Water Air Soil Pollut (2021) 232: 221
https://doi.org/10.1007/s11270-021-05101-7

In Vivo Effects on Stress Protein, Genotoxicity, and Oxidative Toxicity Parameters in *Oreochromis niloticus* Tissues Exposed to Thiamethoxam

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Received: 17 January 2021 / Accepted: 29 March 2021 / Published online: 18 May 2021

Abstract This study aimed to observe the effect of toxicity of the pesticide thiamethoxam (TMX) at sublethal concentrations in the liver and brain of *Oreochromis niloticus*. In the experiment, fish were exposed to 50, 100, and, 150 mg/L with thiamethoxam for 48 h and 15 days. The superoxide dismutase (SOD), catalase (CAT), glutathione S-transferaz (GST), glutathione peroxidase (GPx), and ethoxyresorufin-*O*-deethylase (EROD) activities; and thiobarbituric acid reactive substance (TBARS), heat shock proteins 70 (HSP70), glutathione (GSH), and genotoxicity parameter 8-hydroxy-2′-deoxyguanosine (8-OHdG) were analyzed by spectrophotometric methods and ELISA techniques. Depending on time and dose in TMX exposure in liver tissue, a significant decrease in GSH level; an increase in SOD, GST, GPx, and EROD enzyme activities; and HSP70, TBARS, and 8-OHdG levels was determined. In brain tissue, SOD, GST, and EROD enzyme activities, an increase in HSP70, TBARS, and 8-OHdG levels, and a decrease in CAT enzyme activity and GSH levels were determined. In this study, TMX in the concentrations used showed that changes in oxidative stress biomarkers, genotoxicity parameter 8-OHdG levels, and HSP70 levels caused toxic effects in the model organism. As a result of the study, the changes and protective effects of the antioxidant system and stress proteins at the cellular level were determined in sublethal doses of toxic effects caused by TMX in the vital organs of the organism. In this toxicological study, TMX exposure resulted in toxicity to *O. niloticus* liver and brain tissues, in addition, responses of biomarkers to time and concentrations were determined. As a result of this study, the potential toxic effects of the commonly used pesticide TMX will reveal both the ecological risks of the aquatic organism and the basic data of the safety and risk assessments of *O. niloticus* consumed as food for human health.

Keywords Pesticide · Thiamethoxam · *Oreochromis niloticus* · HSP70 · 8-OHdG

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1 Introduction

The neonicotinoids, the newest class of insecticides, have significant toxic effects on the environment and human health (El-Gendy et al., 2019). Thiamethoxam (TMX) is one of the most common varieties of neonicotinoids used in agricultural activities and public health (Bargańska et al., 2013). As a result of agricultural activities, thiamethoxam dissolves in the soil in

low amounts and reaches the aquatic environment through groundwater. It is a potential contaminant in groundwater and open water due to its very high solubility (Ali et al., 2020; EFSA, 2013; Hintze et al., 2020; Zhao et al., 2012). According to a study, TMX with high water solubility was determined to dissolve 4.1 g/L (Banerjee et al., 2008).

It is also used for pest control in human habitats such as homes and green spaces (Kurwadkar et al., 2013). Exposure to neonicotinoids causes carcinogenic and mutagenic effects (Karabay & Oguz, 2005). It is known that pesticides have toxic effects on fish by increasing the amount of reactive oxygen species (ROS). In general, oxidative system biomarkers detoxify ROS radicals and prevent toxicity (Vasylkiv et al., 2010). Oxidative stress develops in cells due to the insufficiency of antioxidant systems damaged by toxicity in biological systems. In addition, oxidative stress biomarkers are used to assess pesticide contamination in water (Lushchak, 2011).

Oxidative stress biomarkers such as SOD, CAT, GPx, and GST provide antioxidant protection by working together to effectively protect against oxidative stress and tissue-specific damage. SOD is an enzyme that scavenges oxidants such as superoxide (O^{-2}), hydrogen peroxide (H_2O_2), and oxygen (Figueiredo-Fernandes, Fontaínhas-Fernandes, Peixoto, et al., 2006a). Subsequently, CAT is effective in catalyzing the formation of hydrogen peroxide into water and oxygen (Chelikani et al., 2004). GST is an enzyme group that catalyzes the conjugation of GSH xenobiotics that have reactive electrophilic groups. The GST enzyme, which belongs to group II biotransformative enzymes, uses GSH as a substrate and binds electrophilic xenobiotics to prevent oxidative damage and provide detoxification. While the GPx enzyme provides H_2O_2 detoxification, the reduced GSH is oxidized and converted to oxidation of GSH. This process prevents damage caused by the overproduction of the ROS variable H_2O_2 (Fernandes et al., 2008). The metabolic processes of biotransformation are required for the detoxification and excretion of pesticide detoxification enzymes, including EROD and GST. EROD activity is widely used in the determination of toxicity of xenobiotics in combination with oxidative stress biomarkers in fish tissues (Puch-Hau et al., 2018).

Heat shock proteins (HSPs), called stress proteins, were discovered in 1962 from *Drosophila melanogaster* larvae (Ponomarenko et al., 2013; Ritossa, 1962). HSPs are induced by environmental and biological stressors such as cell metabolism, growth, cell differentiation, and apoptosis (Schmitt et al., 2007). HSPs act as a molecular chaperon in all organisms and cells, enabling proteins to regain their clustered and misfolded structures for rearrangement (Feder & Hofmann, 1999; Purandhar et al., 2014).

The stress response stimulates heat shock protein function as molecular chaperones that protect cells during stress. HSPs are highly conserved and cytoprotection protein groups (Sreedhar & Csermely, 2004). Among the HSP groups of different sizes, HSP70 is the most strongly induced after stresses such as anticancer drugs and oxidative stress. The HSP70 is the most conserved and studied HSP class among the stress proteins (Schmitt et al., 2007).

Thiobarbituric acid reagents (TBARS) are the most widely used assay for lipid peroxidation and are a good indicator for pesticides and heavy metal pollution in aquatic organisms (Nogueira et al., 2003; Temiz, 2019). Lipid peroxidation occurs when oxidative stress biomarkers are probably insufficient to detoxify excess ROS produced during the biotransformation process (Modesto & Martinez, 2010). In genotoxicity studies, 8-OHdG measurement, produced as a result of oxidative stress in DNA and RNA structures, is a widely used biomarker that shows DNA oxidation (Arslan et al., 2017). As a result of the interaction of the hydroxyl ion (HO-) of the ROS with the nucleobase guanine in the DNA structure, the formation of C8-hydroxyguanine (8-OHGua) or the nucleoside deoxyguanosine (8-hydroxy-2′)-deoxyguanosine is formed. It causes an increase in radical structures as a result of its reaction with HO^{-}, after which the electron separation forms the 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Dizdaroglu et al., 2002; Kasai, 1997).

In the organism, free radicals formed as a result of toxicity interacting with antioxidant system elements and providing protection at the cellular level with a chain reaction. Thus, it becomes important that vital organs such as the brain and liver are prevented from being damaged. The brain is an important physiological and neurological significance in fish and is a vital organ in toxicology, especially in the nervous system of fish exposed to pesticides (Mishra & Devi, 2014). In the organism, the liver is one of the vital organs that controls important processes such as the metabolism of various xenobiotics, detoxification, and excretion (De Boeck et al., 2003; Temiz & Kargin, 2019). It is an important

organ that contains necessary enzymes for the repair of macromolecules damaged during normal metabolism or the formation of oxidative stress (Nwani et al., 2013).

In the rapidly growing world, it is more likely than ever that the monitoring of aquatic system pollution with biochemical parameters is needed to protect the ecosystem and human health.

In the aquatic system, fish are used as an important bioindicator in the determination of water pollution (Van der Oost et al., 2003). Fish are crucial ecological species for monitoring and evaluating the safety of pesticides because they are both in the food web in the aquatic ecosystem and are consumed as a food source by humans. *Oreochromis niloticus* is widely used as a biological model in studies dealing with the negative effects of various pesticides and forms an excellent model in aquatic pollution studies (Cogun et al., 2017; Üner et al., 2014). In this study, effects of TMX in the liver and brain; SOD, CAT, GPx, GST, and EROD enzyme activities; and GSH, HSP70, TBARS, and 8-OHdG levels were studied in *O. niloticus* exposed the pesticide over 48 h and 15 days.

2 Materials and Methods

2.1 Experimental Design

The experiments were carried out at 50, 100, and 150 mg/L different nominal concentrations of thiamethoxam preparations in static-renewal system (APHA, AWWA, WEF, 1998). In addition, the results were discussed concerning the sublethal concentrations. *O. niloticus* was taken from Çukurova University Faculty of Aquaculture ponds and acclimated to laboratory conditions in the experimental laboratory for 2 months. Mean lengths of fish were 13.7 ± 1.22 cm, and weights 36.4 ± 3.05 g. At end of this period, water quality characteristics in glass aquarium were as follows: pH: 8.3 ± 0.7, temperature: 25 ± 1 °C, dissolved oxygen: 7.4 ± 0.3 mg/L, total hardness: 195.5 ± 6.4 $CaCO_3$ mg/L, total alkalinity: 275.2 ± 7.2 $CaCO_3$ mg/L. The study was approved by the Animal Experimentation Ethics Committee of Çukurova University (Protocol 18-6/2018). A total of 4 glass aquariums, each 40 × 40 × 40 cm in size, were used. The first three aquariums were filled with 40 L of one of the TMX solutions tested, and the fourth one was filled with tap water and used as control. Fish were added into each pesticide concentration and the control contained a total of 6 fish per aquarium for 48 h and 15 days of exposure. At the end of the 15th day, all fish were made paralyzed by cutting the spinal cord.

2.2 Biochemical Analysis

Liver and brain tissues were removed, washed with 0.9% saline, then weighed and stored at – 80 °C until biochemical analysis. The tissues were homogenized for 3 min with 1/10 (w/v) volume of ice at pH 7.4 containing 1.17% KCl in 0.1 M phosphate buffer using a glass-Teflon homogenizer (Ultra Turax T-18). Homogenates were centrifuged at 16,000x*g* for 20 min in an Eppendorf at 4 °C (Nuve NF 800R). The supernatants obtained after centrifugation were taken into clean Ependorf tubes and used for the determination of SOD, CAT, GPx, GST, and EROD enzyme activities and GSH, TBARS, HSP70, and 8-OHdG levels. The remaining portion of the liver and brain homogenates were prepared according to the methods of Kennedy and Jones (1994) for the measurement of EROD enzyme activity by preparing a microsomal supernatant.

2.3 Protein Level

By applying the Bradford (1976) method, protein level in supernatants was calculated in milligrams per milliliter using a standard regression curve using bovine serum albumin in a microplate reader at a wavelength of 595 nm.

2.4 SOD Enzyme Activity

Measurement of SOD activity was determined by measuring iodo-p-nitro tetrazolium (INT) inhibition of superoxide anion radicals produced by xanthine oxidase in tissues at 37 ° C for 3 min at 505 nm (McCord & Fridovich, 1969).

2.5 CAT Enzyme Activity

CAT activity was determined by Beutler (1975) as the activation of H_2O_2 at 37 °C for 2.5 min in Tris-HCl buffer (pH 8.0). CAT activity was measured spectrophotometrically at 230 nm.

2.6 GPx Enzyme Activity

GP_X enzyme activity, using t-butyl hydroperoxide as substrate, was determined by the difference in absorbance values during oxidation to $NADP^+$ on NADPH (Beutler, 1984).

2.7 GSH Level

Dithionitrobenzoic acid (DTNB) is a disulfide compound that is reduced by sulfhydryl compounds to form a yellow complex. The color intensity of the yellow complex formed by supernatant and DTNB is directly proportional to the concentration of GSH in the medium. It was evaluated spectrophotometrically at 412 nm (Beutler, 1975).

2.8 GST Enzyme Activity

Glutathione S-transferase catalyzes the reaction of the thiol group of GSH with the electrophilic compounds. GST activity is the change in GSH and CDNB (1-chloro-2,4-dinitrobenzene) conjugation at a wavelength of 340 nm for 2 min at 30 °C (Habig et al., 1974).

2.9 EROD Enzyme Activity

EROD enzyme activity was based on kinetic measurement of 7-ER (7-ethoxyresorufin) using NADPH as the substrate, resulting in pink coloration of the final product of resorufin. 1/10 diluted microsomal sample was added to EROD buffer (0.1 M Tris and NaCl, pH 8.0) and 2 μM 7-ER (7-ethoxyresorufine dissolved in methanol), NADPH (dissolved in 25 mg/ml homogenisation buffer and freshly prepared). A change in wavelength of 572 nm was detected for 3 min at 30 °C. EROD activity was calculated based on a pre-prepared standard curve of resorufin. The spectrophotometric measurement of EROD activity was based on the method of Klotz et al. (1984).

2.10 HSP70 Level

The levels of HSP70 in the supernatants were diluted with coating buffer to give a total protein concentration of 35 μg/ml by a non-competitive ELISA method. The samples were boiled for 5 min and seeded in a 96-well microplate. After 12 h of incubation at 4 °C, the sample-seeded wells were washed with wash buffer and incubated with blocking buffer for 2 h at 37 °C. The wells washed with rewash buffer were incubated at 37 °C for 2 h, with monoclonal anti-heat shock protein 70. Again, the wells were washed with wash buffer and incubated with anti-mouse IgG incubated for 1 h at 37 °C. After incubation, wells washed with rewash buffer were incubated with OPD (o-phenylenediamine dihydrochloride) as substrate for 2 h at 37 °C. Absorbance values calibrated to standard at 405 nm (De Boeck et al., 2003).

2.11 TBARS Level

Thiobarbituric acid reagents (TBARS) were secondary products of lipid peroxidation, an important parameter used in the determination of lipid peroxidation. Under aerobic conditions, the supernatants from the tissues were vortexed with 8.1% SDS, 20% acetic acid, and 0.8% TBA (pH 3.4) buffers and incubated at 95 °C for 30 min. After incubation, it was allowed to cool down and stop the reaction. The samples were centrifuged again by the addition of n-butanol/pyridine (14:1). The pink color formed in the samples was converted to absorbance spectrophotometrically at 532 nm. The levels of TBARS were measured via absorbance values measured using 1,1,3,3-tetraethoxypropane as standard (Ohkawa et al., 1979).

2.12 8-OHdG Level

Each sample was studied in duplicate with competitive enzyme-linked immunosorbent assay (ELISA) to determine levels of 8-OHdG, a genotoxicity biomarker. BioTek ELX800 Microplate reader at 450-nm wavelength Yin et al. (1995) was studied in the ELISA kit (Sunred, 201-00-0041) by method modification.

2.13 Statistical Analysis

The results of biochemical analysis were analyzed by SPSS 22.0 package program. One-way ANOVA (Duncan test; $p < 0.05$) test was used for the difference between exposure groups, the independent *t*-test was used for the time-dependent difference between the same exposure groups, and the difference was evaluated at the $t < 0.05$ significance level.

3 Results

In this study, sublethal concentrations of TMX in the liver and brain tissues of *O. niloticus* and time-dependent changes in SOD, CAT, GPx, GST, and EROD enzyme activities and GSH, TBARS, HSP70, and 8-OHdG levels are given in Figs. 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a, and 9a and Figs. 1b, 2b, 3b, 4b, 5b, 6b, 7b, 8b, and 9b.

3.1 SOD Enzyme Activity

Liver SOD enzyme activity (Fig. 1a) and brain SOD enzyme activity (Fig. 1b) were given. Liver SOD enzyme activity (Fig. 1a) increased to 15% and 25% in TMX concentrations of 100 and 150 mg/L, respectively, at 48 h compared to control. In 15 days according to the control, a significant increase in TMX concentrations of 50 mg/L, 100 mg/L, and 150 mg/L was 32%, 42%, and 57%, respectively. Liver SOD enzyme activity, TMX exposure depending on time, on the 15th day, 21%, 19%, and 22% increases in 50-mg/L, 100-mg/L, and 150-mg/L exposures relative to 48 h were statistically significant. Brain SOD activity (Fig. 1b) increased by 47%, 70%, and 84% for 48 h compared to control of TMX concentrations of 50, 100, and 150 mg/L, respectively. SOD activities were increased by concentrations of 50, 100, and 150 mg/L of TMX according to the control of 36%, 50%, and 58%, for 15 days, respectively. Decreased time-dependent brain SOD enzyme activity in TMX exposure is statistically significant at 10%, 13%, and 15% at 50-, 100-, and 150-mg/L effects, respectively.

3.2 CAT Enzyme Activity

Liver CAT enzyme activity (Fig. 2a) and brain CAT enzyme activity (Fig. 2b) were given. Liver CAT enzyme activity (Fig. 2a) was reduced by 3%, 6%, and 11% at 50-, 100-, and 150-mg/L effect, respectively, at 48 h compared to control. A decrease of 2%, 5%, and 9% at 50-, 100-, and 150-mg/L exposures compared to control at 15 days was statistically significant. Liver CAT enzyme activity, time-dependent change in TMX effect 2%, 2%, and 1% increase in 50-, 100-, and 150-mg/L effects, respectively, is statistically significant. Brain CAT enzyme activity (Fig. 2b) was reduced by 9% and 11% in TMX concentrations of 100 and 150 mg/L compared to control at 48 h, respectively. A decrease of 35%, 44%, and 51% at 50-, 100-, and 150-mg/L exposures relative to control at 15 days was statistically significant. Brain CAT enzyme activity, time-dependent change in TMX exposure at 30%, 38%, and 44% increase in 50-, 100-, and 150-mg/L exposures at 15 days, is statistically significant.

3.3 GPx Enzyme Activity

Liver GPx enzyme activity (Fig. 3a) and brain GPx enzyme activity (Fig. 3b) were given. There was no statistically significant difference in liver GPx enzyme activity (Fig. 3a) at 48 h compared to control. On the 15th day, an increase of 12%, 13%, and 24% in the effect of 50, 100, and 150 mg/L compared to control was statistically significant. Liver GPx enzyme activity, time-dependent change in the effect of TMX concentrations, was not statistically significant at 15 day. A decrease of 22% and 24% in brain GPx enzyme activity (Fig. 3b) at 100- and 150-mg/L TMX exposure compared to control at 48 h was found to be a statistically significant difference, respectively. No change in TMX exposure compared to control was detected at 15 days. Brain GPx enzyme activity, time-dependent change in TMX exposure, was statistically significant at a 17%, 28%, and 29% increase in 50-, 100-, and 150-mg/L effects, respectively.

3.4 GSH Level

Liver GSH level (Fig. 4a) and brain GSH level (Fig. 4b) were given. Liver GSH level (Fig. 4a) was determined to significantly decrease in TMX concentrations of 100 mg/L and 150 mg/L 32% and 47% for 48 h, and 22% and 64% for 15 days, respectively. Forty-eight hours and 15 days of exposure to 100 and 150 mg/L TMX decreased liver GSH levels significantly. Liver GSH level, at the TMX concentration of 150 mg/L, determined decrease in 32% reduction on time dependence. Brain GSH levels (Fig. 4b) were statistically significant at 11%, 19%, and 25% reduction in 50-, 100-, and 150-mg/L effect, respectively, at 48 h compared to control. On the 15th day, a reduction of 50, 100, and 150 mg/L of the effect was determined by 19%, 27%, and 34%, respectively. Brain GSH level, the time-dependent change in TMX exposure, was reduced by 14%, 14%, and 17% in 50-, 100-, and 150-mg/L effects, respectively.

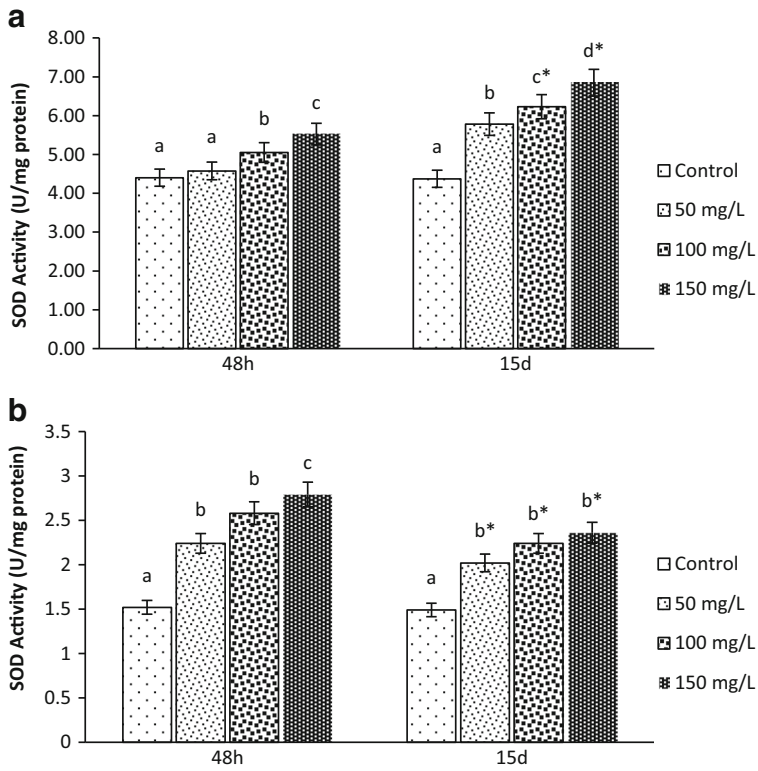

Fig. 1 SOD activity (U/mg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

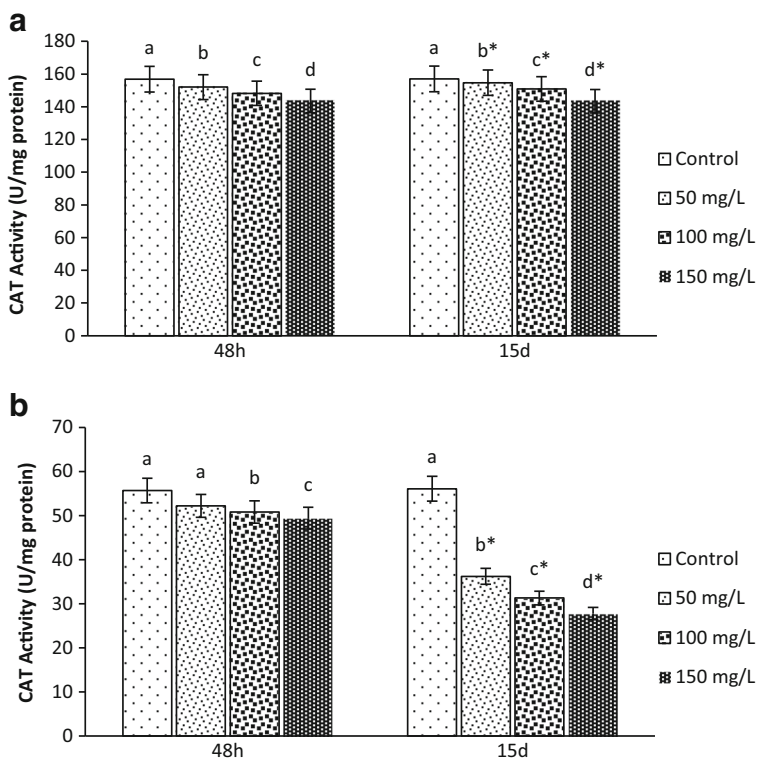

Fig. 2 CAT activity (U/mg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

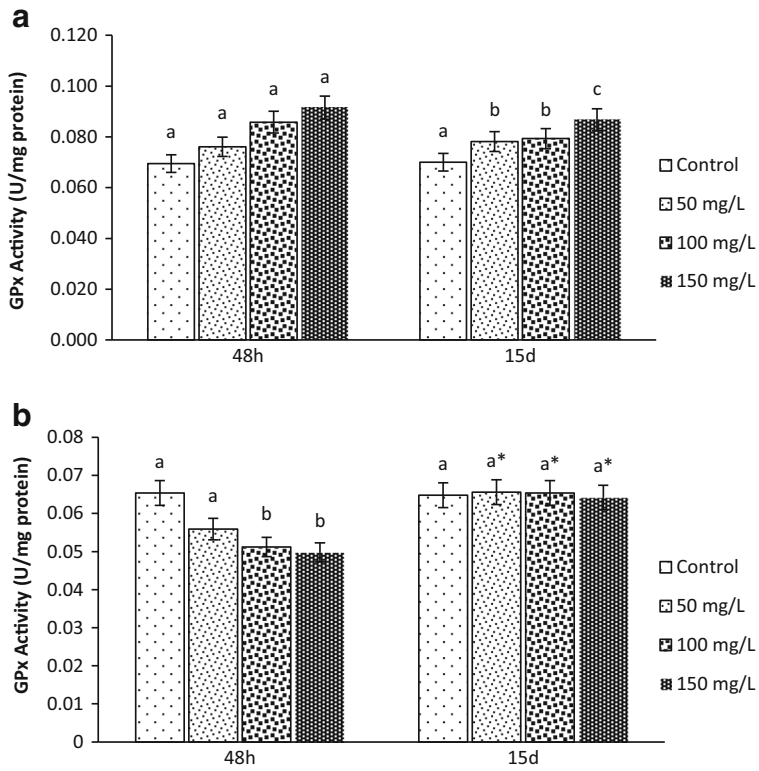

Fig. 3 GPx activity (U/mg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, and c indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

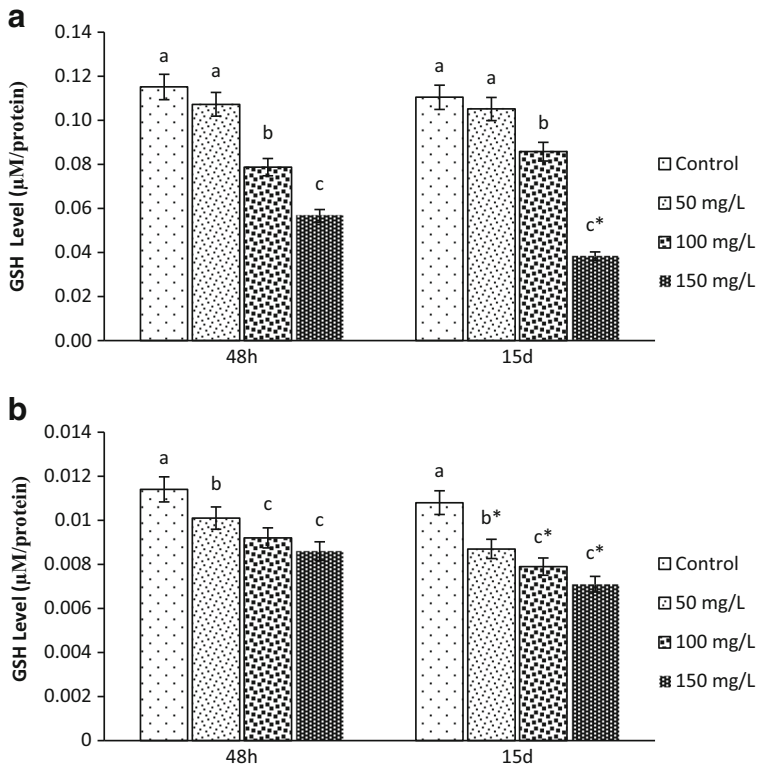

Fig. 4 GSH level (µM/protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, and c indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

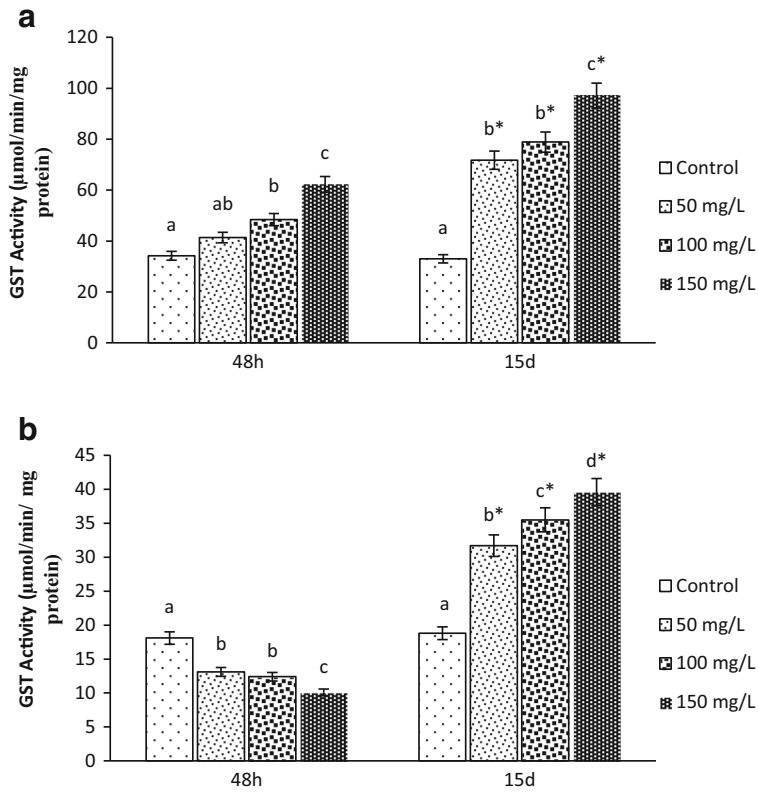

Fig. 5 GST activity (µmol/min/mg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

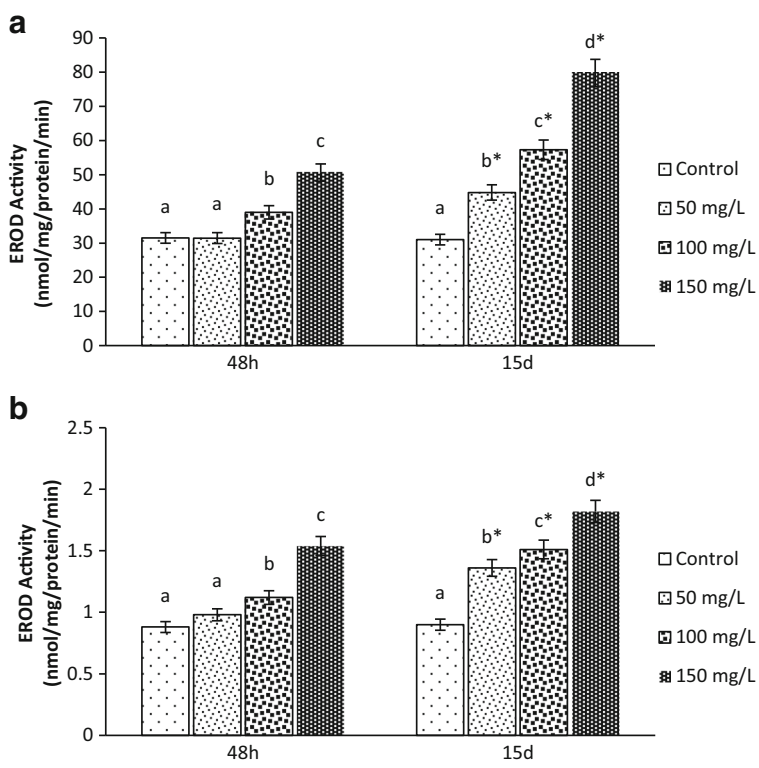

Fig. 6 EROD activity (nmol/mg protein/min) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

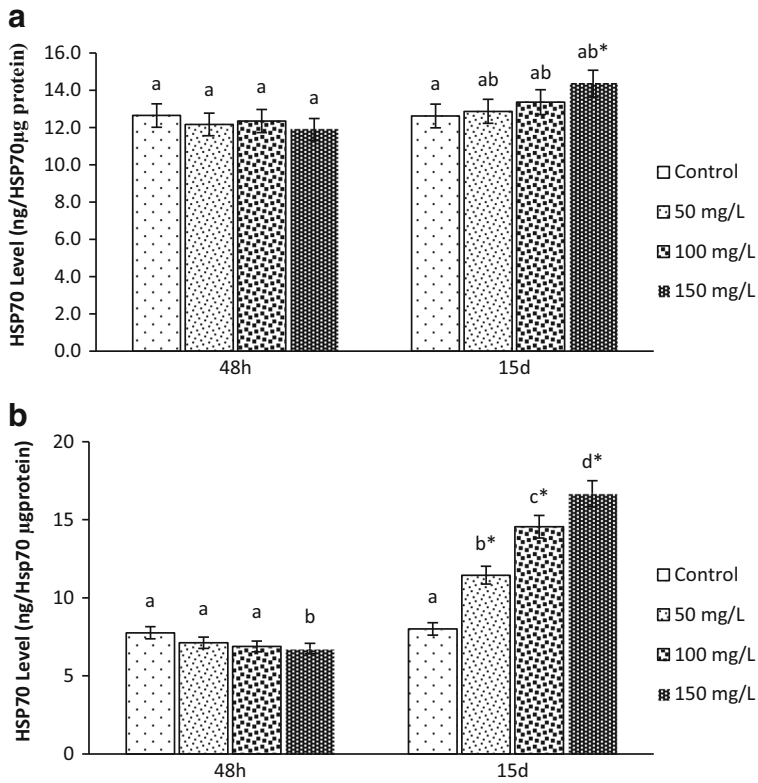

Fig. 7 HSP70 level (ng/Hsp70 µg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

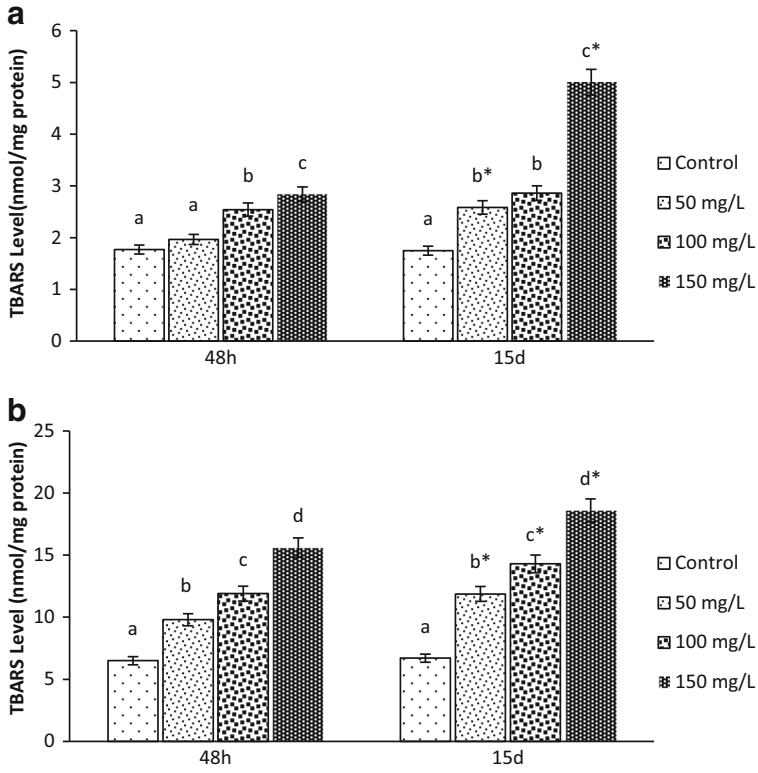

Fig. 8 TBARS level (nmol/mg protein) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

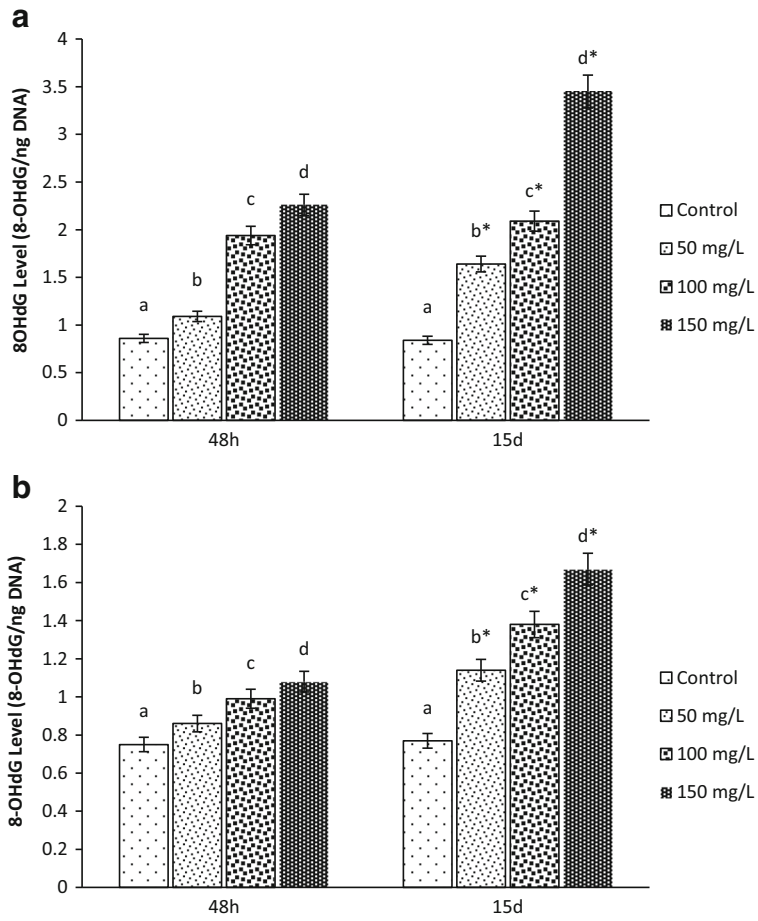

Fig. 9 8-OHdG level (8-OHdG/ng DNA) in liver (**A**) and brain (**B**) of *O. niloticus* after exposure to sublethal doses of TMX for 48 h and 15 days. The letters a, b, c, and d indicate differences between concentrations, and the asterisk (*) indicates differences between exposure periods. Data showing different letters are significantly different at the $p < 0.05$ level ($N = 6$)

3.5 GST Enzyme Activity

Liver GST enzyme activity (Fig. 5a) and brain GST enzyme activity (Fig. 5b) was given. Liver GST enzyme activity (Fig. 5a) in the liver increased compared to 50, 100, and 150 mg/L TMX concentrations, by 21%, 42%, and 82% for 48 h and 117%, 139%, and 194% for 15 days, respectively. Liver GST enzyme activity, the time-dependent change in TMX exposure, increased by 74%, 63%, and 56% in 50-, 100-, and 150-mg/L effects, respectively. Brain GST enzyme activity (Fig. 5b) was statistically significant at a reduction of 28%, 31%, and 44% at 50-mg/L, 100-mg/L, and 150-mg/L effects, respectively, at 48 h compared to control. In 15 days according to the control, a significant increase in the 50-, 100-, and 150-mg/L effects was determined with 68%, 89%, and 111%, respectively. Brain GST enzyme activity, a reduction of 141, 186, and 292% in the effects of 50 mg/L, 100 mg/L, and 150 mg/L compared to time, was determined in TMX exposure, respectively.

3.6 EROD Enzyme Activity

Liver EROD enzyme activity (Fig. 6a) and brain EROD enzyme activity (Fig. 6b) were given. Liver EROD enzyme activity (Fig. 6a) incerased to 24% and 61% respectively in fish exposed to 100 and 150 mg/L TMX concentrations for 48 h. The activity of this enzyme was also significant in fish exposed to 50 mg/L, 100 mg/L, and 150 mg/L TMX concentrations 15 days, being 45%, 85%, and 157%, respectively ($p < 0.05$). Exposure time-dependent increases were observed in liver EROD enzyme activity at 50 mg/L, 100 mg/L and 150 mg/L TMX concentrations being 42%, 47%, and 57% higher at 15 days compared to 48 h. Brain EROD activity (Fig. 6b) was statistically significant at 27 and 75% increase in concentrations of 100 and 150 mg/L, respectively, at 48 h compared to control. A significant increase in the effect of 50 mg/L, 100 mg/L, and 150 mg/L compared with control was determined at 15 days with 51%, 68%, and 102%, respectively. Brain EROD enzyme activity,

time-dependent change in TMX exposure, was 38%, 35%, and 18% increase in 50-mg/L, 100-mg/L, and 150-mg/L effects, respectively.

3.7 Stress Protein Level

Liver HSP70 level (Fig. 7a) and brain HSP70 level (Fig. 7b) were given. Liver HSP70 level was not significantly different from the control for 48 h. It was determined that 14% increase in the exposure of 150 mg/L during the 15 days was statistically significant ($p < 0.05$). For liver HSP70 level, a significant difference was observed with a 20% increase in the exposure of 150 mg/L TMX concentration depending on the time (Fig. 7a). Brain HSP70 level (Fig. 7b) was statistically significant at a 13% reduction in 150 mg/L TMX concentration at 48 h compared to control. For brain HSP70 level, 43%, 82%, and 108% in 50-mg/L, 100-mg/L, and 150-mg/L effects compared to control, a significant increase was determined at 15 days, respectively. Brain HSP70 level, the time-dependent change in TMX exposure increased by 61%, 111%, and 147% in 50-mg/L, 100-mg/L, and 150-mg/L effects, respectively.

3.8 TBARS Level

Liver TBARS level (Fig. 8a) and brain TBARS level (Fig. 8b) were given. The liver TBARS level (Fig. 8a) increased to 44% and 60% in TMX concentrations of 100 and 150 mg/L, respectively, at 48 h compared to control. The increase in 50-mg/L, 100-mg/L, and 150-mg/L effects compared to the control in 15 days was 48%, 64%, and 186%, respectively. Liver TBARS level, time-dependent change in TMX exposure, was statistically significant at a concentration of 50 and 150 mg/L at a 31% and 76% increase, respectively ($p < 0.05$). Brain TBARS levels (Fig. 8b) increased by 51%, 83%, and 140%, respectively, at 50-, 100-, and 150-mg/L effects over 48 h compared to control. On the 15th day, an increase of 50 mg/L, 100 mg/L, and 150 mg/L compared to control was statistically significant in 77%, 113%, and 178%, respectively. Brain TBARS level, time-dependent change in TMX exposure, was statistically significant at 50-mg/L, 100-mg/L, and 150-mg/L effect increase of 21%, 20%, and 18%, respectively ($p < 0.05$).

3.9 8-OHdG Levels

The liver 8-OHdG level is shown in Fig. 9a and the brain TBARS level is shown in Fig. 9b. Liver 8-OHdG level (Fig. 9a) increased to 27%, 126%, and 163% at TMX concentrations of 50 mg/L, 100 mg/L, and 150 mg/L, respectively, at 48 h compared to control. At 15 days, the increase in effects of 50 mg/L, 100 mg/L, and 150 mg/L relative to control was 95%, 149%, and 311%, respectively. Liver TBARS level was statistically significant at 50, 100, and 150 mg/L concentration, with time-dependent change in TMX exposure increasing 34%, 7%, and 36%, respectively ($p < 0.05$). Brain TBARS levels (Fig. 9b) increased by 15%, 32%, and 44% at an effect of 50, 100, and 150 mg/L at 48 h, respectively, compared to control. On the 15th day, 50-mg/L, 100-mg/L, and 150-mg/L increases compared to control were statistically significant at 48%, 79%, and 117%, respectively. Brain TBARS level was statistically significant with a time-dependent increase of 33%, 39%, and 55% in TMX exposure in the 50-mg/L, 100-mg/L, and 150-mg/L groups, respectively ($p < 0.05$).

4 Discussion

Oxidative stress biomarkers, such as SOD and CAT, play an important role in the elimination of ROS produced during the biotransformation of xenobiotics; moreover, the first defense mechanism against ROS is induction of the SOD/CAT system (Lushchak, 2016). Pollutants have been reported to affect SOD and CAT activity in tissues (Dinu et al., 2010).

For SOD enzyme activity in zebrafish livers compared to control groups, a significant increase in 7-day and 14-day exposure was detected in all the thiamethoxam-treated groups (Yan et al., 2016). In this study, SOD activity increased in TMX-exposed brain and liver tissues. Brain tissue decreased in all exposure groups with time. This result has been reported to cause SOD enzyme inactivation due to the overproduction of superoxide radicals. SOD is the oxidative stress biomarker that affects superoxide radicals, the precursor of ROS in cells (Luo et al., 2008). In the cell, SOD activity regulates the steady-state level of H_2O_2 and oxygen radicals of the two species that are superoxide radicals. In the case of SOD pollution, the enzyme protein de novo synthesis activity increases as a result of the rise of radicals (Herrera et al., 2019). It was

reported that SOD activity in rainbow trout brain tissue showed an increase in the concentration of 30 μg/L linuron, but activity decreased at 120 μg/L and 240 μg/L compared to control (Topal, Alak, Altun, et al., 2017a). Imidacloprid at 125-μg/L and 1250-μg/L exposures has been reported to increase liver tissue SOD activity in *Prochilodus lineatus* (Vieira et al., 2018). CAT is a major enzyme that involves cellular homeostasis and in the conversion of hydrogen peroxides into water and oxygen as an oxidative stress biomarker (Sharifinasab et al., 2016). In this study, CAT activity decreased in TMX exposure in brain and liver tissues and it was shown that CAT enzyme activity increased in both tissues over time. Neonitinoid imidacloprid exposure has been reported to reduce liver tissue CAT activity in *P. lineatus* (Vieira et al., 2018). Malev et al. (2012) reported that increased hydroxyl radicals as a result of exposure to pollutants may inhibit the CAT enzyme and consequently cause oxidative stress. As a result of toxicity in different tissues, the CAT enzyme has been reported to give different physiological responses during detoxification. Increased CAT enzyme activity in liver and kidney tissues of *Carassius auratus* was reported in exposure to penconazole and mancozeb (Atamaniuk et al., 2014; Husak et al., 2017). GPx, H_2O_2, and lipid peroxides play an important role in catalyzing GSH. An important feature of GPx enzyme is also involved in keeping the GSH at a certain level (Farkhondeh et al., 2020). In this study, for GPx enzyme activity, a 15-day increase in TMX exposure in liver tissue and a time-dependent increase in brain tissue were found. No significant change in GPx enzyme activity was reported in *O. niloticus* 0.2-μg/L methomyl exposure (Meng et al., 2014). In *C. auratus* liver, an increase in GPx activity has been reported in 2,4-dichlorophenol exposure (Zhang et al., 2004). In *Channa punctatus*, deltamethrin exposure was reported to increase GPx activity in liver and kidney tissue (Sayeed et al., 2003). Superoxide anion radicals, catalyzed by SOD activity, are detoxified by CAT and GPx. It is thought that the increase in GPx enzyme activity, which varies depending on the amount of GSH in the cell, is induced by CAT inhibition (Li et al., 2009; Nordberg & Arnér, 2001; Zhang et al., 2008). It has been reported that GSH provides resistance to the toxic effects of pesticide and electrophilic pesticide metabolites by conjugation reactions and GPx-mediated reduction reactions (Peña et al., 2000). It has been stated that it is directly related to GSH level oxidative stressors in fish (Leggatt et al., 2007). In this study, a decrease in liver and brain tissue was determined in GSH level TMX exposure. It is thought that the decrease in GSH level is tried to eliminate toxicity by detoxification by increasing GPx enzyme activity. GSH-dependent enzymes and 96-h effect of methyl-parathion *Brycon cephalus* in the muscle and gill tissues were found to reduce the levels of GSH (Monteiro et al., 2006). The high amount of H_2O_2 accumulation before peroxidase inactivation caused by exposure to imidazole increases lipid peroxidation and causes a decrease in the amount of GSH. In addition, the role of GSH apart from GPx catalysis is used as a substrate for GST enzyme activities, allowing the removal of electrophilic compounds from the cell (Monteiro et al., 2006; Trivedi et al., 2005). GST acts as a family of cytosolic multifunctional enzymes and as a detoxification enzyme in different tissues of fish. GST and GSH are indicated in phase II conjugation reactions on the metabolism of pesticides in fish. Their essential functions are the defense against oxidative damage (Bacchetta et al., 2014). Under the effect of endosulfan, GST enzyme activity decreased in various tissues of *Jenynsia multidentata* (Ballesteros et al., 2009). Martinez-Lara et al. (1996) found that in gilthead seabream (*Sparus aurata*), a decrease of GST activity in the exposition of pesticides occurred. According to the results of our study, a decrease in liver tissue in 48 h and an increase in 15 days was determined in TMX exposure. In brain tissue, unlike liver, 48-h decrease in effect, 15-day increase was determined. It is thought that this change in GST activity in tissues affects different physiological processes on pollutants and may change the response in GST activity according to the GSH level in the tissue. Increased GST enzyme activity in liver and kidney tissues has been reported with deltamethrin exposure in *Channa punctatus* (Slaninova et al., 2009). It was reported that serum GST enzyme activity increased significantly in *Clarias garipenus* species in 30 days after poisoning with tiametoxam compared to control groups (El Euony et al., 2020). In biochemical steps, the damage caused by pollutants such as pesticides is measured in fish species by phase I, i.e., CYP1A (measured as EROD), and phase II, namely GST enzyme (Teles et al., 2005).

Studies demonstrated that a number of pesticides increase EROD enzyme activity in tissues, a significant biomarker for pesticides (Figueiredo-Fernandes, Fontaínhas-Fernandes, Rocha, & Reis-Henriques, 2006b; Van der Oost et al., 2003). In this study, EROD activity in liver and brain tissue increased with TMX

exposure. It was reported that the change of EROD enzyme activity varies according to the time and type of pesticide. González et al. (2009) showed that EROD activities in liver tissues of different fish species increased after exposure to albendazole. However, no difference was observed in EROD activity after exposure to diazinon (Trídico et al., 2010). For changes in phase I and II enzymes in tissues exposed to toxicants, it is thought that by binding to -SH groups with GST, EROD activity decreases and biotransformation enzymes create a specific response for each toxicant.

HSP70 is known to play under normal and stressed conditions vital molecular roles in protein assembly, correct folding, and translocation; moreover, it interacts between hormones and their receptors in a variety of fish species (Welch, 1993). Ceyhun et al. (2010) found that HSP70 expression increased by deltamethrin in rainbow trout. Studies also show that oxidative stress caused by pollutants such as pesticide can increase HSP70 expression (Lee & Corry, 1998; Sanders, 1993). In the research that gives a parallel result, it was reported that HSP70 levels increased in different concentrations of ethiprole and fipronil pesticides in *Oreochromis niloticus* liver (Guedes et al., 2020). Our study determined that HSP70 levels increased by dose and time dependence.

Especially, a significant increase in liver tissue compared to brain tissue was determined. The change in HSP70 levels in our study supported the use of it as a biomarker in organisms as a result of stress caused by pesticide exposure. This situation may be due to the amount of HSP70 stimulated to protect cells against structural defects of proteins under stress (Sanders, 1993). HSP70 is in the cell under normal conditions; it works as a molecular chaperone and is outside of the protection and repair of proteins and lipids.

It provides protection by increasing the amount with its cytoprotective feature under stress conditions (Moreira-de-Sousa et al., 2018; Temiz, 2020).

One of the molecular mechanisms including pesticide toxicity is lipid peroxidation. Lipid peroxides are one of the end products produced by chemical reactions on polyunsaturated fatty acids (PUFAs) by attacking membrane phospholipids of ROS by chain reaction. Lipids as macromolecules can cause serious apoptosis or necrosis when they are damaged in permeability due to the destruction of the cell membrane as a result of oxidative damage (Hermes-Lima, 2004; Toroser et al., 2007). In the present study, we observed that thiamethoxam exposure increased TBARS levels in liver and brain tissues of *O. niloticus*. The results concerned that TBARS levels may indicate a compensatory response of the fish after thiamethoxam-induced oxidative stress. There are many studies that also observed elevated levels of TBARS in aquatic animals (Huang et al., 2003; Li et al., 2003; Sayeed et al., 2003). For example, Li et al. (2003) showed that TBARS levels increased in the liver of *Carassius auratus* exposed to 3,4-dichloroaniline. The results of the present study indicated that the exposure of fish to sublethal concentrations of these pesticides causes significant changes in TBARS levels in liver tissues. This elevation in TBARS levels may suppress antioxidant defense enzyme activities because of oxidative stress generated by water pollutants such as pesticides. TBARS levels accumulating as a result of the increase in lipid peroxidation can cause DNA damage by causing an increase in ROS (Cooke et al., 2003). Another important biomolecule affected by free oxygen radicals is DNA; it can cause serious damage at the nucleotide level of DNA. DNA damage can be determined by using comet assay; DNA double-strand breaks parameter and 8-OHdG level, which are different methodical methods to determine the genotoxicity (Berkoz et al., 2019; Pandey et al., 2018). In our study, an increase in 8-OHdG levels in the liver and brain tissues of *O. niloticus* was determined in all concentrations and periods of thiamethoxam exposure. As a similar result, thiamethoxam exposure was reported to produce DNA damage determined by comet assay in zebrafish liver at concentrations of 0.30, 1.25, and 5.00 mg/L (Yan et al., 2016). In the exposure of neonicotinoid insecticide imidacloprid, 8-OHdG levels in the brain tissue of *Gobiocypris raru* were reported to increase with an effect of 2.0 mg/L (Tian et al., 2018). Topal, Alak, Ozkaraca, et al. (2017b) reported that rainbow trout exposed to neonicotinoid imidacloprid (5 mg/L, 10 mg/L, 20 mg/L) for 21 days caused neurotoxic effects in the brain tissues. Research shows that the increase in 8-OHdG levels in tissues has been reported to be a response to oxidative stress (Anjana Vaman et al., 2013; Karataş et al., 2019).

5 Conclusion

Biochemical parameters were used as biomarkers at the cellular level under the toxic effect of sublethal doses in an in vivo study. In this study, it was determined that TMX toxicity had a significant negative effect in

O. niloticus liver and brain tissues. It will provide a new perspective for the effects of stress protein and DNA oxidation parameters on different biochemical processes as parameters indicating oxidative damage together with antioxidant system enzymes. In SOD, CAT, GPx, GST, and EROD activities, GSH, TBARS, and 8-OHdG levels, the changes occurring as biological markers are evidence of toxic effect. HSP70, which is a stress protein, could not show an adequate protective mechanism in tissues, although its level increased with its chaperone property. Environmental protection studies in the world and pollution monitoring programs will provide information on the usability of these parameters as biomarkers.

The results of this study will guide in demonstrating the toxic effects of TMX and pesticides belonging to the same chemical groups in aquatic organisms. Changes in the activity or levels of biomarkers with different pathways in determining toxic effects will hint the effects that occur in human and environment in the studies performed.

Acknowledgements This research was carried out with technical and financial assistance in the Ecophysiology Research Laboratory of Çukurova University, Faculty of Arts and Sciences, Department of Biology. The authors appreciate all the support they receive from Çukurova University.

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