EFFECTS OF METHIDATHION ON ANTIOXIDANT SYSTEM AND EXPRESSION OF HEAT SHOCK PROTEIN 70 (HSP70) GENE IN THE LIVER OF *Oreochromis niloticus* L. 1758

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ABSTRACT

In this study, effects of sublethal concentrations of methidathion on catalase (CAT), superoxide dismutase (SOD), glutathion peroxidase (GSH-Px) activities and lipid peroxidation with expression of heat shock protein (HSP70) gene in liver tissues of *Oreochromis niloticus* were investigated. The 96-h LC_{50} value for methidathion was determined as 0.1045 mg/L for *O. niloticus* in this study. 0.013 (1/8 of LC_{50}) and 0.026 mg/L (1/4 of LC_{50}) sublethal concentrations were applied for 4 and 10 days in this experiment. CAT and GSH-Px activities in liver tissue were increased, in relation to both the time and doses applied. SOD activities were increased under the influence of both concentrations at the 4th day, but on 10th day, it was decreased with influence of 0.026 mg/L methidathion concentration. The tissue MDA levels were significantly increased in relation to both the time and dose applied. The relative mRNA levels of the HSP70 gene were detected by real time polymerase chain reaction (RT-PCR). The mRNA levels of HSP70 gene by methidathion increased significantly on 4th days compared to control, but decreased significantly on 10th days.

KEYWORDS:
Oreochromis niloticus, methidathion, antioxidant enzyme, HSP70

1. INTRODUCTION

The aquatic ecosystem is contaminated with toxic chemicals from domestic, industrial and agricultural activities. Pesticides are one of the major classes of toxic compounds used in agricultural activities [1]. Exposure to environmental factors, such as pesticides and other chemicals, can increase reactive oxygen species (ROS) production and affect normal cellular redox status. When the production of ROS is greater than the cells' ability to remove them, damage can occur in DNA, lipids and protein molecules [2]. Organophosphate pesticides (OPs) are the most widely used synthetic chemicals for controlling a variety of pests. The main target of OPs action is the central and peripheral nervous system in animals [3]. Besides, many authors claim that these compounds in both acute and chronic intoxication disturb the redox processes, changing the activities of anti-oxidative enzymes and causing enhancement of lipid peroxidation (LPO) in many organs in organisms [4, 5].

ROS, such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are continuously formed in oxygen-consuming organisms. Exposure to xenobiotics or toxic chemical pollutants may produce an imbalance between these endogenous and exogenous ROS, and can subsequently induce a decrease in antioxidant defenses or cause oxidative damage in organisms [6]. Defence systems that tend to inhibit ROS formation include the antioxidant enzymes, such as catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1) and glutathione peroxidase (GSH-Px; EC 1.11.1.9) [7]. LPO has also been used as a bio-indicator of oxidative damage in aquatic organisms exposed under polluted environmental conditions. LPO can be increased in the tissues of fish, as indicated by an increased production of malondialdehyde (MDA). The most widely used test for oxidative stress is the measurement of MDA, a product of lipid peroxidation, by the thiobarbituric acid (TBA)-reacting substances assay [6].

Heat shock proteins (HSPs) also known as stress proteins consist of a family of molecules that play an important role in the cellular stress response [8, 9]. HSPs have been demonstrated to increase markedly in all animals, in response to a group of stressors including temperature, exposure to heavy metals, pesticides, oxidizing and sulfhydryl reagents, chelating drugs, inhibitors of gene expression, etc. [8, 9]. The synthesis of this protein may assist the cells to deal with various stressors and to protect cells from subsequent stressors [10]. HSP70 family has been most widely used as a biomarker due to its rapid and significant increase

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during cellular stress [11]. The induction of HSP70 by different pesticides has been demonstrated in several aquatic organisms, both under laboratory and field conditions [11, 12].

Methidathion (MD; 0,0-dimethyl S-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl) phosphorodithioate) is one of the OPs. MD is a non-systemic insecticide, registered for the control of a wide range of agricultural mite and insect pests in terrestrial food crops [13]. MD treatment in previous studies resulted in increased oxidative stress and altered activities of superoxide scavenging enzymes in different tissues of organisms [14, 15].

The liver plays an important role in several vital functions of basic metabolism, and it is also the major organ of accumulation, biotransformation and excretion of contaminants in fish, including degradation and bioactivation of pesticides [1]. The evaluation of biochemical changes in fish liver has become an important tool for monitoring environmental to contaminants in experimental studies [1, 16].

Fish have been widely used as models to evaluate the health of aquatic ecosystems in toxicological pathology [17]. In the present study, *Oreochromis niloticus* was chosen as an experiment model, because of its wide availability and suitability for toxicity testing [5]. We have determined the 96-h LC_{50} value for MD for *O. niloticus*. And also, we have studied the effects of sublethal concentrations of MD (0.013 and 0.026 mg/L) on oxidative stress biomarkers and HSP70 gene mRNA level in the liver tissue of *O. niloticus* for 4 and 10 days.

2. MATERIALS AND METHODS

2.1 Chemicals

High Pure RNA Tissue Kit (Roche) and Transcriptor First-Strand cDNA Synthesis Kit (Roche) were used for RNA isolation and cDNA synthesis**.** Probes were obtained using the Assay Design Center (Roche Applied Science, http://www. Roche-applied-science.com). The organophosphate insecticide methidathion (0,0-dimethyl S-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3 ylmethyl) was purchased as Pestanal grade chemical (95.6 % purity; Sigma Aldrich). The chemicals used for enzyme activity measurements were also purchased from Sigma-Aldrich.

2.2 Toxicity test

For the toxicity tests, groups containing 10 fish were placed into a glass aquarium. Fish were starved 2 days before the beginning of the experiments. Fish were divided into control and test groups. Different concentrations of MD were applied (0.005, 0.01, 0.05, 0.10, 0.15, 0.25, 0.50 and 1.00 mg/L). During the 96-h experiment, the water was aerated continuously. Each test solution was renewed daily. The dead fish were removed and recorded. This assay was applied twice. At the end of the experiment, median lethal concentrations (LC_{50}) were determined for a 96-h period. The LC_{50} values were calculated by probit analysis using SPSS Version 15.0 software. The 96-h LC_{50} value for *O. niloticus* was determined as 0.1045 mg/L in this study.

2.3 Test animals and treatment

Juvenile *O. niloticus* (mean weight: 28.00 ± 0.88 g; mean length:11.00 ± 0.20 cm) were obtained from Mersin University, Fisheries Faculty, Aquaculture Department and transferred to the laboratory where the temperature was kept at 24 ± 2 °C (12:12 L:D). Throughout the experiments, dechlorinated tap water with pH value of 7.85, an alkalinity of 326 mg/L $CaCO_3$, and oxygen concentration of 6.70 mg/L was used. The fish were allowed to acclimatize to these conditions for 2 weeks. The fish were fed at a rate of 2% body weight/day with a commercial pellet diet (Çamlı-Yem, Izmir-Turkey) during the acclimation period. Experiments were conducted in glass aquaria containing 100 L of test solution. Fish were exposed to 0.013 (1/8 of LC_{50}) and 0.026 (1/4 of LC_{50}) mg/L sublethal concentrations of MD for 4 and 10 days. Stock solution was prepared by MD and diluting it in acetone to give the dosing concentrations. The water was refreshed every 2 days to compensate for the pesticide lost in the exposure medium. Seventy-two fish were divided into 3 experimental groups, each with 24 fish, as follows: Control (Cont) group; 0.013 mg/L MD1 group (13 µg/L MD1); 0.026 mg/L MD2 group (26 µg/L MD2) group. The control group was exposed to acetone at the highest concentration of stock solution used in the MD-exposed groups (the absence of MD). Thirty-six fish were used for oxidative stress parameters and 36 fish were used for gene expression. At the end of each exposure period, 12 fish of each treated group as well as of the control group were removed from each tank and killed by transaction of the spinal cord. The liver tissues of both control and treated fish were dissected. Tissue samples were obtained from an individual fish and prepared for analysis.

2.4 Enzyme assays

2.4.1 Preparation of the homogenate

The liver tissues were homogenized to 1/5 (w/v) ratio in physiological saline solution (0.8% NaCl) with homogenizer, and then centrifuged at 13500 rpm for 10 min in a Sigma 2–16 K centrifuge at +4 °C, and supernatant was used for biochemical analyses.

2.4.2 Determination of enzyme activities

The CAT activities of liver tissues were determined according to the method of Aebi [18]. The enzymatic decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity.

The SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O_2 generated by the xanthine/xanthine oxidase system [19]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The reduction in

NBT by superoxide anions to blue formazan was measured at 560 nm. The enzyme activities are given in U/mg protein.

The total GSH-Px activity was assayed by Jocely method [20], using H_2O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) as substrates. The conversion of NADPH to nicotinamide adenine dinucleotide phosphate (NADP) was followed by recording the changes in absorption intensity at 340 nm, and one unit of GSH-Px activity was defined as the amount of protein that oxidizes 1 molar NADPH per min, and is expressed as unit of tissue protein content.

2.5 Measurement of MDA levels

The levels of MDA homogenized tissues, as an index of LPO, were determined by TBA reaction using the method of Yagi [21]. MDA and other aldehydes when boiled with thiobarbituric acid at acidic pH give a pink-colored product that can be assayed spectrophotometrically. Briefly, a 50 µL sample of tissue homogenate was mixed with 750 µL of TBARS reagent. The mixture was incubated for 30 min in a boiling water-bath. After cooling, the mixture was centrifuged at 3500 rpm for 15 min. Absorption was measured at 532 nm, and the values are expressed as nanomoles of MDA/mg protein.

2.6 Protein determination

The tissue protein contents were measured only to determine the specific activity of antioxidant enzymes according to the method developed by Lowry *et al.* [22] using bovine serum albumin as standard. Absorbances of samples were measured at 750 nm wavelength by a spectrophotometer.

2.7 Real-time PCR assay

2.7.1 RNA isolation and cDNA synthesis

Total RNA was isolated from fast-frozen liver tissues of both control and exposed groups using High Pure RNA Tissue Kit (Roche®) according to the manufacturer's protocol. Following isolation, cDNA synthesis was performed using Transcriptor First-Strand cDNA Synthesis Kit (Roche®) according to the manufacturer's protocol. All cDNA were stored at -70 °C until use. Beta-Actin (β-Actin) was used as a positive control to relative for PCR procedure.

Primers were designed in Primer3 web (version 4.0.0) (http://primer.ut.ee) using *O. niloticus* HSP70 (GenBank accession number FJ213839) and β-Actin (GenBank accession number AY116536) (Table 1). UPL (Universal Probe Library) probes were used for RT-PCR using the Roche Light Cycler 480 RT-PCR System. UPL probes were designed using the Assay Design Center (Roche Applied Science, http://www. Roche-applied-science.com).

Quantification of the gene expression by RT-PCR analysis was performed using a thermal cycler. For PCR, 0.4 µL HSP forward primer; 0.4 µL HSP reverse primer; 0.4 UPL µL probe; 10 µL probe master; 3.8 µL PCR grade water were mixed in a total reaction volume of 15 µl. For each sample, 5 µl cDNA was added to the mixture and total reaction volume of 20 µl was performed on a Light Cycler 480 PCR System. Thermal cycler conditions were as follows: 1 cycle at 95 °C for 10 min, 45 cycle at 60 ºC for 30 s, 72 °C for 1 s, and 1 cycle at 40 °C for 60 s. The same method was applied for the β-Actin gene.

TABLE 1 - Sequence of primers used in the amplification of HSP and Actin cDNA

Gene	Primer Sequence
HSP70	Forward 5´-GTGTGGGGGTTTTCCAACAT-3´ Reverse 5´-ATTTTGGGCTTCCCTCCGTC-3´
β-Actin	Forward 5´-AAAAATCAAGCGCCCACAGC-3´ Reverse 5´-CAAACACCGGTTTTAGCGCA-3´

2.8 Statistical analysis

The LC_{50} values were calculated by probit analysis using SPSS Version 15.0 software (SPSS Inc., USA). Data were presented as means ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests at $P<0.05$.

3. RESULTS

3.1 Toxicity assay

In the present study, different concentrations of MD were administrated to *O. niloticus*. The mortality rates of fish were calculated as a percentage after 96-h of MD treatment. The mortality of *O. niloticus* increased depending on the dose of MD. The data were obtained from the toxicity test evaluating using the Probit Analysis Method. The LC_{50} 96-h value for *O. niloticus* was found to be 0.1045. 95% confidence limits were between 0.10022-0.10787 mg/L (Table 2).

TABLE 2 - 96 hours toxicity results of the methidathion bioassay on *O. niloticus* (LC: Lethal concentration; SE: Standard Error)

Points of Lethal Concentration	Concentration (mg/L)	95% Confidences Limits (mg/L)	Intercept±SE
LC 1.00	0.0932	0.0796-0.0981	46.0967± 12.7422
LC 5.00	0.09646	0.0855-0.1005	
LC 10.00	0.09819	0.0887-0.1018	
LC 30.00	0.10190	0.09570-0.1051	
LC 50.00	**0.1045**	**0.1002-0.1078**	
LC.80.00	0.1089	0.1057-0.1149	
LC.90.00	0.1113	0.1079-0.1197	
LC 99.00	0.1171	0.1122-0.1332	

3.2 Biochemical assay

The CAT activity in liver tissues of fish are given in Fig. 1. The CAT activity in liver tissues of fish were increased significantly (P<0.05) by exposing the concentrations of 13 and 26 µ/L. These rate were 23 and 29% on 4th days, 28 and 47% on 10th days, respectively (Fig. 1).

The SOD activity in liver tissues of fish are shown in Fig. 2. MD1 did not make any significant changes in SOD activity compared to control, but in MD2 was increased significantly (P<0.05) on 4th day. 13 and 26 µg/L concentrations of MD caused a significant inhibition (P<0.05) by 31 and 50%, respectively, in liver of fish on 10th day (Fig. 2).

The GSH-Px activity in liver tissues are given in Fig. 3. There was no significant change in GSH-Px activity in MD1 group compared to control, but in MD2 group was significantly increased (P<0.05) on 4th days. 13 and 26 µg/L of MD concentrations caused a significant increase (P<0.05) by 36% and 77%, respectively, in liver of fish on 10th day (Fig. 3).

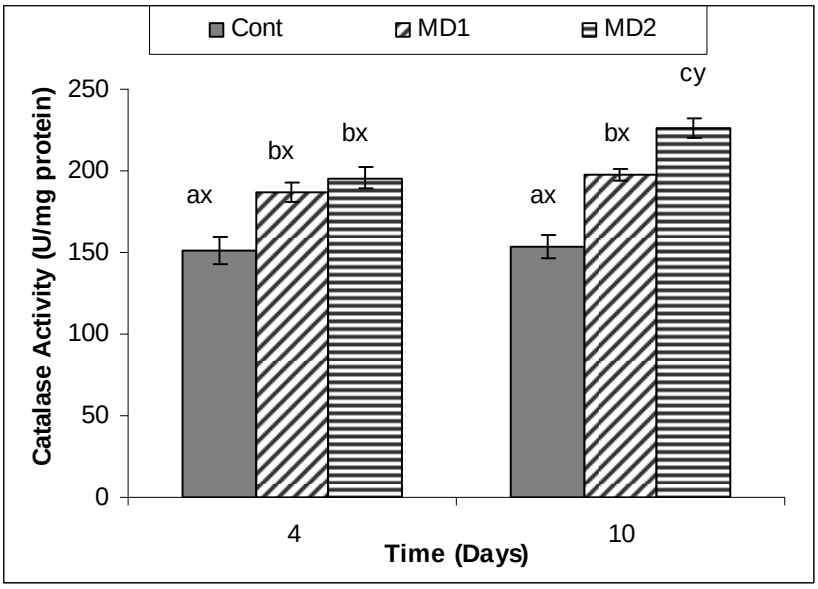

FIGURE 1 - CAT activity in liver tissue of *O. niloticus* exposed to sublethal concentrations of methidathion: 13 µ/L (MD1) and 26 µ/L (MD2). Each value is the mean ± SE (n = 6). a, b, c: indicate a significant difference (P<0.05) between means recorded for the same day in each group. x, y: indicate a significant difference (P<0.05) between means recorded for the different days in same group.

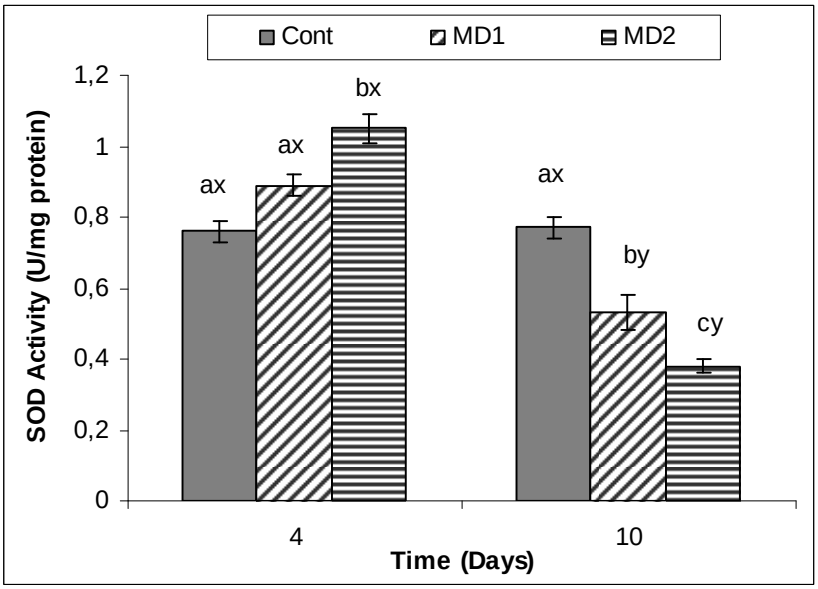

FIGURE 2 - SOD activity in liver tissue of *O. niloticus* exposed to sublethal concentrations of methidathion: 13 µ/L (MD1) and 26 µ/L (MD2). Each value is the mean ± SE (n = 6). a, b, c: indicate a significant difference (P<0.05) between means recorded for the same day in each group. x, y: indicate a significant difference (P<0.05) between means recorded for the different days in same group.

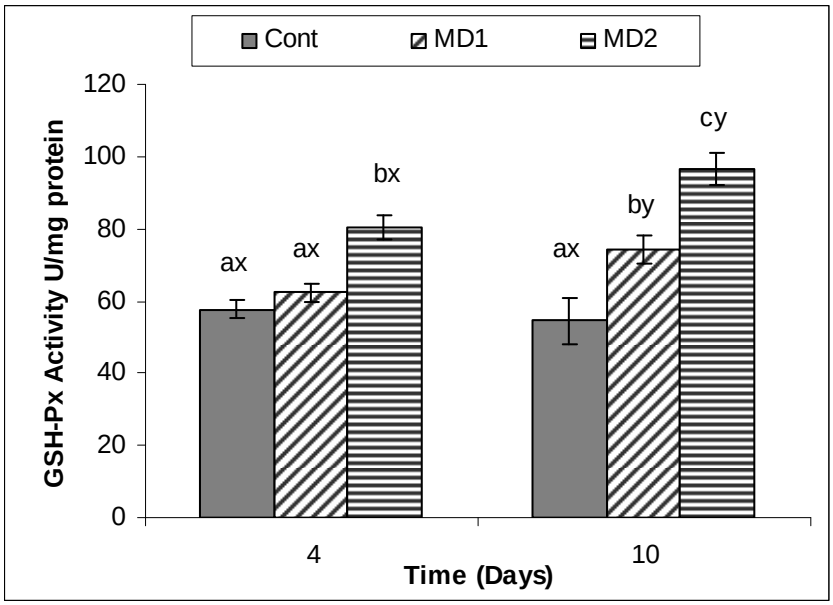

FIGURE 3 - GSH-Px activity in liver tissue of *O. niloticus* exposed to sublethal concentrations of methidathion: 13 µ/L (MD1) and 26 µ/L (MD2). Each value is the mean ± SE (n = 6). a, b, c: indicate a significant difference (*P*<0.05) between means recorded for the same day in each group. x, y: indicate a significant difference (*P*<0.05) between means recorded for the different days in same group.

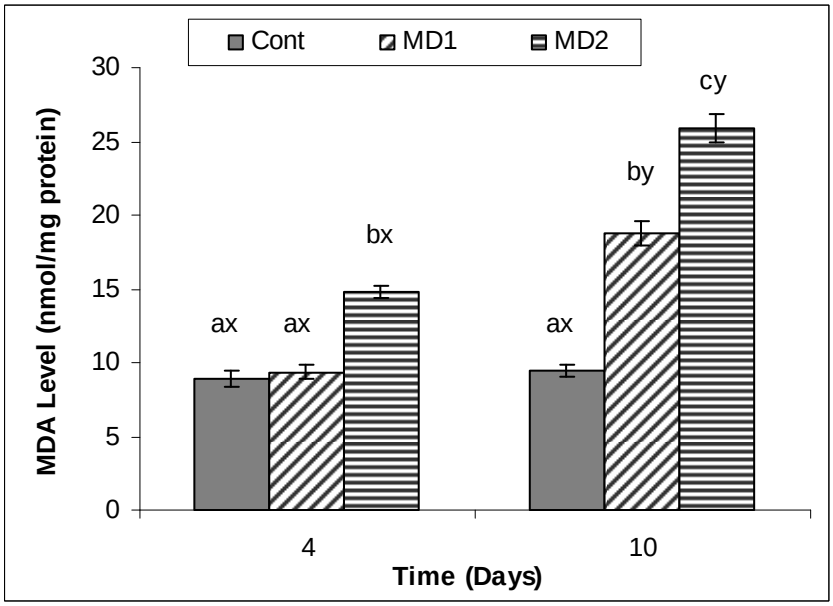

FIGURE 4 - MDA level in liver tissue of *O. niloticus* exposed to sublethal concentrations of methidathion: 13 µ/L (MD1) and 26 µ/L (MD2). Each value is the mean ± SE (n = 6). a, b, c: indicate a significant difference (*P*<0.05) between means recorded for the same day in each group. x, y: indicate a significant difference (*P*<0.05) between means recorded for the different days in same group.

The levels of MDA in liver tissues are given in Fig. 4. There was no significant change in tissue MDA level in MD1 group compared to control, but tissue MDA level in MD2 group was increased significantly ($P<0.05$) on 4th days. MDA level of liver tissues were increased significantly ($P<0.05$) in MD1 (98%) and in MD2 groups (175%), compared with control on 10th days exposure (Fig. 4).

The mRNA levels of HSP70 gene in liver tissues are given in Fig. 5, and β-actin was used as the internal control gene. 13 and 26 µg/L of MD concentrations caused a significant increase by 84 and 200% ($P<0.05$), respectively, in the liver of fish on the 4th day. In contrast, the mRNA levels of HSP70 gene in the liver tissues were decreased significantly ($P<0.05$) in MD1 (41%) and MD2 (56%) groups on 10th days when compared to control (Fig. 5).

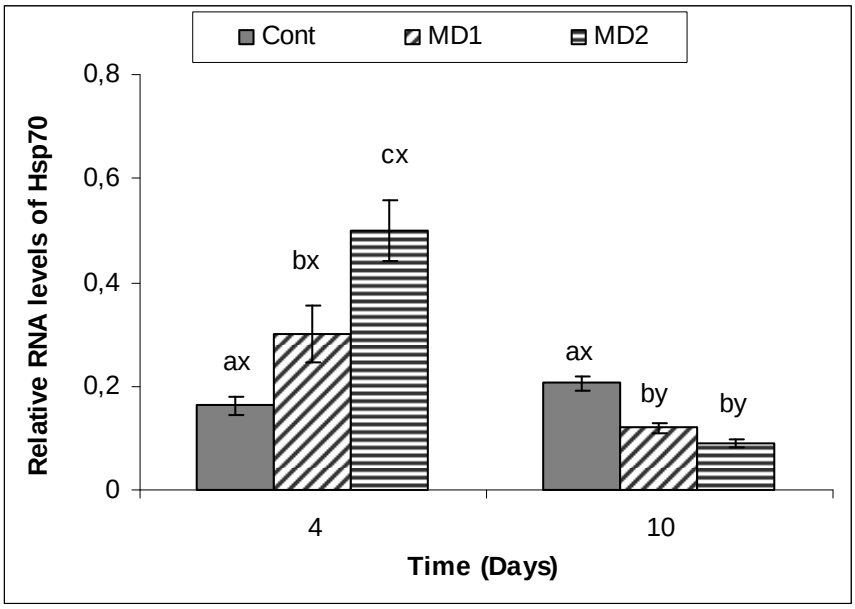

FIGURE 5 - Relative mRNA levels of HSP70 to β-actin in liver tissue of *O. niloticus* exposed to sublethal concentrations of methidathion: 13 µ/L (MD1) and 26 µ/L (MD2). Each value is the mean ± SE (n = 6). a, b, c: indicate a significant difference (*P*<0.05) between means recorded for the same day in each group. x, y: indicate a significant difference (*P*<0.05) between means recorded for the different days in same group.

4. DISCUSSION AND CONCLUSION

MD is one of the more toxic OPs. The 96-h LC_{50} values of methidathion for fish have been determined to be 6.6–14 µg/L for rainbow trout, 2.2–9 µg/L for bluegill, and 6.8 µg/L for goldfish [23]. Balint *et al.* [24] reported that 96-h LC_{50} value of MD for *Cyprinus carpio* was 6 mg/L MD (approx.) concentration. There is no data about LC_{50} values of MD for *O. niloticus* in previous studies. The 96-h LC_{50} value for MD was determined as 0.1045 mg/L for *O. nil*oticus in this study. The different sensitivity of fish may be due to differences of sensitivity of enzymes activation [13] and species, strain, age, and sex of the animal, the dose and route of exposure, and the effect of various environmental, nutritional and physiological factors [25].

The present study showed that CAT and SOD activities increased in the liver tissues of fish on 4th day by MD concentrations while CAT activity was enhanced, SOD activity was decreased on 10th days. Zhang *et al.* [26] observed that CAT activity in the liver of *Carassius auratus* was increased, although SOD activity was inhibited gradually with 2,4-dichlorophenol concentration increasing. Thomaz *et al.* [5] reported that CAT activity was increased and SOD activity was decreased in the liver of *O. nil*oticus exposed to the insecticide trichlorfon for 96 h. Also, Lushchak *et al.* [27] found that the activity of CAT in the liver of *C. auratus* was elevated but SOD activity was inhibited by exposure to roundup. Exposure to methyl parathion resulted in a significant induction of CAT activity in *Brycon cephalus* liver [28]. CAT and SOD enzymes have related functions. SOD catalyzes the dismutation of the superoxide anion radical to H_2O and H_2O_2, which is detoxified by both CAT and GSH-Px activities. Due to the inhibitory effects on ROS formation, the SOD-CAT system provides the first defense line against oxygen toxicity, and is usually used as an indirect biomarker indicating ROS production [7]. An increase in CAT enzyme activity is probably a response toward increased ROS generation in pesticide toxicity [28]. Usually, an induction of hepatic SOD activity was observed when exposed to organic pollutants [29]; however, the excess production of superoxide radicals or after their transformation to H_2O_2 causes an oxidation of the cysteine in the enzyme and deactivates SOD [30]. The decreased antioxidant enzymes result in increased oxidative stress, an indication of impaired antioxidant defense mechanism due to excessive generation of free radicals generated by insecticides [4]. The toxicity of MD in the present study may be caused by the unbalance between free radicals and antioxidants, which might have resulted in inhibition of SOD activity.

In this study, we found that GSH-Px activities were increased in the liver of MD-treated fish. Similar results have also been reported in other fish species exposed to pesticides. Özkan *et al.* [31] showed that GSH-Px activity was increased in the liver of *O. niloticus* exposed to chlorpyrifos. Likewise, GSH-Px activities were elevated in the liver of *C. auratus* exposed to 2,4-dichlorophenol [26] and to malathion [32]. The biological function of GSH-Px is to reduce H_2O_2 and lipid hydroperoxides [33]. These data in our study suggest that MD treatment may result in increased formation of oxygen-free radicals that could stimulate GSH-Px activity to cope with this increased oxidative stress and protecting membranes from damage due to LPO products [7].

MDA levels were increased in the liver of MD-treated fish in the present study. MDA is a main oxidation result of peroxidized polyunsaturated fatty acids and increased MDA is a key indicator of LPO. LPO is one of the main processes induced by oxidative stress, and the first step of cellular damage caused by OP insecticides [4]. Previous investigations have reported on the induction of LPO in the liver by different OP insecticides [34, 35]. The induction of ROS could increase the oxidation of polyunsaturated fatty acids and lead to peroxidation [36]. The increased MDA content might have resulted from an increase of free radicals as a result of stress conditions in the fish with insecticide intoxication [34]. The increased lipid peroxidation in the present study suggests that ROS induced damage may be one of the main toxic effects of MD.

In this study, it was shown that the mRNA levels of HSP70 gene in the liver were increased by exposed MD concentrations on 4th days. In contrast, it was decreased on 10th days. HSP induction is observed as a general marker for cellular stress associated with a wide range of cellular injuries [37]. Many studies have indicated that environmental stressors alter HSP70 expression in Oncorhynchus mykiss [38], Oncorhynchus tshawytscha [39] and C. carpio [40]. In general, increased mRNA levels of HSP70 are recognized as results from the proteotoxic action of included xenobiotics [41]. Gene expression is regulated by physiological factors, such as hormones and cytokines, or by environmental factors, such as xenobiotics or physical parameters. Oxidative stress is a key component of both of these mechanisms [42, 43]. Oxidative stress can induce mRNA levels of HSP70 gene, indicating that oxygen radicals, especially superoxide anions, contributes to HSP70 expression [44]. The authors explained that the expression of HSP70 in the liver with toxicants correlated with altered metabolic capacity, suggesting that higher HSP70 expression in tissues may be at the expense of other biochemical pathways [10, 12, 40]. The stimulation of mRNA levels of HSP70 gene in liver may response to concern of the cellular redox status and play an important role in protecting organism from oxidative stress [45, 46]. In the present study, the increased mRNA levels of HSP70 gene in the liver by exposure to MD may indicate its important role as a molecular chaperone under oxidative stress caused by MD [46].

In fish, the type and duration of exposure to a stressor, the tissue type and species specific heat shock factors may control HSP70 induction [47, 48]. It was explained in a study that on the responses of coho salmon to zinc and temperature demonstrated that the expression of liver and gill HSP70 decreased in parallel with increased dietary zinc and increased water temperature [49]. The decline in tissue HSP70 could have occurred as energy redirected away from cellular protein synthesis and towards other metabolic processes needed to cope with the stressors [49, 50]. The reduction in the cellular energy status may limit HSP induction [47]. The possible explanation for this is cellular energy deficiency due to the mitochondrial dysfunction in organisms exposed to the pesticide stress. Exposure to pesticide can limit the amount of ATP available for HSPs synthesis and function [51]. Besides, the resulting oxidative stress is a reason of enhanced lipid peroxidation and changes in structure and function of other important cellular components, such as protein and DNA [52]. The regulated of gene expressions may be related to oxidative stress or to changes in structure of cellular component by exposure to pesticide [37].

In conclusion, the present study showed that sublethal concentrations of MD can alter antioxidant enzyme (CAT, SOD and GSH-Px) activities and increase in MDA level in a dose and time-depended in the liver of *O. niloticus*. The induced antioxidant enzyme activities may indicate a response towards increased ROS generation in pesticide toxicity. Our study showed that significant increases in the mRNA level of HSP70 gene in the liver on 4th days, although inhibition was observed on 10th days. The mRNA level of HSP70 gene may be related to oxidative stress or to changes in structure of cellular component by exposure to MD. Overall results indicate that exposure of MD caused oxidative stress and altered enzyme activities with expression of gene of *O. niloticus*.

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The authors have declared no conflict of interest.

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