Article

Effect of Chronic Exposure to Pesticide Methomyl on Antioxidant Defense System in Testis of Tilapia (*Oreochromis niloticus*) and Its Recovery Pattern

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Abstract: The chronic effect of environmental methomyl on the antioxidant system in testis of Nile tilapia (*Oreochromis niloticus*) and its recovery pattern was investigated. Tilapia were exposed to sublethal concentrations of 0.2, 2, 20 and 200 $\mu\text{g L}^{-1}$ methomyl for 30 days and thereafter moved to methomyl-free water for 18 days. Antioxidant levels in testis, including glutathione peroxidase, catalase, glutathione-S-transferase, glutathione reductase, superoxide dismutase, reduced glutathione, oxidized glutathione were measured every 6 days during the period of exposure, and at 18 days after being transferred to methomyl-free water. The results showed that lower methomyl concentration (0.2 $\mu\text{g L}^{-1}$) had no effect on the above antioxidants, thus 0.2 $\mu\text{g L}^{-1}$ could be seen as NOAEL for methomyl to tilapia. However, higher methomyl concentration of 2, 20 and 200 $\mu\text{g L}^{-1}$ could significantly influence the above antioxidants. Glutathione peroxidase and oxidized glutathione increased significantly. On the contrary, reduced glutathione decreased significantly. Catalase, superoxide dismutase, glutathione reductase, glutathione-S-transferase increased at lower methomyl (2 and 20 $\mu\text{g L}^{-1}$), but decreased at higher methomyl (200 $\mu\text{g L}^{-1}$). The recovery test showed that oxidative damage caused by lower methomyl of 2 and 20 $\mu\text{g L}^{-1}$ was reversible, and oxidative damage caused by higher methomyl of 200 $\mu\text{g L}^{-1}$ was irreversible within 18 days of recovery period.

Keywords: tilapia; biomarkers; catalase; glutathione peroxidase; glutathione reductase; glutathione-S-transferase; oxidized glutathione; reduced glutathione; superoxide dismutase

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1. Introduction

Methomyl ($\text{C}_5\text{H}_{10}\text{N}_2\text{O}_2\text{S}$) is a carbamate pesticide yet is widely used and has greatly contributed to pest control and promoted the harvest of agricultural products. Because of its high solubility in water (57.9 g L^{-1} at 25 °C) and only weak to moderate adsorption in soils, methomyl has been detected in surface waters and food crops [1], with residues as high as 55.3 $\mu\text{g L}^{-1}$ reported in environmental water [2]. Owing to its frequent detection in natural water bodies and its endocrine disruption potential in animals [3], the toxicity of methomyl to aquatic animals has attracted considerable attention [4–6].

Reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical, are generated in aerobic cells during normal metabolism, especially through mitochondrial oxidative metabolism, and some of these intermediates are thought to be harmful to cells, and leads to a state of oxidative stress and causing oxidative damage [7]. Oxidative damage can result in hemolysis, muscle degradation, nervous system impairment, cellular metabolism deterioration, and even cell death [7]. Nevertheless, aerobic organisms have evolved mechanisms to activate antioxidant defense systems in various organs and tissues; these involve

antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), as well as antioxidant scavengers (e.g., glutathione), to protect from damage caused by high levels of ROS [4]. Antioxidant defenses protect against attack from either exogenous (xenobiotic) or endogenous (physiological) sources of ROS. Thus, there is mediation in aerobic cells between antioxidant scavengers and ROS. ROS can be eliminated by antioxidant defenses during normal physiological processes; however, severe oxidative stress will suppress the activities of antioxidant enzymes and lead to oxidative damage [8]. Antioxidant enzymes can be sensitive to pollutants and are capable of being induced quickly; hence, they are relatively sensitive indicators of environmental harm compared with other toxicity parameters [3,9]. The antioxidant enzymes GPx, CAT, GST, SOD and GR, and the non-enzymatic antioxidants reduced glutathione (GSH) and oxidized glutathione (GSSG), are often used as biomarkers for early detection of harmful effects as well as chronic damage caused by exposure to toxic substances [10].

Acute toxicity of the pesticide methomyl to aquatic animals has been reported [11]; however, its sublethal toxicity to aquatic animals, especially fish, has been scarcely examined. Therefore, the present study investigated sublethal toxicological effects of the endocrine disruption pesticide methomyl to Nile tilapia (*Oreochromis niloticus*) by analyzing antioxidant defense parameters in testis, namely, the activities of GST, GPx, CAT, SOD and GR, and the contents of GSH and GSSG, to better understand the harmful effects of methomyl to fish and to protect the quality of our water bodies.

2. Materials and Methods

2.1. Fish and Chemicals

Male Nile tilapia were supplied by the fish farm at the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Science (Wuxi, China). The average weight and length of the tilapia were 112.24 ± 9.48 g and 17.06 ± 0.91 cm. Prior to the experiment, the fish were cultured in 200-L glass aquaria for 4 weeks to acclimate to laboratory conditions. The experiment used dechlorinated tap water, with a pH, mean temperature, and dissolved oxygen content of 7.0 ± 0.5 , 25 ± 0.5 °C, and $6.3\text{--}7.0$ mg L⁻¹, respectively. Fish were fed a commercial feed (Ningbo Tech-bank Co., Ltd., China) at the daily rate of 2% body weight. The photoperiod was 12-h light/12-h dark. Methomyl (CAS 16752-77-5) of 97% (w/w) purity was used. All other reagents were of analytical grade and were bought from Sangon Biotech (Shanghai, China) or Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental Design

Thirty tilapia were randomly stocked into glass aquaria with a water volume of 200 L. The concentrations of methomyl tested were 0 (control), 0.2, 2, 20 and 200 µg L⁻¹, and each concentration had three replicates. The concentration range was chosen according to information obtained from our previous research to determine the acute toxicity of methomyl to tilapia (i.e., 96-h LC₅₀ was 430 µg L⁻¹), the methomyl residue levels found in environmental water ($0\text{--}55.3$ µg L⁻¹) [2], and the maximum permissible methomyl concentration in drinking water (200 µg L⁻¹) established in 2012 by the United States Environmental Protection Agency. The actual concentrations of methomyl in the experimental water were measured using ultra-performance liquid chromatography-tandem mass spectrometry, following a method established by our research team [12].

Half the aquarium water was renewed daily, and methomyl was added as needed to maintain the given concentrations. The experiment lasted for 48 days, with 30 days for methomyl exposure and 18 days for recovery in methomyl-free water. Testis ($n = 6$ per group) were sampled at 10 min (day 0), and at days 6, 12, 18, 24 and 30 in the exposure period (note: only the samples taken at day 30 were used here), and at 18 days after the fish were moved to methomyl-free water. Fish were euthanized using 250 mg L⁻¹ MS-222, and then measured and weighed. Thereafter, the testis was immediately sampled, snap-frozen using liquid nitrogen and stored at -80 °C until analysis. An electric homogenizer

(PRO200; PRO Scientific Bio-Gen, USA), refrigerated centrifuge (Sigma 2-16K, Germany), and ultraviolet-visible spectrophotometer (UV-Vis 759S, China) were used to homogenize, centrifuge, and quantify the samples.

2.3. Biochemical Analysis

Tilapia testes were homogenized using an electrical homogenizer. About 0.30 g of testis 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 testes tissue was homogenized after addition of 1.0 mL of 1.0 mmol/L EDTA and 10 μ L HClO_4 for measurement of reduced glutathione and oxidized glutathione. The extracts were centrifuged at $10,000\times$ 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.

Glutathione peroxidase (GPx) activity was measured according to the method described by Hafeman [13], and one unit was defined as a decrease in reduced glutathione of 1 μ mol/min after the per-minute decrease in non-enzyme reaction was subtracted. Catalase (CAT) activity was assayed by ultraviolet spectrophotometry [14], and one unit of enzyme activity is defined as the amount of enzyme which decreased the concentration of H_2O_2 by 50% in 100 s at 25 °C. Glutathione-S-transferase (GST) activity was assessed using 1-chloro-2, 4 -dinitrobenzene (CDNB) as substrate, according to the method described by Habig [15], and one unit was defined as the amount of enzyme catalyzing the formation of 1 μ mol 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 [16], and one unit was defined as the amount of enzyme oxidizing 1 μ mol of NADPHmin^{-1} under the condition of the specific assay. Superoxide dismutase (SOD) was assayed according to the method described by Marklund and Marklund [17], and one unit of SOD activity is defined as the amount of the enzyme which gave 50% inhibition of the oxidation rate of 0.1 mM pyrogallol in one ml of solution at 25 °C. Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were measured by the method of Hissin and Hilf [18], and GSH and GSSG contents were expressed in μgmg^{-1} protein. Protein levels were estimated by the method of Bradford [19] using bovine serum albumin as a standard.

2.4. Statistical Analysis

The activities of SOD, CAT, GST, GPx and GR, and the contents of GSH and GSSG in testis of methomyl-exposed fish were compared with the levels in testis of the controls for each sampling day; results were expressed as percentage of the control. All data were expressed as mean $\pm$ SD ($n = 6$). ANOVA was used for statistical comparisons, with $\alpha = 0.05$.

3. Results

The actual methomyl concentrations in the 0 (control), 0.2, 2, 20 and 200 $\mu\text{g L}^{-1}$ groups were 0, 0.23, 2.12, 21.50, 182.0 $\mu\text{g L}^{-1}$ respectively, just after the fish were exposed, and 0, 0.21, 1.92, 18.52, 179.0 $\mu\text{g L}^{-1}$ respectively, after exposure for 24 h. And the results were discussed in relation to the nominal concentrations.

The results of effect of chronic exposure to pesticide methomyl on antioxidant defense system in testis of tilapia (*Oreochromis niloticus*) and recovery pattern were showed in Figure 1 and Table S1. No significant changes ($p > 0.05$) were observed in the antioxidant enzyme activities and contents in testis of tilapia exposed to 0.2 $\mu\text{g L}^{-1}$ methomyl, whereas the testis of fish exposed to concentrations of 2, 20 or 200 $\mu\text{g L}^{-1}$ showed significant changes ($p < 0.05$): GPx and GSSG significantly increased, and GSH significantly decreased ($p < 0.05$). Moreover, dose-response relationships were found between methomyl and GPx, GSSG or GSH. Specifically, GPx and GSSG increased with increasing concentrations of methomyl; GSH decreased with an increase in methomyl concentration. CAT, SOD, GR

and GST were induced in testis of tilapia exposed to 2 and 20 $\mu\text{g L}^{-1}$; however, they were inhibited in testis of fish exposed to 200 $\mu\text{g L}^{-1}$.

Figure 1. Cont.

Figure 1. SOD (a), CAT (b), GST (c), GR (d), GPx (e) activities and GSH (f), GSSG (g) contents in testis of tilapia exposed to methomyl for 30 days, and after an 18-day recovery period in methomyl-free water (R18). Note: All the data were expressed as Average $\pm$ SD ($n = 6$). “*” means significant difference from the control. Different lower-case letters indicate significant difference among concentrations at the same exposure period, and different upper-case letters indicate difference between exposure and recovery periods at the same methomyl concentration, and with $p < 0.05$ being considered significant.

When the tilapia were transferred to methomyl-free water to recover for 18 days, SOD, GR, GST, CAT, GPx, GSSG and GSH in testis of fish in each group recovered to some extent. There were no significant differences in SOD, GR, GST, CAT and GPx activities, nor in GSSG and GSH contents, in fish exposed to $2 \mu\text{g L}^{-1}$ and $20 \mu\text{g L}^{-1}$ compared with the controls. Conversely, there were significant changes ($p < 0.05$) in the $200 \mu\text{g L}^{-1}$ treatment group compared with the control group.

4. Discussion

4.1. Effect of Methomyl on Antioxidant Defenses in Tilapia Testis

GR, GPx, GST, GSSG and GSH are components of the glutathione-related antioxidant system and play important roles in intercellular defense against oxidative damage [20]. Various relationships exist between GST, GR, GPx, GSSG and GSH. GPx can catalyze GSH to GSSG, accompanied by the detoxification of hydroperoxides and ROS, and GSSG is then reduced to GSH during GR catalysis with the consumption of NADPH that is recycled into the pentose phosphate pathway [7]. The conjugation of GSH to xenobiotics can be catalyzed by GST, which facilitates chemical excretion by the addition of more polar groups [21]. Therefore, all these antioxidants work together to mitigate and prevent oxidative damage.

GSH is a major low-molecular-weight sulfhydryl compound in the cytosol, and acts as a cellular reducing, protective reagent against numerous pollutants through the sulfhydryl ($-\text{SH}$) group. As an antioxidant enzyme substrate and a direct scavenger of oxyradical, GSH plays an important role in detoxification and the elimination of oxidative damage through the $-\text{SH}$ group [7,22]. Our experiment showed that GSH levels in testis of tilapia exposed to methomyl decreased significantly, especially at the high concentrations (Figure 1f), which might be explained as follows. First, because of the conjugation of GSH to methomyl catalyzed by GST, GSH could conjugate with electrophilic intermediates catalyzed by GST, which will decrease GSH levels [21]. A similar decreasing trend in GSH content accompanied by a rise in the GST level was reported by other researchers [7,23]. In our experiment, a significant increase in GST was accompanied by a decrease of GSH in testis of fish exposed to 2 and $20 \mu\text{g L}^{-1}$ methomyl (Figure 1c). Second, GSH can scavenge ROS through the oxidation of GSH to GSSG catalyzed by GPx, which will decrease the level of GSH [24]. Previous researchers have found decreasing levels of GSH accompanied by increases in GSSG and GPx in Nile tilapia exposed to methomyl [11], in rainbow trout

(*Oncorhynchus mykiss*) exposed to carbamazepine [22], and in Nile tilapia exposed to domoic acid [25]. In the present study, the steady rise in GSSG and GPx was accompanied by a decrease in GSH in testis of the tilapia exposed to methomyl concentrations 2, 20, and 200 $\mu\text{g L}^{-1}$ (Figure 1g). Overall, these results suggest that the transformation of GSH to GSSG happens under the oxidative stress caused by toxic materials [22]. Additionally, a loss of adaptive mechanisms and damage to GSH synthesis during the stress condition caused by methomyl exposure might be other reasons for declines in GSH content [22]. For instance, similar changes in GSH levels were observed in goldfish (*Carassius auratus*) exposed to 2-chlorophenol [21] and in rainbow trout exposed to the fungicide propiconazole [23]. GR can reduce GSSG to GSH with the consumption of NADPH, thereby effectively regulating the formation of ROS during exposure to exogenous pollutants. Under oxidative stress conditions, an increase of GR activity always reestablished the level of GSH, which is oxidized and effectively regulates ROS formation [26]. Likewise, in our own experiment, increases in GR were found at 2 $\mu\text{g L}^{-1}$ and 20 $\mu\text{g L}^{-1}$ methomyl. A similar trend in GR levels was found in liver of Nile tilapia exposed to methomyl [11] and in liver of snakehead (*Channa punctatus*) exposed to atrazine [27]. Moreover, this compensatory response of GR appears to invariably be accompanied by increases in CAT and SOD, which help prevent the production and accumulation of free radicals caused by toxic materials [28]; comparable trends in the changes in GR in relation to SOD or CAT were observed in our experiment.

SOD can protect an organism's cells from the oxidative stress caused by free radicals; it can catalyze free radical of superoxide anion to O_2 , and then H_2O_2 is catalyzed to H_2O with the help of CAT or GPx. SOD could reportedly be induced during high production of free radical of superoxide anion [29]; therefore, the significant increase in SOD ($p < 0.05$) in our study (Figure 1a) could be considered a direct response to the free radical of superoxide anion in response to methomyl, just as reported previously in tilapia exposed to methomyl [11] and in rainbow trout exposed to selenite [30]. CAT catalyzes hydrogen peroxide to O_2 and H_2O ; hence, the increase of CAT in the present study (Figure 1a) could reduce the oxidative stress of hydrogen peroxide produced by the methomyl exposure [28].

In our experiment (Figure 1a–d), the activities of SOD, CAT, GST and GR significantly increased ($p < 0.05$) in testis of tilapia exposed to 2 $\mu\text{g L}^{-1}$ or 20 $\mu\text{g L}^{-1}$ methomyl; however, the antioxidant enzymes decreased significantly ($p < 0.05$) in fish exposed to a concentration of 200 $\mu\text{g L}^{-1}$. A similar trend was described for topmouth gudgeon (*Pseudorasbora parva*) exposed to methomyl [31]. It has been reported that the dose influences the activity of antioxidant enzymes in fish exposed to pollutants [11]. It could be speculated that, because the methomyl could not be metabolized and was not completely removed from the tilapia body by the cells under conditions of chronic exposure and a high dose, the methomyl accumulated in the body and consequently destroyed the protein structures of the antioxidant enzymes, and finally manifested as decreased antioxidant enzyme activity, namely that of SOD, CAT, GST and GR [11,32].

No significant changes in the activities of SOD, CAT, GST, GR, and GPx or the contents of GSSG and GSH were found in testis of the fish exposed to 0.2 $\mu\text{g L}^{-1}$ compared with the controls (Figure 1a–g), which means that 0.2 $\mu\text{g L}^{-1}$ methomyl could not induce oxidative stress. In our concomitant tests, we also found no tissue damage or apoptosis of the testis of tilapia exposed to 0.2 $\mu\text{g L}^{-1}$ methomyl [33]. Therefore, the methomyl concentration of 0.2 $\mu\text{g L}^{-1}$ could be considered the NOAEL (No Observed Adverse Effect Level) for tilapia. Our previous acute toxicity test showed that the 96 h LC_{50} of methomyl to tilapia was 430 $\mu\text{g L}^{-1}$ [33], and it is 2150 times of NOAEL. It is always thought that one-tenth of 96 h LC_{50} can be considered as safety concentration of harmful materials to fish [34], however, the present test showed that maybe shorter than one-thousandth of 96 h LC_{50} can be considered as NOAEL of harmful materials to fish.

4.2. Recovery Pattern

When the tilapia were transferred to methomyl-free water to recover for 18 days, SOD, GR, GST, CAT, GPx, GSSG and GSH in testis of fish in each group recovered to some extent.

There were no significant differences in SOD, GR, GST, CAT and GPx activities, nor in GSSG and GSH contents, in fish exposed to 2 $\mu\text{g L}^{-1}$ and 20 $\mu\text{g L}^{-1}$ compared with the controls, which means that these antioxidant parameters could recover to normal levels and were reversible at methomyl concentrations of no more than 20 $\mu\text{g L}^{-1}$ given a recovery period of at least 18 days. Conversely, there were significant changes ($p < 0.05$) in the 200 $\mu\text{g L}^{-1}$ treatment group compared with the control group; thus, the antioxidant parameters could not recover to normal levels and appeared irreversible following methomyl exposure at 200 $\mu\text{g L}^{-1}$, even after an 18-day recovery period.

5. Conclusions

No significant changes ($p > 0.05$) were observed in antioxidant enzyme activities and contents in testis of Nile tilapia exposed to the low methomyl concentration of 0.2 $\mu\text{g L}^{-1}$. Thus, together with those of our synchronous work about effect of methomyl on gonad tissue damage to the same fish [33], 0.2 $\mu\text{g L}^{-1}$ methomyl might be considered the NOAEL for tilapia.

Oxidative damage occurred when tilapia were exposed to 2, 20 or 200 $\mu\text{g L}^{-1}$ methomyl. GPx and GSSG increased significantly ($p < 0.05$). Conversely, GSH decreased significantly ($p < 0.05$). CAT, SOD, GR and GST increased at the relatively low concentrations of 2 $\mu\text{g L}^{-1}$ and 20 $\mu\text{g L}^{-1}$ but decreased at the highest concentration tested (200 $\mu\text{g L}^{-1}$).

Oxidative damage caused by the lower methomyl levels of 2 and 20 $\mu\text{g L}^{-1}$ was reversible within 18 days after exposure, but the damage caused by 200 $\mu\text{g L}^{-1}$ methomyl was irreversible.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11083332/s1>, Table S1: SOD, CAT, GST, GR, GPx activities and GSH, GSSG contents in testis of tilapia exposed to methomyl for 30 days, and after an 18-day recovery period in methomyl-free water (R18).

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