability affected by CPF, forty fertilized eggs were selected and exposed to 100 mL of 10, 30, 100, 300 μg/L, respectively, in glass beakers (size: 250 mL), with four replicates for each treated concentration. At 60 and 96 hours post fertilization (hpf), the hatched larvae were counted in each treated group, and the hatchability was analyzed by the ratio of hatched numbers/total exposed numbers × 100. And the body lengths of the larval zebrafish in all CPF treated groups were measured at the time point of 96 hpf. The heart rates were monitored with the Zebralab Video-Track system (ViewPoint Life Science, France) at the time points of 48 hpf of 30 larval fish in each CPF treated group. The abnormal and dead zebrafish embryos and larval were monitored at the points of 96 hpf exposure to CPF.

2.4. Locomotion analysis

To determine the locomotion affected by CPF, fertilized eggs were exposed to 10, 30, 100, 300 μg/L CPF in a 96-well plate (1 egg per well), respectively, for 96 h. The data of ten eggs were collected and designed as one CPF treated group, with four replicates for each concentration (totally forty eggs were used in each CPF treated group). Before the locomotion analysis, the exposure solutions were changed to clean water. Free swimming activities during 30 min visible light and swimming in response to a 10 min light-to-dark photoperiod stimulation were recorded after the exposure of the larvae to various concentrations of CPF for 96 hpf. The tested larval fish was monitored with the Zebralab Video-Track system (ViewPoint Life Science, France) equipped with a PointGray IEEE-1394 camera (Model GRAS-03K2M-C, 30 fps) and an infrared filter. The entire record hardware is linked to the computer control program and kept insulated from lab environment in a sealed opaque plastic box (ViewPoint Life Science, France).

2.5. Determination of AChE activity, MDA and GSH contents, and the activities of SOD, CAT, GPX and GST in the larval fish

To determine the kinetics of AChE and antioxidant enzyme activities induced by CPF, fifty fertilized eggs were selected and exposed to 10, 30, 100, 300 μg/L CPF in 100 mL of each of the above solutions in glass beakers (size: 250 mL) for 96 hpf, with four replicates for each CPF concentration. After the exposure, about 50 larvae fish in each group were defrosted and homogenized on ice with 180 μL ice-cold saline, and totally four replicate pools were prepared for each treatment. The homogenate was centrifuged at