# EFFECTS OF DIETARY SUPPLEMENTATION OF VITAMIN C ON LEVEL OF PROTEIN CARBONYL IN LIVER AND NUCLEAR ABNORMALITIES IN PERIPHERAL ERYTHROCYTES OF *Oreochromis niloticus*

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## ABSTRACT

The aim of this study was to investigate the propensity of chlorpyrifos (CPF) to induce formation of protein carbonyl (PC) in the liver and genotoxic effects in peripheral erythrocytes of *Oreochromis niloticus* by using micronucleus (MN) and nuclear abnormalities (NAs) tests. Also, possible attenuation by vitamin C (Vit C) on these parameters was investigated. Fish were exposed to 12 and 24 µg/L chlorpyrifos for 96 hours. The liver of *O. niloticus*, exposed to 24 µg/L CPF, showed higher protein carbonyl levels than control. Administration of vitamin C was effective in reducing PC level in CPF+Vit C group. The MN and NAs frequencies in peripheral erythrocytes of fish were observed to be increased depending on CPF dose applied. Administration of Vit C ameliorated the increase in formations of MN and NAs. These data indicated the protective role of ascorbic acid against chlorpyrifos-induced genotoxicity and suggested a significant role of its antioxidant property to these beneficial effects.

**KEYWORDS:** Chlorpyrifos, *Oreochromis niloticus*, protein carbonyl, micronuclei, nuclear abnormalities

## 1. INTRODUCTION

Exposure to environmental factors, such as pesticides and other chemicals, can increase reactive oxygen species (ROS) production and affect normal cellular redox status. When the production of ROS is greater than the cells' ability to remove them, damage can occur in DNA, lipids and protein molecules [1]. The damage to membrane lipids, protein and DNA is the endpoint biomarker of oxidative stress-inducing effects of pesticides [2]. Organophosphorous compounds (OPs) are among the most widely used pesticides, either as insecticides or herbicides. The main toxic effect of OPs is the inhibition of acetylcholinesterase (AChE) [3]. In addition, it has been recently reported that OPs induce oxidative stress in organisms, and different types of DNA lesions have been demonstrated to arise as a result of oxidative attack in different tissues, including single- and double-DNA strand breaks, cross-links, chromosomal aberrations, and DNA base oxidation [4]. Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphate pesticide used heavily throughout the world for agricultural and domestic purposes [5].

Protein carbonyls are normally used as a biomarker for protein damage caused by oxidized amino acid residues in stress conditions [6]. The formation of carbonyl proteins is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting, owing to increased susceptibility to protease action, in protein breakdown by proteases [7].

The micronuclei (MN) test detects the effects of mutagenic substances in chromosomes by identifying acentric fragments or chromosomes that remain outside the nucleus and are identified as MN. This technique allows us to identify both clastogenic and aneugenic agents. The formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes by Carrasco et al. [8]. NAs, including lobbed (LB), blebbed (BL), and notched (NT) nuclei as well as binucleated (BN) cells, have been used by several authors as possible indicators of genotoxicity [9, 10]. Several studies have shown that erythrocytes of fish present a high frequency of micronuclei and nucleus abnormalities, after exposition to different chemicals under both field and laboratory conditions [11]. The detection of MN and NAs in fish help us to know the status of water quality as well as the health of particular species and potential risks, it might have after consumption [11].

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Vit C (ascorbic acid) is one of the non-enzymatic antioxidants that can protect ROS, thus preventing tissue damage. Several authors have reported that Vit C supplementation in diets for aquatic organisms has prevented the negative effects of stress, minimized toxicity by water contaminants, and increased immune response [12].

Fish have been widely used as models to evaluate the health of aquatic ecosystems in toxicologic pathology. In the present study, *O. niloticus* was chosen as an experimental model, because of its wide availability and suitability for toxicity testing [13]. The present study aimed to investigate effects of sublethal concentrations of chlorpyrifos (12 and 24 µg/L) for 96 hours on protein oxidation of *O. niloticus* liver. Also, genotoxic effects in peripheral erythrocytes by using the MN and NAs tests were investigated. In addition, we have studied the protective role of Vit C on the tested parameters.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

A technical formulation of the organophosphate insecticide chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) was used (purity 99.9%). All chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation (USA).

### 2.2. Tested animals and treatments

Juvenil *O. niloticus*, each weighting 13.01 ± 0.94 g and measuring 11.08 ± 0.97 cm in length were obtained from Mersin University, Fisheries Faculty, Aquaculture Department and transferred to laboratory at 24 ± 2 °C (12:12 L:D). Throughout the experiments, dechlorinated tap water with pH value of 7.85, an alkalinity of 326 mg/L $CaCO_3$, and oxygen concentration of 6.70 mg/L was used. The fish were allowed to acclimatize to these conditions for two weeks and fed at a rate of 2 % body weight/day with a commercial pellet diet (Çamlı-Yem, İzmir-Turkey) during acclimation period. Commercial diet composition: 44% protein, 18% lipids, 3% cellulose, 12% ash (commercial diet containing 160 mg/kg Vit C).

Experimental diets were prepared in the laboratory from commercial pellet diet. Vit C was obtained as powder from Sigma (*2-Phospho-L-ascorbic acid trisodium salt).* The Vit C-supplemented diet was prepared by adding 200 mg/100 g to the commercial pellet, and then sealing the vitamin to the pellet by spraying 2.5 ml fish oil/100 g feed [12, 14]. The control diet was prepared by spraying 2.5 ml fish oil/100 g on the commercial pellet diet.

Experiments were conducted in glass aquaria containing 100 L test solution. Fish were exposed to 12 and 24 µg/L sublethal concentrations of chlorpyrifos (CPF) for 96 hours chosen according to 96-h $LC_{50}$ value previously determined for juvenile *O. niloticus* (98.67 µg/L) [15]. Stock solution was prepared by technical CPF and diluting it in acetone to give the dosing levels. No mortality was observed during the experiments. The water was refreshed every two days to compensate for the pesticide lost in the exposure medium. The experimental fish were divided into five groups (n: 8 in each group):

1) Control: The fish of control group was exposed to tap water plus an equal amount of solvent (acetone) and was fed with control diet.
2) Vit C: The fish of Vit C group was exposed to tap water and fed with ascorbic acid-supplemented diet.
3) CPF1: The fish of CPF1 group was exposed to 12 µg/L CPF and fed with control diet.
4) CPF2: The fish of CPF2 group was exposed to 24 µg/L of CPF and fed with control diet.
5) CPF+Vit C: The fish of this group was exposed to 24 µg/L CPF and fed with ascorbic acid-supplemented diet.
6) Positive control: the fish of positive control group was exposed to 4.0 mg/L cyclophosphamide (CP) and fed with control diet. This group was only used for comparison in genotoxicity assay [16].

The fish were killed by transaction of the spinal cord and the livers were dissected. Tissue was homogenized to 1/5 (w/v) ratio in physiological saline solution (0.8 % NaCl) with homogenizer, and then centrifuged at 13,500 rpm for 10 min in a Sigma 2-16K centrifuge at +4 °C, and supernatant was used for biochemical analyses.

### 2.3. Protein determination

Tissue protein content was determined according to the method developed by Lowry et al. [17] using bovine serum albumin as standard.

### 2.4. Measurement of protein carbonyl concentration

The oxidative damage of proteins was assessed spectrophotometrically by determination of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as described by Levine et al. [18]. In aliquots of 200 µl, proteins were precipitated by the addition of 100 µl 20% trichloroacetic acid (TCA) for 5 min on ice, and centrifuged at 4000×g for 5 min. The pellet was redissolved in 100 µl 0.2 M NaOH, and 100 µl of 2 M HCl or 10 mM DNPH in 2 M HCl added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room temperature. Proteins were precipitated with TCA, and washed three times with 500 µl 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess DNPH. Samples were redissolved in 200 µl 20 mM $KH_2PO_4$, pH 2.3, and absorbance was read at 370 nm. The carbonyl content in nmol mg protein$^{-1}$ was calculated using a molar extinction coefficient of 22,000 $M^{-1}$ $cm^{-1}$ at 370 nm after subtraction of the blank absorbance.

### 2.5. Micronucleus and nuclear abnormalities assays

Peripheral blood samples were obtained from the caudal vein of fish on 4th day of exposure period. Fish blood was processed for MN and NAs tests. Fresh blood samples were dropped on clean microscopic slides, fixed in pure ethanol for 10 min and left to air-dry at room temperature, then followed by 10% Giemsa (w/v) staining for 10 min. Five slides were prepared for each fish, and in each preparation, 1000 erythrocytes were examined under the light microscope using 1000× magnification to determine the frequencies of micronucleus and nuclear abnormalities in erythrocytes. For the scoring of micronuclei, the following criteria were adopted from Al-Sabti and Metcalfe [10]. MN must be smaller than one-third of the main nuclei and clearly separated from the main nuclei. NAs were classified according to Carasso et al. [8].

### 2.6. Statistical analyses

Data were expressed as means ± standard error and analyzed with SPSS 10.0 for Windows PC program. ANOVA and Duncan's multiple range tests were used to analyse differences between groups. The differences were defined as statistically significant when $P<0.05$.

## 3. RESULTS

### 3.1. Protein carbonyl content

Protein carbonyl levels of liver were significantly ($P< 0.05$) elevated in 24 µg/L CPF-treated group when compared with control group (Fig. 1). Vit C treatment significantly decreased PC level in CPF+Vit C group as compared to 24 µg/L treated group.

### 3.2. Micronucleus and Nuclear Abnormalities

The frequencies of MN and NAs observed in exposed groups are shown in Table 1. Treatment with positive genotoxic agent, CP caused a significant increase in the frequencies of MN and NAs in erythrocytes of *O. niloticus*. Treatment with CPF exposure also caused a significant increase in MN and NAs frequencies in a concentration-dependent manner. The shape of micronucleus and other nuclear abnormalities are presented in Fig. 2.

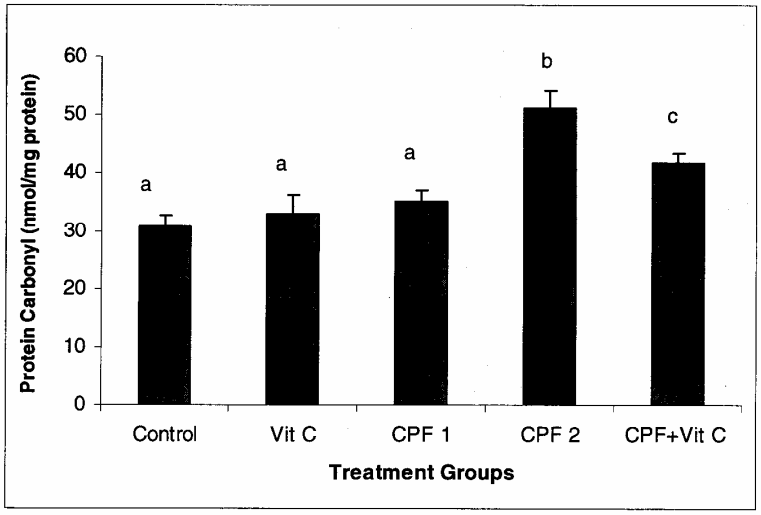


**FIGURE 1 - Effects of chlorpyrifos sublethal concentrations (12 and 24 µg/L) on protein carbonyl level in liver of *O. niloticus* and protective role of Vitamin C. The values are means ± standard error (SE) (n: 8). Groups with different letters are significantly different (P<0.05).**

**TABLE 1 - Effects of chlorpyrifos sublethal concentrations (12 and 24 µg/L) for 96 hours on micronucleus and nuclear abnormalities frequencies (‰) in peripheral erythrocytes of *O. niloticus*.**

| Treatment Groups | Nuclear Abnormalities | | | |
|---|---|---|---|---|
| | Micronuclei | Blebbed Nuclei | Notched Nuclei | Lobbed nuclei |
| Control | 0.44±0.11 [a] | 0.77±0.11 [a] | 1.55±0.29 [a] | 1.55±0.22 [a] |
| Vit C | 0.66± 0.19 [a] | 0.55±0.22 [a] | 1.78±0.39 [a] | 1.77±0.11 [a] |
| Positive Control | 4.22±0.11 [b] | 4.33±0.19 [b] | 5.99±0.19 [b] | 6.11±0.39 [b] |
| CPF1 | 2.22±0.11 [c] | 1.66±0.19 [c] | 2.77±0.11 [c] | 3.55±0.29 [c] |
| CPF2 | 3.77±0.29 [b] | 3.55±0.22 [d] | 4.99±0.19 [d] | 4.55±0.11 [d] |
| CPF+Vit C | 2.22±0.40 [c] | 2.11±0.22 [c] | 2.99±0.19 [c] | 2.88±0.11 [c] |

The values are means ± standard error (SE). Groups with different letters are significantly different (ANOVA, post hoc, Duncan SPSS 10.00, $P<0.05$).

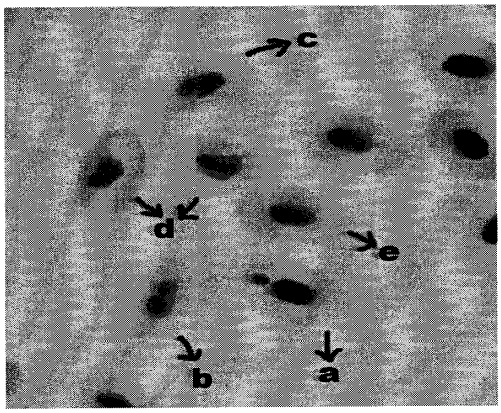


**FIGURE 2 - Micronuclei and nuclear abnormalities in peripheral blood erythrocytes of *O. niloticus* exposed to chlorpyrifos: a) Micronucleated erythrocyte, b) Blebbed nucleus (BL), c) Notched nucleu (NT), d) Lobbed nucleus (LB), e) Normal shaped nucleus (Magnification 1000×).**

## 4. DISCUSSION AND CONCLUSION

Fish erythrocytes have been proposed as a tool for studying several aspects of toxicology and a useful model to investigate oxidative stress, because their membranes are rich in long-chain n-3 polyunsaturated fatty acids, which are oxidized under oxidative stress conditions induced by different compounds [19]. Similarly to the liver, the fish erythrocyte is considered as a main location for the production of ROS due to its role in the oxygen transport via hemoglobin as well as its $O_2$ utilization. Furthermore, since toxic chemicals are absorbed by gills, skin, and gut, and transported to other tissues through the bloodstream, they come into direct contact with the erythrocytes, which, in turn, are among the first cells to suffer from toxic effects [20].

In the present study, we observed the increase in protein carbonylation in dose-dependent manner as a result of CPF exposure. Protein carbonyl served as a validated marker for protein oxidation. Our results are in agreement with other studies which showed that several pesticides (including OPs) induced ROS production and caused protein oxidative damage in different tissues [1]. The increase of PC level observed in liver following CPF exposure was probably ascribed to the excessive production of ROS, which could be related to hepatocyte enzyme leakage [21]. The induction of an excess of ROS production can lead to oxidative injuries of important cellular macromolecules, such as lipids, proteins, and nucleic acids [22]. In addition, our present results showed substantial reduction in the level of protein oxidation in CPF+Vit C group when compared with the CPF2 group. Ascorbic acid has been established to scavenge superoxide, hydroxyl, and peroxyl radicals efficiently [23]. The antioxidant activity of ascorbic acid is based on its ability to react with free radicals. This reaction is likely to be of fundamental importance in all aerobic cells, which must defend against the toxicity of the very element depended upon as the terminal electron acceptor for energy production via the respiratory chain enzymes [24].

In the present study, both concentrations of CPF induced significantly higher numbers of MN and NAs than the control and their frequencies increased dependent on concentrations. Several studies were suggested that alkylation of DNA is the essential step for mutation induction by some OP pesticides. Blasiak et al. [25] demonstrated the ability of methyl-parathion to interact directly with double-stranded DNA disturbing its stability and conformation. Padungtod et al. [26] reported chromosomal damage expressed as increased frequency of aneuploidy after occupational exposure to a mixture of OP. On the other hand, Wellman and Kramer [27] demonstrated that the OPs and their metabolites have insufficient affinity for DNA to bind and, thereby, produce genotoxicity in animals exposed to the methyl-parathion and CPF. Oxidative stress has been associated to DNA strand breaks, DNA base oxidation and chromosomal aberrations [28, 29]. According to the above information, induction of MN and NAs might be caused by ability of CPF to protein oxidation which caused oxidative stress. Vit C prevents genetic damage caused by toxicants by several mechanisms, and is known as an antimutagen that acts mainly by interfering with free radical generation and the formation of toxic metabolites [30]. Our present results showed substantial reduction in frequencies of MN and NAs in CPF+Vit C group when compared with CPF group.

The present findings indicate that increase in protein oxidation by CPF concentrations might cause to increase MN and NAs frequencies of erythrocytes. Among the potential molecular mechanisms of genotoxicity of OPs are induction of oxidative stress and alkylation. Administration of vitamin C was effective in reducing PC level and formations of MN and NAs. These data indicated the protective role of ascorbic acid against CPF-induced genotoxicity, and suggested a significant role of its antioxidant property to these beneficial effects.

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