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Effects of atrazine and chlorpyrifos on the production of nitric oxide and expression of inducible nitric oxide synthase in the brain of common carp (*Cyprinus carpio* L.)

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ABSTRACT

The study aimed to investigate the effects of atrazine (ATR), chlorpyrifos (CPF), and the mixture of them on nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in the brain of common carp. The triazine herbicide ATR and the organophosphorus insecticide CPF are frequently and extensively applied in agriculture all over the world. 220 Carps were averagely divided into eleven groups according to the different treatments and concentration, including the exposure and recovery experiments. In the present study, we investigated production of NO, iNOS activity and iNOS mRNA and protein expression in the brain of the common carp after a 40 d exposure to ATR, CPF, alone or in combination, and a 40 d recovery treatment. The results showed that the activity of iNOS and production of NO were significantly higher in all groups of fish exposed to high doses ATR, CPF and their mixture compared to control fish. After a 40 d recovery treatment, iNOS activity and production of NO were lower than in the corresponding exposure groups in all the recovery groups. The mRNA and protein levels of iNOS were significantly higher in the high-dose group of ATR and CPF compared to control group, but were significantly lower in the group of the mixture of ATR and CPF compared to control group. Results indicated that NO and iNOS were involved in oxidative stress and brain tissue damage induced by ATR, CPF, and their mixture. Thus, the information presented in this study is helpful to understand the mechanism of ATR-, CPF- and ATR/CPF-mixture-induced neurotoxicity in fish.

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1. Introduction

The triazine herbicide atrazine (ATR) and the organophosphorus insecticide chlorpyrifos (CPF) are frequently and extensively applied in agriculture all over the world (Saulsbury et al., 2009; Warnemuende et al., 2007). They could contaminate the aquatic environment by agriculture runoff and irrigation waters. Several studies indicated that ATR and CPF were the contaminants of surface and ground water in many countries (Banks et al., 2005; Du Preez et al., 2005; Miller et al., 2000; Murphy et al., 2006). Annual sales of ATR in the United States are approximately 33–36 million kg (Tillitt et al., 2010). And the increasing use of ATR has contaminated the surface waters in a majority areas of the United States (Capel and Larson, 2001; Solomon et al., 1996). Investigation of the Yang River and Guanting Reservoir in China demonstrated that large portions of these two water bodies were contaminated with ATR, with concentrations ranging from 0.22 to 26 μg/L (Jin and Ke, 2002). In addition, the CPF residues in river water samples were raised in agricultural areas in the Choluteca river basin of Honduras (Kammerbauer and Moncada, 1998).

Some studies have demonstrated that ATR and CPF can promote toxic effects on aquatic animals (Solomon et al., 2008; Tillitt et al., 2010; Wang et al., 2011). It has been shown that ATR could cause biochemical and histopathological changes (Paulino et al., 2012), genotoxicity (de Campos Ventura et al., 2008), endocrine disruption (Moore and Waring, 1998), oxidative stress (Jin et al., 2010b) in fish. Researchers have demonstrated that CPF is also genotoxic (Ali et al., 2008), immunotoxic (Eder et al., 2008) and neurotoxic (Eddins et al., 2010) in fish. In addition, CPF alters synaptic neurotransmission and inhibits neurite outgrowth in neural cell differentiation (Das and Barone, 1999). In our lab, we have demonstrated that ATR, CPF, and their mixture could cause adverse effect on the brain of carp, including acetylcholinesterase

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; ATR, atrazine; CPF, chlorpyrifos.

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(AChE) activity and carboxylesterase activity (Xing et al., 2010b), the expression of AChE (Xing et al., 2010a) and heat shock proteins (Xing et al., 2012a) and histopathological changes and antioxidant response (Xing et al., 2012b).

NO, a highly versatile and ubiquitous signaling molecule, is generated in the body by the nitric oxide synthase enzyme (NOS), an enzyme that exists in three isoforms encoded by distinct genes: neuronal NOS, endothelial NOS (eNOS), and inducible NOS (iNOS). Both neuronal NOS and eNOS are constitutive isoforms, which generate small quantities of NO. In contrast, iNOS can be induced by various cytokines or lipopolysaccharides and produces large amounts of NO over long time periods. NO is thought to play diverse physiological functions including vasodilation, neurotransmission, and host cell defense (Moncada and Higgs, 1995). It is also known that NO plays an important role in the toxicity of pesticides and heavy metals (Ortiz-Ortiz et al., 2009; Pi et al., 2003). In recent years, NO and iNOS are believed to have important roles in fish (Conte, 2003; Gonzalez et al., 2007; Saeij et al., 2000). The location of iNOS in the central nervous system in goldfish and trout was demonstrated (Virgili et al., 2001). However, reports on NO/NOS pathway are relatively less in common carp exposure to ATR and CPF.

Compared to the many studies on the physiological and toxicological effects of ATR or CPF in mammals, carp ATR or CPF metabolism is still an unexplored topic. Moreover, carp belong to higher trophic levels in the biosphere and food chain, and thus may play an important role in ATR or CPF circulation in the ecosystem in the same way as mammals do. In this context, we investigated the production of NO, iNOS activity and the mRNA and protein expression levels of iNOS gene in the brain of common carp following the exposure to ATR, CPF, and their mixture.

2. Materials and methods

2.1. Chemicals

ATR (purity 98.0 percent) and CPF (purity 99.5 percent) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Stock solutions of ATR and CPF were prepared by dissolving in acetone (purity 99 percent). All working solutions were taken from this stock solution. The concentration of acetone was kept less than 0.05 percent in all pesticide and herbicide solutions used.

2.2. Fish

Common carp (mean body length, 12.5 ± 1.29 cm; mean body weight, 190 ± 10 g) used in this study were purchased from an aquarium operating freshwater fish species. The fish were maintained in the laboratory tanks ($90 \times 55 \times 45$ cm^3) with continuous aeration. Acclimatization to experimental conditions for 15 d was done using dechlorinated tap water ($CaCO_3$: 230 mg/L, Ca: 42.5 ± 1.2 mg/L, dissolved oxygen concentration remained above 7 mg/L and pH 7.4 ± 0.2). The water temperature was adjusted to 20 ± 1 °C, and the photoperiod was 12 h light and 12 h dark.

2.3. Experimental design

2.3.1. Toxicity test

The concentrations of toxicant were determined according to 1/500, 1/50 and 1/5 of 96 h LC_{50} values according to Xing et al. (2012c). 220 Fish were randomly divided into eleven groups: 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 mixture-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, with water and pesticide completely replaced once every 2 d by transferring fish to freshly prepared pesticide solutions. The exposure treatments were detailed in Supplement Table 1.

At the end of the exposure, ten fish were killed in each group and the brain was 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 the northeast agriculture university.

2.3.2. Recovery test

The method of recovery experiment in the present study was similar to Xing et al. (2012c). But the recovery time interval was longer. Ten fish from exposed fish of each batch were kept in pesticide-free water for 40 d in another set of large fresh 200-L glass aquaria provided with a filter and continuous aeration. The condition (water quality parameters, water temperature and photoperiod) during the recovery experiment was the same as that in the exposure experiment (Supplement Table 1).

2.4. NO and iNOS activity assay

The brain homogenates of each treatment group were used for NO and NOS activity assay. The NO and iNOS activities were determined using NO and iNOS activity assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The method used in the present study was according to the procedure by our group (Zhang et al., 2011).

2.5. Gene expression analysis

Total RNA was isolated from each of the organs of each fish using Trizol reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The RNA concentrations were determined using GeneQuant 1300 (GE Healthcare Biosciences, Piscataway, NJ). Reverse transcription reaction (40 μL) consisted of the following: 10 μg of total RNA, 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL), 1 μL of RNase inhibitor (40 U/μL), 4 μL of deoxynucleoside triphosphate (10 mM), 2 μL of Oligo dT (50 μM), 4 μL of dithiothreitol (0.1 M), and 8 μL of 5 × reverse transcriptase buffer. The procedure of the reverse transcription was according to the instructions of the manufacturer (Invitrogen). The reverse transcription products (cDNA) were then stored at −20 °C for PCR.

We used the common carp iNOS mRNA GenBank sequence with an accession number of **AJ242906**. Common carp β-actin (GenBank accession no. **M24113.1**) as housekeeping gene was used as an internal reference. The PCR primer sequences used for real-time quantitative reverse transcription PCR (qPCR) are listed as described previously (Gonzalez et al., 2007; Xing et al., 2012c). Primers were synthesized by Invitrogen Biotechnology Co. Ltd. in Shanghai, China. The following set of primers were used: for iNOS: forward 5′-**AAC AGG TCT GAA AGG GAA TCC A**-3′; and reverse 5′-**CAT TAT CTC TCA TGT CCA GAG TCT CTT CT**-3′; for β-actin: forward 5′-**GAT GGA CTC TGG TGA TGG TGT GAC**-3′; and reverse 5′-**TTT CTC TTT CGG CTG TGG TGG TG**-3′. The amplification products were: iNOS-100 bp, β-actin-167 bp. The PCR products were electrophoresed on two percent agarose gels, extracted, cloned into the pMD18-T vector (Takara, Ohtsu, Japan), and sequenced.

qPCR was used to detect the expression of the iNOS gene in the brain by using SYBR Premix Ex Taq (Takara), and qPCR work was performed on an ABI PRISM 7500 Detection System (Applied Biosystems, USA). The program was one cycle at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and at 61 °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 primer specificity and product purity. The amplification efficiency for each gene was determined by using the Data Analysis for Real-Time PCR (DART-PCR) program (Peirson et al., 2003). The mRNA relative abundance was calculated according to the method of Pfaffl (2001), accounting for gene-specific efficiencies and was normalized to the mean expression of β-actin.

2.6. Western blot analysis

An equivalent amount of tissue (depending on the tissue examined, between 50 and 150 mg) was homogenized in 800 μL of ice-cold grind buffer (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 30 mM NaF, 30 mM sodium pyrophosphate, 0.1 percent SDS, one percent Triton X-100 and protease inhibitor cocktail). The sample was then centrifuged for 10 min at 10,000*g* at 4 °C, and supernatant was collected. Protein content was measured according to Bradford's procedure (Bradford, 1976). Equal amounts of total protein (40 μg/condition) were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on ten percent gels. Separated proteins were then transferred to nitrocellulose membranes using a tank transfer for 2 h at 200 mA in Tris–glycine buffer containing 20 percent methanol. Membranes were blocked with five percent skim milk for 16–24 h and incubated overnight with diluted primary rabbit antibody against iNOS (1:200, Abcam, USA) followed by a horse-radish peroxidase (HRP) conjugated secondary antibody against rabbit IgG (1:1000, Santa Cruz, USA). To verify equal loading of samples, the membrane was incubated with monoclonal β-actin antibody (1:1000, Santa Cruz, USA), followed by a HRP conjugated goat anti-mouse IgG (1:1000). The protein bands were visualized by enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China). The signal was detected by X-ray films (TransGen Biotech Co., Beijing, China). The optical density (OD) of each band was determined by Image VCD gel imaging system (Beijing Sage Creation Science And Technology Co. Ltd., Beijing, China), and the iNOS expression were expressed as the ratio of OD of iNOS and OD of β-actin, respectively.

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