

Pesticide-Contaminated Feeds in Rainbow Trout (*Oncorhynchus mykiss* W. 1792) Aquaculture: Oxidative Stress and DNA Damage

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Abstract. Although chlorpyrifos (CPF) is an organophosphate medium-toxicity pesticide, it is highly toxic to fish and aquatic animals. This study was conducted with young rainbow trouts (n=20) raised on twelve floating cages on the Atatürk Dam Lake. The fish were divided into four groups and were fed no insecticide (control groups), 0.02, 0.04 and 0.08 mg/l CFP-contaminated feed orally for 30 days. The oxidative status of trout blood tissues and the comet assay was used to measure DNA strand breaks in blood erythrocytes. No significant differences were observed at all the doses of CFP between the values of hematocrit levels and total antioxidant status. On the other hand, there was a significant correlation of increased CFP doses with leucocyte levels (P<0.05), total oxidant status (P<0.01), oxidative stress index (P<0.05) and DNA damage (P<0.01).

Keywords: Chlorpyrifos, DNA damage, comet assay, oxidative stress, *Oncorhynchus mykiss*.

INTRODUCTION

The coastal areas of Atatürk Dam Lake have a long tradition of agricultural activities. To increase yields, agricultural management in this region includes the use of high quality seeds and chemical fertilizers as well as herbicides, insecticides, rodenticides and fungicides.

In this study, we focused on a pesticide-chlorpyrifos (O,O-diethyl-3,5,6-trichloro-2-pyridylphosphorothionate; CPF), a widely used organophosphate (OP) insecticide to control a variety of insects and pests in agricultural field, industrial area, domestic properties and landscape (Wu *et al.*, 2011). It has been demonstrated that CPF is neurotoxic owing to its irreversible inhibition of acetylcholinesterase (AChE) of animals (Oruc, 2010; Chen *et al.*, 2012). In recent years, there have been a large number of reports regarding CPF existence in surface waters around the world, for example, CPF has been detected in Portugal surface and ground waters (Cerejeira *et al.*, 2003; Palma *et al.*, 2009). Moreover, CPF has also been detected in

a wide range of aquatic ecosystems in the US including natural and urban streams, rivers, lakes, and sloughs (Gilliom *et al.*, 2006). Although mechanisms of off-site migration of CPF to aquatic environments are not fully elucidated, its migration is generally linked to the transport of suspended sediment in agricultural and urban runoff (Gebremariam and Beutel, 2010).

Although CPF belongs to medium-toxicity pesticide, it is highly toxic to fish and aquatic animals (Barron and Woodburn, 1995; Venkateswara Rao *et al.*, 2005), threatening the survival of fish and other aquatic animals and influencing the stability or balance of aquatic ecosystems. Now there have been a great deal of reports available about CPF toxicity on fish, for example, Nile tilapia (*Oreochromis niloticus*) (Chandrasekara and Pathiratne, 2007), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*) (Ambreen and Javed, 2015), and zebrafish (*Danio rerio*) (Levin *et al.*, 2003), indicating that it is important to evaluate CPF toxicity on fish and its possible impact on aquatic ecosystem.

While pesticide contamination via water inflow to lake has been studied elsewhere (Lamers *et al.*, 2011, Anyusheva *et al.*, 2012), the import

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pathway of pesticides to fish via contaminated feeding material and their bioaccumulation in fish has not yet been considered in environmental studies, although it may also constitute a significant potential threat to integrated aquaculture. Therefore, CPF existing in aquatic environment may be a threat to aquatic organisms, including fish.

Oxidative stress has become an important item for aquatic toxicology. Factors of stress in the aquatic organisms can be tolerable with antioxidant molecules. Recently, many investigations have been concerned over the different nutritional products due to their antioxidant potential to prevent or treat the diseases of aquatic animals (Marghitas, 2009; Li *et al.*, 2009). In recent years, several organic forms of antioxidant molecules have been studied as possible natural therapeutic and preventive agents (Sekhon-Loodu *et al.*, 2013). Antioxidant ability has usually been attributed to the activity of antioxidant enzymes as well as to the content of low molecular antioxidants such as carotenoids, tocopherols and phenolic substances (Gulhan *et al.*, 2012).

Since presence of genotoxins in the aquatic environment is a well-known fact, attempt to develop sensitive biomarkers to evaluate genotoxic effects in aquatic organisms has gained importance. The single cell gel electrophoresis (SCGE), known as comet assay, is recognized as one of the most sensitive and reliable methodologies available for DNA strand break detection with the advantages of being fast, simple and applicable to any eukaryotic cell type, *in vivo* as well as *in vitro* (Mitchellmore and Chipman, 1998).

Oncorhynchus mykiss (W. 1792) is a freshwater fish preferring well oxygenated, clean and cold water. Because of its importance in fishing and food industry, it is one of the sensitive test organisms commonly used as an early warning of biological changes caused by pollutants before physiological problems arise. Also, it is recommended to use in acute and chronic toxicity studies as bioindicator (OECD, 1992). Therefore the present study was undertaken to investigate the protective effect of garlic against oxidative stress inducing potential of Aluminum in blood tissue of *O. mykiss* together with genotoxic effect by using alkaline comet assay in erythrocytes exposed to same concentrations *in vivo*.

MATERIALS AND METHODS

Fish and chemicals

This study was conducted in 2014, with 20 young rainbow trout (1 year old and 155.00 ± 5.00 g) raised on twelve floating cages (4x4x4 m) on the Atatürk Dam Lake at $18.08 \pm 1.10^\circ\text{C}$. Rainbow trout (*O. mykiss*) was obtained from local fish culture pools (Malatya, Turkey). During this period, the remade pellets that had 48% protein and 14% lipid were given to the fish manually at a rate of approximately 2% fish body weight per day.

Experimental setup

The fish were divided into four groups. The first group was fed with pure feed as a control group. The fish in group 2 received 0.02 mg/l CFP-contaminated feed orally for 30 days. The fish in group 3 were fed with 0.04 mg/l CFP-contaminated feed for 30 days. In group 4 fish were fed with 0.08 mg/l CFP-contaminated feed for 30 days. The entire experiment was repeated three independent times; each replicated group contained five fish, for a total of 60 fish. No fish mortality occurred during this period. Comet assay was used to measure DNA strand breaks in blood erythrocytes.

Sample collection

At the end of the experiment, blood was collected from the caudal vein of the individual fish after they were anesthetized with benzocaine. These blood samples were collected in anticoagulated (heparin) tubes for hematologic evaluation. Immediately after the blood samples were collected, the hematologic analyses were determined on the same day from the fish as below.

Blood analysis

Leucocyte

Leukocytes counting was performed by transporting blood sample (diluted in Turck solution) with an leukocytes pipette onto counting lamella and examined as for erythrocytes (Blaxhall and Daisley, 1973).

Hematocrit

The microhematocrit method was utilized in hematocrit determination. Non-clotted blood was

transferred into microhematocrit pipette and centrifuged at 12,500 rpm for 5 min and the ratio of shaped blood components in plasma was determined (Jewett *et al.*, 1991).

DNA damage measurements

The comet assay was performed as described by Singh *et al.* (1988) using the following modifications. Ten μL of fresh erythrocyte cell suspension (around 20,000 cells) was mixed with 80 μL of a 0.7% low-melting agarose in a phosphate buffered saline (PBS) at 37°C. Subsequently, 80 μL of the mixture was layered on a slide pre-coated with thin layers of a 1% normal melting point agarose (NMA), and immediately covered with a coverslip. The slides were waited for 5 min at 4°C to allow the agarose to solidify. After removing the coverslips, slides were then immersed in a freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM Na₂EDTA; 10 mM Tris-HCl, pH 10–10.5; 1% Triton X-100 and 10% DMSO added just prior to use) for at least 60 min. The slides were then plunged in a freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na₂EDTA, pH > 13) at 4°C for unwinding (40 min), then electrophoresed (25 V/300 mA, 10 min). All of the steps were executed with minimal lighting. Following electrophoresis, slides were neutralized (0.4 M Tris-HCl, pH 7.5) for 5 min. Dried microscope slides were stained with ethidium bromide (2 $\mu\text{g}/\text{mL}$ in distilled H₂O; 70 $\mu\text{L}/\text{slide}$), spread over a coverslip, and analyzed using a fluorescence microscope (Olympus BX51, Japan) at a 400 \times magnification with epifluorescence and equipped with a rhodamine filter (with an excitation wavelength of 546 nm; and a barrier of 580 nm). Fifty cells were randomly scored by eye in each sample, on a scale of 0–4, based on fluorescence beyond the nucleus, as described by Kobayashi *et al.* (1995). The following scale was utilized: 0, no comet; 1, comet < 0.5 times the width of the nucleus; 2, comet equal to the width of the nucleus; 3, comet greater than the width of the nucleus; 4, comet > twice the width of the nucleus. Scoring cells in this manner has been shown to be as definite and precise as using computer image analyses, and has been shown to be time-efficient Kobayashi *et al.* (1995) and Dikilitaş

et al. (2009). Individual scoring of slides was blinded for demographic or biochemical aspects of the blood sample. The visual score for each class was evaluated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score, which characterized the degree of DNA damage in the studied groups, was the sum of the scores in the five comet classes. Therefore, the total visual score could range from 0 [all undamaged] to 400 [all maximally damaged] arbitrary units (AU). The visual scoring method was developed by Collins *et al.* (1997). All of the procedures were completed using the same staff, and DNA damage was detected using a single observer that was not aware of the subject's status. Comets were scored independently by two investigators.

Measurement of total oxidant status

Plasma TOS levels were evaluated using a novel automated measurement method, suggested by Erel (2005). In this method, oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity level, which can be determined spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are articulated in terms of micromolar hydrogen peroxide equivalent per liter [$\mu\text{mol H}_2\text{O}_2$ equiv/L]. This method was utilized to an automated analyzer, Aereset (Abbott, USA).

Measurements of total antioxidant status

The TAS of plasma was measured using an automated analyzer (Abbott, Aereset, IL, USA) with a TAS measurement kit developed by Erel (2004). In this assay, a standardized solution of Fe²⁺ + *o*-dianisidine complex reacts with a standardized solution of hydrogen peroxide via a Fenton-type reaction, producing hydroxyl radicals. At a low pH, these potent reactive oxygen species (ROS) oxidize decreased colorless *o*-dianisidine molecules to yellow-brown colored dianisidyl radicals. Oxidation

reactions progress among the dianisidyl radicals and further oxidation reactions occur. Color formations are raised with additional oxidation reactions. Antioxidants in the sample keep down oxidation reactions and color formation. In the past, the assay has observed a precision of less than 3% CV. The results are expressed as mmol of Trolox equivalent/L.

Oxidative stress index (OSI)

To calculate the oxidative stress index (OSI) of the samples; primarily, TOS and TAS units are accepted as μmol . Then, OSI is calculated according to OSI (AU: arbitrary units) = $[(\text{TOS } \mu\text{mol/L}) / (\text{TAS } \mu\text{mol/L})] \times 100$ formula.

Statistical analysis

The results obtained for the groups expressed as the mean \pm standard deviation. ANOVA was used to determine whether there were analyzed for statistical significance between the control and experimental groups with an analysis of variance. Duncan's test was performed using the SPSS 10.1 computer program (SPSS). All differences were expressed significant at P-values less than 0.05.

RESULTS

Table I shows the effect of different concentration of CPF on the oxidative status, DNA damage and some blood parameters of rainbow trout. Dose of 0.08 mg/l CPF significantly ($p < 0.05$) increased the leucocyte level. The highest level of TOS was found in the 0.08 mg/l CPF group (8.90 ± 0.67) when compared to control (5.32 ± 0.35) ($p < 0.05$). Plasma OSI levels in 0.08 mg/l CPF group (0.46 mg/l) was significantly higher than control (0.17 ± 0.02) and the other groups ($p < 0.05$). Similarly, DNA damage level in 0.08 mg/l group ($18.20 \pm 1.96 \text{ AU}$), was significantly higher than control (1.00 ± 0.58) and the other groups ($p < 0.01$).

DISCUSSION

In this study, the WBC levels of trout were significantly increased at the dose of 0.08 mg/l CPF. Similarly, Ural (2013) determined leucocyte counts of *Cyprinus carpio* as 42.30 ± 8.15 and 44.74 ± 6.72 ($\times 10^3$) in the 0.04 mg/l and 0.08 mg/l dose of CPF.

Table I.- Effect of different concentrations of chlorpyrifos (CPF) on the oxidative status, DNA damage and some blood parameters of rainbow trout.

Parameters & CPF Doses (mg/l)	Mean \pm SEM (n=5)	Minimum – Maximum	Significance
Leucocyte ($\times 10^3$)			
Control	25.60 \pm 5.29 ^a	24.600-26.400	P<0.05
0.02	24.70 \pm 8.88 ^a	22.200-26.400	
0.04	23.00 \pm 1.14 ^a	20.600-25.400	
0.08	30.80 \pm 2.18 ^b	23.800-36.200	
Hematocrit HTC (%)			
Control	24.00 \pm 2.52	19-27.00	-
0.02	25.25 \pm 1.65	22-29.00	
0.04	23.00 \pm 0.55	21-24.00	
0.08	24.00 \pm 0.32	23-25.00	
Total antioxidant status (TAS)			
Control	0.32 \pm 0.04	0.25-0.40	-
0.02	0.21 \pm 0.02	0.15-0.26	
0.04	0.33 \pm 0.04	0.20-0.42	
0.08	0.31 \pm 0.04	0.23-0.45	
Total oxidant status (TOS)			
Control	5.32 \pm 0.35 ^a	4.68-5.90	P<0.01
0.02	6.32 \pm 0.26 ^a	5.76-7.00	
0.04	7.19 \pm 0.50 ^b	6.10-8.37	
0.08	8.90 \pm 0.67 ^c	7.30-10.52	
Oxidative stress index (OSI)			
Control	0.17 \pm 0.02 ^a	0.15-0.22	P<0.05
0.02	0.22 \pm 0.01 ^b	0.18-0.25	
0.04	0.23 \pm 0.05 ^b	0.18-0.31	
0.08	0.46 \pm 0.09 ^c	0.28-0.70	
DNA Damage (AU)			
Control	1.00 \pm 0.58 ^a	0.00-2.00	P<0.01
0.02	1.75 \pm 0.48 ^a	0.00-2.00	
0.04	4.40 \pm 1.17 ^b	2.00-8.00	
0.08	18.20 \pm 1.96 ^c	12.00-24.00	

Same superscript shows that the values are not significantly different from each other.

Okechukwu *et al.* (2007) and Yonar *et al.* (2012) found increasing leucocyte counts in African catfish (*Clarias gariepinus*)-Teugels and *Cyprinus carpio* after CPF treatment. Oral and dermal applications of OPs could increase (Areechon and Plumb, 1990; Reddy *et al.*, 1991) or decrease (Siwicki *et al.*, 1990) the leucocyte counts in fishes. Besides that, pesticides induced the immunotoxicity. So immunosuppression occurred in fish which results in increased bacterial infections (De Guise *et al.*, 1994).

It was determined that TOS is increasing, while TAS is decreasing. This situation could be resulted from lipid peroxidation rise. It is shown that in CFP-intoxicated animals the reduced glutathione (GSH), CAT, SOD, G6PD activities decreased (Goel *et al.*, 2005; Verma *et al.*, 2007). GSH is naturally occurring antioxidant and prevents the formation of free radicals. It is in the center of cellular oxidation (Mascio *et al.*, 1991; Hayes *et al.*, 2005; Aly *et al.*, 2010).

Pesticides induce oxidative stress. They cause DNA damage, changes in membrane viscosity and lipid peroxidation (Bindhumol *et al.*, 2003; Banudevi *et al.*, 2006; Zama *et al.*, 2007). Lipid peroxidation is one of the molecular mechanisms of pesticide-induced toxicity. Pesticides can break the biochemical structures and physiological functions of red blood cells (RBC) (Mansour and Mossa, 2009). CFP-ethyl (CE) decreases the antioxidant defense system, while increasing the oxidative stress and *in vivo* lipid peroxidation in erythrocytes of rats (Gultekin *et al.*, 2000-2001; Altuntas *et al.*, 2002). CFP induces the lipid peroxidation with free radical generation by affecting the antioxidant mechanism in cells (Gultekin *et al.*, 2000; Demir *et al.*, 2011). Also, intoxication of CFP *in vivo* rat erythrocytes corrupts the cellular antioxidant mechanism with free radicals generation by inducing oxidative stress. As a result of that SOD, CAT and GPx activities are reduced by CFP in rat erythrocytes (Demir *et al.*, 2011).

In this study, DNA damage of trout erythrocytes exposed to the dose of 0.08 mg/l CPF increased by measuring with single cell gel electrophoresis. Srivastava (2014) showed that *in vitro* exposure of rat lymphocytes with CPF dose-dependent increase in the DNA damage obtained by single cell gel electrophoresis. CFP can induce oxidative stress resulting in tissue damage, including lipid peroxidation, nuclear DNA damage, protein kinase C activation and altered gene expression (Bagchi *et al.*, 2006). It was found that OP induced oxidative DNA damage in treating human lymphocyte cultures with azinphos methyl (AZM) was determined by the Comet assay (Muniz *et al.*, 2008).

The role of ROS tissue and DNA damage in liver and brain are reported in nitrogenous base

(Azqueta *et al.*, 2009). The purine and pyrimidine damages have been shown by modified comet assay (Srivastava (2014). Antioxidant enzymes such as CAT, SOD, GPx, G6PD, and GST have mitigating toxic effect (Adali *et al.*, 1999; Aly *et al.*, 2010). Oxidative stress occurs when the balance is broken between antioxidant defense and ROS production. It has potential damaging effect on biomolecules like DNA (Halliwell and Gutteridge, 2007). It is reported that CPF changes enzyme-related antioxidant defense mechanism and increases the ROS reproduction in different rat tissues (Aly *et al.*, 2010; Goel *et al.*, 2005).

This study obviously showed that high CPF dose (0.08 mg/l) increases TOS level, but decreases TAS level in rainbow trout plasma. Also, excessive ROS production can lead to DNA damage.

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