Original Article

Vitamin C and Chitosan Alleviate Toxic Effects of Paraquat on Some Biochemical Parameters in Hepatocytes of Common Carp

*Zeinab Sharifinasab [1], Mahdi Banaee[*1], Mohammad Mohiseni [1], Ahmad Noori [2]*

Received: 29.06.2015 *Accepted: 04.08.2015*

ABSTRACT

Background: Paraquat is nonselective bipyridyl herbicide that induces hepatotoxicity through oxidative stress. Vitamin C and chitosan have antioxidant as well as radical scavenger properties and show protective effects against reactive oxygen species (ROS). In the present study, hepatoprotective effects of chitosan and vitamin C were evaluated in common carp exposed to paraquat.

Methods: While exposed to 0.02 mg. L^{-1}paraquat for 21 days, common carp were fed chitosan (1000 mg. kg^{-1} feed), vitamin C (1000 mg. kg^{-1} feed), and vitamin C combined with chitosan. At the end of the experiment, activities of hepatic enzymes and oxidative stress biomarkers were evaluated.

Results: Paraquat induces changes in the activity of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, alkaline phosphatase and lactate dehydrogenase in liver tissue of fish. However, these enzymes were restored to normal levels in fish fed with vitamin C and vitamin C combined with chitosan following exposure to paraquat. Increased levels of malondialdehyde were observed in liver after exposure to paraquat, while glucose-6-phosphate dehydrogenase and catalase activities and the total antioxidant levels decreased. Administration of vitamin C combined with chitosan significantly reduced malondialdehyde levels and increased the total antioxidant capacity, glucose-6-phosphate dehydrogenase and catalase activities.

Conclusion: Administration of vitamin C is effective in reducing liver toxicity of paraquat. However, administrating both vitamin C and chitosan is more effective. In other words, chitosan and vitamin C have a synergic effect. They could be used as hepatoprotective agents against paraquat-induced hepatotoxicity in fish.

Keywords*:* Biochemical parameters, Chitosan, Oxidative stress, Paraquat, Vitamin C.

IJT 2016; 31-40

INTRODUCTION

Paraquat (1, 1′-dimethyl-4 4′-bipyridinium dichloride) is one of the most important bipyridylium herbicides with unusual toxicological properties. This herbicide is one of the most common active agrochemicals used in Iranian agriculture. Contamination of the aquatic environment by paraquat is mainly via runoff from farmland. However, only a small fraction of the paraquat applied to the agricultural fields may ultimately enter surface waters. Paraquat exerts its effect as an electron donor and transforms molecular oxygen to active radicals, which are responsible for lipid peroxidation. Paraquat and its metabolites can affect some physiological and biochemical processes in fish by influencing the activities of several enzymes [1]. Fishes have evolved a complex antioxidant system composed by both non-enzymatic and enzymatic components to prevent the harmful effects of ROS. In recent years, however, the emphasis has been shifted to studying agents that can have either protective or ameliorative effects on biochemical parameters of fish exposed to various xenobiotics [2, 3]. Reduced bioavailability of paraquat in water or decreased biological effect of paraquat may be effective in maintaining the health of fish living in polluted waters.

Chitosan, a deacetylated derivative of chitin, is a naturally occurring polysaccharide

1. Department of Aquaculture, Behbahan Khatam Alanbia University of Technology, Iran.
2. Department of Aquaculture, Hormozgan University, Bandar Abbas, Iran.
*Corresponding Author: E-mail: banaee@bkatu.ac.ir

found abundantly in marine crustaceans, insects and fungi. Chitosan can be used in the treatment of water contaminated with pesticides [4-6]. The potential of chitosan to remove paraquat from polluted water has been proven [7]. Therefore, chitosan can reduce the bioavailability of paraquat in water. Moreover, chitosan and its derivatives are used in delivery of drugs, hormones, vaccines and vitamins [8, 9]. Chitosan may also be effective in healing tissue damage caused by toxicity of pesticides.

Vitamin C (ascorbic acid) is a naturally occurring antioxidant. It is an essential nutrient for the biosynthesis of collagen, L-carnitine, and the conversion of dopamine to norepinephrine [10]. Vitamin C neutralizes reactive oxygen species (ROSs) by reducing their amount. Thus, by increasing the bioavailability of vitamin C in the body of organisms, the biological effects of this vitamin would be augmented [11, 12]. Chitosan nanoparticles are capable of carrying Vitamin C in biological systems [11]. Therefore, chitosan can increase the bioavailability of vitamin C [12].

Hepatoprotective effects related to the antioxidant activity of chitosan on paraquat-induced hepatic injury have not previously been studied. However, the potential antioxidant effects of vitamin C against hepatotoxicity and oxidative stress caused by other pesticides were reported by some authors [13].

Therefore, in this study we aimed to find out the efficacy of vitamin C, chitosan and the combination of them against toxicity of sub-lethal concentrations of paraquat in common carp.

MATERIALS AND METHODS

Chemical Materials

Low weight chitosan (80% deacetylated) purchased from Aldrich Chemical Company Inc., USA. Paraquat (Gramoxone) at 20% concentration 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 Merck Chemical Company. Germany.

Fishes Treatment

One hundred and eighty juvenile Common carp, *Cyprinus carpio*, weighing (38.50 ± 5.80 g) were used according to the National Ethical Framework for Animal Research in Iran [14]. The fishes were purchased from a commercial farm in Behbahan, Khuzestan Province, Iran, and were transferred to the Aquaculture Laboratory of Aquaculture Department, Natural Resources and Environment Faculty, Behbahan Khatam Alanbia University of Technology.

The specimens were randomly distributed in 18 plastic tanks (80 l) 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 use. During the acclimatization period, the fishes were fed two times per day with commercial diet from Beyza Feed Mill, Shiraz, Iran. The fishes were randomly assigned to six groups. Group I: the specimens were fed a normal diet for 21 days and were considered the control group; Group II: the specimens were exposed to 0.02 $mg.L^{-1}$ paraquat; Group III: the fishes were fed a diet enriched with 1000 mg chitosan per 1 kg feed for 21 days; Group IV: the specimens were exposed to 0.02 $mg.L^{-1}$ paraquat and were fed 1000 mg chitosan per 1 kg feed for 21 days; Group V: the specimens were exposed to 0.02 $mg.L^{-1}$ paraquat and were fed a diet enriched with 100 mg vitamin C per 1 kg feed for 21 days; Group VI: the specimens were exposed to 0.02 $mg.L^{-1}$paraquat and were fed a diet enriched with 100 mg vitamin C combined with 1000 mg chitosanper 1 kg feed for 21 days. Each treatment was applied to triplicate groups in completely randomized design. To prevent the build-up of metabolic wastes and to keep concentrations of paraquat near the nominal level, the water was changed daily.

On 21 days of the study, the specimens were euthanized by decapitation and their livers were carefully removed, washed repeatedly in ice-cold physiological saline and accurately weighed. Tissue samples were homogenized in ice cold phosphate buffer for two min (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 supernatants were immediately used to measure biochemical parameters via spectrophotometric assays.

Biochemical Parameters Analysis

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

in a coupled reaction with malate dehydrogenase in the presence of NADH. In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to form glutamate and pyruvate. Lactate dehydrogenase converts pyruvate to lactate and NAD^+. All these activities were monitored by measuring changes in absorbance at 340 nm. Alkaline phosphatase (ALP) assay was based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm. Gamma-glutamyl transferase (GGT) activity was determined by a coupled enzyme assay, in which the GGT transfers the g-glutamyl group from the substrate L-g-Glutamyl*p*-nitroanilide, and liberates chromogen *p*-nitroanilide proportional to the GGT present. GGT activity was recorded during 3 min at 418 nm [15]. In G6PDH assay, this enzyme catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phospho-D-gluconate, along with the concomitant reduction of $NADP^+$ to NADPH. The rate of NADPH formation is proportional to G6PDH activity and is measured using spectrophotometry as increase in absorbance at 340 nm [16]. Protein levels in tissues were determined by standard procedures used in clinical biochemistry laboratories according to thebiochemical kits user manuals (Pars Azmoun Co, Iran) [17].

CAT activity was determined according to Góth [18], with some modifications. Catalase activity was measured by hydrogen peroxidase assay based on 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.

$$\text{Catalase activity } (kU.L^{-1}) = \frac{A(\text{sample}) - A(\text{blank }1)}{A(\text{blank}2) - A(\text{blank}3)} \times 271$$

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 (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. Aliquots (100 µL) 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. The calculations were performed using a calibration curve of $FeSO_4 \cdot 7H_2O$ (100 to 1000 µM/L) [19].

Malondialdehyde (MDA) content was assessed by modified thiobarbituric acid assay and was expressed as µmol/g tissue [20]. Briefly, 500 µl of the supernatant was transferred to a Pyrex tube and mixed with 2500 µl trichloroacetic acid (20%) and 1000 µmL thiobarbituric acid (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 by spectrophotometry 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 whole body samples are expressed in µM per g protein. All biochemical parameters were measured by UV/VIS spectrophotometer (model UNICCO 2100).

Statistical Analysis

All data were examined for normality (Shapiro-Wilk test). Statistical tests were performed with SPSS (IBM, Release 19) software by means of one way analysis of variance, followed by Duncan multiple comparison test ($P<0.01$). Data are presented as mean ± SD in each experimental group. Significant differences between values were characterized by alphabetical symbols ($P<0.05$).

RESULTS

No mortality was observed during the experiment. Alterations in the biochemical parameters of liver tissue are presented in Figure 1-9.

Aspartate aminotransferase (AST) activity significantly decreased in liver tissue of fishes exposed to paraquat as compared to control group. However, administration of vitamin C and vitamin C combined with chitosan showed an increase in the activity of AST as compared to the group exposed to paraquat alone (Figure 1).

Paraquat decreased alanine aminotransferase (ALT) activity. Combined treatment of vitamin C and chitosan resulted in a significant improvement in ALT activity in liver tissue of paraquat-exposed fishes (Figure 2).

Exposure to paraquat decreased alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) activities in liver tissue. Administration of vitamin C and chitosan did not significantly affect ALP and GGT activities in liver tissue of fishes exposed to paraquat (Figure 3 & 4).

Lactate dehydrogenase (LDH) activity significantly increased in fishes exposed to paraquat alone. Simultaneous administration of chitosan and vitamin C resulted in a marked normalization of LDH activity when compared to the paraquat treated groups (Figure 5).

There was a significant decrease in catalase activity in liver of fishes exposed to paraquat ($P<0.05$), whereas CAT activity was elevated in fishes fed a diet enriched with vitamin C and vitamin C combined with chitosan as compared to the group exposed to paraquat alone. Chitosan administration did not significantly modify the activities of the CAT in liver tissue of fishes exposed to paraquat (Figure 6).

Activity of G6PDH significantly decreased in liver tissue of fish exposed to paraquat as compared to control group. However, its activity in fish treated with paraquat along with vitamin C and the group which were treated with paraquat along with chitosan, was significantly ($P<0.05$) lower than that of control group (Figure 7).

Levels of malondialdehyde (MDA) in liver significantly increased ($P<0.05$) in the group exposed to 0.02 $mg.L^{-1}$ of paraquat when compared with the non-exposed group on day 21. The MDA levels in hepatocytes reduced significantly in groups, which ascorbic acid and ascorbic acid combined with chitosan, were used. However, MDA levels in hepatocytes of fishes exposed to paraquat and treated with chitosan were reduced compared with non-treated group exposed to paraquat. The MDA level in liver of fishes exposed to paraquat and treated with chitosan was greater than that of control group (Figure 8).

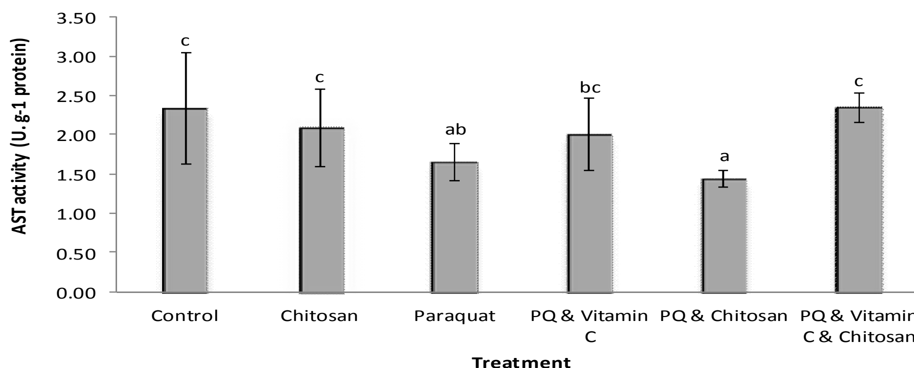

Figure 1. Ameliorative effect of chitosan and vitamin C on the AST activity in the liver tissue of common carp exposed to paraquat.

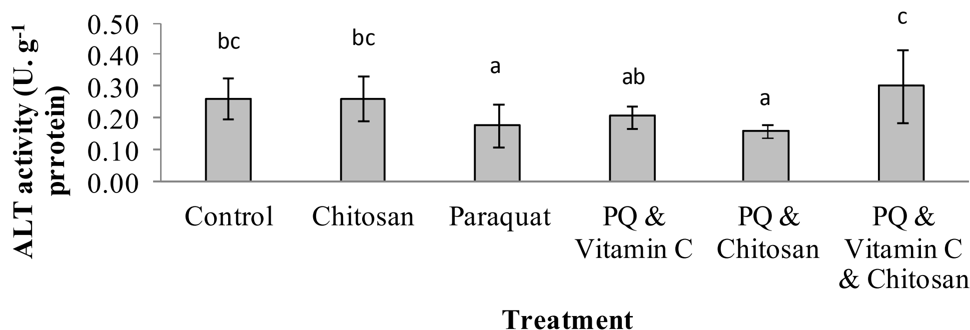

Figure 2. Ameliorative effect of chitosan and vitamin C on the ALT activity in the liver tissue of common carp exposed to paraquat.

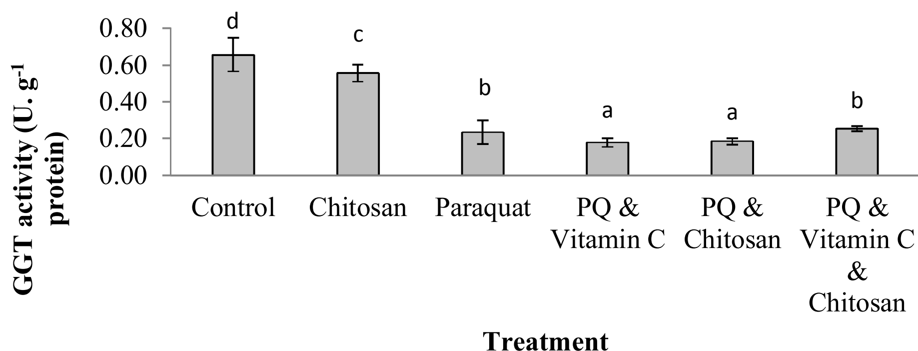

Figure 3. Ameliorative effect of chitosan and vitamin C on the GGT activity in the liver tissue of common carp exposed to paraquat.

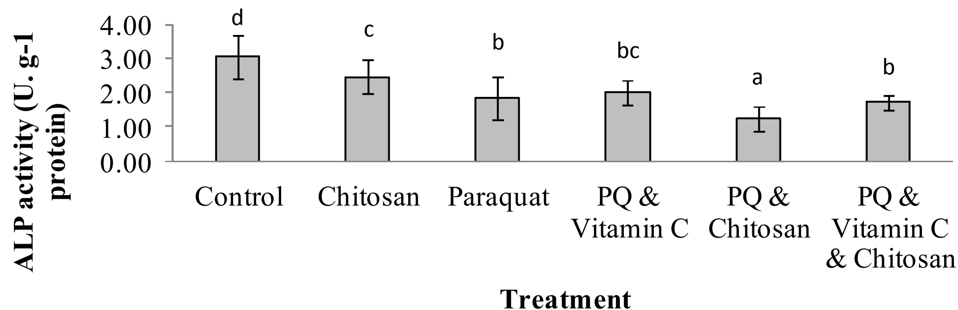

Figure 4. Ameliorative effect of chitosan and vitamin C on the ALP activity in the liver tissue of common carp exposed to paraquat.

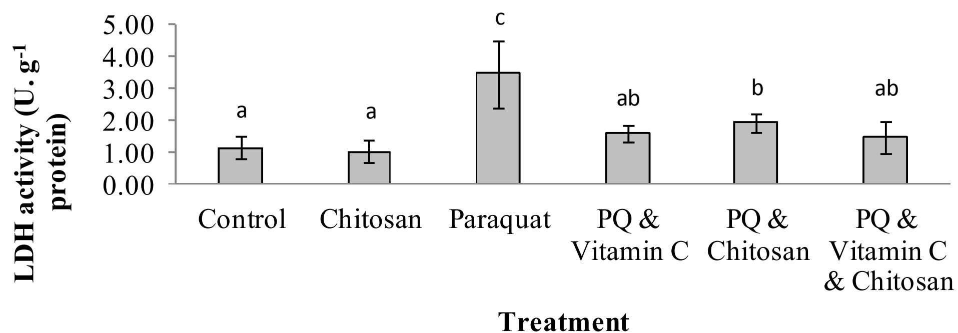

Figure 5. Ameliorative effect of chitosan and vitamin C on the LDH activity in the liver tissue of common carp exposed to paraquat.

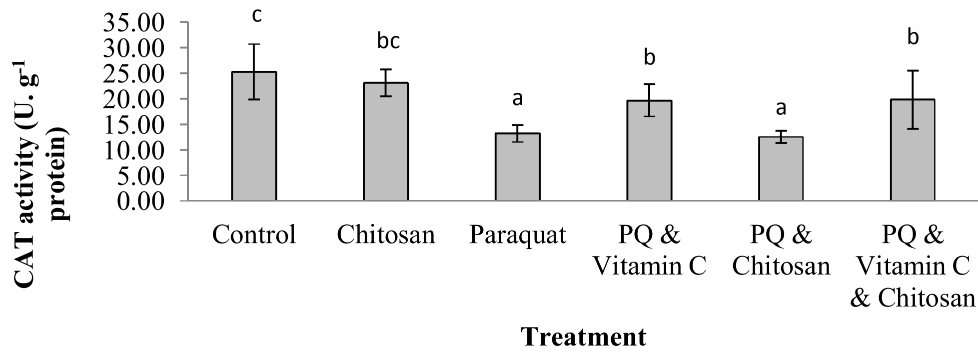

Figure 6. Ameliorative effect of chitosan and vitamin C on the CAT activity in the liver tissue of common carp exposed to paraquat.

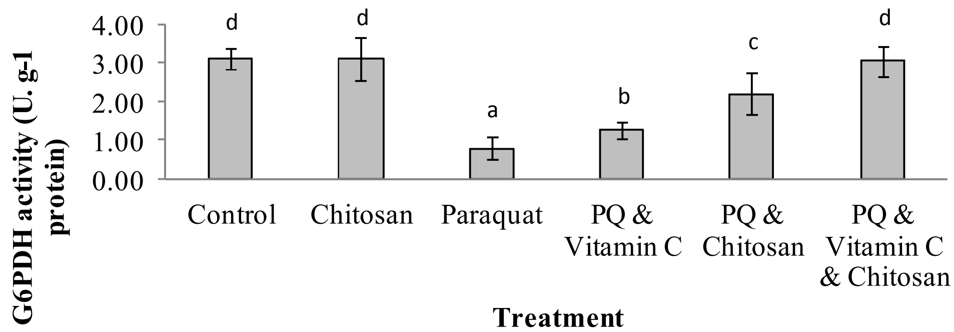

Figure 7. Ameliorative effect of chitosan and vitamin C on the G6PDH activity in the liver tissue of common carp exposed to paraquat.

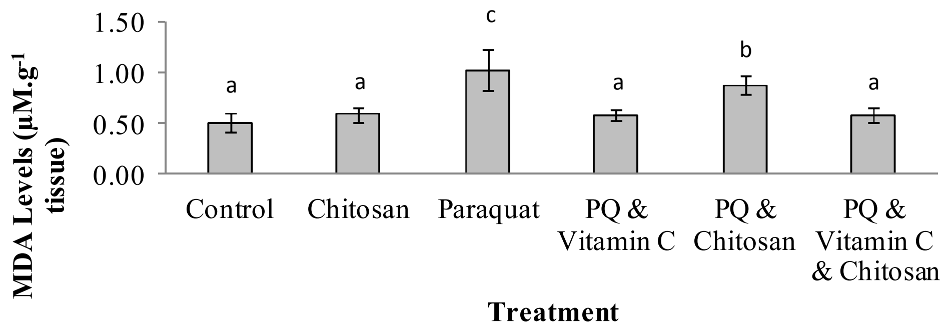

Figure 8. Ameliorative effect of chitosan and vitamin C on the MDA levels in the liver tissue of common carp exposed to paraquat.

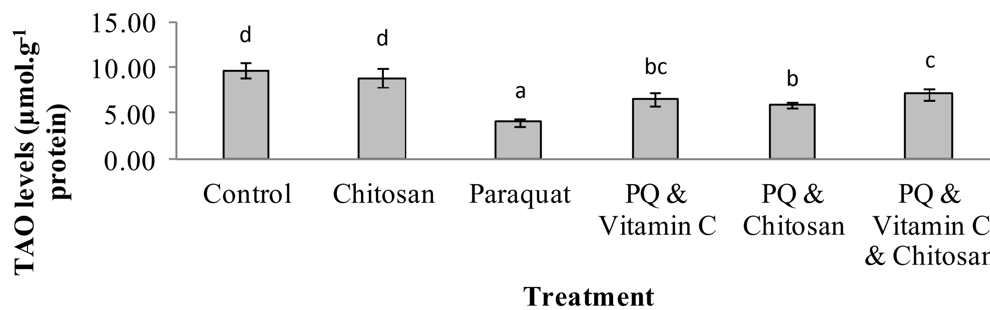

Figure 9. Ameliorative effect of chitosan and vitamin C on the TAO levels in the liver tissue of common carp exposed to paraquat.

Total antioxidant levels reduced significantly in liver of fishes exposed to paraquat with no supplementary feeding. However, ascorbic acid and ascorbic acid combined with chitosan treatment increased total antioxidant levels in liver tissue of paraquat-exposed fishes. Chitosan treatment also improved total antioxidant levels (Figure 9).

DISCUSSION

In the present study, the protective effects of ascorbic acid (vitamin C) and chitosan against paraquat-induced toxicity in the liver of common carp and the resulting hepatic oxidative damage were investigated. Biochemical parameters of the treated fishes were compared to those of untreated controls (Table 1). Mussi and Calcaterra suggested lipid peroxidation as an important parameter of the toxicity of paraquat [21].

Our results indicated a significant decrease in the total antioxidant levels and an increase in MDA levels in the fishes exposed to paraquat as compared to controls ($P<0.05$). Therefore, the increase in MDA levels in the present study could be related to an increase in the generation of ROS in the liver of the fishes exposed to paraquat. An increase in MDA levels was reported in different tissues of fish exposed to fenpropathrin [22], Deltamethrin [23], methyl parathion [24], chlorpyrifos [24], carbamazepine [25], and atrazine [26]. This leads to the loss of cellular membrane integrity resulting in pores in the membranes. Due to cell membrane lipid peroxidation of unsaturated fatty acids, short chain fatty acids containing R-COOH, R-OOH, R-CHO, and R-OH are created which seriously affect the cellular membrane functions such as the activity of hormone receptors and neural mediators, ion transport channels and the activity of membrane enzymes and transportation of specific molecules. On the other hand, the formation of malondialdehyde (MAD) during peroxidation of fatty acids with double bonds can create covalent bonds and polymerize cellular membrane components.

Total antioxidant levels reduced significantly in liver of fish exposed to paraquat with no supplementary feeding. The cellular total antioxidant capacity plays a crucial role in intracellular protection against the reactive oxygen species [27]. The overproduction of reactive oxygen species during pesticide detoxification may be associated with decrease in the hepatic total antioxidant capacity [28, 29]. Impairment in the synthesis of enzymatic and non-enzymatic antioxidants may be the most important factor in reducing levels of cellular total antioxidant capacity. Therefore, decline in the cellular antioxidant capacity makes the cells more vulnerable to oxidative stress damage. Concomitant supplementation with vitamin C and chitosan treatment elevate the level of total antioxidant in hepatocytes. This enhanced cellular total antioxidant capacity seems to be essential in maintaining the redox state and to cope with the oxidative stress in the fish exposed to paraquat.

Significant decrease in the activity of catalase was observed in liver tissue of fish after exposure to paraquat ($P<0.05$). Catalase plays an important role in the elimination of hydrogen peroxide in cells [29]. Catalase acts on H_2O_2 by decomposing it, and protects the cell against hydrogen peroxide mediated lipid peroxidation. Therefore, significant decrease in CAT activity in liver tissue of fish exposed to paraquat might be a biochemical response to overproduction of H_2O_2 in liver cells. Hence, reduced CAT activity in hepatocytes may indicate a defect in the antioxidant defense system. Increased catalase activity in liver tissue of fish fed a diet supplemented with vitamin C or Vitamin C combined with chitosan may reflect the effects of vitamin C on reducing levels of reactive oxygen species. Our result is supported by study of Mehrpak et al. [13], where vitamin C and chitosan was demonstrated to alleviate cadmium-induced oxidative stress in common carp.

Glucose-6-phosphate dehydrogenase (G6PDH) was significantly inhibited by paraquat. Decreased G6PDH led to reduced biosynthesis of nicotinamide-adenine dinucleotide phosphate (NADPH) and cellular reduced glutathione (GSH) level [30]. In addition, NADPH is required for the generation of reduced glutathione, which is important for protective effect against oxidative damage. Therefore, the results of this study show that decreased G6PD activity is sufficient to increase oxidative stress. However, oral administration of chitosan or vitamin C supplementation significantly increased G6PDH activity as

compared to fishes exposed to paraquat. Administrating combination of vitamin C and chitosan had more preventive effect on the decrease of G6PDH caused by paraquat and improved the diminished activity of G6PDH in liver tissue of fishes exposed to paraquat.

Aminotransferase enzymes such as AST, ALT, and GGT are important in cellular nitrogen metabolism, oxidation of amino acids, and liver gluconeogenesis [31]. In stress situations, increased activity of liver enzymes has stimulatory effects on gluconeogenic mechanism [31, 32]. After 21 days, the results showed that paraquat treatment significantly decreased AST, ALT and GGT activities in liver tissue. Thus, alterations in aminotransferase activities may indicate interference with the cellular energy supply for fish exposed to paraquat. Similar changes were observed in *Labeo rohita* and *A. mossulensis* after exposure to fenvalerate and fenpropathrin, respectively [22, 33]. However, oral administration of vitamin C combined with chitosan to fish exposed to paraquat reverses the activity of AST, and ALT to normal levels. Similar findings were reported by Merhpak et al. [13] who observed that vitamin C and chitosan reduces cadmium hepatotoxicity in fish.

The increased LDH activity caused by paraquat could be explained by changes in the cellular metabolism process during the treatment. Paraquat significantly increased LDH activity in the liver of common carp, which indicated serious liver damage. This suggests impairment in the aerobic capacity of the liver tissue. The observed increase of LDH activity can be attributed to the conversion of accumulated pyruvate into lactate, transported through muscles to liver and regenerated glucose and glycogen to supply energy for fish exposed to insecticides. Banaee et al [27] demonstrated the adverse effects of malathion on liver and a resulting increase in LDH, AST and ALT activities. However, LDH activity returned to normal levels in fish fed a diet supplemented with ascorbic acid and ascorbic acid combined with chitosan. The present results agree with those obtained by Mehrpak et al. [13] who revealed that vitamin C and chitosan are capable of almost completely alleviating liver toxicity in fish exposed to cadmium chloride.

ALP plays a significant role in phosphate hydrolysis and in membrane transport and acts as a good bio-indicator of stress in biological systems. The decrease in ALP activity by paraquat probably indicates altered transport of phosphate and inhibitory effect on the cell growth and proliferation. In contrast, increased ALP activity in the whole body of the freshwater fish *Alburnus mossulensis* in exposure to fenpropathrin has been reported [22]. Moreover, co-treatment of vitamin C and chitosan did not induce any significant changes in ALP activity in hepatocyte of fish exposed to paraquat.

CONCLUSION

Paraquat toxicity in liver could be attributed to the oxidative stress on the hepatic cells, which leads to decrease in cellular total antioxidant levels. The increase in MDA and CAT activity is indicative of reactive oxygen species formation in the hepatocytes of fish exposed to paraquat. Administration of ascorbic acid and ascorbic acid combined with chitosan allowed recovering some enzymatic activities, changed by paraquat treatment. Nevertheless, chitosan supplementation was not sufficiently effective in regulating liver enzymes activities in fish exposed to paraquat. Nonetheless, ascorbic acid and ascorbic acid combined with chitosan could be useful in decreasing paraquat toxicity by inhibiting the paraquat-induced oxidative stress.

ACKNOWLEDGMENT

This study was supported by grant from Behbahan Khatam Alanbia University of Technology. In addition, the authors are grateful to Maryam Banaee, our English editor, for proofreading the manuscript. The authors declare that there is no conflict of interests.

REFERENCES

1. Parvez S, Raisuddin S. Effects of paraquat on the freshwater fish *Channa punctata* (Bloch): non-enzymatic antioxidants as biomarkers of exposure. Arch Environ Contam Toxicol 2006; 50: 392–7.
2. Yan Y, Wanshun L, Baoqin H, Bing H, Chenwei F. Protective effects of chitosan oligosaccharide and its derivatives against carbon tetrachloride-induced liver damage in mice. Hepatol Res 2006; 35: 178–84.
3. Li R, Zhou Y, Ji J, Wang L. Oxidative damages by cadmium and the protective effects of low-molecular-weight chitosan in the freshwater crab

(*Sinopotamon yangtsekiense* Bott 1967). Aqua Res 2011;42: 506-15.
4. Yoshizuka K, Lou Z, Inoue K. Silver-complexed chitosan micro-particles for pesticide removal. Reactive & Functional Polymers 2000; 44: 47–54.
5. Jaiswal M, Chauhan D, Sankararamakrishnan N. Copper chitosan nanocomposite: synthesis, characterization, and application in removal of organophosphorous pesticide from agricultural runoff. Environ Sci Pollut Res 2012; 19: 2055–62.
6. Moradi Dehaghi S, Rahmanifar B, Mashinchian Moradi A, Aberoomand Azar P. Removal of permethrin pesticide from water by Chitosan-Zinc oxide nanoparticles composite as an adsorbent. J Saudi Chem Soc 2014; 18(4): 348-55.
7. Tanada S, Kyotani S, Nakamura T, Nishioka Y.In vitro paraquat removal with granular chitosan.Journal of Environmental Science and Health. Part A: Environmental Science and Engin Toxicol 1993; 28(3): 671-82.
8. Bernkop-Schnürch A, Dünnhaupt S. Chitosan-based drug delivery systems. Europ J Pharma Biopharma 2012; 81(3): 463-9.
9. Nallamuthu I, Devi A, Khanum F.Chlorogenic acid loaded chitosan nanoparticles with sustained release property, retained antioxidant activity and enhanced bioavailability. Asian J Pharma Sci 2015; 10: 203-11.
10. Li Y, Schellhorn HE.New Developments and Novel Therapeutic Perspectives for Vitamin C. J Nutr 2007; 137(10): 2171-84.
11. Alishahi A, Mirvaghefi A, Tehrani MR, Farhmand H, Koshio S, Dorkoosh FA.Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (*Oncorhynchus mykiss*). Carbohydrate Polymers 2011; 86: 142-6.
12. Jiménez-Fernández E, Ruyra A, Roher N, Zuasti E, Infante C, Fernández-Díaz C. Nanoparticles as a novel delivery system for vitamin C administration in aquaculture. Aquaculture 2014; 432: 426-33.
13. Mehrpak M, Banaee M, Nematdoost Haghi B, Noori A. Protective effects of vitamin C and chitosan against cadmium-induced oxidative stress in the liver of common carp (*Cyprinus carpio*). Iran J Toxicol 2015;. 9(30): 1360-7.
14. Mobasher M, Aramesh K, Aldavoud SJ, Ashrafganjooei N, Divsalar K, Phillips CJC, Larijani B. Proposing a national ethical framework for animal research in Iran. Iran J Public Health 2008; 37(1):39–46.
15. Moss DV, Henderson AR. Clinical enzymology In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia: W.B. Saunders Company, 1999; p 617-721.
16. Gómez-Milán E, Lozano MJSM.Daily and annual variations of the hepatic glucose 6-phosphate dehydrogenase activity and seasonal changes in the body fats of the gilthead seabream *Sparus aurata*. J Exp Zool Part A: Ecological Genetics and Physiology 2007; 307A: 516–26.
17. Johnson AM, Rohlfs EM, Silverman LM.Proteins. In: Burtis CA, Ashwood ER, editors. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia: W.B. Saunders Company, 1999; p 477-540.
18. Góth L. A simple method for determination of serum catalase and revision of reference range. Clinica Chimica Acta 1991; 196: 143-152.
19. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. Anal Biochem 1996; 239: 70–6.
20. Placer ZA, Cushman L, Johnson BC.Estimation of products of lipid peroxidation (malonyldialdehyde) in biological fluids. Anal Biochem 1966; 16: 359-64.
21. Mussi MA, Calcaterra NB.Paraquat-induced oxidative stress response during amphibian early embryonic development. Compara Biochem Physiol Part C 2010; 151: 240–7.
22. Banaee M, Sureda A, Zohiery F, NematdoustHagi B,Garanzini DS. Alterations in biochemical parameters of the freshwater fish, *Alburnus mossulensi*s, exposed to sub-lethal concentrations of Fenpropathrin. Int J Aqua Biol 2014; 2(2): 58-68.
23. Yonar ME, Sakin F. Ameliorative effect of lycopene on antioxidant status in *Cyprinus carpio* during pyrethroid deltamethrin exposure. Pesticide Biochem Physiol 2011; 99: 226–31.
24. Sharbidre AA, Metkari V, Patode P. Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, *Poecilia reticulate*. Pesticide Biochem Physiol 2011; 101: 132–41.
25. Li ZH, Velisek J, Zlabek V, Grabic R, Machova J, Kolarova J, Randak T. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine. Chem-Biol Interact 2010; 183: 98–104.
26. Paulino MG, Sakuragui MM, Fernandes MN. Effects of atrazine on the gill cells and ionic balance in a neotropical fish, *Prochilodus lineatus*. Chemosphere 2012; 86: 1–7.

27. Banaee M, Sureda A, Shahaf S, Fazilat N. Protective effects of silymarin extract on malthion-induced zebra cichlid (*Cichlasoma nigrofasciatum*) hepatotoxicity. Iran J Toxicol 2015; 9(28): 1239-46.
28. Monteiro DA, de Almeida JA, Rantin FT, Kalinin AL. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). Compara Biochem Physiol Part C 2006; 143: 141–9.
29. Banaee M, Sureda A, Mirvaghefi AR, Ahmadi K. Biochemical and histological changes in the liver tissue of Rainbow trout (*Oncorhynchus mykiss*) exposed to sub-lethal concentrations of diazinon. Fish Physiol Biochem 2013; 39: 489-501.
30. Singh S, Anand A, Srivastava PK. Regulation and properties of glucose-6-phosphate dehydrogenase: A review. Int J Plant Physiol Biochem 2012; 4(1): 1-19.
31. Banaee M. Physiological dysfunction in fish after insecticides exposure: Insecticides often Undesired but still so Important, Edited by Stanislav Trdan, Published by InTech. 2013; Chapter 4, p 103-42.
32. Banaee M. Adverse effect of insecticides on various aspects of fish's biology and physiology: Insecticides – Basic and Other Applications Book, Edited by Sonia Soloneski and Marcelo Larramendy, Published by InTech. 2012; Chapter 6, p 101-26 .
33. Prusty A.K, Kohli MPS, Sahu NP, Pal AK, Saharan N, Mohapatra S, Gupta SK (2011) Effect of short term exposure of fenvalerate on biochemical and haematological responses in *Labeo rohita* (Hamilton) fingerlings. Pesticide Biochem Physiol 100: 124-9.