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Original Article

Amelioration of cadmium-induced changes in biochemical parameters of the muscle of Common Carp (*Cyprinus carpio*) by Vitamin C and Chitosan

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Abstract: The aim of this study was to investigate the effects of administering antioxidants, including vitamin C and chitosan on oxidative stress markers in muscle as edible tissues of *Cyprinus carpio* exposed to cadmium chloride. In this experiment, by exposing to 0.2 mg/L cadmium chloride for 21 days, fish were fed a normal diet, diet containing chitosan (1000 mg/kg diet), 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. Fish exposure to cadmium chloride significantly increased AST, LDH, CPK, catalase, and MDA activity, while it significantly decreased AST and AChE activity, and levels of total antioxidant in muscle cells. Administration of chitosan or vitamin C alone or in combination with each other to fish exposed to cadmium chloride was effective in regulating ALT, CPK, and catalase activity. Although administration of vitamin C and chitosan caused a significant decrease in MDA, AST and LDH, these enzymes were still significantly higher than those in the control group. Administration of vitamin C and chitosan had no significant effects on the activity of AChE and levels of total antioxidant. Although, chitosan alone could not prevent oxidative stress damages in muscle tissues of cadmium-treated fish, administration of vitamin C combined with chitosan may increase the efficiency of antioxidant defense system and improve the detoxification system in the muscles of fish exposed to cadmium chloride.

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Introduction

With increasing pollution of aquatic ecosystems with heavy metals such as cadmium, fish exposure to these compounds and their consequent biological effects on the health of species inhabiting polluted waters are inevitable (Choi et al., 2013; Banaee et al., 2015a). When cadmium exists in water, it may enter the fish body through the gills, skin or the digestive system and then distribute in varied tissues (Choi et al., 2013; Banaee et al., 2015a). Cadmium can affect the physiological activity of cells in different ways. It can disrupt the metabolic processes in cell mitochondria, disrupt the electron transport chain, and cause lipid peroxidation of cell membranes and affect the permeability of cell membrane, inhibit oxidative phosphorylation and protein synthesis, and disturb ion transfer (Wang et al., 2004; Gonzalez et al., 2006). Cadmium binds to thiol groups of proteins, causes oxidant-antioxidant imbalance and destroys cell membrane proteins. Therefore, the first stage in the oxidative stress caused by cadmium toxicity can be attributed to cadmium binding to glutathione sulfhydryl and oxidation of cell membrane proteins. Reactive oxygen species produced during fish exposure to cadmium can be omitted by cellular antioxidant defense system. Cadmium is known as an exacerbations Sarcopenia disease, the degenerative loss of muscle mass, in

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humans (Papa et al., 2014). Changes in biochemical markers of smooth muscle cells in vitro condition indicates the effect of cadmium on cell homeostasis (Kaji et al., 1994). Mould and Dulhunty, (2000) observed that disorder in muscle contraction can be caused by changes in electrical voltage in the membranes of cells after exposure to cadmium.

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 the consumers' food safety. Natural and synthetic antioxidants such as vitamins (Ozturk et al., 2009; Ibrahim and Banaee, 2014; Banaee et al., 2015b) and antioxidants like chitosan with unique biological properties (e.g. drug delivery systems) (Sun et al., 2011; Yoon et al., 2011; Alishahi et al., 2011a, b), can be effective in this regard (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. Therefore, vitamin C is really effective in protecting protein thiol groups against oxidation (Naziroğlu et al., 2010). Vitamin C (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, immunostimulant and many other biological 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, b), antibiotics (Kavaz et al., 2010) and vitamins (Alishahi et al., 2011a, b). Recently, several researchers have become interested in the antioxidant activity of chitosan (Santhosh et al., 2007; Sun et al., 2011; Yoon et al., 2011).

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/100gr)	<45

TVN: Total volatile nitrogen

Regarding chitosan properties in vitamin transfer in biological systems and the antioxidant properties of vitamin C and chitosan, we hypothesize that administration of vitamin C and/or chitosan may reduce the toxic effects of cadmium and reinforce antioxidant defense system in skeletal muscle of fish which were exposed to cadmium chloride. Therefore, this study aimed to investigate the protective effects of vitamin C and chitosan against the oxidative stress in skeletal cells in common carp exposed to cadmium. 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 activity of intracellular enzymes in skeletal muscles were measured.

Materials and Methods

Chmical materials: Low weight chitosan (80% deacetylated) were purchased from Aldrich Chemical Company Inc. (USA). All other chemical materials were obtained from Merk Chemical Company (Germany). Ascorbic acid (vitamin C) was purchased from Rooyan Daroe Company (Iran).

Fish treatment: A total of 180 juvenile common carp, *Cyprinus carpio*, weighting 37.65±4.40 g were obtained from a private fish farm in Behbahan, Khuzestan Province, Iran (December 2014) and used according to National Ethical Framework for Animal Research in Iran (Mobasher et al., 2008). Specimens were randomly introduced into 18 plastic tanks (80 liter) and acclimatized in aerated freshwater (24±2°C, pH=7.4±0.2, 16L/8D, and 40% water

exchange rate/day) for two weeks before experiment. During the acclimatization period, fish were fed twice a day with commercial diet from Beyza Feed Mill, Shiraz, Iran (Table 1).

Fish were randomly assigned to six groups, including specimens (I) fed with a normal diet for 21 days considering as control group, (II) exposed to 0.2 $mg.L^{-1}$ cadmium chloride, (III) fed a diet enriched with 1000 mg chitosan per 1 kg feed for 21 days, (IV) exposed to 0.2 $mg.L^{-1}$ cadmium chloride and were fed a diet enriched with 1000 mg chitosan per 1 kg feed for 15 days, (V) exposed to 0.2 $mg.L^{-1}$ cadmium chloride and were fed a diet enriched with 100 mg vitamin C per 1 kg feed for 21 days, and (VI) exposed to 0.2 $mg.L^{-1}$ cadmium chloride and were fed 100 mg vitamin C combined with 1000 mg chitosan per 1 kg feed for 21 days.

The water was exchanged daily to reduce the buildup of metabolic wastes and to keep concentrations of cadmium chloride near the nominal level. At the end of the experiment, specimens 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: During activity measurement, creatine phosphokinase (CPK) reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH. 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 (Moss and Henderson, 1999). Acetylcholinsetrase (AChE) activity was determined by adding an adequate volume of sample into a cuvette containing 0.1 M phosphate pH 8.0, and acetylcholine iodide (0.015 M) and dithiobis nitrobenzoic acid (0.01 M) as substrates. AChE activity was recorded during 180 s at 405 nm (Knedel and Boetteger, 1967).

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

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

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) that was prepared freshly. 100 µL aliquots of the supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-

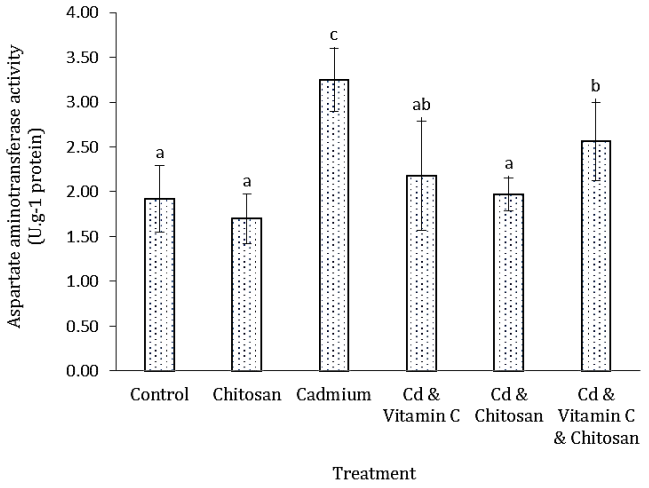

Figure 1. Protective effect of vitamin C and chitosan on the muscle AST activity of fish.

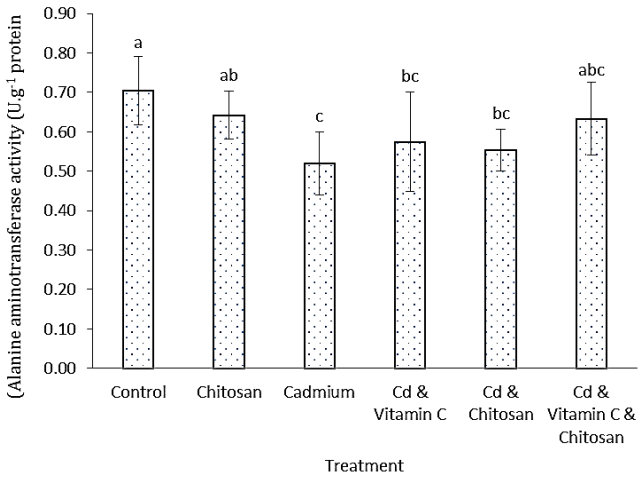

Figure 2. Protective effect of vitamin C and chitosan on the muscle ALT activity of fish.

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 $FeSO_4 \bullet 7H_2O$ (100 to 1000 µM/L) (Benzie and 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 was transferred to a Pyrex tube and mixed with 2500 µl trichloroacetic acid (20%) and 1000 µmL thiobarbituric acid (67%). Then, the tubes were 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 (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

Changes in biochemical parameters of muscle cells are presented in Figures 1-6. During the experiment, no mortality was observed in different groups of the experiment. The activity of aspartate aminotransferase (AST) in muscle cells of fish exposed to cadmium chloride was significantly more than that of the control group. The administration of chitosan or vitamin C alone had a significant effect to reduce the activity of AST and restoring it to the normal level. Administering both chitosan and vitamin C to the fish exposed to cadmium chloride had no significant effects on restoring this enzyme to the normal levels (Fig. 1).

No significant difference was found in the activity of alanine aminotransferase (ALT) in muscles of the control or chitosan-treated group. However, fish exposure to cadmium chloride caused a significant decrease in the activity of ALT. The results indicate that combined administration of chitosan and

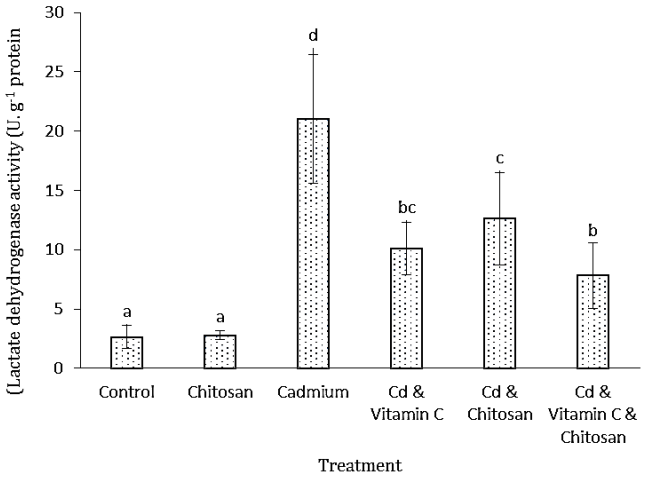

Figure 3. Protective effect of vitamin C and chitosan on the muscle LDH activity of fish.

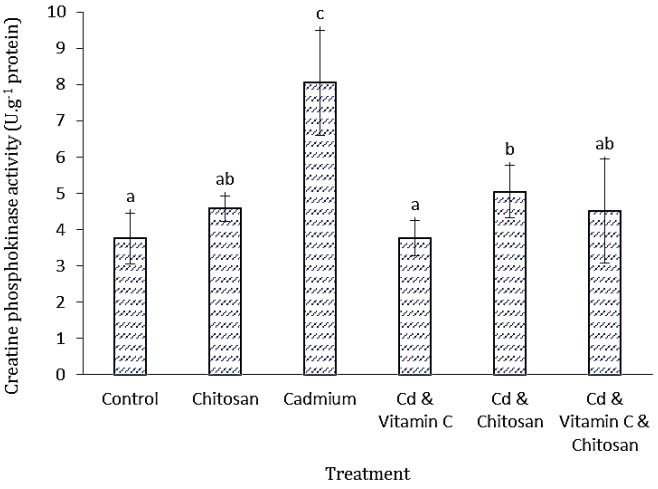

Figure 4. Protective effect of vitamin C and chitosan on the muscle CPK activity of fish.

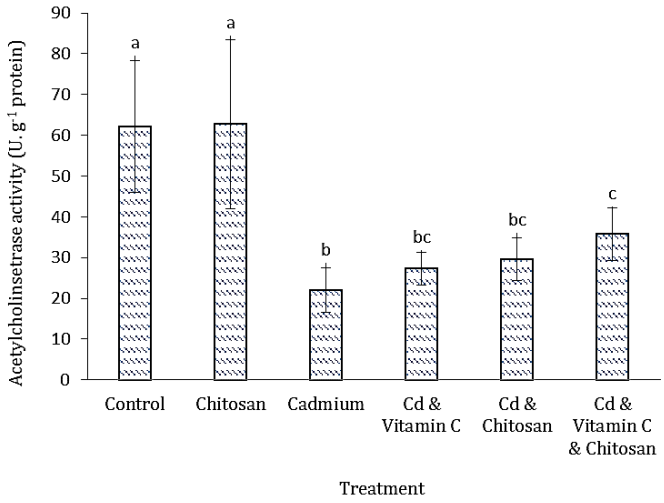

Figure 5. Protective effect of vitamin C and chitosan on the muscle AChE activity of fish.

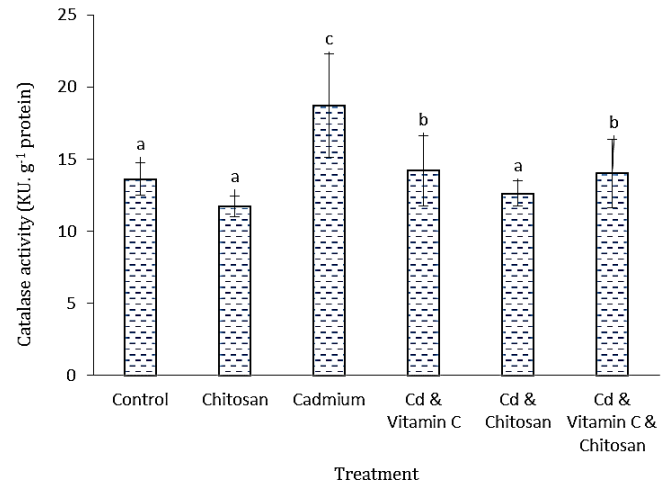

Figure 6. Protective effect of vitamin C and chitosan on the muscle CAT activity of fish.

vitamin C to the fish treated with cadmium chloride restored the activity of ALT (Fig. 2).

Common carp exposure to cadmium chloride increased lactate dehydrogenase (LDH) activity in muscle cells, though the administration of vitamin C and/or chitosan to cadmium chloride-exposed fish regulated the enzyme's activity. Yet, LDH activity in treated fish was significantly higher than that of the control group (Fig. 3).

The increased activity of creatine phosphokinase (CPK) was observed in muscle cells of fish exposed to cadmium chloride. The administration of vitamin C and/or chitosan reduced the activity of this enzyme (Fig. 4).

The results showed that acetylcholinesterase (AChE) activity in muscles of exposed fish to cadmium chloride was significantly lower than that of the control group. Vitamin C and/or chitosan administration had no effects on the activity of this enzyme (Fig. 5).

Compared with the control group, a significant elevation was found in catalase activity in fish which were exposed to cadmium chloride. This is while the activity of this enzyme reduced after the administration chitosan and/or vitamin C. However, catalase activity returned to normal only in edible tissues of fish which were treated with chitosan and cadmium chloride (Fig. 6).

We found that chitosan administration alone had no significant effects on the cellular total antioxidant

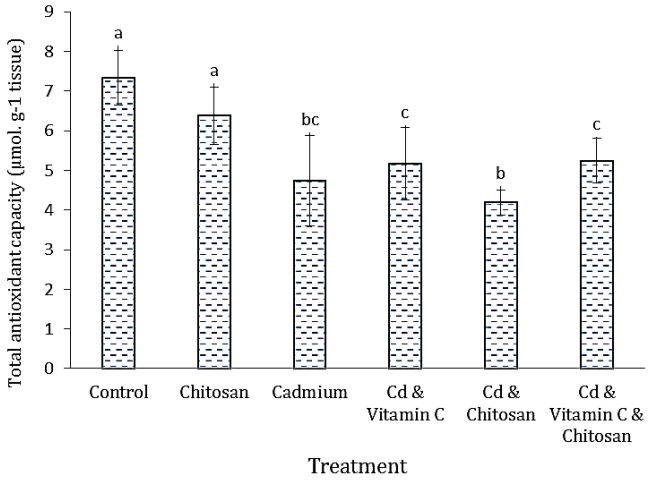

Figure 7. Protective effect of vitamin C and chitosan on the muscle total antioxidant capacity of fish.

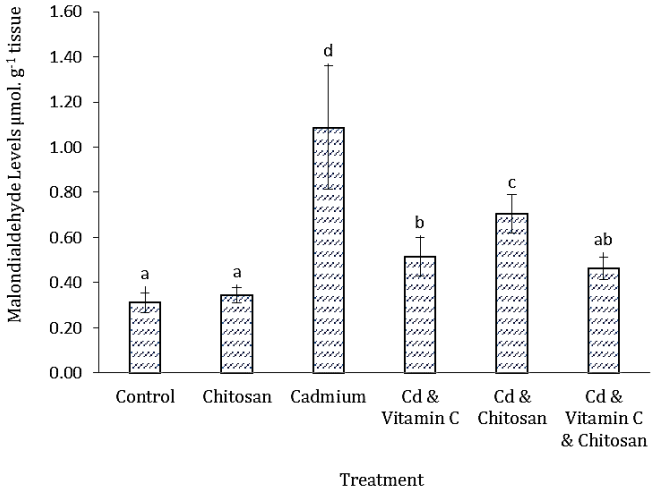

Figure 8. Protective effect of vitamin C and chitosan on the muscle MDA levels of fish.

capacity in muscle of fish compared to the control group. Fish exposure to cadmium chloride is caused a significant decrease in total antioxidant level of muscle cells. Also, administration of chitosan and/or vitamin C to the fish exposed to cadmium chloride had no significant effects on increasing total antioxidant level of muscle cells and their return to normal levels (Fig. 7).

A significant increase in malondialdehyde (MDA) was observed in muscle tissues of fish which were exposed to cadmium chloride, compared to the control group. According to the results, the combined administration of chitosan and vitamin C reduced MDA in exposed fish and returned the enzyme to the normal level. Although administration of chitosan or vitamin C alone caused a significant reduction in MDA in muscle tissues compared with that in fish which were just treated with cadmium chloride, MDA level was still high in the former groups compared to that of the control group (Fig. 8).

Discussion

Cadmium is one of the non-biological trace elements with very active chemical properties. Pervious study indicate that one possible molecular mechanism involved cadmium toxicity is the disruption of delicate oxidant/antioxidant balance, which can cause histopathological alternations via oxidative damage (Wang et al., 2004; Gonzalez et al., 2006). The findings of this study show that fish exposure to cadmium chloride increases free radicals significantly (Zahedi et al., 2013). Therefore, the significant elevation in catalase activity in these fish may be a response to the increased hydrogen peroxide radicals. A significant increase in MDA as well as the decrease in total antioxidant levels were observed in the present study that may be related to high production of reactive oxygen species during the detoxification of cadmium chloride. Due to the presence of long-chain polyunsaturated fatty acids, phospholipids, glycolipids, glycerides and sterols in the biochemical structure of cell membrane, cells are too sensitive to lipid peroxidation. Therefore, damage to the cell membrane can affect the activity of intracellular enzymes. Pervious authors demonstrated that cadmium can increase lipid peroxidation and cause oxidative stress (Defo et al., 2015), inducing the generation of free radicals (Jin et al., 2015). Reduced levels of catalase may signify the influence of vitamin C and chitosan administration in reducing hydrogen peroxide. However, administration of vitamin C and chitosan had no significant effects on total antioxidant capacity of cells or preventing lipid peroxidation of muscles cell membrane in fish exposed to cadmium chloride.

The increased activity of AST may be a physiological response to the use of amino acids in

oxidation or glycogen process in cells to provide them energy to cope with the toxic effects of cadmium (Banaee et al., 2011). Fish exposure to cadmium may inhibit ALT synthesis or activity. The elevated LDH in muscles of exposed fish may indicate impairment of oxidative phosphorylation in mitochondria and development of cellular hypoxia, providing energy in the absence of oxygen and re-oxidation of NADPH by lactate dehydrogenase (Murray et al., 2003). A significant decreases in muscle energy sources, and increases in muscle lactate were reported in silver carp (*Hypophthalmichthys molitrix*) exposed to cadmium (Zhang et al., 2013).

A significant increase in CPK in fish muscles may indicate the cell response to the increasing energy need to cope with cadmium chloride toxicity. The transfer of phosphate group from creatine phosphate to ATP in order to regenerate ATP is done by CPK (Murray et al., 2003). Vitamin C can regulate the activity of cellular enzymes as a radical scavenger and due to its effect on the cellular glutathione level. Vitamin C efficiency improves by chitosan which acts as its carrier. Therefore, simultaneous administration of vitamin C and chitosan to the fish treated with cadmium chloride regulated the activity of ALT, ALP and CPK. Although chitosan and/or vitamin C affected LDH activity, its activity is still high compared with that in the control group. Vitamin C and chitosan administration had no effects on returning LDH activity to the normal level in fish exposed to paraquat (Sharifinasab et al., 2016). Although chitosan has antioxidant properties (Yan et al., 2006; Ramasamy et al., 2014) and acts as a radical scavenger (Samarakoon et al., 2013; Ozcelik et al., 2014), the results of our study show that chitosan alone could not prevent oxidative stress damages in muscle tissues of cadmium-treated fish.

Oxidative stress has an important role in changing levels of AChE (Frasco et al., 2005; Rodríguez-Fuentes et al., 2008). Activation or deactivation of acetylcholinesterase (AChE) activity is attributed to the effect of hydrogen peroxide on this enzyme (Schallreuter et al., 2004). Reduced AChE activity may be caused by the inhibitory effect of cadmium and the increased level of free radicals on cadmium chloride-treated fish. The administration of vitamin C and chitosan did not return this enzyme to its original state.

In conclusion, although administration of antioxidants such as vitamin C and chitosan may reduce cadmium toxicity by increasing the efficiency of antioxidant defense system and detoxifying muscles of fish exposed to cadmium chloride. Administration of antioxidants may not always have a protective effect on the muscles of fish exposed to contaminants. Therefore, regarding the great importance of muscle tissues for the 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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چکیده فارسی

تاثیر حفاظتی ویتامین C و کیتوزان بر شاخص‌های زیستی استرس اکسیداتیو در عضله ماهی کپورمعمولی (*Cyprinus carpio*) در معرض کلراید کادمیوم

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چکیده:

هدف از این مطالعه بررسی تاثیر تجویز آنتی‌اکسیدان‌ها شامل ویتامین C و کیتوزان بر شاخص‌های استرس اکسیداتیو در عضله ماهی‌های در معرض کلراید کادمیوم است. در این آزمایش ماهی‌ها به مدت ۲۱ روز با جیره نرمال، جیره واجد کیتوزان (۱۰۰۰ میلی‌گرم به ازای هر کیلوگرم غذا)، ویتامین C (۱۰۰۰ میلی‌گرم به ازای هر کیلوگرم غذا) و ویتامین C همراه با کیتوزان تغذیه شدند و به طور همزمان در معرض ۰/۲ میلی‌گرم بر لیتر کلراید کادمیوم قرار گرفتند. در این آزمایش، شاخص‌های استرس اکسیداتیو شامل فعالیت آنزیم کاتالاز، آنتی‌اکسیدان کل و مالون‌دی آلدهید (MDA) و پارامترهای بیوشیمیایی سلولی نظیر فعالیت آنزیم‌های آسپارتات آمینوترانسفراز (AST)، آلانین آمینوترانسفراز (AST)، کراتین فسفوکیناز (CPK)، لاکتات دهیدروژناز (LDH) و استیل‌کولین استراز (AChE) اندازه‌گیری شد. تماس ماهی‌ها با کلراید کادمیوم سبب افزایش معنی‌دار در سطح فعالیت آنزیم AST، LDH، CPK، CAT و همچنین سطح MDA و کاهش معنی‌دار سطح فعالیت آنزیم ALT، AChE و سطح TAO سلول های عضله گردید. تجویز کیتوزان و ویتامین C به تنهایی و یا توام با یکدیگر به ماهی‌های تحت تیمار کلراید کادمیوم، در تنظیم سطح فعالیت آنزیم ALT، CPK و CAT مؤثر بود. اگرچه تجویز ویتامین C و کیتوزان سبب کاهش سطح MDA و سطح فعالیت AST و LDH گردید، اما همچنان سطح آنها در این گروه‌ها در مقایسه با ماهی‌های گروه کنترل به طور معنی‌داری بالا بود. در حالی که تجویز ویتامین C و کیتوزان تاثیر معنی‌داری بر سطح فعالیت AChE، و سطح TAO نداشت. اگرچه کیتوزان به تنهایی نتوانست از آسیب‌های ناشی از استرس اکسیداتیو در بافت عضله ماهیان تحت تیمار کادمیوم پیشگیری کند، اما تجویز آنتی‌اکسیدان‌های نظیر ویتامین C و کیتوزان ممکن است کارایی سیستم دفاع آنتی اکسیدانی و سم‌زدایی سلول‌های عضله ماهی‌های در معرض کلراید کادمیوم را افزایش دهد.

کلمات کلیدی: آنتی‌اکسیدان‌ها، کادمیوم، شاخص‌های زیستی، استرس اکسیداتیو، بافت خوراکی.