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Assessment of pesticide residues and gene expression in common carp exposed to atrazine and chlorpyrifos: Health risk assessments

Houjuan Xing [a,b,1], Zhilei Wang [b,1], Hongda Wu [c], Xia Zhao [a], Tao Liu [a], Shu Li [a], Shiwen Xu [a,*]

[a] College of Veterinary Medicine, Northeast Agricultural University, 59 Mucai Street, Harbin 150030, PR China
[b] Animal Health Supervision Institute of Heilongjiang Province, 243 Haping Road, Xiangfang District, Harbin 150069, PR China
[c] Institute of Animal Science, Academy of Agricultural Sciences of Heilongjiang Province, 368 Xuefu Road, Xiangfang District, Harbin 150086, PR China

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ABSTRACT

This study assessed the impacts of atrazine (ATR), chlorpyrifos (CPF) and combined ATR/CPF exposure on the kidney 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 expression levels of heat shock proteins genes (HSP60, HSP70 and HSP90) and pesticide residues in the kidney tissue. The results revealed that the mRNA and protein levels of HSP60, HSP70 and HSP90 were induced in the kidney of common carp by ATR, CPF, and ATR/CPF mixture. The accumulated amounts of ATR, CPF, and their metabolites in the kidney tissues exhibited dose-dependency. These results exhibited that increasing concentration of ATR and CPF in the environment causes considerable stress for common carp, suggesting that the expression levels of HSP60, HSP70 and HSP90 may act as potential biomarkers for assessing the environmental ATR and CPF risk for carp.

1. Introduction

In agricultural areas worldwide, there is an increasing concern about watershed contamination due to the widespread use of pesticides. Atrazine (ATR) is one of the most widely used pesticides in the world, mainly due to its relatively low cost and ease of application. Chlorpyrifos (CPF) is a conventional organophosphorous insecticide and is widely used to control a variety of pests in agriculture and animal farm (Saulsbury et al., 2009). ATR and CPF are considered a moderately persistent chemical in the environment with a halflife ranging from a few days to months (Song et al., 2009; Palma et al., 2009). ATR is found in relatively low levels in the environment, usually less than 1 ppb, but can be found at levels as high as 21 ppb in groundwater, 42 ppb in surface waters, 102 ppb in river basins in agricultural areas, and up to 224 ppb in Midwestern streams during May–August (Kolpin et al., 1998; Powell et al., 2011). ATR concentrations of up to 108 μg/L have been reported in rivers of North America (USEPA, 2002). CPF has been detected in surface waters at average levels of 0.01–1.95 μg/L (Cerejeira et al., 2003; Palma et al., 2009). Direct application of CPF to water bodies to control mosquitoes or agricultural run-off from treated areas can result in CPF contamination of up to 4.3 μg/L in streams and lakes (Thomas and Nicholson, 1989; Richards and Baker, 1993; Wood and Stark, 2002). Because of extensive usage and moderate solubility in water, they have been commonly detected as a contaminant in surface and ground-water of many countries (Banks et al., 2005; Du Preez et al., 2005; Zhou et al., 2009). With intensive use of these agricultural chemicals in recent years, the toxic effects have become a great threat to the health of human and aquatic animals (Nakadai et al., 2006; Rowe et al., 2007; Moore et al., 2007; Ali et al., 2008; Ernst et al., 2014). ATR has induced severe hormonal disturbances in amphibians (Hayes et al., 2002) and tumors in rats (Peyre et al., 2014). ATR has been classified as a possible human carcinogen by the International Agency for Research on Cancer (1999).

In recent years, the highly conserved family of heat shock proteins (HSPs) has received extensive attention for their roles in response to stress. Members of different HSP families are grouped according tomolecular size and perform varying and different roles in the cell. The HSP60 is involved in protein stability and folding (Cechetto et al., 2000), the HSP70 family is necessary for translocation and protein folding (Xing et al., 2013), and the HSP90 family is involved in steroid receptor formation and protein folding (Liu et al., 2013a). These HSP families are important for immune function (Wallin et al., 2002) and have been demonstrated to be upregulated in fish during stress (Xing et al., 2013). Numerous reports analyzed the effects of environmental stressors on the expression of HSP60, HSP70 and HSP90 genes in fish, including pesticide accumulation (Dowling et al., 2006; Yang et al., 2010a),

* Corresponding author. Fax: +86 451 55190407.
E-mail address: shiwenxu@neau.edu.cn (S. Xu).
[1] These authors contributed equally to this work.

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PCBs (Na et al., 2009), PAHs (Abdel-Gawad and Khalil, 2013) and heavy metal accumulation (Rajeshkumar and Munuswamy, 2011). However, there are only a few papers on the expression alterations of HSP60, HSP70 and HSP90 genes in fish challenged by pesticide (Liu et al., 2013a; Yang et al., 2010b). Of particular interest is the joint toxicity of CPF and ATR, which has been shown to exhibit greater than additive toxicity to several aquatic invertebrate species (Schuler et al., 2005). There are only a few reports focusing on the joint toxicity of ATR and CPF on vertebrate species, and the present study focused on aspects of acute toxicity (Wacksman et al., 2006).

Among many aquatic organisms, fish is a valuable biomonitor of environmental pollution, including common carp (*Cyprinus carpio L.*). The major function of the kidney in fish is to maintain body fluid homeostasis, the same as higher vertebrates, but additionally, it is a major lymphoid organ (Press and Evensen, 1999). Thus, a study of the primitive kidney of vertebrates as exemplified by fish is of particular importance to fully understand the immune system of all vertebrates. Here, we examined the mRNA and protein levels of HSP60, HSP70 and HSP90 genes in the kidney of common carp exposed to ATR and CPF alone and in combination by quantitative real-time PCR and western blot.

2. Materials and methods

2.1. Fish

All procedures used in the present study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. The fish model was developed as described in our previous article (Xing et al., 2012). The 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 specializing in freshwater fish species and maintained in laboratory tanks ($90 \times 55 \times 45$ cm^3) with continuous aeration. The fish were acclimated to experimental conditions for 15 d using dechlorinated tap water ($CaCO_3$: 230 ± 3.16 mg/L, Ca: 42.5 ± 1.2 mg/L; dissolved oxygen concentration remained above 7 mg/L; pH 7.4 ± 0.2). The water temperature was adjusted to 20 ± 1 °C and the photoperiod was 12 h of light and 12 h of dark. Fish were fed once a day with commercial food (Jiaji Co., Zhenjiang, China) according to the manufacturer's guidelines during the acclimation and experimental period.

2.2. Chemicals

ATR (purity 98.0%) and CPF (purity 99.5%) were purchased from Sigma-Aldrich Chemical Co. (USA). Stock solutions of ATR and CPF were prepared in analytical grade acetone (purity 99%), and all working solutions were made from this stock solution. The concentration of acetone was kept at $< 0.05\%$ in all pesticide solutions used.

2.3. Experimental design

2.3.1. Exposure test

Experimental 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 ATR/CPF combination treatment groups (1.13, 11.3 and 113 μg/L), one solvent control (acetone) group, and one water control group. The binary mixtures were composed of a 1:1 mass ratio of ATR and CPF. Each treatment group was 20 fish and 2 replicates. ATR and CPF are stable in water and have a long half-life. The concentrations used in the present study have been found in the environment. The fish were exposed under semi-static conditions for 40 d to water and pesticide, which were completely replaced once every 2 d by transferring fish to freshly prepared pesticide solutions.

At the end of the exposure, fish were sacrificed by decapitation and then bled. The kidneys were then excised immediately on an ice-cold plate and washed in physiological saline solution (0.86% NaCl). The tissues were stored at −80 °C for the RNA and protein isolation and the determination of tissue pesticide contents.

2.3.2. Recovery test

Ten fish from each exposure group were kept as a set in fresh, pesticide-free water for 40 d in large 200 L glass aquaria with filters and continuous aeration. The conditions during the recovery experiment were the same as those described above.

In this study, there were ten fish per group killed at the two sampling events, and five fish per group were used in official test. The remaining tissues were used in preliminary experiment and served as standby tissues. During the experiment, no mortality was observed over the course of the experiment in either the control fish or in any of the treatment groups.

2.4. Tissue pesticide content analysis

2.4.1. Tissue ATR content analysis

Tissue samples were homogenized and extracted by the method of Wang et al. (2013). The extracts were filtered and combined, and then 3 g of NaCl was added successively to each sample and dissolved by shaking the suspension. The filtrate was re-extracted three times with 30 mL of trichloromethane. The extracts were combined, passed through anhydrous Na_2SO_4 columns, and collected. The eluates were concentrated into a triangular flask by rotary evaporation, dissolved with 1.5 mL of methanol, and filtered. The concentrations of ATR in extracts were analyzed with an Agilent high-performance liquid chromatography (HPLC) system. HPLC was performed with Waters equipment, equipped with a diode array using an ODS 5-micron Hypersil capillary column (250 mm long, 4.6 mm in diameter) following the procedures described by Muňoz and Rosés (2000). Mobile phases used in the isocratic elution were distilled water and methanol (v/v: 20/80). The column head temperature was 25 °C and flow rate was held constant at 1 mL/min. The volume injected was 10 μL. The eluents were monitored by UV detection of a wavelength of 222 nm for ATR, atrazine-2-hydroxy, and atrazine-desethyl.

2.4.2. Tissue CPF content analysis

For CPF content analysis, approximately 2 g (wet wt) of stored soft tissues samples was weighed in a centrifuge tube (50 mL). Each sample received 1 g of anhydrous Na_2SO_4 and 10 mL of acetidin and was homogenized using an IKA homogenizer at 6000 rev/min for 2 min. Then, the tool bit was washed using 10 mL of acetidin. The homogenate was centrifuged at 5000 rev/min for 5 min to obtain the supernatant. The residue in the centrifuge tube was re-extracted once with 10 mL of acetidin and centrifuged. The two supernatants were concentrated into a rotary evaporation flask by rotary evaporation at 40 °C, and the precipitate was dissolved with 5 mL of acetonitrile by convolution agitate and supersonic ablution. The extracts were passed through Acor aluminum oxide columns and collected. The eluates were evaporated to dryness under a nitrogen stream at 50 °C. The residue was finally dissolved in 2 mL of N-hexane and filtered for the HPLC analysis of the concentrations of CPF in extracts. The HPLC procedure was similar to the method used by Abu-Qare and Abou-Donia (2001). Briefly, mobile phases used in the isocratic elution were distilled with water and acetonitrile (v/v: 40/60). The column head temperature was 25 °C, and the flow rate was held constant at 1 mL/