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Effects of chronic exposure of methomyl on the antioxidant system in liver of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The chronic effect of methomyl on the antioxidant system in tilapia (*Oreochromis niloticus*) was investigated. Fish were exposed to sub-lethal concentrations of 0.2, 2, 20 and 200 μ gL^{-1} for 30 days, and then transferred to methomyl-free water for 18 days. Hepatic antioxidant parameters, including Glutathione-S-transferase (GST), Glutathione peroxidase (GPx), Glutathione reductase (GR), Reduced glutathione (GSH) and oxidized glutathione (GSSG), were measured at 10 min (day 0), 6, 12, 18, 24 and 30 days after starting the experiment and at 18 days after transferring to methomyl-free water. There were no significant changes in enzymatic activity and content of antioxidants in liver of tilapia exposed to 0.2 μg L^{-1} methomyl compared to controls. However, the results showed significant increases in activities of GST, GR, GPx and levels of GSSG accompanied by a decrease in GSH levels following methomyl exposure in tilapia to 2, 20 or 200 μg L^{-1} over the 30-day exposure period and the highest induction rates in GST, GR, GPx and GSSG were 150.87%, 163.21%, 189.76%, and 179.56% of the control respectively, and the highest inhibition rate in GSH was 50.67% of the control, suggesting the presence of oxidative stress. Thus it would appear that the 0.2 μg L^{-1} methomyl might be considered as the no observed adverse effect level (NOAEL). Recovery data showed that the effects produced by lower concentration of methomyl 20 μg L^{-1} were reversible but not at the higher 200 μg L^{-1} concentration.

1. Introduction

Pesticides are used worldwide in agricultural activity, mostly to promote the harvest of products. However, these compounds are released into the environment and due to their physico-chemical properties, such as water solubility, vapor pressure or partition coefficients between organic matter (in soil or sediment) and water, they can disperse in various environmental media provoking serious health problems (Gramatica and Di Guardo, 2002). Carbamates are systemic and contact pesticides used as substitutes for organochlorine insecticides because of their high efficiency and relative low persistence in the environment (Ribera and Narbonne, 2001). Methomyl ($C_5H_{10}N_2O_2S$), *S*-methyl-1-*N*-[(methylcarbamoyl)-oxy]-thioacetimidate, is an insecticide belonging to the family of carbamate pesticides, and it is one of the environmental estrogens having endocrine disrupting effects. Because of its broad biological activity, relatively rapid disappearance and high efficiency against insects, methomyl is widely used in many agricultural countries for crop protection and soil or plant treatment (WHO, 1996). Methomyl has high water solubility (57.9 g L^{-1} at 25 °C) and a weak-to-moderate adsorption to soils, and therefore poses a contamination risk to surface and groundwater, especially the methomyl applied in the agricultural area are expected to infiltrate into the groundwater and threatens the safety of the resource for drinking water (Strathmann and Stone, 2001).

In aerobic cells, reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical etc., are generated during normal metabolism, particularly as a result of oxidative metabolism at mitochondrial membranes, and these intermediates might be detrimental to the cell, leading to a state called oxidative stress (Zhang et al., 2004). Most components of cellular structure and function are likely to be the potential targets of oxidative damage, and the most susceptible substrates for autoxidation are polyunsaturated fatty acids of the cell membrane, which undergo peroxidation rapidly. This may lead to muscle degradation, impairment of the nervous system, hemolysis, general deterioration of the cellular metabolism and eventual cell death (Zhang et al., 2004). However, Aerobic organisms have evolved defense system

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to protect themselves from the toxic effects of the increased ROS production, activating the antioxidant system, such as antioxidant scavengers, e.g. glutathione, and specific antioxidant enzymes, e.g. GPx, GR and GST (Freeman and Crapo, 1982; Winston and Di Giulio, 1991; Frei, 1999; Hogg and Kalyanaraman, 1999). Defense systems protect against attack from either endogenous (physiological production) or exogenous (xenobiotic-related) sources of ROS. Thus, there is mediation between ROS production and antioxidant scavengers in aerobic cells. Under normal physiological situation, the production of ROS and other oxygen reactive species are thought to be removed by antioxidant defense systems; however, a severe oxidative stress suppresses the activities of these enzymes and lead to oxidative damage (Livingstone, 2001). Hence, the use of biochemical measurements in organisms as pollution indicators gives valuable information about deleterious responses of organisms (Tortelli et al., 2006; Lushchak, 2011). Biochemical effects of pollutants occur more quickly, thus they provide earlier warning signal before other toxicological endpoints become evident (Livingstone, 1998; Stara et al., 2013). Monitoring of the biomarkers in living organisms including fish is a validated approach and serves as early warning of adverse changes and damage resulting from chemical exposure (Van der Oost et al., 2003).

There were some reports about the acute toxicity of methomyl on aquatic organisms (Hashimoto and Nishiuchi, 1981; Farré et al., 2002; Pereira and Gonçalves, 2007; Li et al., 2008), however, the chronic toxic effects of methomyl on aquatic organisms, especially on fish, were scarcely investigated. Thus, the aim of the present study was to investigate the chronic toxic effects of the pesticide methomyl on tilapia *Oreochromis niloticus*, by analyzing the responses of the fish liver antioxidant defense system, comprising GST, GPx, GR, GSH and GSSG.

2. Materials and methods

2.1. Fish and chemicals

Male Nile tilapia, *O. niloticus*, was chosen for this study because methomyl is one of the environmental estrogens having endocrine disrupting effects and tilapia is commonly available in most fish farms worldwide. Specimens of *O. niloticus* with an average weight of 150.7 ± 9.7 g and length of 19.0 ± 1.4 cm were supplied by the fish farm of Freshwater Fisheries Research Center, Chinese Academy of Fishery Science (Wuxi, China). Before the experiments fish were acclimated under laboratory conditions for 4 weeks at a population density of 30 specimens in 200-L glass aquaria supplied with dechlorinated tap water. The physicochemical characteristics of the water used in the aquaria were analyzed according to methods in "Standard method for the examination of water and wastewater" (State Environmental Protection Agency of China, 2002). The water had a pH of 7.3 ± 0.3 and a temperature of 25 ± 0.5 °C. Water hardness was 107 mg L^{-1} (as $CaCO_3$), and dissolved oxygen concentration was 6.5–7.0 mg L^{-1}. The stocks fish and experimental fish were all fed two percent body mass daily, with a commercial fish feed (Ningbo Tech-bank Co., Ltd., China) and submitted to a 12-h light and 12-h dark photoperiod. Fish were used when no mortality was observed in the acclimation population. Methomyl (97% w/w) was produced by Shanghai Focus Biological Technology Co., Ltd., China. All other chemicals used were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) and Sangon (Shanghai, China).

2.2. Experimental design

Male tilapia were randomly distributed into 200-L glass aquaria containing different concentrations of methomyl (control, 0.2, 2, 20 and 200 μg L^{-1}), with 3 replicates per treatment, and the range of exposure concentrations was based on the information from the previous study on 96 h LC_{50} (430 μg L^{-1}) for tilapia (with an average weight of 3.9 ± 0.4 g and length of 6.3 ± 0.6 cm), the residue level (0–55.3 μg L^{-1}) of methomyl in environmental water (Van Scoy et al., 2013) and the U.S. EPA on drinking water quality established a maximum permissible concentration for methomyl of 200 μg L^{-1} (U.S. EPA, 2012). The actual methomyl concentrations in the test water were measured by the method of Chen et al. (1996). The actual methomyl concentrations of control, 0.2, 2, 20, 200 μg L^{-1} groups were 0, 0.24, 2.06, 21.55, 205.57 μgL^{-1} respectively at 0 h of exposure (the initial concentrations), and were 0, 0.20, 1.95, 19.55, 198.57 μgL^{-1} respectively after 24 h of exposure. And the results were discussed in relation to the nominal concentrations.

Thirty fish were introduced into each concentration in a semi-static system and water was renewed daily. The experiment last for 48 days, and after 30 days of exposure period the left fish were transferred to methomyl-free water for 18 days and then the same parameters were carried out for studying the recovery response. sampling of exposed and control fish (n=6 per group, 2 tilapia per replicate) was done at 10 min (day 0), 6, 12, 18, 24, and 30 days after starting the experience and at 18 days (R18) after transferring to methomyl-free water for recovery. Feed was withheld 24 h prior to sampling. Tilapia were euthanized in approximately 250 mg L^{-1} ethyl 3-aminobenzoate methanesulfonate salt (MS-222, TCI Inc., Japan), and thereafter measured and weighed. Then, fish were rapidly killed by decapitation and the liver immediately removed and processed, and then snap-frozen in liquid nitrogen and stored at −80 °C for later assay.

Samples were stored at −80 °C with an Ultra-low temperature freezer (Forma-86C, America). Samples were homogenized using an electrical homogenizer (Pro-200, America). Centrifugations were done with a refrigerated centrifuge (Sigma 2-16K, Germany). Spectrophotometric readings were carried out with a UV–vis spectrophotometer (UV-759S, China).

2.3. Measurement of enzyme activity parameters

2.3.1. Samples preparation

Tilapia livers were homogenized using an electrical homogenizer. About 0.30 g of liver tissue was homogenized after addition of 3.0 ml of 10.0 mM Tris buffer (pH 7.5) for detection of enzyme activities. About 0.10 g of liver tissue was homogenized after addition of 1.0 ml of 1.0 mmol/l EDTA and 10 μl $HClO_4$ for measurement of GSH and GSSG. The extracts were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was stored at 4 °C and used immediately as the enzyme analysis. All the above operations were carried out at 4 °C.

2.3.2. Biochemical analysis

Glutathione peroxidase (GPx) activity was measured by the method of Hafeman et al. (1974), and one unit was defined as a decrease in GSH of 1 μmol/min after the per-minute decrease in non-enzyme reaction was subtracted. Glutathione-S-transferase (GST) activity was assessed using 1-chloro-2, 4 -dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974), and one unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under the condition of the specific assay. Glutathione reductase (GR) activity was measured according to the method described by Carlberg and Mannervik (1975), and one unit was defined as the amount of enzyme oxidating 1 μmol of NADPH min^{-1} under the condition of the specific assay. Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were measured by the method of Hissin and Hilf (1976), and GSH and GSSG contents were expressed in μg mg^{-1} protein. Protein levels were estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Statistical analysis

The activities of GST, GPx, GR and the content of GSH, GSSG of treated tilapia were compared with control group in each sampling day, including recovery group (R18), and expressed as percent of the control. All data were expressed as means ± standard deviation (n=6). One-way analysis of variance (ANOVA) was used for statistical comparisons with $P<0.05$ being considered significant.

3. Results

Neither mortality nor visible disease signals were observed in the tilapia exposed to sublethal concentrations of methomyl during the performance of the experiment. Changes in the activities of antioxidant enzymes GST, GR and GPx in experimental fish were shown in Figs. 1–3 correspondingly, and changes in the contents of non-enzymatic antioxidant parameters GSH and GSSG in experimental fish were shown in Figs. 4 and 5 respectively.

Exposure of tilapia to methomyl altered the normal functioning of hepatic antioxidants activities. Compared with control, no significant ($P>0.05$) changes in GST, GR, GPx activities were observed in 0.2 μg L^{-1} group. GST, GR and GPx activities were significantly ($P<0.05$) increased in 2 μg L^{-1} group after 18 days of methomyl exposure. As the dose was increased to 20 μg L^{-1}, significant ($P<0.05$) elevation was noted for GR and GPx at 12 days. With respect to GST, significant ($P<0.05$) increase in 20 μg L^{-1} group occurred after 18 days of methomyl exposure.