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# THE PROTECTIVE ROLE OF VITAMIN C AND CHITOSAN AGAINST PARAQUAT-INDUCED OXIDATIVE STRESS IN MUSCLES OF COMMON CARP (*Cyprinus carpio*)

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## ABSTRACT

The purpose of this study was to examine the effects of antioxidants, including vitamin C, chitosan or a combination of both, on oxidative stress markers in muscles, as edible tissues of fish, exposed to paraquat. Fish exposed to 0.02 mg/L paraquat for 21 days were fed different diets: a normal diet, a diet containing chitosan (1000 mg/kg diet), a diet with vitamin C (1000 mg/kg diet) or both vitamin C and chitosan. Oxidative stress markers, including the activity of catalase, total antioxidant and malondialdehyde (MDA), as well as biochemical parameters including the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and acetylcholinesterase (AChE), were measured in muscles. Fish exposure to paraquat increased LDH, CPK, catalase and MDA activity significantly, while it significantly decreased AST, ALT and AChE activity and total antioxidant capacity in muscles. Administration of vitamin C, combined with chitosan, to fish exposed to paraquat was effective in regulating AChE, AST, ALT, LDH, CPK and catalase activity. A significant increase in the total antioxidant status and a significant decrease in MDA levels were observed in fish fed chitosan-vitamin C complex. In conclusion, it is suggested that combined supplementation with vitamin C and chitosan may improve the detoxification system in the muscles of fish and protect common carp from paraquat toxicity.

***How to Cite***

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## INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is one of the most widely used herbicides, especially in developing countries (Gil et al., 2014). There are many pathways by which paraquat is distributed throughout aquatic ecosystems. This herbicide may enter surface waters primarily via surface runoff, spray drift and drainage. The

fish inhabiting aquatic ecosystems close to agricultural fields are the most important non-target organisms that can be affected by herbicides. In surface waters, paraquat may be absorbed through the gill, skin and digestive system of fish and distribute in different tissues via the blood. Due to the lipophilic property of this herbicide, it accumulates mainly in fatty tissues. Often, paraquat toxicity is attributed to its redox activity and subsequent generation of reactive oxygen species (ROS) (Gil et al., 2014).

Several enzymes such as NADPH-cytochrome P450 reductase, xanthine oxidase, NADH-ubiquinone oxidoreductase and nitric oxide synthase play an important role in metabolism and detoxification of paraquat (Gil et al., 2014). A paraquat monocation radical ($PQ^{\cdot+}$) is the first metabolite of paraquat detoxification process in cells. Inside the cell, $PQ^{+}$ rapidly gets re-oxidized to $PQ^{2+}$ by accepting an electron from NADPH and in the process it generates superoxide ($O_2^{\cdot-}$). This further gives rise to formation of hydrogen peroxide and hydroxyl radical, which plays an essential role in the PQ's cytotoxicity. Fish may tolerate a mild oxidative stress induced by paraquat but a higher disturbance between the generation of reactive oxygen species (ROS) and the activity of the cellular antioxidant defense system leads to oxidative stress, which is associated with the pathogenesis of several fish diseases (Parvez and Raisuddin, 2006).

In recent years, extensive studies have been conducted to find out simple and appropriate strategies to reduce the effects of environmental pollutants on the health of organisms and to improve food safety for the consumers. Natural and synthetic antioxidants such as vitamins (Ozturk et al., 2009; Banaee et al., 2015b) and chitosan with unique biological properties (e.g. drug delivery systems) (Sun et al., 2011; Yoon et al., 2011; Alishahi et al., 2011a; Alishahi et al., 2011b) can be effective in reducing these adverse effects (Mehrpak et al., 2015). Physiologically, vitamin C is one of the most potent antioxidants which is soluble in water. Furthermore, it has a key role in the regeneration of vitamin E and increasing cellular stores of glutathione. Vitamin C is really effective in protecting protein thiol groups against oxidation (Naziro lu et al., 2010). This vitamin (ascorbic acid) can act as a natural antioxidant in negating free radicals and preventing lipid peroxidation of unsaturated fatty acids in cell membrane, both during the normal functioning of cells and when the organism is exposed to a toxic compound (Ozturk et al., 2009).

Chitosan is another natural compound of great interest to researchers due to its pharmacological properties including anti-cancer, anti-ulcer, anti-bacterial and immunostimulant properties. As a drug and hormone carrier, chitosan is used as vaccine for peptide and protein antigens (Chua et al., 2012), growth factors, anti-cancer drugs (Wei et al., 2013), analgesics and anti-inflammatory drugs (Agrawal et al., 2010; Grenha et al., 2010a; Grenha et al., 2010b), as well as antibiotics and vitamins (Alishahi et al., 2011a; Alishahi et al., 2011b). Recently, several researchers have become interested in the antioxidant activity of chitosan (Santhosh et al., 2007; Sun et al., 2011; Yoon et al., 2011).

Therefore, in this study, we hypothesize that administration of vitamin C and/or chitosan may reduce the toxic effects of paraquat and reinforce the antioxidant defense system in muscles of fish which were exposed to paraquat. This study aimed to investigate the protective effects of vitamin C and chitosan against the oxidative stress in muscle cells in common carp exposed to paraquat. Also, the activity of antioxidant defense system and biochemical properties of skeletal muscles in exposed fish, the effects of vitamin C and chitosan administration on causing a balance between lipid peroxidation rate and the antioxidant capacity of the cell, as well as the activities of intracellular enzymes in skeletal muscles were measured.

## MATERIALS AND METHODS

***Chemicals:*** Low weight chitosan (80% deacetylated) was purchased from Aldrich Chemical Company Inc. (USA). Paraquat (20%) (Gramoxone) was obtained as a commercial preparation (Jiangsu Hai Bang Company, China, imported by Iran). Vitamin C (Ascorbic acid) was bought from Rooyan Darou Company, Iran. All biochemical kits were purchased from Pars Azmoun Co, Iran. Other chemical materials were obtained from Merk Chemical Company (Germany).

***Fish treatment:*** A total of 180 juvenile common carp *Cyprinus carpio* weighting 37.7±4.4 g were obtained from a private fish farm in Behbahan, Khuzestan Province, Iran (December 2014) and used according to the National Ethical Framework for Animal Research in Iran (Mobasher et al., 2008). Fish were randomly introduced into 18 plastic tanks (80 liter) and acclimatized in aerated freshwater (24±2°C; pH, 7.4±0.2; 16 L/8D; 40% water exchange rate/day) for two weeks before the experiment. During the acclimatization period, fish were fed twice a day with a commercial diet from Beyza Feed Mill, Shiraz, Iran (Table 1).

**Table 1.** Composition of commercial diet

| Nutrients | Value |
|---|---|
| Gross energy (Kcal/Kg) | 3500 |
| Crude protein (%) | 35-37 |
| Crude lipid (%) | 9-11 |
| Crude fiber (%) | 5% |
| Moisture (%) | <10 |
| Ash (%) | <10 |
| TVN (mg/100 gr) | <45 |

TVN: Total volatile nitrogen

Fish were randomly assigned to six groups: (I) fish were fed with a normal diet for 21 days and were considered as the control group, (II) fed a diet enriched with 1000 mg chitosan per 1 kg feed for 21 days (Rad et al., 2014), (III) exposed to 0.02 mg $l^{-1}$ paraquat (Eisler, 1999), (IV) exposed to 0.02 mg $l^{-1}$ paraquat and were fed a diet enriched with 1000 mg vitamin C per 1 kg feed for 21 days (Mirvaghefi et al., 2016), (V) exposed to 0.02 mg $l^{-1}$ paraquat and were fed a diet enriched with 1000 mg chitosan per 1 kg feed for 21 days, and (VI) exposed to 0.02 mg $l^{-1}$ paraquat and were fed 1000 mg vitamin C combined with 1000 mg chitosan per 1 kg feed for 21 days.

Paraquat stock solution was prepared daily in a 1 liter beaker, in dechlorinated tap water, to a concentration of 200 mg. This stock solution was then diluted in dechlorinated tap water in a tank to have the appropriate nominal concentration (0.02 mg $l^{-1}$). The water was exchanged daily to reduce the buildup of metabolic wastes and herbicide was added to keep concentrations of paraquat constant.

At the end of the experiment, fish were euthanized by decapitation and muscles were carefully removed, washed repeatedly in ice-cold physiological saline and accurately weighed. Tissue samples were homogenized for two minutes in ice cold phosphate buffer (pH 7.4; 1:10 w/v) using a glass homogenizer and then centrifuged for 15 min at 15000 g at 4°C in a refrigerated centrifuge. The resulting supernatants were immediately used to measure the biochemical parameters by using spectrophotometric assays (Mehrpak et al., 2015).

***Biochemical Parameters Analysis:***

Creatine phosphokinase (CPK) reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{Glucose-6P} + \text{ADP}$$

$$\text{Glucose-6P} + \text{NADP} \xrightarrow{\text{G6PDH}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$$

Lactate dehydrogenase (LDH) activity was measured based on the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH.

$$\text{2-Oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{Oxaloacetate}$$

$$\text{Oxoglutarate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and -ketoglutarate to form glutamate and pyruvate; pyruvate is converted to lactate by lactate dehydrogenase and NADH is oxidized to $NAD^+$.

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

All these activities were monitored by measuring changes in absorbance at 340 nm (Moss & Henderson, 1999).

Acetylcholinesterase (AChE) or cholinesterase hydrolyzed butyrylthiocholine to butyrate and thiocholine. Thiocholine reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 5-mercapto-2- nitrobenzoic acid (5-MNBA) according the following reactions:

$$\text{Butyrylthiocholine} + \text{H}_2\text{O} \xrightarrow{\text{Cholinesterase}} \text{Butyrate} + \text{Thiocholine}$$

$$\text{Thiocholine} + \text{DTNB} \xrightarrow{\text{Oxidase}} \text{thiocholine} + \text{5-MNBA} + \text{H}^+$$

The rate of 5-MNBA formation, measured at 405 nm, is proportional to the enzymatic activity of cholinesterase in the plasma (Knedel & Boetteger, 1967).

Protein levels in tissues were determined by standard procedures used in clinical biochemistry laboratories according to the user manuals of biochemical kits (ParsAzemon Co, Iran) (Johnson et al., 1999).

$$\text{Protein} + \text{Cu}^{+2} \xrightarrow{\text{alkaline solution}} \text{Cu-Protein-complexs}$$

Catalase (CAT) activity was determined according to Góth (1991), although with some modifications. Catalase activity was measured by hydrogen peroxidase assay based on the formation of its stable complex with ammonium molybdate. 200 µL of the supernatant was incubated in working solution including 1000 µL hydrogen peroxide and 500 µL phosphate buffer (pH: 7.4) at 25°C for 60 S. Then 1000 µL of 32.4 mmol.$L^{-1}$ ammonium molybdate was added to the reaction solution and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm wavelengths.

$$\text{Catalase activity (kU.L}^{-1}) = \frac{\text{A(sample)-A(blank 1)}}{\text{A(blank2)-A(blank3)}} \times 271$$

Blank 1 contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL distilled water; blank 2 contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL buffer; blank 3 contained 1.0 mL buffer, 1.0 mL molybdate and 0.2 mL buffer.

Total antioxidant capacity was estimated according to the ferric reducing ability of plasma (FRAP). Briefly, the FRAP reagent contained 5 mL of a (10 mmol/L) TPTZ (2,4,6-tripyridyl- s- triazine) solution in 40 mmol/L HCL plus 5 mL of $FeCl_3$ (20 mmol/L) and 50 mL of acetate buffer (0.3 mol/L, pH=3.6), and was prepared freshly. 100 µL aliquots of the supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-triazine ($Fe^{3+}$-TPTZ) complex to ferrous tripyridyl-s-triazine ($Fe^{2+}$-TPTZ) at pH 3.6 and 25°C is directly proportional to the concentration of total antioxidant in the sample. $Fe^{2+}$-TPTZ has an intense blue color that can be monitored for up to 5 min at 593 nm by a UV/VIS spectrophotometer. Calculations were performed using a calibration curve of $FeSO_4 \cdot 7H_2O$ (100 to 1000 µM/L) (Benzie & Strain, 1996).

Malondialdehyde (MDA) content was assessed by modified thiobarbituric acid assay and was expressed as µmol/g tissue (Placer et al., 1996). Briefly, 500 µl of the supernatant were transferred to a Pyrex tube and mixed with 2500 µl trichloroacetic acid (20%) and 1000 µL thiobarbituric acid solution (67%). The tubes were then placed in boiling water (100°C) for 15 min. After cooling, the chromogenic substrate was extracted into the organic phase with 1000

µL of distilled water and 5000 µL *n*-butanol: pyridine (15: 1). The mixture was then centrifuged at 2000 g for 15 min at 4°C. The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels. MDA concentration was calculated using MDA standard. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards. Concentrations of MDA in muscle samples are expressed in µM per g tissue. All biochemical parameters were measured by UV/VIS spectrophotometer (UNICO 2100).

***Statistical analysis:*** All data were examined for normality (Shapiro-Wilk test). Statistical tests were performed with SPSS (IBM, 19) software by means of one-way analysis of variance (ANOVA). The significant means were compared by Tukey test and $P < 0.05$ was considered statistically significant. Data are presented as mean ± S.E.M in each experimental group. Significant differences between values were indicated by $P < 0.05$.

## RESULTS

Alterations in biochemical parameters in muscles are shown in Figures 1-8. Paraquat significantly decreased acetylcholinesterase activity in muscles of fish when compared with the control group ($P < 0.05$). Administration of vitamin C (alone) did not show any effect on the activity of AChE in muscles as compared with paraquat treated group. However, administration of chitosan or combination of vitamin C with chitosan ameliorated paraquat-induced alterations in AChE activity, restoring its activity to normal levels (Figure 1).

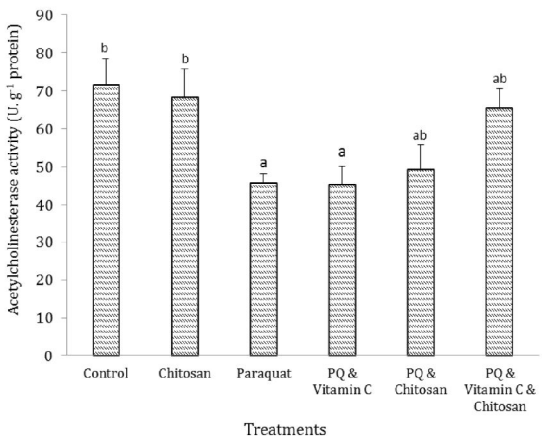


**Fig 1.** Ameliorative effects of chitosan and vitamin C on acetylcholinsetrase (AChE) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

A significant ($P < 0.05$) increase in the activity of CPK was observed in muscles of fish exposed to paraquat when compared with the control group (Figure 2). Administration of chitosan supplement to fish exposed to paraquat significantly decreased CPK activity to near normal levels (Figure 2). Following oral administration of vitamin C+chitosan, CPK activity increased in muscles of fish exposed to paraquat. However, administration of vitamin C shows a significant decrease in the activity of CPK in muscles of fish exposed to paraquat.

Analysis of biochemical parameters showed that paraquat induced a significant increase in LDH activity (Figure 3). Oral administration of vitamin C and chitosan complex to fish exposed to paraquat restored LDH activity in muscles to near control levels. Although administration of vitamin C and chitosan alone remarkably decreased LDH activity, there was a significant difference in LDH activity between these groups and the control group.

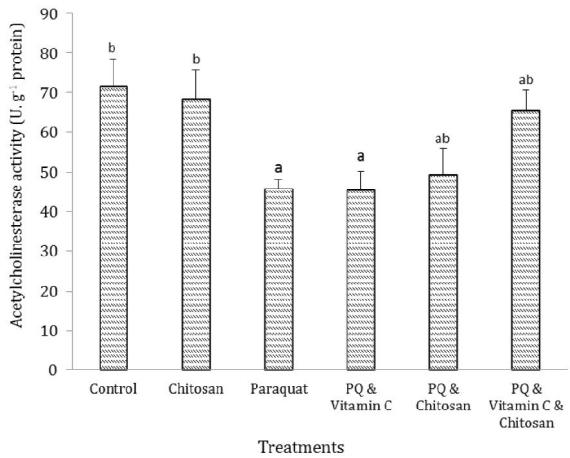


**Fig 2.** Ameliorative effects of chitosan and vitamin C on creatine phosphokinase (CPK) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

Paraquat treatment significantly reduced AST and ALT activities compared with those in the control group ($P < 0.05$). Oral administration of vitamin C alone considerably restored AST and ALT activities to control values in muscles of fish exposed to paraquat ($P < 0.05$) (Figures 4 and 5). Oral administration of vitamin C+chitosan increased AST and ALT activity in muscles of fish exposed to paraquat.

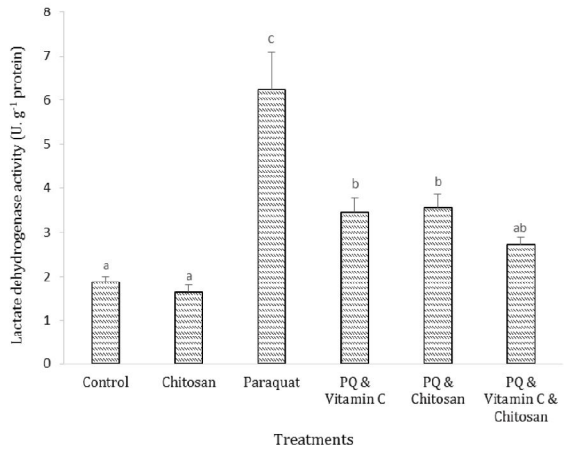


**Fig 3.** Ameliorative effects of chitosan and vitamin C on lactate dehydrogenase (LDH) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

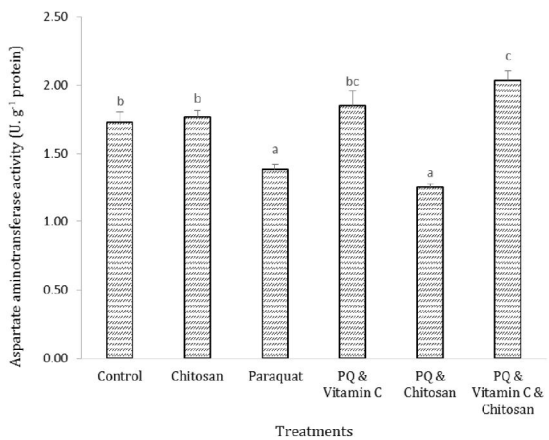


**Fig 4.** Ameliorative effects of chitosan and vitamin C on aspartate aminotransferase (AST) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

Paraquat-induced oxidative stress in muscles was examined by measuring malondialdehyde (MDA) and total antioxidant levels. Levels of MDA in the muscles of paraquat-treated fish were significantly increased compared with those in the control group (Figure 6). Administration of vitamin C alone and chitosan alone remarkably decreased MDA levels in muscles of fish exposed to paraquat. Administration of vitamin C and chitosan complex significantly decreased MDA levels; however, MDA contents in muscles of fish fed vitamin C and chitosan were significantly higher than the control group.

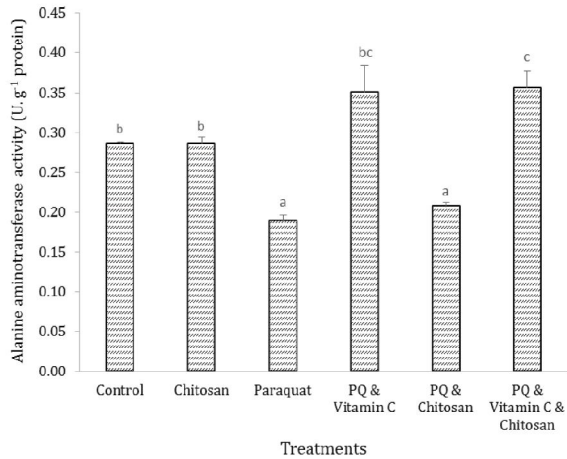


**Fig 5.** Ameliorative effects of chitosan and vitamin C on alanine aminotransferase (ALT) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

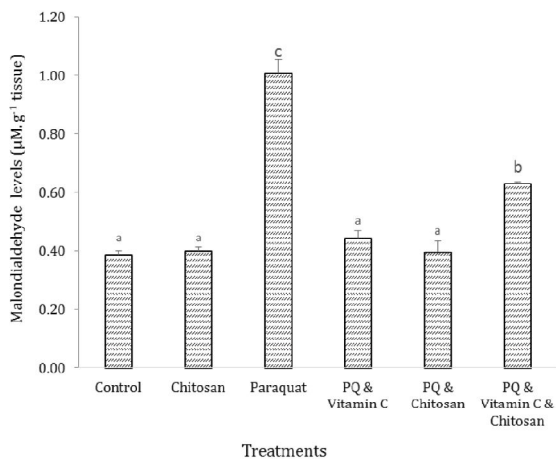


**Fig 6.** Ameliorative effects of chitosan and vitamin C on malondialdehyde (MDA) levels in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

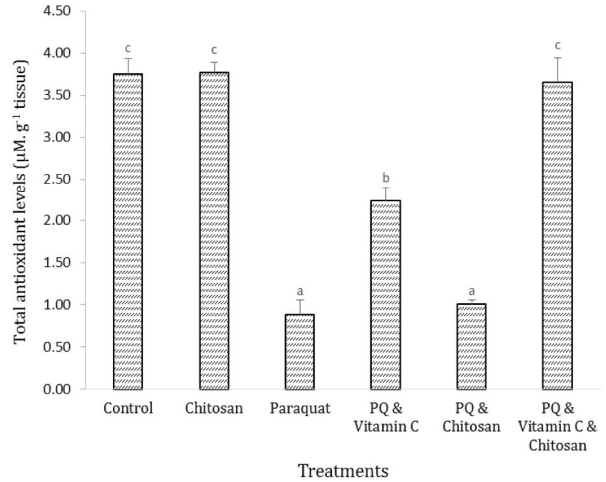


**Fig 7.** Ameliorative effects of chitosan and vitamin C on the total antioxidant levels in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

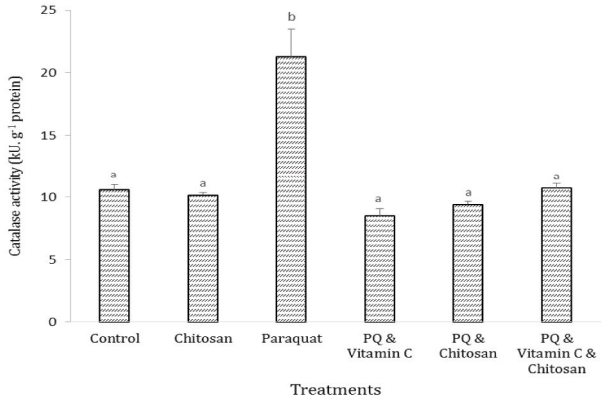


**Fig 8.** Ameliorative effects of chitosan and vitamin C on catalase (CAT) activity in the muscle tissue of common carp exposed to paraquat Significant differences between values when compared with control groups were shown by alphabet letters ($p < 0.05$); similar alphabet letters indicated no significant difference between experimental groups. Error bars represent the mean + S.E.M

A significant decrease in total antioxidant levels was observed in the muscles of fish exposed to paraquat compared with the control group ($P < 0.05$). Our data shows that vitamin C and a combination of vitamin C and chitosan can reduce oxidative stress effects of paraquat (Figure 7). Oral administration of chitosan had no significant effects on the cellular total antioxidant levels.

As shown in Figure 8, the activity of CAT in muscle tissue was significantly increased after paraquat exposure compared with that in the control group ($P < 0.05$). The results suggest that vitamin C and chitosan decreased CAT activity in muscles of fish exposed to paraquat.

## DISCUSSION

This study was planned to investigate the protective effects of vitamin C, chitosan or a combination of both on paraquat-induced oxidative damage in the muscle tissue of common carp. Various biochemical parameters considered as useful biomarkers in clinical diagnosis of damage to muscle tissues were measured to understand the protective effects of vitamin C, chitosan or a combination of both to the toxicity caused by paraquat in the muscles.

In this study, a significant decrease in AChE activity was observed in paraquat-treated fish. The decrease in AChE activity could be due to the decreased enzyme synthesis by the inhibitory nature of paraquat (Alcaro et al., 2007). Decreased AChE activity in *Alburnus mossulensis* exposed to fenpropathrin has been reported (Banaee et al., 2014). A decrease in AChE activity in different tissues of fish was observed after exposure to chlorpyrifos (Halappa and David, 2009), malathion (Patil and David, 2008), monocrotophos (Rao, 2006), atrazine (Santos and Martinez, 2012), permethrin and deltamethrin (Goulding et al., 2013). Our results indicate that dietary vitamin C combined with chitosan significantly increased AChE activity in the muscles of *C. carpio* exposed to paraquat. The effect of chitosan administration on AChE activity is due to its neuroprotective effect (Pangestuti and Kim, 2010). However, Banaee et al. (2015a) found that administration of vitamin C and chitosan had no significant effects on the activity of AChE in muscles of common carp after exposure to cadmium chloride. By an alteration in acetylcholinesterase structure, interaction with amino acid residue serine, blockage of AChE active site (Sabullah et al., 2014), as well as interference in transfer of calcium, cadmium may affect AChE activity (Carageorgiou et al., 2005). Therefore, administration of vitamin C and chitosan may not counteract all possible effects of cadmium on acetylcholinesterase. In this study, administration of chitosan and vitamin C and chitosan complex may have facilitated excretion of paraquat and its metabolites.

AST and ALT have a key role in cellular nitrogen metabolism, oxidation of amino acids and liver gluconeogenesis (Murray et al., 2003). In stressful conditions, increased activity of liver enzymes such as AST and ALT has stimulatory effects on gluconeogenic process (Murray et al., 2003). In the present study, fish exposed to paraquat for 21 days showed a decrease in AST and ALT activities when compared with the control group. Impairment in the synthesis of AST and ALT may be the most important factor in reducing the activity

of these enzymes. A decrease in AST activity was observed in muscle tissues of *Clarias gariepinus* exposed to paraquat (Chimela et al., 2014). The antioxidant property and radical scavenger activity of vitamin C could have a key role on regulating AST and ALT activities. Ergul et al. (2010) and Ismail (2013) reported that vitamin C prevents an increase in the activities of AST and ALT which is the primary evidence of their cytoprotective activity. In fish exposed to paraquat, administration of chitosan and vitamin C resulted in a significant alternation of AST and ALT activity higher than normal levels. Increased AST and ALT activity may indicate the rearrangement of protein building blocks and regeneration of muscle tissues in fish fed vitamin C combined with chitosan (Murray et al., 2003).

LDH participates in the anaerobic pathway of carbohydrate metabolism. The increase in LDH activity is a commonly used diagnostic index for identifying increases of anaerobic metabolism resulting from depletion of energy under anaerobic and environmentally stressful conditions (Murray et al., 2003). The results in the present study also reported a significant increase in LDH activity in the muscles of paraquat treated group which may be to provide energy to cope with the stress induced by paraquat. An increase in LDH activity was observed in *A. mossulensi*s and *C. carpio* exposed to fenpropathrin (Banaee et al., 2014) and chlorpyrifos (Banaee et al., 2013a), respectively. Banaee and Ahmadi (2011) reported an increase in LDH activity in crayfish exposed to endosulfan. The administration of vitamin C and chitosan in combination countered LDH activity by regulating glycolysis (Lekka et al., 2001; Uetaki et al., 2016).

Creatine phosphokinase (CPK) activity depends on age, gender, muscle mass, physical activity and environmental conditions (Slenzka et al., 1993; Baltusnikas et al., 2015, Banaee et al., 2016). Muscle mass damage may increase CPK activity in fish after exposure to paraquat. Banaee et al. (2014) found that damage to connective tissues and a reduction of muscle mass are the main reasons for alterations in the activity of CPK. So, the increased activity of CPK in fish exposed to paraquat may be indicative of a disorder in muscle fibres. These results agree with a previous study carried out on *C. carpio* exposed to bifenthrin (Velisek et al., 2008) and chlorpyrifos (Banaee et al., 2013a). Administration of chitosan had positive effects on the CPK activity in muscles of fish exposed to paraquat. The effect of chitosan administration on the CPK activity could be due to chitosan's antioxidant and ROS scavenger activity. Banaee et al. (2015a) reported that administration of vitamin C and chitosan complex normalized CPK activity which was increased following cadmium-induced muscle damage.

Paraquat toxicity could be attributed to $H_2O_2$ production, which could be eliminated by CAT activity. Hydrogen peroxide may play a role in increasing MDA levels in the fish exposed to paraquat. An increase in CAT activity was observed in different tissues of fish exposed to paraquat (Sharifinasab et al., 2016), atrazine (Paulino et al., 2012), fenpropathrin (Banaee et al., 2014) and cadmium chloride (Banaee et al., 2015a). Decreased activity of catalase may signify the effect of vitamin C and chitosan on reducing hydrogen peroxide. Banaee et al. (2015a) reported that administration of chitosan had a significant effect on CAT activity in muscles of fish exposed to cadmium chloride.

Both vitamin C and chitosan can act as a radical scavenger (Banaee et al., 2015a). Chitosan can also function as a carrier for vitamin C and therefore improve its efficiency (Banaee et al., 2015a). Consequently, administration of vitamin C and/or chitosan alone or as a complex may have a protective role in regulating the aforementioned intercellular enzymes against paraquat toxicity.

The decrease in total antioxidant levels observed in muscles following paraquat exposure was probably because of the excessive production of reactive oxygen species. Some authors report that paraquat-induced toxicity may be due to increased ROS, increased lipid peroxidation and oxidative damage (Sharifinasab et al., 2016). So, neutralizing ROS and preventing lipid peroxidation are important strategies in treatment and prevention of oxidative damage (Sharifinasab et al., 2016). Moreover, impairment in the synthesis of enzymatic and non-enzymatic antioxidants could be the most important factor in reducing the levels of cellular total antioxidant. Therefore, the decline in the cellular antioxidant capacity makes the cells more vulnerable to oxidative stress damage. Similar results were observed in rainbow trout and carp after exposure to diazinon and cyfluthrin (Sepici-Dinçel et al., 2009; Banaee et al., 2013b). The overproduction of free radicals during pesticide detoxification may be associated with a decrease in the cellular total antioxidant capacity (Monterio et al., 2006). Our data show that feeding fish with chitosan-vitamin C complex normalized total antioxidant levels and inhibited lipid peroxidation. Similar results are reported by Mehrpak et al. (2016) and Banaee et al. (2015a).

The present study indicated that paraquat treatment induced oxidative stress, as demonstrated by depleted total antioxidant capacity and increased MDA in muscle tissue. Similar results were observed in the common carp exposed to cadmium (Banaee et al., 2015a). An increase in MDA levels was reported in different tissues of fish exposed to diazinon (Oruç and Usta, 2007; Isik and Celik, 2008), deltamethrin (Yonar and Sakin, 2011), methyl parathion (Monteiro et al., 2006; Isik and Celik, 2008; Sharbidre et al., 2011), chlorpyrifos (Sharbidre et al., 2011), carbamazepine (Li et al.,, 2010) and atrazine (Paulino et al.,, 2012). The results of the present study indicated that vitamin C alone and vitamin C combined with chitosan ameliorated the lipid peroxidation of muscle cell membrane in fish exposed to paraquat. These findings are in accordance with Banaee et al. (2015a) and Mehrpak et al. (2016).

## CONCLUSION

The present study shows that increased oxidative stress is apparent in paraquat-induced fish. Based on the aforementioned results, it is suggested that vitamin C and chitosan, and a complex of them, have a significant protective effect on paraquat-induced muscle damage. Vitamin C and chitosan reduce lipid peroxidation, and vitamin C and chitosan complex improve cellular total antioxidant capacity. Restoration of biochemical parameters in the fish fed with vitamin C and chitosan diet was probably due to the increased efficiency of antioxidant defense system and detoxifying muscles of fish exposed to paraquat and radical scavenger activity of vitamin C combined with chitosan. This study provides biological evidence that supports using vitamin C, chitosan and a complex of them in treating paraquat-induced toxicity. Therefore, with regard to the importance of muscle tissues for human consumers, an appropriate strategy must be found to maintain the quality and health of edible tissues of fish which are exposed to environmental pollutants.

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### *Sažetak*

## ZAŠTITNA ULOGA VITAMINA C I KITOZANA PROTIV OKSIDATIVNOG STRESA U MIŠIĆIMA ŠARANA (*Cyprinus carpio*) UZROKOVANOG PARAKVATOM

Svrha ovog istraživanja je ispitati učinak antioksidansa, uključujući vitamin C, kitozan ili kombinaciju obaju, na oksidativni stres markera u mišićima, kao jestivog tkiva riba, izloženih parakvatu. Ribe izložene 0,02 mg/L parakvata tijekom 21 dana hranjene su različitim tipovima prehrane: normalnom prehranom, hranom koja sadrži kitozan (1000 mg/kg po obroku), vitaminom C (1000 mg/kg po obroku) ili vitaminom C i kitozanom. Markeri oksidativnog stresa mjereni su u mišićima, a uključivali su aktivnost katalaze, ukupne antioksidanse i malondialdehide (MDA), kao i biokemijske parametre, uključujući aktivnost aspartat aminotransferaze (AST), alanin aminotransferaze (ALT), kreatin fosfokinaze (CPK), laktat dehidrogenaze (LDH) i acetilkolinesteraze (AChE). Izlaganje riba parakvatu značajno povećava aktivnost LDH, CPK, katalaze i MDA, dok se značajno smanjuje AST, ALT i aktivnost AChE te ukupni antioksidativni kapacitet u mišićima. Davanje vitamina C u kombinaciji s kitozanom ribama izloženim parakvatu pokazalo se učinkovito u reguliranju AChE-a, AST-a, ALT-a, LDH-a, CPK-a i aktivnosti katalaze. Značajno povećanje ukupnog antioksidativnog statusa i značajan pad razine MDA uočeni su kod riba koje se hrane kompleksom kitozan-vitamin C. Kao zaključak izražavamo mišljenje da kombinirani dodatak vitamina C i kitozana može poboljšati sustav detoksikacije u mišićima ribe i štititi šarana od parakvatne toksičnosti.

**Ključne riječi:** antioksidansi, kitozan, vitamin C, parakvat, biomarkeri, oksidativni stres, mišić

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