Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 51, No. 2, June 2022
pages (167-177)

sciendo

ISSN 1730-413X
eISSN 1897-3191

Assessment of 8-hydroxy-2-deoxyguanosine activity, apoptosis, acetylcholinesterase and antioxidant enzyme activity in *Capoeta umbla* brain exposed to chlorpyrifos

by

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DOI: **https://doi.org/10.26881/oahs-2022.2.05**
Category: **Original research paper**
Received: **March 14, 2022**
Accepted: **April 26, 2022**

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Abstract

In this study, neurotoxic responses to exposure to chlorpyrifos (CPF) at different doses (55 and 110 µg l^{-1}) and at different time intervals (24 and 96 h) were investigated in Siraz fish (*Capoeta umbla*) using 8-hydroxy 2-deoxyguanosine (8-OHdG) activity, caspase-3, acetylcholinesterase (AChE) and oxidative stress parameters [malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR)]. In this study, the LC_{50} value of CPF was determined for the first time for *C. umbla* and calculated as 440 µg l^{-1}. In this study, 12.5% (55 µg l^{-1}) and 25% (110 µg l^{-1}) of the LC_{50} value were used. The obtained data indicate a significant increase in the MDA level and inhibition of antioxidant enzymes in the brain ($p < 0.05$). Considering DNA damage and the apoptotic process, no significant changes were found in 8-OHdG and caspase-3 activity at both doses exposed for 24 h, but a significant increase was detected in both markers at 96 hours compared to the control group ($p < 0.05$). In the case of AChE activity, which is one of the neurotoxic markers in the brain, while inhibition was determined only at the high concentration (110 µg l^{-1}) at the end of 24 hours, a decrease in enzyme activity was observed at the end of 96 hours in both concentration groups. In the light of all these results, we can say that CPF showed inhibitory effects on enzyme activity and inducing effects on MDA, caspase-3 and 8-OHdG levels. Based on these results, it can be concluded that CPF contributes to oxidative stress in fish and may have neurotoxic effects.

Key words: fish, brain, neurotoxicity, pesticides, apoptosis, toxicity mechanism

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1. Introduction

Pesticides widely used in agriculture have adverse effects on non-target ecosystems and ecosystem components around the world. Especially in aquatic ecosystems, their adverse effects on physiological and behavioral characteristics in fish are of major concern (Yonar et al. 2011; Topal et al. 2017a). One pesticide that is commonly used on a daily basis, chlorpyrifos (O, O-diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate; CPF) is a broad-spectrum organophosphate insecticide. Since its commercial introduction in 1965, CPF has been the insecticide of choice for pest control in homes as well as agricultural areas (Deb & Das 2013). Therefore, CPF poses a serious threat to the health of humans and aquatic organisms. Furthermore, to protect the ecological balance and human health, the effects of pesticide pollution on fish (Fig. 1) should be investigates (Xing et al. 2012a).

Environmental pollutants, especially pesticides, cause oxidative stress by producing highly reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and singlet oxygen species ($^1O_2$) (Kirici et al. 2017). There are many protective antioxidant mechanisms against oxidant degradation caused by oxygen-free radicals (Gutteridge 1995). A decrease in antioxidant protection causes an increase in ROS and some negative changes in the organism (Golovanova et al. 1999). Harmful effects of ROS are prevented by antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR)], reduced glutathione (GSH) and some vitamin groups (A, C, and E). Antioxidant levels can be used as an indicator of the antioxidant status of the organism and serve as biomarkers of oxidative stress. Oxidative stress resulting from the deterioration of the oxidant/antioxidant balance may cause DNA damage, enzymatic inactivation, and peroxidation of cell components, especially lipid peroxidation (Yonar et al. 2016). Malondialdehyde (MDA), a product of lipid peroxidation caused by reactive oxygen species and free radicals, is widely used as an important indicator in the assessment of oxidative stress (Alak et al. 2017a, b; İspir et al. 2017).

Many oxidative DNA damage products have been identified. Among them, the guanine derivative 8-OHGua and its deoxynucleoside 8-OHdG are very important in detecting DNA damage (Atamanalp et al. 2021). The 8-OHdG is formed by enzymatic cleavage following 8-hydroxylation of the guanine base in DNA after attack by hydroxyl radicals under oxidative stress (Xu et al. 2004). Caspase, which releases endonuclease by destroying the endonuclease cross-linked proteins of DNA, is also known as cysteine-aspartic acid proteases. The central component of the apoptotic program are caspases. Caspase activation is cell specific and caspase inhibitors have been shown to inhibit effector caspases and apoptosis (Tomatır 2003). These enzymes, which have a large family of homologs with each other, are produced as inactive granules that are activated by proteolytic degradation (Hengartner 2000; Donepudi & Grutter 2002). Several studies have revealed that apoptosis deregulation affects the balance between cell proliferation and cell death and contributes to cell growth (Jalili-Nik et al. 2020; Soukhtanloo et al. 2020; Zeinali et al. 2020). When DNA is damaged, the cell is eliminated by being directed to apoptosis. However, changes in the acetylcholinesterase (AChE) enzyme are known to play an important role in apoptosis, regulation of cell-cell interaction, cell proliferation and differentiation (Lazarevic-Pasti et al. 2017). AChE, on the other hand, plays an important role in the cholinergic system, which involves nerve impulse transmission at synapses (Modesto & Martinez 2010), but also separates acetylcholine into choline and acetate. The AChE enzyme in the brain can be a target for toxic chemicals (Schmidel et al. 2014) and these chemicals inhibit the AChE enzyme, resulting in impaired nerve function and excessive AChE accumulation (Bhattacharya 1993). Therefore, AChE is used as a biomarker in assessing neurotoxic changes (Topal et al. 2015; Topal et al. 2017a, b; Alak et al. 2019a, b).

The brain and neurological system are among the most preferred tissues and systems in aquatic toxicology due to the damage caused by pesticides in the fish nervous system (Mishra & Devi 2014). Similarly, fish are often used today as experimental models to measure environmental pollution

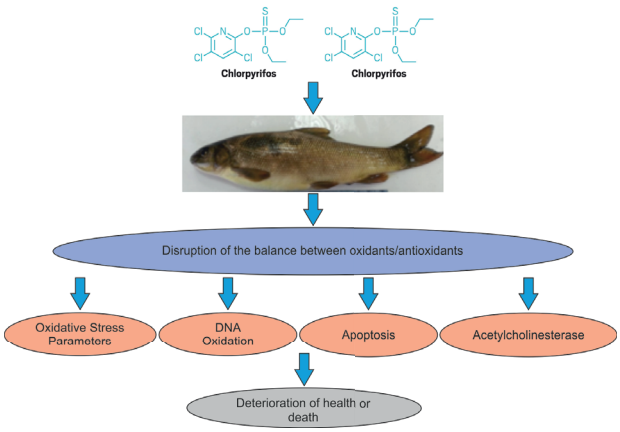

Figure 1

Susceptibility of fish to exposure to chlorpyrifos

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and related biochemical changes in terms of the well-being of aquatic ecosystems. Therefore, data on the toxic effects of this pesticide in fish can assist in toxicological risk assessment (Topal et al. 2017b). Although studies on CPF–fish interactions have been conducted in recent years using biochemical, histopathological, and oxidative stress markers (Uçar et al. 2020a), there are still inadequacies in assessing the CPF toxicity mechanism. The objective of this study is to address the knowledge gaps identified through the literature review. To this end, the effects of different concentrations and durations of CPF on oxidative damage in *Capoeta umbla* brain tissue were evaluated by reliable markers (8-OHdG, caspase-3, AChE, GR, SOD, CAT, GPx and MDA) in pollution studies.

2. Materials and methods

2.1. Study materials and design

Capoeta umbla individuals (weight 92.7–121.4 g; length 18–23 cm) caught in the Murat River (Bingöl, Turkey; 38°46'24.0"N; 40°35'40.7"E) were used in this study. The fish were transferred to 600 l fiberglass tanks at Bingöl University, Faculty of Agriculture, Aquaculture Facility. Prior to the experiment, the fish were acclimatized (21 days) following the fluid analysis procedure. During acclimation, they were fed a commercial diet ad libitum. The physicochemical properties of the water used in the study are presented in Table 1.

The insecticide used in this study was obtained from a commercial institution (Turkey). The CPF concentrations of 200, 300, 400, 500 and 1000 µg/l were used to determine the LC_{50} value of CPF. Ten fish were randomly selected for each group from 600 l tanks and five groups were formed. The fish were placed in 60 l aquariums with aeration stones placed there beforehand. The effects of CPF were observed by the researchers for 96 h and changes were noted. Dead fish were immediately removed from the aquarium. Probit analysis was performed to calculate the LC_{50} value after the 96-hour period.

Table 1

Physicochemical parameters of water

Physicochemical parameters	Values
Dissolved oxygen	8.02 ± 1.04 mg l^{-1}
pH	8.0 ± 0.5
Temperature	15 ± 3°C
Alkalinity	137 ± 9 mg l^{-1}
Total hardness	151 ± 34 mg l^{-1}

After calculating the 96 h LC_{50} value, *C. umbla* specimens (n = 180) were assigned to three groups (n = 30 per aquarium). The experimental groups were established in duplicate. The stock solution of CPF was prepared by dissolving in water. The following groups were established:

I. Control Group

II. 12.5% of the LC_{50} 96 value of CPF (55 µg l^{-1})

III. 25% of the LC_{50} 96 value of CPF (110 µg l^{-1})

Fish were exposed to the test solutions for 96 h. Fish samples were collected at 24 and 96 hours in accordance with the test procedure for the established application volumes. No fish deaths were observed at this stage of the experiment.

2.2. Determination of the DNA damage (8-OHdG) level

A commercial off-the-shelf Fish ELISA kit (Catalog No. 201-00-0041; SunRed) was used to determine the level of 8-OHdG in brain tissue homogenates of *C. umbla* fish. Homogenates were incubated for 10 min in the dark at 37°C on a plate shaker. Stop Solution (50 µl) was then added and the measurement was performed in an ELISA plate reader at an absorbance of 450 nm within 10 min (Alak et al. 2021).

2.3. Determination of the caspase-3 level

A commercially available off-the-shelf fish ELISA Kit (CASP3; Catalog No. 201-00-0031; SunRed) was used to determine caspase-3 enzyme activity in control and experimental group samples. The main point of this analysis is to determine the product formed as a result of the reaction of the substrate with the caspase-3 enzyme. Measurements were performed in the ELISA plate reader at an absorbance of 450 nm within 10 min (Alak et al. 2021).

2.4. Measurement of AChE enzyme activity

The AChE activity was determined by the modified method of Ellman et al. (1961). Brain tissues were homogenized in 0.1 M phosphate buffer (1% v/v, pH 7.4) containing Triton-X 100 and centrifuged at 13,000 x g for 30 min (4°C). The obtained supernatants were used as a source of enzymes for the determination of AChE activity (Rosenfeld et al. 2001; Alak et al. 2019b).

2.5. Determination of antioxidant enzyme activity and the lipid peroxidation level

2.5.1. Preparation of homogenates

Brain tissues were immediately removed from fish with broken necks and washed with saline solution (0.9% NaCl). Subsequently, 50 mM KH_2PO_4 buffer (pH 7.4) was added to the tissue samples that were cut into small pieces and homogenized. The homogenates were centrifuged at 13,000 g for 30 min and used to measure supernatant enzyme activity after the homogenization process (Beutler 1971).

2.5.2. Measurement of lipid peroxidation and enzyme activity

The level of malondialdehyde (MDA) in *C. umbla* brain tissue was applied as a lipid peroxidation (LPO) indicator using thiobarbituric acid reactions according to the method by Placer et al. (1966). GR activity was measured at 25°C by modifying the method of Carlberg & Mannervik (1975). The method of Sun et al. (1988) at 560 nm and 20°C was used for SOD measurement. CAT activity was determined by the method of hydrogen peroxide decomposition at 20°C and 240 nm (Aebi 1983). Finally, GPx measurement was carried out by observing the oxidation rate of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Beutler 1971).

2.6. Protein determination

Protein concentration was determined spectrophotometrically at 595 nm according to the Bradford method with bovine serum albumin as a standard (Bradford 1976).

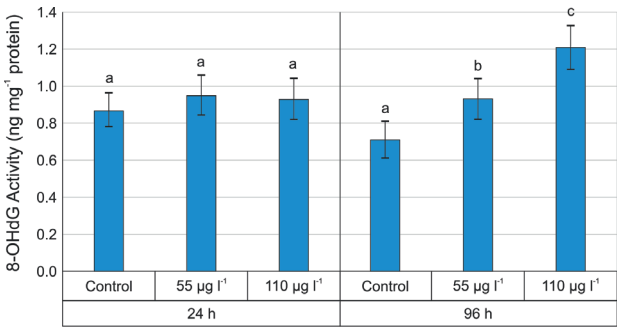

Figure 2

Effects of different exposure times and concentrations of CPF on 8-OHdG activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

2.7. Statistical analysis

One-way ANOVA was used for data analysis, and the significant difference between the control and experimental groups was determined with the Duncan test and SPSS 23.0 software. The required level for statistical significance was set as $p < 0.05$. The mean ± standard error (SE) form was used to represent the data (n = 15).

3. Results

At the end of the study, a probit analysis was performed to determine the LC_{50} 96 h value. As a result of the probit analysis, the LC_{50} 96 h value of CPF for *C. umbla* fish was calculated as 440 µg l^{-1}.

While 8-OHdG (Fig. 2) and caspase-3 (Fig. 3) activity did not change in the brain tissue of fish exposed to 55 and 110 µg l^{-1} CPF for 24 h, it increased significantly ($p < 0.05$) in the brain tissue of fish exposed to 55 and 110 µg l^{-1} CPF for 96 h.

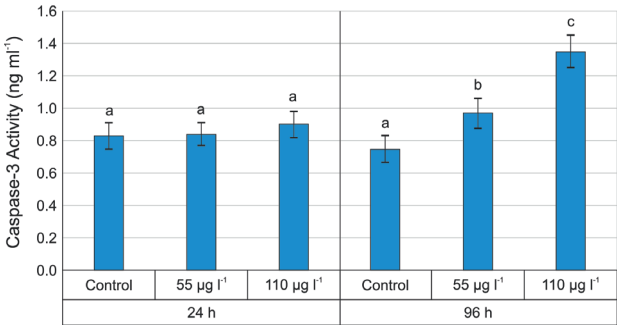

Figure 3

Effects of different exposure times and concentrations of CPF on caspase-3 activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

When evaluating the enzyme activity in the *C. umbla* brain tissue, while no significant changes in AChE enzyme activity were observed in fish brain tissue exposed to 55 µg l^{-1} CPF for 24 h, exposure to 110 µg l^{-1} for 24 h and exposure to 55 and 110 µg l^{-1} for 96 h caused a statistically significant decrease in AChE enzyme activity ($p < 0.05$; Fig. 4).

Antioxidant enzyme parameters were determined in fish brain tissue after exposure of *C. umbla* fish to 55 and 110 µg l^{-1} CPF for 24 and 96 h. The obtained results indicate that while SOD, CAT, GPx, and GR activity decreased according to a dose and exposure time, the MDA levels generally increased. The differences

between the results were found to be statistically significant ($p < 0.05$; Figs 5–9).

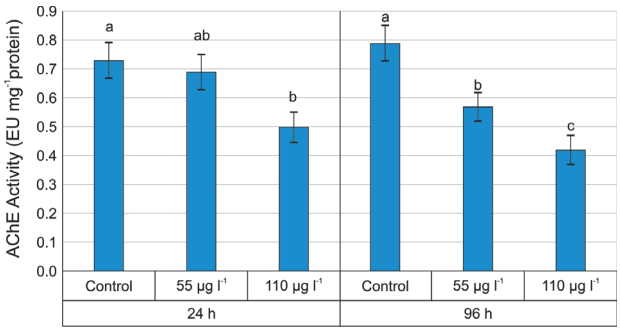

Figure 4

Effects of different exposure times and concentrations of CPF on AChE activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

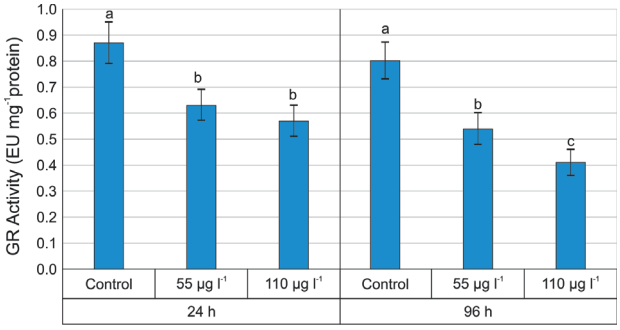

Figure 5

Effects of different exposure times and concentrations of CPF on GR activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

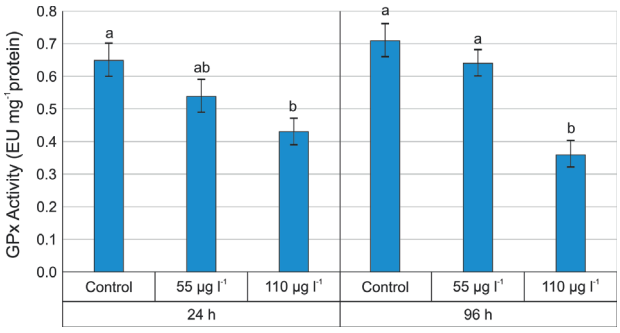

Figure 6

Effects of different exposure times and concentrations of CPF on GPx activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

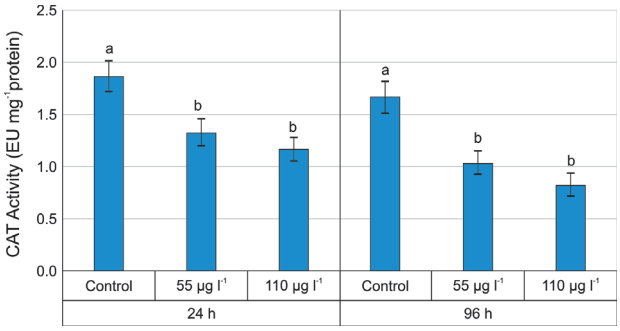

Figure 7

Effects of different exposure times and concentrations of CPF on CAT activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

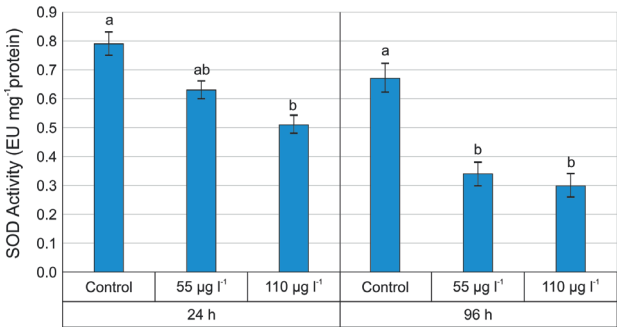

Figure 8

Effects of different exposure times and concentrations of CPF on SOD activity in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

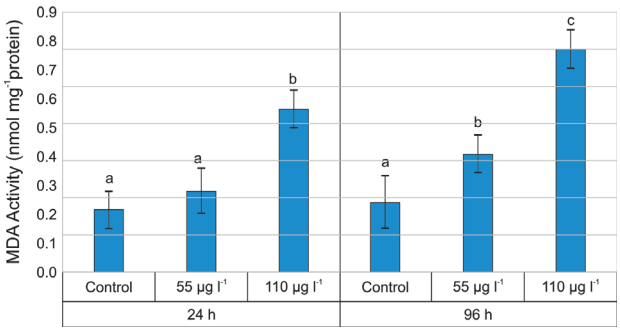

Figure 9

Effects of different exposure times and concentrations of CPF on the MDA level in *C. umbla* brain. Different letters (a, b, c) indicate significant differences between the groups ($p < 0.05$). Each value is the mean ± SE.

4. Discussion

Aquatic environments are the main recipients of domestic, industrial, and agricultural wastes; therefore, aquatic organisms are directly affected by pollutants. These pollutants accumulate in fish tissues and cause changes in their metabolic and physiological processes, and consequently cause their death. Therefore, the determination of antioxidant enzymes, as well as metabolic and physiological processes in aquatic organisms is of great importance in assessing the level of environmental pollution (Uçar et al. 2020a). Pesticides are the most important environmental pollutants, especially in aquatic ecosystems. CPF, one of these pesticides, is highly toxic to aquatic organisms because it inhibits acetylcholine, one of the most important neurotransmitters in the parasympathetic nervous system (Topal et al. 2014; Uçar et al. 2020a). CPF induces different harmful effects, including neurotoxicity through inhibition of acetylcholinesterase in fish species (Kokushi et al. 2015), biochemical and histopathological changes (Xing et al. 2012a; 2012b; Topal et al. 2014), oxidative stress (Xing et al. 2012a; Kavitha & Rao 2008) and genotoxicity (Ali et al. 2009). However, studies on the toxicity effects of CPF on fish, and especially its effects on the nervous system are insufficient. In this study, neurotoxic responses were investigated by observing the levels of caspase-3, 8-OHdG, AChE and antioxidant defense parameters such as GR, SOD, CAT, GPx, and MDA in *C. umbla* brain exposed to CPF.

4.1. 8-OHdG

DNA is the most important biological target for oxidative attacks, and the 8-OHdG level is a commonly used marker for the evaluation of the genotoxic effect of pollutants (Alak et al. 2019b,c; Uçar et al. 2020b). Several published studies have reported that simulative pesticide toxicity triggers oxidative stress with ROS production at the 8-OHdG level and causes DNA damage (Alak et al. 2017a; Alak et al. 2019a, b; Uçar et al. 2020a; Atamanalp et al. 2021). This is because the effect of pesticides directly as an oxidizing agent can be induced by a wide variety of DNA lesions in the presence of cellular reductants (Song et al. 2009). In our study, CPF significantly induced 8-OHdG activity in the brain tissue of fish in parallel with the exposure time, which had a significant effect on 8-OHdG activity. In different studies conducted in parallel with our research, it has been reported that different pesticides increase the level of 8-OHdG in fish brain tissue (Topal et al. 2015; Topal et al. 2017a; Alak et al. 2021). Shi et al. (2010) stated that chemicals cause apoptosis and are associated with DNA fragmentation and caspase-3 activation.

4.2. Caspase-3

Caspase-3, a member of the cysteine-dependent aspartate-specific protease family, is a central apoptosis agent that contributes to the morphology of apoptotic cells and endamages several molecules (Monteiro et al. 2009). Caspase-3 is an important parameter in the progression of apoptosis, which catalyzes the cleavage of certain essential cellular proteins (Teng et al. 2019). Alak et al. (2019a) reported that caspase-3 activation causes DNA fragmentation and cell death in the final application of apoptosis. In this study, however, it has been determined that the caspase-3 activation level in *C. umbla* brain tissue increased as a function of dose and time (96 h, 55 and 110 µg l^{-1}). The source of this increase may be the induction of apoptosis due to CPF toxicity. The determined increase in the caspase-3 level at a high dose and long exposure time is supported by parameters such as a decrease in GR, GPx, SOD and CAT enzyme activity, high MDA levels, and an increase in 8-OHdG levels. Caspases, as effectors of apoptosis, play an important role in many physiological processes such as maintenance of homeostasis, development of tissues and organs, maturation of cytokines, and immune responses (Qu et al. 2018). Apoptosis has a vital effect on the development and homeostasis of organisms. High levels of oxidative stress caused by ROS generation stimulate a variety of cellular signaling pathways, often associated with apoptosis. Stimulation of apoptosis by environmental toxicants is associated with a change leading to oxidative stress in the redox balance of the antioxidant defense system. It can be said that this change causes oxidative stress, DNA damage and apoptosis in fish. In addition, it can be considered that this increase occurs in apoptotic cells due to the deterioration of the body enzyme balance and the alteration of the apoptosis pathway (Alak et al. 2020; Uçar et al. 2020b).

4.3. AChE

AChE is one of the enzymes responsible for cholinesterase activity in nervous systems of vertebrates, and when exposed to pollutants, AChE can be used as a biomarker for the assessment of neurotoxic changes (Gholami-Seyedkolaei et al. 2013; Topal et al. 2017b). AChE is an important enzyme involved in the breakdown of the neurotransmitter acetylcholine into choline and acetate and may be a target for toxic chemicals (Schmidel et al. 2014).

These chemicals, by inhibiting the AChE enzyme, lead to impaired nerve function and excessive ACh accumulation (Bhattacharya 1993). AChE inhibition occurring in the brain causes changes in behavior (Kirby et al. 2000). In this study, while no significant change was found in fish brain tissue exposed to 55 µg l^{-1} CPF for 24 h, a significant decrease in AChE enzyme activity was determined in fish brain tissue exposed to 110 µg l^{-1} for 24 h and 55 and 110 µg l^{-1} for 96 h. When AChE activity decreases, acetylcholine accumulates in the synapses and this causes physiological deterioration in many functions such as feeding, swimming and behavior (Glusczak et al. 2006). The occurrence of AChE inhibition may be associated with lower AChE expression (Xing et al. 2013) and cause neurotoxic changes in the nervous system (Da Cuna et al. 2011). Furthermore, CPF affects this enzyme inhibition by disrupting the antioxidant defense system and inducing oxidative stress (Adedara et al. 2018). Previous studies have shown that pesticides can cause AChE inhibition in different fish tissues (Almeida et al. 2010; Xing et al. 2010; Xing et al. 2013; Topal et al. 2017a, b; Parlak 2018; Alak et al. 2019a).

4.4. Antioxidant parameters

Endogenous antioxidants such as GR, GPx, SOD and CAT, which are the first step of the defense mechanism, can effectively remove the produced free radicals (Bhattacharjee & Sil 2006) and protect cells against oxidative damage. Reduced CAT activity changes the redox state of the cell (Stara et al. 2012). The CAT enzyme is widely distributed in biological tissues and breaks down hydrogen peroxide into oxygen and water to protect tissues against oxidative damage (Vasylkiv et al. 2011; Parlak 2018). GPx catalyzes the reduction of hydrogen peroxide and lipid peroxides and acts as an effective protective enzyme against lipid peroxidation for the sake of using GSH (İspir et al. 2017). GR activity serves to maintain the cytosolic concentration of reduced glutathione. Stimulation of GR activity is a potential biochemical marker of oxidative stress (Cazenave et al. 2006; Kirici et al. 2021). If reduced GR and GPx activity is not compensated for by the synthesis of new glutathione molecules, it can lead to GSH depletion (Stara et al. 2012; Kirici et al. 2021). GSH is the substrate of GR and GPx enzymes. Reduction in GSH levels results in increased free radical production and disruption at the cellular level. GSH is important in the assessment of oxidative stress and inhibits hydrogen peroxide (Jurma et al. 1997). Changes in the GSH level under the influence of pollutants are explained by adjustments and activations that can replace the GSH level (Zhang et al. 2005). In this study, the decrease in GR and GPx activity in the CPF treated groups is believed to be associated with an increase in O_2 (Alak et al. 2017a, b). It can be said that the decrease in antioxidant enzyme activity as a result of CPF application suppresses the adaptation response of fish to this pesticide and cannot neutralize the ROS produced as a result of pollutants. Furthermore, the decrease in antioxidant levels can be attributed to the increase in free radicals by CPF and the excessive use of antioxidants to maintain this balance.

The SOD-CAT system acts as the first line of defense against oxygen toxicity due to its role in preventing oxidant formation. Therefore, changes in SOD and CAT activity are used as biomarkers to indicate ROS production (Tabassum et al. 2015). SOD is responsible for the removal of hydrogen peroxides, which is considered an important enzyme in the formation of lipo-peroxides and in the prevention of cell membrane damage (Oliva et al. 2010). In this study, reduction in GR, SOD, CAT and GPx activity in fish brain tissue showed that CPF exposure interrupted the defense line against toxicity and inhibited enzyme activity in tissues. Therefore, the increase in the lipid peroxidation level in target tissues is due to the accumulation of OH. This can be confirmed by the sustained increase in MDA levels in our study. MDA, one of the end products of lipid peroxidation that occurs under the influence of toxic substances, is an important indicator showing that the damaged structure of cell membranes is stable (Toroser et al. 2007). The decrease in antioxidant parameters sensitized cells to the adverse effects of oxidative stress and consequently increased lipid peroxidation supported the MDA production (Fırat & Aytekin 2018). Studies have shown that oxidative stress caused by synthetic pyrethroids leads to different acute toxicities and weakening of the immune system of fish at the later stages (Ullah et al. 2018; Uçar et al. 2020b; Atamanalp et al. 2021).

In this study, the LC_{50} value of CPF was determined for the first time for *C. umbla* fish. Furthermore, based on the results, it was determined that CPF causes neurotoxic changes in fish brains by affecting physiological and biochemical functions. It was shown that CPF can be neurotoxic to fish through inhibition of antioxidant enzymes, increase in the MDA level, inhibition of AChE enzyme, increase in 8-OHdG and caspase-3 activity. These data may provide useful information in understanding the molecular mechanism involved in CPF toxicity and in understanding future ecotoxicological studies. In addition, the possible relationship between oxidative stress and biochemical-physiological response should be investigated in further studies with different aquatic organisms using long exposure time.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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