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Toxicity mechanisms of chlorpyrifos on tissues of rainbow trout and brown trout: Evaluation of oxidative stress responses and acetylcholinesterase enzymes activity

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Abstract

Chlorpyrifos (CPF) is used intensively as an insecticide. There is a high risk of interference with the aquatic environment due to unconscious use and has a negative effects especially fish. In this study, the responses of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta fario*) exposed to the same pollutants have been evaluated and compared in terms of target organs. Fish were exposed to different concentrations of CPF (0.25, 0.5, and 1 µg L^{-1}) through 21 days. After the process oxidative stress [superoxide dismutase (SOD), catalase (CAT glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx)], acetylcholinesterase (AChE) and malondialdehyde (MDA) have been measured in gill, kidney and liver tissues. CPF exposure led to a significant changes in the enzyme activities and decreased AChE in all tissues ($p<0.05$). All antioxidant enzyme activities and MDA levels showed tissue-specific alterations ($p<0.05$). These results put forward a close relationship between AChE inhibition and chlorpyrifos concentrations. In addition to, CPF concentrations caused oxidative stress and inhibition in all enzyme activities of two different trout species' gill, liver and kidney tissues.

Keywords: AChE, Chlorpyrifos, Fish, Oxidative stress responses, Pesticide

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Introduction

Pesticides are among the most chemicals, and important for environment and health (Siemering *et al.,* 2005). Chlorpyrifos (CPF) is an organophosphate insecticide and it has been most commonly used to control harmful insects on plants (Jeon *et al*., 2016; Xing *et al*., 2015). Chlorpyrifos is highly toxic to aquatic organisms because of it inhibits acetylcholine which one of the most important neurotransmitter in the parasympathetic nervous system (Narr, 2014; Tam *et al.*, 2015; Xing *et al*., 2015). In recent years, there are too much researches about CPF – fish interactions using the biochemical, histopathological and oxidative stress markers (Díaz-Resendiz and Girón-Pérez, 2014; Jeon *et al*., 2016; Xing *et al*., 2015; Topal *et al.*, 2016). Investigation of metabolic and enzymatic activities in aquatic organisms is important because it allows understanding the ecological effects of pollutants. Enzymes, which are physiological and biochemical markers, have been used to determine environmental pollution in aquatic organisms (Jimenez and Stegeman 1990). There are many protective antioxidant mechanisms against oxidant degradation caused by free oxygen radicals (Gutteridge, 1995). The decrease in antioxidant protection causes an increase in reactive oxygen species (ROS) and some adverse changes in the organism (Golovanova *et al.,* 1999). ROS increase also accumulates free radicals. This accumulation is often toxic for polyunsaturated fatty acids, proteins, and DNA oxidation. This process is defined as oxidative stress and damages normal functions in the body (Alak *et al.,* 2017a, b).

Due to their place in the food chain and their importance as nutritional sources, fish are used as indicators of the pollution in aquatic systems. Thus, it is important for the future of the ecosystem to determine the possible physiological and biochemical effects of both trace and toxic pollutants on fish (Zarei *et al.,* 2013). Salmonids are used extensively as test organisms for studies of ecotoxicology (AI-Sabti and Metcalfe, 1995; Sandahl and Jenkins, 2002). The same stress factor in a comparison between Salmonidae species showed a wide variation in biochemical responses (Ruane *et al*., 1999; Parlak, 2018)

In this study, the effects of chlorpyrifos exposition on MDA level, antioxidant enzyme (SOD, CAT, GPx, GR, GST and G6PD) and AChE activity of mature rainbow and brown trout' s gill, kidney and liver tissues were determined. This study will provide a better understanding of the toxic mechanisms that occurs in the different tissues of the trout exposed to chlorpyrifos. In the literature search, there are no studies with comparing the responses to pesticide toxicity of two trout species. So the present study was aimed to determining the oxidative damage by using the antioxidant enzyme activities, AChE and MDA levels in different trout tissues species.

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Materials and methods

Fish maintenance and experimental design

The experiments were carried out in the Toxicology Experiment Unit in the Aquarium Fish Research and Application Center and the Laboratories of the Atatürk University Fisheries Faculty, Erzurum, Turkey. 400 liters' fiberglass tanks were used as experiment medium. Concomitantly, average weight 200±15 g rainbow (*O. mykiss*) and brown trout (*S. t. fario*) fish were stocked as treatment material. The amount of water entering to the tanks was at least 0,5 L min^{-1} per kg of fish. Water quality criteria has been measured (water temperature 10.5±1.5 ^{o}C, dissolved oxygen 9.3 mg L^{-1} and pH 7.2) during the study. Total 96 fish were distributed to 8 tanks. One of *O. mykiss* and one of *S. t. fario* tanks were designed as control groups (no pesticide treatment). The other 6 tanks (three of them *O. mykiss* and the others *S. t. fario*) had exposed to different CPF concentrations. CPF doses (0.25, 0.5, and 1 µg L^{-1}) were determined according to the level of LC_{50} (9, 8 µg L^{-1}) (Extoxnet, 1996) for trout. At the end of the experiment period, control and all treatments group fish were sampled for analyses.

Chemical material

Chlorpyrifos (chlorpyrifos, O,O-diethyl-O-(3,5,6-trichlor-2-pyridyl) phosphorothioate) was obtained from Akdeniz Chemistry Company (Turkey).

Preparation of the homogenate and enzyme activity determination

Tissue samples (gill, kidney and liver) were taken from all fish. After washing up with the physiological saline (0.9% NaCl), the tissues were stored at -20 °C until analysis.

At the end of experiment gill, liver and kidney tissues were frozen in liquid nitrogen. Tissues homogenates (gill, liver and kidney) were prepared with a few minor modifications according to Alak *et al*. (2013). The tissues were homogenized with potassium phosphate buffer. Later, the prepared homogenates were centrifuged at 13000 rpm (1 hour at 4 °C). The MDA levels in tissues were assessed according to Luo *et al.* (2006). After the processing procedure the homogenate samples were measured at 532 nm by spectrophotometer. The protein content of each homogenate has been measured as standard by Bradford (1976). Nitro-blue tetrazolium (NBT) is used as marker in the measurement SOD activity. The homogenate samples were measured as spectrophotometric at 560 nm. One enzyme unit was defined as SOD activity that inhibits 50% of NBT reduction (Sun *et al.,* 1988). CAT activity was measured at 240 nm spectrophotometrically according to Aebi (1974). For assaying of GPx activity was performed at 340 nm spectrophotometrically, GR activity was measured using NADPH and GSSG as substrates, and G6PD activity was measured at 340 nm spectrophotometrically according to Beutler (1984). GST activity was measured at 340 nm spectrophotometrically according to (1-

chloro-2, 4 dinitrobenzene (CDNB), Sigma) Habig *et al.* (1974).

AChE assay optimization

O. mykiss and *S. t. fario* gill, liver and kidney tissues were homogenized with phosphate buffer (pH 7.4). Then supernatants were used as the enzyme source for determination of the AChE activity (Ellman *et al.,* 1961; Botté *et al*., 2012). The reaction mixture included 10 mM acetylthiocholine iodide, 0.5 Mm DTNB in 1% sodium citrate, 0.5 M phosphate buffer (pH 8) and water in a total volume of 0.1 ml. The reaction absorbance was measured at 412 nm for 5 min and expressed as mmol substrate hydrolyzed min^{-1} g^{-1} tissue (Botté *et al.,* 2012; Jeon *et al.*, 2016).

Statistical analyses

The results were assessed using SPSS 20.0 software. The one-way analysis of variance (ANOVA) and Duncan tests were performed according to the differences between the experimental groups at level of $p \leq 0.05$. The data were expressed as the mean±SD.

Results

Antioxidant enzyme activities

Statistical analysis showed that AChE activity in rainbow trout and brown trout tissues change in stress response at different concentrations of CPF. AChE and antioxidant enzyme activities have changed at a significant level in the all tissues ($p<0.05$) (Tables 1,2).

Table 1: The effects of different concentrations of CPF on gill, liver and kidney antioxidant enzyme, AChE and MDA levels of rainbow trout (*Oncorhynchus mykiss*).

Concentrations of CPF (µg L^{-1})	Tissue Types	*SOD	*CAT	*GPX	*GR	*GST	*G6PD	*AChE	*MDA
0,25	Gill	0.51±0.03^{Aa}	49.52±9.25^{Ca}	0.48±0.02^{Db}	0.44±0.05^{Aa}	0.29±0.00^{Ba}	0.25±0.01^{Bb}	0.001±0.000^{Ba}	0.33±0.03^{Ca}
	Liver	0.28±0.01^{Ab}	8.42±0.46^{Cb}	0.44±0.08^{Dc}	0.37±0.04 ^{Aa}	0.35±0.06^{Ba}	0.19±0.01^{Bc}	0.005±0.001^{Bc}	0.22±0.02^{Cc}
	Kidney	0.34±0.04^{Aa}	58.65±7.92^{Cc}	0.56±0.04^{Da}	0.61±0.03 ^{Aa}	0.09±0.01^{Bb}	0.33±0.02^{Ba}	0.005±0.001^{Bb}	0.39±0.03^{Cb}
0,5	Gill	0.41±0.03^{Ba}	7.60±0.40^{Aa}	0.61±0.01^{Cb}	0.34±0.07 ^{Aa}	0.42±0.04^{Ba}	1.21±0.01^{Ab}	0.004±0.001^{Ca}	0.27±0.01^{BCa}
	Liver	0.22±0.07^{Bb}	86.19±4.70^{Ab}	0.54±0.08^{Cc}	0.25±0.01 ^{Aa}	0.65±0.04^{Ba}	0.76±0.16^{Ac}	0.008±0.001^{Cc}	0.16±0.01^{BCc}
	Kidney	0.40±0.06^{Ba}	150.90±26.4^{Ac}	0.50±0.07^{Ca}	0.51±0.06 ^{Aa}	0.16±0.02^{Bb}	0.37±0.04^{Aa}	0.002±0.00^{Cb}	0.38±0.05^{BCb}
1	Gill	0.60±0.06^{ABa}	17.40±1.88^{Ba}	0.55±0.13^{Bb}	0.29±0.04 ^{Aa}	0.26±0.04^{Ca}	0.45±0.12^{Bb}	0.002±0.00^{Ca}	0.43±0.04^{Aa}
	Liver	0.43±0.04^{ABb}	29.25±0.36^{Bb}	0.51±0.11^{Bc}	0.34±0.06 ^{Aa}	0.35±0.01^{Ca}	0.21±0.01^{Bc}	0.006±0.00^{Cc}	0.30±0.00^{Ac}
	Kidney	0.39±0.07^{ABa}	33.16±7.06^{Bc}	0.32±0.05^{Ba}	0.46±0.02 ^{Aa}	0.19±0.01^{Cb}	0.37±0.02^{Ba}	0.003±0.00^{Cb}	0.57±0.05^{Ab}
Control	Gill	0.54±0.03^{ABa}	13.66±4.48^{Da}	0.17±0.01^{Ab}	0.05±0.01 ^{Aa}	0.29±0.02^{Aa}	0.35±0.06^{Bb}	0.009±0.001^{Aa}	0.23±0.03^{Ba}
	Liver	0.35±0.00^{ABb}	46.94±1.23^{Db}	0.81±0.07^{Ac}	0.21±0.07 ^{Aa}	0.64±0.07^{Aa}	0.15±0.03^{Bc}	0.012±0.001^{Ac}	0.10±0.02^{Bc}
	Kidney	0.50±0.02^{ABa}	47.00±0.14^{Dc}	0.38±0.08^{Aa}	0.32±0.01 ^{Aa}	0.43±0.06^{Ab}	0.48±0.04^{Ba}	0.010±0.001^{Ab}	0.15±0.04^{Bb}

Lowercase superscripts (a, b, c, d) indicate significant differences among different tissue within each experimental treatment group, whereas superscripts (A, B, C, D) in uppercase show significant differences among concentrations. Each value is the mean±S.D. of 12 fish. *$p<0.05$. All enzymes are EU mg $protein^{-1}$ MDA (nmol mg^{-1} protein)

Table 2: The effect of different concentrations of CPF on gill, liver and kidney antioxidant enzyme, AChE and MDA levels of Brown trout (*Salmo trutta fario*).

Concentrations of CPF (µg L^{-1})	Tissue Types	*SOD	*CAT	*GPX	*GR	*GST	*G6PD	*AChE	*MDA
0,25	Gill	0.47±0.02 ^{Aa}	222.88±27.8^{Ca}	0.43±0.01^{Db}	0.17±0.03 ^{Aa}	0.60±0.05^{Ba}	0.17±0.03^{Bb}	0.004±0.000^{Ba}	0.51±0.06^{Ca}
	Liver	0.40±0.02 ^{Ab}	135.1±17.46^{Cb}	0.33±0.02^{Dc}	0.46±0.06 ^{Aa}	0.21±0.00^{Ba}	0.26±0.003^{Bc}	0.006±0.001^{Bc}	0.23±0.01^{Cc}
	Kidney	0.48±0.07 ^{Aa}	16.25±4.99^{Cc}	0.55±0.06^{Da}	0.72±0.10 ^{Aa}	0.16±0.01^{Bb}	0.77±0.14^{Ba}	0.003±0.001^{Bb}	0.28±0.09^{Cb}
0,5	Gill	0.32±0.05 ^{Ba}	412.50±20.2^{Aa}	0.18±0.01^{Cb}	0.80±0.10 ^{Aa}	0.27±0.07^{Ba}	0.34±0.05^{Ab}	0.000±0.000^{Ca}	0.93±0.03^{BCa}
	Liver	0.31±0.00 ^{Bb}	88.14±12.21^{Ab}	0.21±0.01^{Cc}	0.59±0.05 ^{Aa}	0.13±0.03^{Ba}	0.25±0.04^{Ac}	0.002±0.001^{Cc}	0.07±0.00^{BCc}
	Kidney	0.38±0.09 ^{Ba}	5.59±1.07^{Ac}	0.47±0.08^{Ca}	0.08±0.02 ^{Aa}	0.14±0.00^{Bb}	0.46±0.02^{Aa}	0.002±0.001^{Cb}	0.34±0.01^{BCb}
1	Gill	0.26±0.02 ^{ABa}	206.15±6.26^{Ba}	0.04±0.06^{Bb}	0.84±0.19 ^{Aa}	0.08±0.02^{Ca}	0.40±0.06^{Bb}	0.002±0.001^{Ca}	0.38±0.02^{Ba}
	Liver	0.28±0.03 ^{ABb}	214.86±3.79^{Bb}	0.17±0.03^{Bc}	0.59±0.14 ^{Aa}	0.25±0.01^{Ca}	0.22±0.02^{Bc}	0.002±0.001^{Cc}	0.18±0.02^{Bc}
	Kidney	0.32±0.00 ^{ABa}	67.70±1.45^{Bc}	0.69±0.04^{Ba}	0.46±0.05 ^{Aa}	0.05±0.02^{Cb}	0.66±0.07^{Ba}	0.002±0.000^{Cb}	0.84±0.01^{Bb}
Control	Gill	0.20±0.03 ^{ABa}	15.50±0.47^{Da}	0.59±0.05^{Ab}	0.90±0.06 ^{Aa}	0.49±0.06^{Aa}	0.16±0.04^{Bb}	0.003±0.001^{Aa}	0.05±0.14^{Aa}
	Liver	0.35±0.05 ^{ABb}	15.14±4.73^{Db}	0.63±0.09^{Ac}	0.14±0.01 ^{Aa}	0.05±0.00^{Aa}	0.25±0.00^{Bc}	0.006±0.000^{Ac}	0.03±0.00^{Aa}
	Kidney	0.39±0.06 ^{ABa}	135.64±29.5^{Dc}	0.37±0.03^{Aa}	0.67±0.02 ^{Aa}	0.44±0.04^{Ab}	0.60±0.09^{Ba}	0.006±0.00^{Ab}	0.07±0.38^{Aa}

Lowercase superscripts (a, b, c, d) indicate significant differences among different tissue within each experimental treatment group, whereas superscripts (A, B, C, D) in uppercase show significant differences among concentrations. Each value is the mean±S.D. of 12 fish. * $p<0.05$. All enzymes are EU mg $protein^{-1}$ MDA (nmol mg^{-1} protein)

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Discussion

Aquatic environments are the main receivers of domestic, industrial and agricultural wastes and therefore, aquatic organisms are directly affected by pollutants. These pollutants accumulate in the tissues of fish, and cause stress, alterations in metabolic and physiological activities, and mortality. Thus, the determination of the antioxidant enzymes, metabolic and physiological activities of aquatic organisms has great importance in evaluating the environment's pollution level.

SOD enzyme activity was decreased by CPF exposure for two fish species compared with control group (Tables 1,2) ($p<0.05$). These results had shown parallel findings with previous studies (Oruç *et al.*, 2004; Peixoto *et al*., 2006). The drop in SOD enzyme activities may be resulted from the reactive oxygen species (ROS) increasing which is formed due to interaction with pesticide (Alak *et al.*, 2017a; 2018). SOD and CAT antioxidant enzymes are the most important factors in the defense system of organisms against to toxicity (Pandey *et al.,* 2003). CPF application caused an increase in CAT specific activity compared to the control group (Tables 1,2). However, the statistical analysis showed significant difference among all groups ($p<0.05$). The higher CAT enzyme activity in liver and kidney tissues can be explained by the presence of these enzymes in these organs. Peixoto *et al*. (2006), reported that catalyze enzyme activity had been inhibited or inducted against certain pollutants and unsuitable biomarkers in toxicology studies. According to Kavitha and Rao (2008), there is an inverse correlation between CPF-antioxidant enzyme activities (CAT, SOD, GPx) and lipid peroxidation levels.

Glutathione redox level (GPx and GSH) and lipid peroxidation (MDA) are important biomarkers in toxicology studies (Oruç *et al*., 2004; Alak *et al.*, 2017a). Especially the changes in GSH level are considered to be important indicators of oxidative stress caused by pollutants (Zhang *et al*., 2005; Uçar *et al*., 2016; Alak *et al.,* 2017a). The increase in GSH level can be explained by the regulation of the level of free radicals caused by pollutants and the activation of the enzymes involved in GSH synthesis (Alak *et al*., 2017a). In this study, within the all tissue of the 2 trout species which had been exposed to different dosages of CPF, higher GPx values had been obtained in the control group as compared to treatment groups and the difference between the groups had been found to have a significance of $p<0.05$ (Tables 1,2). GPx activity showed slight increase in 0.5 µg L^{-1} and 1 µg L^{-1} rainbow trout's gill, liver and kidney tissues, but for brown trout's tissue increased only in kidney tissue. The enzyme activity alterations in all tissues was the reason of changing GSH/GSSG rate connected with GSH decreasing and GSSG increasing. The maximum decrease of GPx enzyme activity was observed in liver for two trout species, especially in 0.5 µg L^{-1} CPF group. Reductions in GPx levels were thought to be due to O_2 increase in liver tissue (Matkovics *et al.,* 1987; Alak *et al*., 2017 a, b).

We determined that GR, GST and G6PD activity changes in the gill, liver and kidney tissues after CPF exposure (Tables 1, 2) (p<0.05). Especially in high dose groups, GR and GST activities were decreased in all tissue and fish species. GST activity differs according to fish species and the types of pollutants (Kavitha and Rao, 2008; Botté *et al*. 2012). The levels of G6PD were increased in high concentration (1 µg L^{-1}) in all fish tissues except brown trout liver tissue. These changes could be explained with O_2 production and the enzyme activity inhibition by the effect of pesticide (Topal *et al*., 2014). Although there are many studies in the literature related to pesticide and G6PD activities in different fish species (Hopa *et al*., 2011; Guler *et al*., 2013; Hopa *et al*., 2015) there were no researches on chlorpyrifos and G6PD activity in trout.

It is known that, in some species AChE shows specific sensitivity to tissues, similarly, enzymes vary depending on dose, time, species and age (Narr, 2014). In the present study, CPF caused significant inhibition of AChE activity in all tissues (gill, liver and kidney) (Tables 1,2) and the AChE activity decreased compared with control of rainbow and brown trout. Gill, liver and kidney AChE activity were clearly influenced by the concentration of the exposure to CPF for all treatment fish species. There are many reports on the relationships with chloropyrifos and AChE activities in different species in the literature (Sturm *et al*., 2007; Oruç, 2010; Topal *et al*., 2014; Jeon *et al*., 2016; Renick *et al*., 2015; Tam *et al*., 2015). The present study data showed similarity with all this study results. AChE activity was clearly influenced by the pesticide exposure in fish. There is a lot of data in the literature about relationships between pesticides and AChE activity (Sismeiro-Vivas *et al.*, 2007; Velisek *et al.*, 2007; Tomé *et al.*, 2014; Suvetha *et al.*, 2015; Parlak, 2018). The reason of AChE inhibition of the pesticide is thought to cause excessive ACh accumulation by disrupting nerve function (Topal *et al.*, 2017). In addition, this pesticide is effective on this enzyme inhibition by disrupting the antioxidant defense system and inducing the oxidative stress (Adedara *et al.*, 2018). Measurement of AChE (a sensitive enzyme) activity is also used in some ecotoxicological studies, especially as toxic indicators of pesticides (Badiou and Belzunces, 2008).

Malondialdehyde (MDA), a product of lipid peroxidation caused by reactive oxygen species and free radicals, is widely used as an important indicator for the evaluation of oxidative stress (Alak et al., 2017 a,b; 2018). The lipid hydro-peroxides formed as a result of lipid peroxidation (LPO) are broken down to form aldehydes which are mostly biologically active materials. Pesticides lead to free radical production and LPO via increasing oxidative stress (Kırıcı *et al*., 2017). We can explain the increase of ROS in the effect of pesticides and this decreases the antioxidant enzymes by causing oxidative stress, which

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increases the formation of LPO and damages the membrane structure can be explained with increasing amount of MDA (Akhgrari *et al*., 2003; Fetoui *et al*., 2010).

Malondialdehyde (MDA) is used for determining the damage of pollutants and it can be frequently measured with thiobarbituric acid. MDA is not a specific or a quantitative indicator for some oxidation process, but it shows a good correlation with lipid peroxidation. The highest MDA values were obtained in gill, liver and kidney tissues of brown trout in all treatment groups and the differences between the groups had been found to be statistically meaningful ($p<0.05$) (Tables 1, 2). Various studies with different fish species, tissues and pollutants display parallel results with the obtained data (Durmaz *et al*., 2006; Oruç and Usta, 2007; Barım and Karatepe, 2010). In the CPF applied groups, it was clearly observed that there was a significant change in the MDA levels. Besides, the reason of lipid peroxidation increase was CAT activity rise. In all tissues and treatment concentrations, obtaining different MDA values can be explained with the free radical increase resulted from CPF exposure (Topal *et al.,* 2016; Alak *et al.,* 2018; Parlak, 2018).

According to our results, CPF can cause oxidative stress in trout tissues (gill, liver and kidney) by causing AChE inhibition, changes in antioxidant enzyme (SOD, CAT. GPx, GR, G6PD and GST) activity and MDA level. The results of this study can be helpful in understanding the mechanism of pesticide toxicity comparing with the rainbow and brown trout. From these findings, it can be said that rainbow trout is more resistant against to pesticide toxicity than the brown trout.

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