

Original Article**Protective Effects of Vitamin C and Chitosan against Cadmium-Induced Oxidative Stress in the Liver of Common Carp (*Cyprinus carpio*)**Milad Mehrpak¹, Mahdi Banaee*¹, Behzad Nematdoost Haghi¹, Ahmad Noori²

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ABSTRACT

Background: Cadmium (Cd) intoxication can cause oxidative stress and involve the antioxidant defense system in hepatocytes. Administration of vitamin C, chitosan, or a combination of both may prevent Cd-induced oxidative damage.

Methods: *Cyprinus carpio* were distributed into six groups. The control group received normal feed (Group I). Group II was exposed to 0.2 mg.L⁻¹ cadmium chloride. Group III were fed 1000 mg chitosan per 1 kg feed. Group IV was exposed to 0.2 mg.L⁻¹ cadmium chloride and fed with 1000 mg chitosan per 1 kg feed while Group V was exposed to 0.2 mg.L⁻¹ cadmium chloride and was fed with 1000 mg vitamin C per 1 kg feed. Group VI, however, was exposed to 0.2 mg.L⁻¹ cadmium chloride and was fed with 1000 mg vitamin C combined with 1000 mg chitosan per 1 kg feed. After 21 days of experiment, activities of hepatic enzymes and oxidative stress biomarkers were evaluated.

Results: Exposure to CdCl₂ caused a significant increase in malondialdehyde levels and altered alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase activities in liver tissues. Hepatic antioxidant capacity, catalase, and Glucose-6-phosphate dehydrogenase activities significantly decreased in fishes exposed to CdCl₂ as compared to the control group. Treatment with vitamin C and vitamin C combined with chitosan significantly ameliorated these changes.

Conclusion: The present findings suggest that the administration of chitosan failed to restore biochemical parameters to normal levels. However, these findings demonstrate that vitamin C and vitamin C combined with chitosan protect the fish against the toxic effects of CdCl₂ on the examined biochemical parameters in liver tissues.

Keywords: Cadmium, Chitosan, Hepatoprotective, Oxidative Stress, Vitamin C.

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INTRODUCTION

Previous studies have shown that oxidative stress [1], reproductive disorders [2], changes in blood biochemical parameters [3], immunosuppression [4], osmotic regulation disorder [5], genetic disorders [6], histopathological damage in different tissues [7] and bioaccumulation of cadmium [8] may occur in fish exposed to waterborne cadmium.

In recent years, scientists have made great efforts to enhance detoxification systems and boost cellular antioxidant systems against xenobiotic-induced oxidative stress [8-10].

Vitamin C is an important vitamin for fish and plays an important role in non-enzymatic antioxidant systems. This vitamin is also known as a valuable free radical scavenger in biological

systems. It can reduce the toxic effects of environmental pollutants on animals [11, 12]. Therefore, increasing the bioavailability of vitamin C may reduce the effects of environmental toxins on fish. The problem, however, lies in the fact that this vitamin is very sensitive to light, temperature, humidity, and pH. Furthermore, vitamin C may quickly be destroyed during preparation or storage of feed. Nevertheless, it may be possible to increase the bioavailability and half-life of vitamin C in feed through using a drug carrier. Vitamin C (L-ascorbic acid) has a simple biochemical structure and small molecular weight despite its high density of negative charges due to the presence of acid and carbonyl groups. Hence, it is combined well with chitosan.

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

Chitosan can be used as a drug carrier [13, 14] in increasing the bioavailability of vitamin C in fish [15, 16]. Its use as a hepatoprotective agent to reduce the toxicity of various chemical compounds in experimental animals has been tested successfully [17]. Containing a hydroxyl group and an amine, chitosan is also used as an adsorbent in wastewater treatment to remove heavy metals and other chemicals [18, 19].

Chitosan may be used alone as an adsorbent, a free radical scavenger, or in combination with vitamin C as a drug carrier to reduce the bioavailability of cadmium or neutralize the reactive oxygen species produced in the detoxification process of cadmium compounds and reduce its toxic effects. Therefore, the present study was performed to evaluate the ameliorative effects of chitosan, vitamin C, or their combination in reducing the toxicity of cadmium chloride in common carp.

MATERIALS AND METHODS

Chemical Materials

Low weight chitosan (80% deacetylated) was purchased from Aldrich Chemical Company Inc. (USA). All other chemicals were provided from Merck Chemical Company (Germany). Ascorbic acid (vitamin C) was purchased from Rooyan Darou Company (Iran).

Fish Treatment

One hundred and eighty juvenile common carp, *Cyprinus carpio*, weighing 37.65 ± 4.40 g were used in the present study according to the National Ethical Framework for Animal Research in Iran [20]. They were randomly distributed to 18 plastic tanks (80 liter) and acclimatized in aerated freshwater ($24 \pm 2^\circ$ C; pH, 7.4 ± 0.2 ; 16 L/8D; 40% water exchange rate/day) for two weeks before the experiment. During the acclimatization period, the fish were fed two times per day with commercial diet from Beyza Feed Mill, Shiraz, Iran.

The fish were randomly assigned to six groups. In Group I (control), the specimens were fed with a normal diet for 21 days. In Group II, the specimens were exposed to 0.2 mg.L^{-1} cadmium chloride. In Group III, the specimens were fed with a diet enriched with 1000 mg chitosan per 1 kg feed for 21 days. In Group IV, the specimens were exposed to 0.2 mg.L^{-1} cadmium chloride and were fed with a diet enriched with 1000 mg chitosan per 1 kg feed for 15 days. In

Group V, the specimens were exposed to 0.2 mg.L^{-1} cadmium chloride and were fed with a diet enriched with 100 mg vitamin C per 1 kg feed for 21 days. In Group VI, the specimens were exposed to 0.2 mg.L^{-1} cadmium chloride and were fed with 100 mg vitamin C combined with 1000 mg chitosan per 1 kg feed for 21 days, respectively.

The water was exchanged daily to reduce the buildup of metabolic wastes and keep cadmium chloride concentrations near the nominal level.

At the end of the experiment, 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 minutes (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 using spectrophotometric assays.

Biochemical Analysis

Creatine kinase reacts with creatine phosphate and ADP to form ATP which is coupled to the hexokinase-G6PD reaction, generating NADPH. In this assay, creatine kinase activity is determined by a coupled enzyme reaction resulting in the production of NADPH, measured at 340 nm, proportionate to the CK activity present in the sample. 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 measured in a coupled reaction with malate dehydrogenase to reduce it with NADH to form malate. In alanine aminotransferase (ALT) assay, alanine and α -ketoglutarate are first converted by the ALT enzyme to glutamate and pyruvate. Pyruvate is converted by lactate dehydrogenase to lactate and NAD^+ . All these activities were monitored by measuring changes in absorbance at 340 nm. Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm [21]. During the 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 the G6PDH activity and it is expressed spectrophotometrically as an increase in absorbance at 340 nm. Levels of protein in the tissues were determined by standard procedures used in clinical biochemistry laboratories based on the manufacturer's instructions of biochemical kits (Pars Azemon Co, Iran) [22]. CAT activity was determined according to Góth method [23], with some modifications. Catalase activity was measured as the rate of decomposition of hydrogen peroxidase based on the formation of its stable complex with ammonium molybdate. 200 µL of the supernatant was incubated in the working solution containing 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.

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

Blank 1 contained 1.0 mL substrate, 1.0 mL molybdate, and 0.2 mL supernatant; 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.

The total antioxidant capacity was determined using the ferric reducing ability of plasma (FRAP) method. 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 FeCl₃(20 mmol/L) and 50 mL of acetate buffer (0.3 mol/L, pH=3.6) which was prepared freshly. A total of 100 µL aliquots of the supernatant was mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-triazine (Fe³⁺-TPTZ) complex to ferrous tripyridyl-s-triazine (Fe²⁺-TPTZ) at pH 3.6 and 25 °C is directly proportional to the concentration of the total antioxidant in the sample. Fe²⁺-TPTZ has an intensive blue color that can be monitored for up to five minutes at 593 nm by a UV/VIS spectrophotometer. The calculations were performed using a calibration curve of FeSO₄·7H₂O (100 to 1000 µM/L) [24].

Malondialdehyde (MDA) content was determined using modified thiobarbituric acid assay expressed as µmol/g tissue [25]. 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 minutes. The tubes were, then, cooled to room temperature and the solution was mixed with a solution containing 1000 µL of distilled water and 5000 µL *n*-butanol: pyridine (15: 1). The mixture was then centrifuged at 2000 g for 15 minutes 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 a standard of MDA. Tetraethoxypropane and absolute ethanol were used to prepare MDA standards. Concentrations of MDA in whole body samples are expressed in µM/gprotein. All biochemical parameters were measured by UV/VIS spectrophotometer (model UNICCO 2100).

Statistical Analysis

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

RESULTS

During the experiment, no deaths were observed in any of the groups. Changes in biochemical parameters of hepatocytes are presented in Figure 1-9. Since drug metabolism and xenobiotic detoxification are done in liver tissue, ameliorative effects of chitosan and vitamin C on biochemical parameters of fish exposed to cadmium chloride were more prominent in hepatocytes than in other tissues.

Although administration of chitosan (as positive control) reduced LDH activity in hepatocytes of fish, no significant changes were observed in other biochemical parameters between control and positive control groups.

A significant decrease was observed in AST, CAT, and G6PDH activities in hepatocytes of fish exposed to cadmium chloride. However, administration of chitosan alone could not account for the enzymes' return to normal levels in fish exposed to cadmium chloride. AST, CAT, and G6PDH activities remained at the normal level in fish fed with a diet enriched with a com-

bination of chitosan and vitamin C. Compared to the control group, no significant changes were observed in CAT and G6PDH activities in hepatocytes of fish treated with vitamin C and cadmium chloride.

ALT activity in hepatocyte of fish exposed to cadmium chloride was significantly higher than that of the control group. Although ALT activity in fish fed on a diet enriched with vitamin C was similar to that of the control group, administration of chitosan in fish exposed to cadmium chloride did not affect the regulation of ALT.

Our results indicate that exposure to cadmium chloride led to a significant increase in ALT and GGT activity in fish. However, compared to the control, other groups indicated no significant changes in these enzymes.

A significant decrease was observed in LDH activity in hepatocytes of fish exposed to cadmium chloride. The results show that admin-

istration of chitosan did not have ameliorative effects on LDH activity in hepatocytes of fish exposed to cadmium chloride. Although administration of vitamin C alone or vitamin C combined with chitosan increased LDH activity in hepatocytes of fish exposed to cadmium chloride, LDH activity in hepatocytes of the treated fish was significantly lower than that of the control group.

The results indicated that exposure to cadmium chloride caused a significant decrease in total antioxidant levels (TAO) in fish. Levels of TAO significantly increased in the presence of vitamin C alone or vitamin C combined with chitosan in diet of fish exposed to cadmium chloride.

Levels of malondialdehyde (MDA) have been shown to indicate endogenous lipid peroxidation. The present results indicated that cadmium chloride increased levels of lipid peroxidation in the liver of the fish.

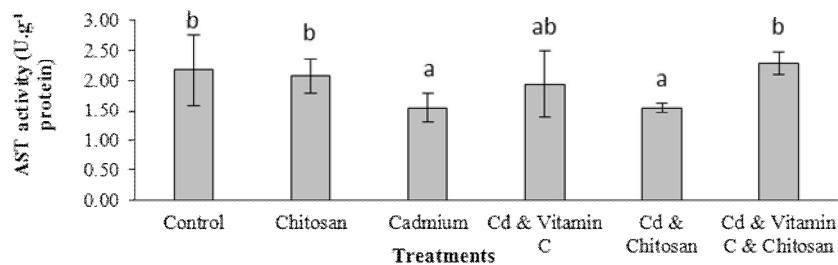


Figure 1. Hepatoprotective effects of vitamin C and chitosan on AST activity in the liver of fish.

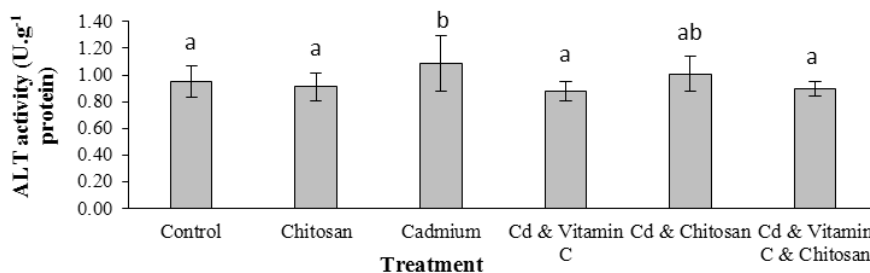


Figure 2. Hepatoprotective effects of vitamin C and chitosan on ALT activity in the liver of fish.

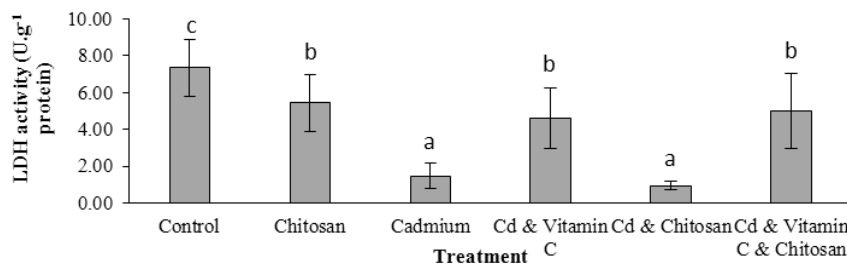


Figure 3. Hepatoprotective effect of vitamin C and chitosan on LDH activity in the liver of fish.

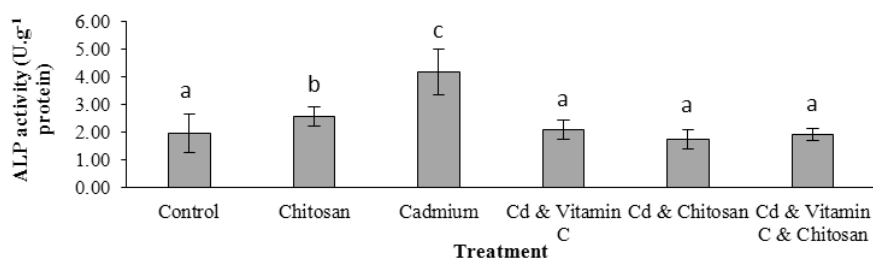


Figure 4. Hepatoprotective effects of vitamin C and chitosan on ALP activity in the liver of fish.

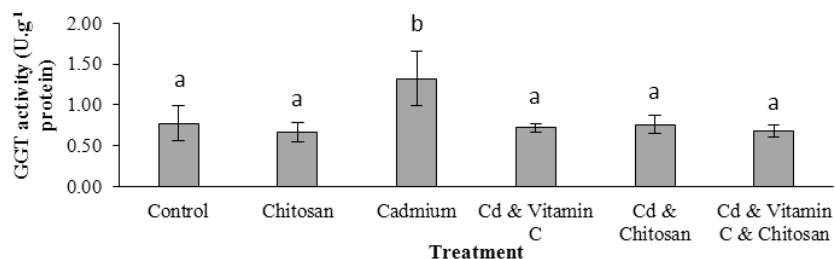


Figure 5. Hepatoprotective effects of vitamin C and chitosan on GGT activity in the liver of fish.

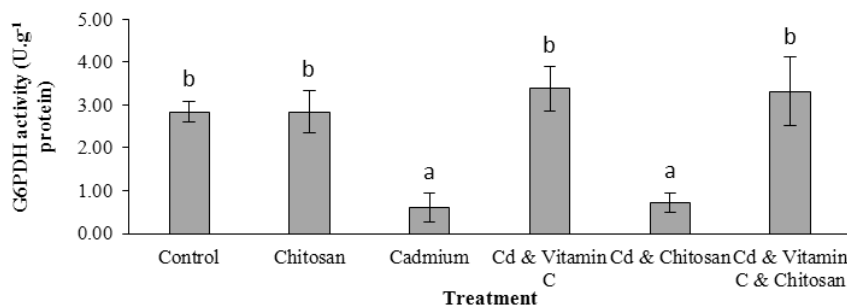


Figure 6. Hepatoprotective effects of vitamin C and chitosan on G6PDH activity in the liver of fish.

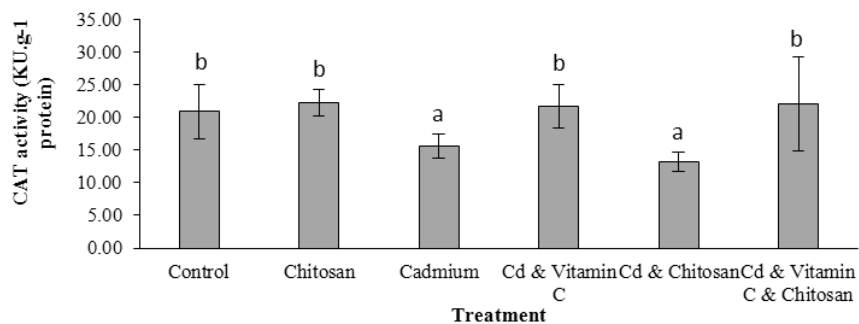


Figure 7. Hepatoprotective effects of vitamin C and chitosan on CAT activity in the liver of fish.

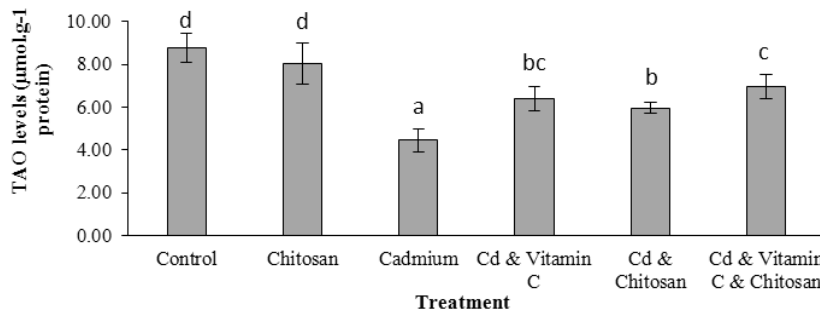


Figure 8. Hepatoprotective effects of vitamin C and chitosan on the total antioxidant levels in the liver of fish.

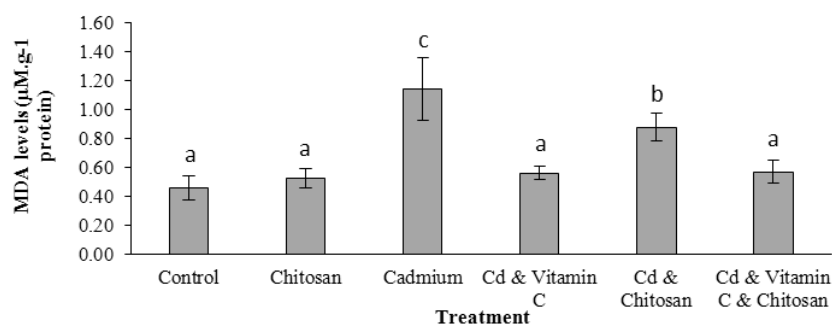


Figure 9. Hepatoprotective effects of vitamin C and chitosan on malondialdehyde levels in the liver of fish.

DISCUSSION

Previous studies have shown that lipid peroxidation of cellular membrane is the main cause of damage of the liver and other organs following Cd exposure. Cadmium is known to enhance the production of reactive oxygen species (ROS), alter the activity of antioxidant enzymes, and decrease the levels of non-enzymatic antioxidants in biological systems [26].

The fish exposed to Cd displayed a tendency toward enhanced oxidative stress and lipid peroxidation, especially when the antioxidant level in the diet was not enough to neutralize free radicals. The present results have clearly demonstrated the ability of Cd to induce oxidative stress in fish liver as evidenced by increased Malondialdehyde. In the present study, elevation in MDA with reduced levels of total antioxidants in liver of Cd-treated fish might be attributed to the overproduction of free radicals and lipid peroxidation end-products, which lead to oxidative stress. Similar findings have also been reported in other fishes, such as Japanese flounder, *Paralichthys olivaceus* [27], catfish, *Clarias gariepinus* [28], and beluga, *Huso huso* [26], which experienced increased MDA levels after Cd exposure. The administration of vitamin C or vitamin C combined with chitosan restored MDA levels, which reveals that vitamin C preserves the integrity of cellular membrane and the normal physiological functions of hepatocytes.

Total antioxidants, including enzyme and non-enzyme antioxidants, play an important role in scavenging cellular reactive oxygen species. According to the present findings, total antioxidant capacity in hepatocytes significantly decreased in Cd-treated fish. Administration of vitamin C or vitamin C combined with chitosan

significantly increased the levels of total antioxidants with a decrease in ROS formation in Cd-treated fish, which could be due to attenuation of MDA by the free radical scavenging activity of vitamin C. The administration of chitosan alone did not notably prevent Cd-induced alterations in levels of total antioxidant and malondialdehyde in the experimental groups. CAT breaks down hydrogen peroxide into oxygen and water to prevent oxidative stress and maintain cell homeostasis. In the present study, CAT activity was significantly inhibited in fish exposed to CdCl₂. In general, decreased CAT activity is related to the binding of Cd to -SH groups of the enzyme, which increased H₂O₂ or superoxide radical [29]. The results of the present study were in agreement with previous reports by Bindhumol *et al.* [30] and Banudevi *et al.* [31]. Administration of vitamin C or vitamin C combined with chitosan protected this enzyme from inhibitory effects of cadmium, which may be due to vitamin C's capacity to react with hydrogen peroxide and oxygen free radicals.

Glucose-6-phosphate dehydrogenase (G6PDH), the first and the rate-limiting enzyme of the pentose phosphate pathway, is essential for maintenance of the cytosolic pool of NADPH and thus the cellular redox balances [32]. Therefore, G6PDH plays a crucial role in the regulation of oxidative stress by regulating the synthesis of NADPH. Our results show that sub-lethal exposure of CdCl₂ caused a significant inhibition in G6PDH activity. Man and Woo [33] reported that there was a significant relationship between Cd-induced oxidative stress and the alteration of G6PDH activity in hepatocytes of fish. This might account for decreased cellular antioxidant capacity and increased fragility of cell membrane upon treatment with Cd. Treat-

ment with vitamin C and vitamin C combined with chitosan showed an increase in G6PDH activity.

In the present study, ALT, GGT, and ALP activities were significantly elevated, whereas AST and LDH activities decreased in hepatocytes of fish exposed to Cd. The increase in ALT, GGT, and ALP activities might be a metabolic mechanism to provide energy for cells against the toxicity of Cd, while the decreased activity of AST and LDH occurs either via the direct effect of Cd on the enzymes or inhibition of their biosynthesis. The administration of vitamin C or vitamin combined with chitosan regulated the activities of AST, ALT, GGT, ALP, CAT, and G6PDH in liver tissues of fish exposed to CdCl₂. This indicates the preservation of hepatocyte from Cd-induced toxic effects. Vitamin C may act as an electron donor cell and neutralize free radicals. Although chitosan has a scavenging effect [34], the present findings suggest that chitosan alone could not reduce the incidence of tissue damage.

CONCLUSION

In conclusion, the findings of the present study clearly indicate that cadmium brings about biochemical changes in the hepatocytes of fish while vitamin C has a partial protective role in Cd-induced hepatic damage. Administration of chitosan was only effective when combined with Vitamin C. In other words, serving as a carrier, chitosan may increase the bioavailability of vitamin C. Hence, it may be useful to use vitamin C combined with chitosan as a supplement to minimize the toxic side-effects of cadmium.

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