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N-Acetylcysteine provides dose-dependent protection against fenthion toxicity in the brain of *Cyprinus carpio* L

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ABSTRACT

N-Acetyl-L-cysteine, a low-molecular weight thiol compound, with two different doses was used to prevent fenthion, an organophosphorus insecticide and acaricide, related oxidative stress in the brain of a model organism, *Cyprinus carpio*. Fish were exposed to sub-lethal and nominal concentration of fenthion after intraperitoneal injection of 0.5 or 400 mg/kg NAC. Brain tissues were then dissected and homogenized to analyse GSH, GSSG, TBARS, and protein contents. Enzymes that constitute the first line antioxidant defence, namely SOD and CAT, GSH-related enzymes, GR and GST, together with AChE activities were also determined spectrophotometrically. Fenthion did not cause any alteration in SOD and CAT activities while increasing GSH content, GSH/GSSG ratio and GST specific enzyme activity and decreasing GSSG, TBARS, and protein contents. Although, the highest induction in SOD and GST enzymes activities and the highest increase in GSH content were observed in the 0.5 mg/kg NAC-injected fish, their protein contents showed a decrease. 400 mg/kg NAC impeded the activation of the GST enzyme and a higher decrease in lipid peroxidation was observed. Fish were also protected against protein depletion by the higher dose NAC application. AChE activity was not influenced by fenthion exposure. Xenobiotic and GSH transporters may cause mild oxidative stress conditions in brain. Cellular redox status could trigger a series of reactions that result in an increase in SOD activity and a decrease in protein content. Based on the present results, it was suggested that the usefulness of NAC against fenthion depends on applied dose and tissue characteristics. Species-specifity and concentration selection should be taken into consideration in studies dealing with anticholinesterases.

1. Introduction

Reduced glutathione (GSH, γ-glutamylcysteinylglycine), a low-molecular-weight sulphur-containing compound, is easily oxidized and can be regenerated rapidly. These properties allow it to play an essential role in many biochemical reactions (Jefferies et al., 2003). Pharmacologic manipulation of GSH synthesis has received much attention due to the critical role of GSH in cellular defence against electrophiles, oxidative stress, and nitrosating species. Supplementing cells with phenolic antioxidants or low molecular weight thiols such as GSH or its esters, dithiothreitol, L-methionine, etc. has been used to replenish GSH contents under GSH-depleting conditions (Griffith, 1999). A clinically relevant GSH precursor, *N*-acetyl-L-cysteine (NAC), has been used in pesticide toxicity studies *in vivo* and *in vitro* in fish species (Sevgiler et al., 2007; Pena-Llopis et al., 2003a,b; Dorval and Hontela, 2003). NAC itself acts as an antioxidant compound against reactive oxygen species (ROS) with its -SH group, and as a cysteine supply to synthesize GSH (Sen, 1997). In contrast to its therapeutic properties, NAC at high doses could be toxic via oxidative stress (Held and Biaglow, 1994; Sprong et al., 1998; Sevgiler et al., 2007). Furthermore, Pena-Llopis et al. (2003b) observed that higher concentrations of NAC were lethal to *Anguilla anguilla*.

Although NAC and GSH can directly scavenge free radicals, the rate constants for their reactions with ROS (0.16 $M^{-1}s^{-1}$ for NAC and H_2O_2, 0.89 $M^{-1}s^{-1}$ for GSH and H_2O_2 [Winterbourn and Hampton, 2008], 68 $M^{-1}s^{-1}$ for NAC and superoxide anion radical [Benrahmoune et al., 2000], 220 $M^{-1}s^{-1}$ for GSH and superoxide anion radical [Jones et al., 2003]) are several orders of magnitude lower than those of antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1; 1.9×10^9 $M^{-1}s^{-1}$ for superoxide anion radical), catalase (CAT; EC 1.11.1.6; 2.6×10^7 $M^{-1}s^{-1}$ for H_2O_2), and glutathione peroxidase (GPx; EC 1.11.1.9; 1×10^7 $M^{-1}s^{-1}$ for H_2O_2) (Halliwell and Gutteridge, 1999). Thus, the direct free radical scavenging activity of NAC is not likely to be of great importance for its antioxidant activity *in vivo* (Atkuri et al., 2007). The three major antioxidant enzymes, which provide a first-line defence against ROS, are SOD, which dismutates superoxide anion radical to H_2O_2 and CAT that converts H_2O_2 to molecular oxygen and water (de Almeida et al., 2007).

Toxicity of organophosphorus compounds (OPs) stems mainly from the accumulation of acetylcholine due to inhibition of acetylcholinesterase (AChE; EC 3.1.1.7). The consequences of excess acetylcholine depend on oxidative stress initiated by the interaction of acetylcholine with cholinergic receptors (Hazarika et al., 2003).

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However, fenthion did not exhibit an anticholinesteratic activity in the liver of *Cyprinus carpio* (Sevgiler et al., 2007) except on butyrylcholinesterase (BChE; EC 3.1.1.8) in 400 mg/kg NAC injected fish. Therefore, the anticholinesteratic action of fenthion and effects of NAC on this action in the brain of *C. carpio* should be evaluated with the same NAC dose and fenthion concentration strategy. Fish brain, however, contains no BChE activity (Chuiko and Podgornaya, 2005).

Fenthion is a contact and systemic OP insecticide and acaricide. Based on avian, fish, and aquatic invertebrate toxicity, U.S. EPA classified all end-use products of fenthion as Restricted Use Products (EPA, 2001). EPA has cancelled all fenthion formulations by the request of its manufacturer, Bayer Chemicals (EPA, 2003), although the production and application of this pesticide in Turkey is still ongoing. It was estimated that about 5194 L of fenthion had been applied against olive and crop pests during the 2007 agricultural period in Çukurova region (pers. Commun.[1]).

A previous study carried out in the liver of *C. carpio* indicated that 400 mg/kg NAC had an additive effect on fenthion- induced oxidative stress by deranging GSH redox status, decreasing GSH-related enzymes activities, and increasing lipid peroxidation (Sevgiler et al., 2007). However, 0.5 mg/kg of NAC provided a limited amelioration on the oxidative effects of fenthion. Same doses of NAC were used in the current study to evaluate the dose-dependent effectiveness of NAC in the brain. The aim of this study was to understand whether the oxidative stress-inducing potential of OP fenthion could be reduced by NAC in the brain of *C. carpio* which was used as a model organism.

2. Materials and methods

2.1. Test animals

All materials and experimental procedures (except SOD and CAT activities) for this work were mentioned in our previous study (Sevgiler et al., 2007). Juvenile specimens of the freshwater fish *Cyprinus carpio* (Cyprinidae), with an average mass of 28.4 ± 1.2 g and an average length of 13.0 ± 0.2 cm, respectively, were acquired from The State Hydraulic Works Fish Culture Pools. The fish were acclimated to laboratory conditions in 100 L glass tanks filled with dechlorinated and gently aerated tap water for one month prior to experiments, in accordance with APHA, AWWA, WPCF (1981) guidelines. The mean measured water quality parameters were: temperature 19.48 ± 0.25 °C, dissolved oxygen 7.05 ± 0.15 mg/L (constant aeration), pH 8.40 ± 0.05, total hardness 284 mg/L $CaCO_3$, alkalinity 235 mg/L as $CaCO_3$ at acceptable levels. The fish were maintained under a 12-h light/12-h dark illumination period. Both experimental and control fish were fed with commercial pellet feed at a rate of 3% of their body weight per day (Çamlı Feed Co., İzmir, Turkey).

2.2. Experimental procedure

A commercial preparation of fenthion ([*O*,*O*-dimethyl-*O*-[3-methyl-4-(methylthio) phenyl] ester (IUPAC), CAS Registry Number: 55-38-9, Lebaycid EC 50, Bayer, 525 g/L) was used in the experiments. Previous concentration-effect experiments indicated that the median lethal concentration of fenthion at 96-h (96-h LC_{50}) was 2.16 mg/L (95% Confidence Interval, 2.00 to 2.39) for *C. carpio* (Sevgiler et al., 2007). A sub-lethal and nominal concentration of fenthion of 1.72 mg/L of the active ingredient, which corresponded to 80% of the 96-h LC_{50}, was selected for 96-h under static-renewal conditions for all the experiments. Water and pesticide were completely replaced once a day, transferring fish to freshly prepared pesticide solutions. There was no fish mortality during the experiments.

[1] Bayer Türk Chemicals Ltd. Co., Turkey, Bayer Türk Kimya San. Ltd. Şti. Çakmak Mah. Balkan Cad. No:53 34770 Ümraniye / İSTANBUL, Tel: (+90 216) 528 36 00, Fax: (+90 216) 527 87 00, E-mail: info@bayercropscience.com.tr.

After the period of acclimatization, the fish were randomly divided into four groups each containing eight individuals and placed in separate glass aquaria (100 L). Prior to toxicity experiments, all the fish, including control were anaesthetized in flaked ice because of the interference of the chemical anaesthesia with the GSH metabolism (Pena-Llopis et al., 2003a). Control and fenthion-only treated fish were intraperitoneally (i.p.) injected with physiological saline solution (0.59% NaCl) to avoid the injection stress. NAC was dissolved in physiological saline solution before injection. NAC-injected fish were received i.p. of 0.5 mg/kg or 400 mg/kg NAC. These four groups will be referred to as the control group, the fenthion-only group (F-only), lower-dose NAC group (LD-NAC) and higher-dose NAC group (HD-NAC), respectively, from this point forward. "All fenthion-treatment groups" indicate the combination of all groups excluding the control group. After injection, a 3-h recovery period in clean water was provided to improve the fish. Control fish were then placed in clean water, while other fish groups were placed in fenthion-water mixture for 96-h.

At the end of the 96-h fenthion treatment period, fish were immediately weighed and their lengths were measured. They were killed by decapitation and brain tissues were dissected out on an ice cold plate. Tissues were then washed in ice cold physiological saline solution, blotted dry, weighed and stored frozen at −80 °C until biochemical analyses.

2.3. Biochemical determinations

2.3.1. Preparation of tissue extracts

The brain homogenates were prepared in 1/10 (w/v) ratios with chilled 0.05 M phosphate buffer that contained 0.25 M sucrose (pH 7.4) using a stainless steel homogenizer (UltraTurrax T-25). All the processes were carried out at 4 °C. Tissue homogenates were centrifuged at 9500 ×*g* for 30 min. (Jouan MR23i) to obtain supernatants, which was then used for biochemical analysis using a spectrophotometer (Shimadzu UV-Mini 1240).

For the determination of GSH and GSSG, 0.5 volumes of ice-cold 10% 5-sulfosalicylic acid were added per mL of brain homogenates and deproteinized homogenates were then centrifuged at 9500 ×*g* for 5 min.

2.3.2. Activities of first-line defence enzymes

SOD activity was measured using the method described by McCord and Fridovich (1969). The inhibition of Iodo-*p*-nitro tetrazolium violet (INT) reduction by $O_2^{-\cdot}$ generated by xanthine–xanthine oxidase was monitored at 505 nm for 3 min. at 37 °C. A standard graphic formed by RANSOD kit was used to evaluate the enzyme activity and one unit of SOD was defined as the capability to cause 50% inhibition of INT reduction.

CAT activity was determined according to Beutler (1984) as the decrease in absorbance of 10 mM H_2O_2 at 37 °C for 2.5 min. ($\varepsilon = 0.71\ M^{-1} \times cm^{-1}$) in Tris–HCl buffer (pH 8.0). The reaction was initiated by adding 20 µL of the sample. The rate of degradation of H_2O_2 by CAT was measured spectrophotometrically at 230 nm. One CAT unit represents the amount of enzyme needed to degrade 1 mmol of H_2O_2/min/mg of total proteins present in the homogenates.

2.3.3. GSH-related enzyme activities

Glutathione reductase (GR; EC 1.8.1.7) activity was assayed according to Carlberg and Mannervik (1975) as the decrease in absorbance of NADPH at 340 nm for 3 min. The reaction medium that contains 0.12 mM NADPH, and 1 mM GSSG was completed to a total volume of 1 mL with phosphate buffer (0.1 M, pH 8) and incubated at 37 °C. Enzyme activity was calculated by using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

Glutathione *S*-transferase (GST; EC 2.5.1.18) was measured by the method described in Habig et al. (1974) using 1-chloro-2,4-