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Pro- and anti-inflammatory cytokine expression in immune organs of the common carp exposed to atrazine and chlorpyrifos

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ABSTRACT

Atrazine (ATR) and chlorpyrifos (CPF) are toxic and subject to long-term in vivo accumulation in different aquatic species throughout the world. The purpose of the present study was to examine the effect of ATR, CPF and combined ATR/CPF exposure on cytokines in the head kidney and spleen of common carp (*Cyprinus carpio* L.). The carp were sampled after a 40-d exposure to CPF and ATR, individually or in combination, followed by a 40-d recovery to measure the mRNA expression of IL-6fam (IL-6), IL-8, TNF-α, IL-10 and TGF-β1 (TGF-β) in the head kidney and spleen tissues. These results showed that the expression of cytokines IL-6, IL-8 and TNF-α in the head kidney and spleen was upregulated following ATR, CPF and mixed ATR/CPF exposure compared with the control group. The expression of IL-10 and TGF-β mRNA was significantly inhibited in both head kidney and spleen of carp exposed to ATR, CPF and the ATR/CPF mixture. The results suggested that long-term exposure of ATR, CPF and the ATR/CPF mixture in aquatic environments can induce the dysregulation of pro-/anti-inflammatory cytokine expression. The information regarding the effects of ATR and CPF on cytokine mRNA expression generated in this study will be important information for pesticides toxicology evaluation.

1. Introduction

Atrazine (ATR) and chlorpyrifos (CPF) are the most widely used pesticides and herbicides, and they have played an important role in agriculture over the past 40 years. Extensive use in the control of weeds and grasses in crops, which can be carried into the aquatic ecosystems by leaching to groundwater [1], has led to environmental pollution. Several studies have also reported that ATR has different degrees of toxic effects on aquatic animals [2] and mammals [3]. ATR has been classified as a current international priority for the control of environmental pollutants. It has been shown that CPF can cause reproductive toxicity in male rats and is also associated with genital malformation and a series of other toxic effects [4].

Cytokine mRNA expression levels in fish have been used as a tool for measuring immune responses. In particular, pro-inflammatory cytokines including IL-6 [5], IL-8 [6] and TNF-α [7] are commonly used immune-regulatory genes in fish. Similarly, the TGF-β and IL-10 inflammatory cytokines have been broadly described in fish, in species such as rainbow trout [8], carp [9], atlantic salmon [10] and grass carp [11]. The mRNA expression analysis of IL-6, IL-8, TNF-α, IL-10 and TGF-β demonstrates the impact of environmental pesticide exposure in the placenta during human pregnancy [12]. The elevation of TNF-α and IL-6 expression was investigated with regard to the effect of $CdCl_2$-polluted drinking water in rats [13]. IL-6, IL-8, IL-10 and TNF-α levels were induced at high concentrations by carboxylate microspheres in mouse lungs [14]. Thalidomide decreased production of fibrogenic cytokine TNF-α, IL-6 and TGF-β1 in a paraquat-induced pulmonary injury model in mice [15]. Abnormal expression of IL-6, TNF-α and TGF-β1 in the seabream head kidney were influenced by stress-related hormones [16].

ATR exposure appears to disrupt the immune system of juvenile mice, with certain effects persisting for a long time after the end of the exposure [3]. The hepatotoxicity and nephrotoxicity of CPF were experimentally studied in egg layer chickens [17]. In addition, oxidative stress and histological changes were investigated in rat retina and kidney after CPF treatment [18]. Our previous studies have shown that ATR, CPF and their mixture can induce oxidative stress [18], inhibit acetylcholinesterase (AChE), carboxylesterase

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(CbE) and glutathione S-transferase isoenzymes (GSTs) [19,20] and lead to immunological abnormalities [21]. However, there are very limited studies on the carp immune organ after exposure to ATR and CPF. In addition, a deep association analysis describing whether the altered cytokine expression can affect immune function has not yet been reported. In the present study, we investigated the mRNA expression levels of selected proinflammatory (IL-6, IL-8, TNF-α) and anti-inflammatory (IL-10, TGF-β1) cytokines in the head kidney and spleen of the common carp following exposure to ATR, CPF and mixed ATR/CPF.

2. Materials and methods

2.1. Chemicals

ATR and CPF were purchased from Sigma Aldrich Chemical Co. (St. Louis, Missouri, USA) and their purity was 98.0% and 99.5%. HEPES stock solutions of ATR and CPF were generated by dissolving the chemicals in acetone (purity: 99%), and the concentration of acetone was maintained at a concentration less than 0.05% in all pesticide working solutions used for treatment. All working solutions were generated from this stock solution.

2.2. Animals

Common carp (*Cyprinus carpio* L.) were purchased from an aquarium specializing in freshwater fish species, and their mean body weight was 190 ± 10 g in this study. The fish were maintained in the laboratory tanks with persistent aeration and acclimatization to experiment conditions for 15 d using dechlorinated tap water (CaCO3: 230 μg/L; Ca: 42.5 ± 1.2 μg/L; the dissolved oxygen concentration remained above 7 μg/L and pH 7.4 ± 0.2). The water temperature was kept at 20 °C, and the photoperiod was 12 h light and 12 h dark. The fish were provided adequate feed with carp pellets and were maintained in a flow through water system.

2.3. Experimental design

2.3.1. Toxicity test

The toxicant concentrations were determined according to 1/500, 1/50 and 1/5 of the 96 h LC_{50} values according to Xing et al. [19]. Two hundred and twenty fish were stochastically divided into eleven groups, including three ATR treatment groups (4.28, 42.8 and 428 μg/L), three CPF treatment groups (1.16, 11.6 and 116 μg/L), three mixed-treatment groups (ATR/CPF; 1.13, 11.3 and 113 μg/L), one solvent control (acetone) and one water control. The binary mixtures were composed of a 1:1 mass ratio of ATR and CPF. The fish were exposed under semi-static conditions for 40 d; water and pesticide were completely replaced once every 2 d by transferring fish to freshly prepared pesticide solutions. At the close of the exposure, ten fish were killed in each group, and the spleen and head kidney were quickly removed, minced and stored at −80 °C until RNA isolation. Animal care and treatment complied with the standards described in the guidelines for the care and use of laboratory animals of Northeast Agricultural University.

2.3.2. Recovery test

The method of recovery in the present study was similar to that described by Xing et al. [22], but the recovery time interval was extended to 40 d. Exposed fish from each group were kept in pesticide-free water for 40 d in another set of large fresh 200-L glass aquaria provided with filtration and continuous aeration. The conditions (water quality parameters, water temperature and photoperiod) during the recovery experiment were the same as those in the exposure experiment.

2.4. Primer design

The primers for real-time amplification of IL-10 and TGF-β1 cDNA were designed using Oligo_6.0 Software (Molecular Biology Insights, Cascade, CO) based on the deposited sequences in GenBank under the accession numbers AB470924.1 and AF136947.1, respectively. β-Actin (GenBank accession no. M24113.1), a housekeeping gene, was used as an internal reference. Pairs of carp-specific primers for IL-6, TNF-α and IL-10 were used in the reaction using a method consistent with that of [23,24]. All primers used (Invitrogen Biotechnology Co. Ltd., Shanghai, China) are listed in Table 1.

2.5. Total RNA isolation and reverse transcription reaction

Total RNA was isolated from each of the organs of each fish using Trizol reagent, according to the manufacturer's instructions (Invitrogen). The reverse transcription reaction (40 μL) consisted of the following: 10 μg of total RNA, 1 μL of Moloney murine leukemia virus reverse transcriptase, 1 μL of RNase inhibitor, 4 μL of deoxynucleoside triphosphate, 2 μL of Oligo dT, 4 μL of dithiothreitol and 8 μL of 5× reverse transcriptase buffer. The reverse transcription procedure was performed according to the manufacturer's instructions (Invitrogen). The reverse transcription products (cDNA) were then stored at −20 °C for PCR.

2.6. Real-time quantitative PCR

RT-PCR was performed to detect the gene expression of IL-6, IL-8, TNF-α, IL-10 and TGF-β in different tissues by using SYBR Premix Ex Taq (Takara, Shiga, Japan). Real-time PCR was performed for each organ. Reaction mixtures were incubated in an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The PCR program included one cycle at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primers specificity and product purity. The amplification efficiency for each gene was determined by using the Data Analysis for Real-Time PCR (DART-PCR) program. The relative mRNA abundance was calculated according to the method of Pfaffl [25], accounting for gene-specific efficiencies and normalized to the mean expression of β-actin.

2.7. Statistical analysis

Statistical analysis of all data was performed using SPSS for Windows (version 13, SPSS Inc., Chicago, IL). One-way ANOVA with a post hoc test was used to elucidate if there were significant differences between the treatment groups and the control group. The difference between the treatment groups and the recovery

Table 1
Primers used for quantitative real-time PCR.

Gene	Accession no[1]	Primer (5′→3′)
β-Actin	M24113.1	Forward: ATGGACTCTGGTGATGGTGTGAC Reverse: TTTCTCTTTCGGCTGTGGTGGTG
IL-6fam	AY102632	Forward: AGCAGCGGGTGGAGGATGTA Reverse: CCTCAGAAATGGCGGTGGAC
IL-8	AB470924.1	Forward: AGCAGCGGGTGGAGGATGTA Reverse: ATGGTTGGCTCTTGAGGTTCC
TNF-α	AJ311800	Forward: GCTGTCTGCTTCACGCTCAA Reverse: CTTGGAAGTGACATTTGCTTTT
IL-10	AB110780	Forward: CGCCAGCATAAAGAACTCGT Reverse: TGCCAAATACTGCTCGATGT
TGF-β1	AF136947.1	Forward:TGCCTGTTGGGATTTGTGC Reverse: AGCCGCCTGCTCTTCATTT

[1] GeneBank accession number for sequence from which primers were designed.