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Research Article

Antioxidant Activity of Vitamin C and E Versus Oxidative Stress Induced by Heavy Metals in Common Carp (*Cyprinus carpio*)

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ABSTRACT

The present study was undertaken to investigate the antioxidant activity of vitamins C and E singly and together in modulating levels of Malondialdehyde (MDA), total protein, and glucose in different organs (gills, liver, & muscles) and plasma of common carp exposed to heavy metals (Pb, Cd, & Hg). The division of fish into two groups (control group and experimental group) was done after acclimatization. Seven days after exposure to heavy metals, the results showed a significant increase in the level of MDA in all organs of the experimental group (B) compared to those of the control group (A). Metal exposure caused a significant increase in the level of glucose in the liver and plasma (group B), while in muscles and gills, it caused a decrease in the amount of glucose (group B). Heavy metals have caused a slight decrease in total protein (gills, liver, & muscles). Seven days after exposure, the fish were split into three groups: one group was fed with vitamin C, another group with vitamin E, and the third group was fed with both vitamins (C & E). Results show that the addition of vitamins C and E as a food supplement resulted in the restitution of MDA and glucose values similar to those of the control group in all three investigated organs. But in terms of the amount of total protein, the results show that the addition of vitamins (C, E, & C+E) could not restore these values. Otherwise, in most cases, these two vitamins (C & E) administered together have shown more ameliorative effects than in the case of separate administration.

Key words: Ameliorative effect, antioxidant, fish, pollution, vitamin

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INTRODUCTION

Environmental pollution is one of the most serious problems not only for humans but also for all living organisms, and its management presents a global challenge that has preoccupied the whole world (Özkara & Akyıl, 2018; Mondal & Palit, 2021). Pollution has affected all ecosystems including aquatic ones. Depending on the size and content, water pollution can have serious consequences for both humans and aquatic organisms. Among all pollutants heavy metals are of particular concern to the environment and no doubt anthropogenic activities are the main culprit of pollution (Briffa *et al*., 2020; Balali-Mood *et al*., 2021). Heavy metals in water are of multiple origins, either from natural sources or from anthropogenic activity such as industrial discharges, sewage discharges, mining, agriculture, marine accidents, shipping traffic, floods, and erosion (Baby *et al*., 2010; Mirmazloomi *et al*., 2015; Gheorghe *et al*., 2017). Some heavy metals in small quantities are essential for biological systems. Although some of them (Pb, Cd, & Hg) in high concentrations show adverse effects on living organisms that may be accompanied by disturbances in metabolic processes, growth, and reproduction with consequences for the entire trophic chain including even humans (Flora *et al*., 2008; Hermenean *et al*., 2015; Elbeshti *et al*., 2018; Marenkov *et al*., 2021; Haseeb *et al*., 2022). Among the species of aquatic animals, fish are permanent residents of this environment, which cannot escape the devastating effects of pollutants (Jacquin *et al*., 2020; Facey *et al*., 2022). Studies in fish on the effect of heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), copper (Cu), nickel (Ni), etc., on their health showed that these metals produce several health disorders, which depend on the dose and time of their exposure (Ahmed *et al*., 2022). These studies have reported that exposure to heavy metals is also manifested by changes in physiological and biochemical parameters in blood and also in fish tissues. Biochemical parameters such as total proteins and glucose levels, which were also investigated in the present study, are widely used as markers of water pollution (Ullah *et al*., 2021; Ngo *et al*., 2022).

Heavy metals also cause oxidative stress, which through redox reactions generates reactive oxygen species (ROS). These highly reactive molecules can cause oxidative damage such as peroxidation of unsaturated lipids on cell membranes, and oxidation of macromolecules such as proteins and DNA. By attacking proteins, they can also inactivate enzymes (Paris-Palacios *et al*., 2000; Varanka *et al*., 2001; Sevcikova *et al*., 2011). Malondialdehyde (MDA) as a product of lipid peroxidation in tissues or even blood used in this study is one of the indicators of oxidative stress. Recently the focus of research has been on enhancing the detoxification system and boosting cellular antioxidant systems through the application of exogenous antioxidants in the form of supplements. Studies in humans and animals (rats, fish, & other animals) have shown that among antioxidants, vitamin supplementation (C and E) has shown an ameliorative and antioxidant effect in cases of heavy metal toxicity (Mosleh, 2013; Jan *et al*., 2015; Poli *et al*., 2022). In addition, research has shown that vitamin E combined with other antioxidants, particularly with vitamin C, has proven to be more effective against the toxicity and oxidative stress caused by heavy metals and xenobiotics (Gupta *et al*., 2004; Rendón-Ramírez *et al*., 2014). The present study aimed to investigate the toxicity of heavy metals (Pb, Cd, & Hg) in common carp (*Cyprinus carpio*) after acute metal exposure and the antioxidant activity of vitamins (C & E) on the liver, gills, muscles, and fish plasma.

MTERIALS AND METHODS

General preparation

The fish (*Cyprinus carpio*) was taken from a fishpond in the village Janjevo (central Kosovo) near Pristina, with approximate weight and length (150 ± 5 g; 20 ± 1 cm), and the gender difference was not taken into account. The aquariums were filled with tap water for fish acclimatization purposes and lasted for 21 days. During the acclimatization, the pH of the water was 7.5 ± 0.5, while the temperature was 17 ± 2 °C. The fish were introduced into the aquarium after the pH and temperature were stabilized. The water in the aquariums was changed every two days, while the fish were fed once a day with commercial food (5% body weight of fish).

Experimental design

After twenty-one days, six fish subjected to acclimatization were divided into the control group - the group not exposed to heavy metals (A). Another twenty-four fish were used as an experimental group - the group that was exposed to heavy metals (B). The control group remained in the aquarium under the same conditions as during the acclimatization until the time of the dissection. The fish of the experimental group (B), after acclimatization, were exposed for seven days to lead acetate ($Pb(CH_3COO)_2$) 0.3 mg L^{-1}, cadmium chloride ($CdCl_2$) 0.4 mg $L^{-1,}$ and mercuric chloride ($HgCl_2$) 0.001 mg $L^{-1.}$ The water in the aquariums was changed daily to avoid contamination of the food. Seven days after exposure to heavy metals (group B), first blood was taken from fish (six), then they were sacrificed by taking their liver, muscles, and gills. The remaining eighteen fish of the experimental group (B) were divided into three other groups, six fish in each group (C, D, & E). These three groups for fourteen days received vitamin supplementation, one group vitamin C (C), the second group vitamin E (D), and the third group vitamin C + E (E). Vitamin C was administered in water (5 mg L^{-1}), while vitamin D through food which in 500 g of food, contained 55 mL of soybean oil and 500 mg of vitamin E (5 mg of vitamin E was in 10 g of food). Fourteen days after taking vitamins (C, E, & C + E), first, blood was taken from the fish of the three groups (C, D, & E) with a syringe then they were sacrificed to get liver, muscles, and gills (Sahiti et al., 2020).

Fish tissue and blood preparation

Blood from the fish was taken by syringe from the caudal vein before sacrifice and added to heparinized test tubes. The plasma obtained after centrifugation was used for the determination of glucose. After sacrifice, fish tissues were isolated separately and frozen together with plasma at -20 °C until homogenization and biochemical analysis. The preparation of tissues for analysis was done by homogenizing the tissues with the homogenizer. The tissues are homogenized by combining with Phosphate Buffer with pH 7.0 in a ratio of 1/10. The obtained homogenates were stored at -20 °C, until the day of analyses.

Biochemical analysis

Lipid peroxidation in tissues homogenates and plasma was measured according to Buege & Aust, 1978. Malondialdehyde (MDA) level was measured as an indicator of lipid peroxidation in tissue. 250 microliters of homogenate, 1.5 mL of 0.75% thiobarbituric acid solution, 1 mL of 30% trichloroacetic acid solution, and 0.2 mL of 5M HCl solution are added to a test tube and heated for at least 15 min at 95 °C, then measured in a spectrophotometer at a wavelength of 532 nm. The determination of protein in tissue homogenates was done by Lowry *et al*., 1951. The number of total proteins was determined by the calibration curve formed by different concentrations of bovine serum albumin (from 20 to 160 mg %). Glucose levels in tissue and plasma were measured with a commercial kit from the Human Company. The principle of the method is based on the action of the enzyme glucose oxidase in the form of β D glucose.

Data analysis

Data were expressed as means ± standard error (SE), p values of $p \leq 0.01$ and $p \leq 0.05$ were considered to be significant. The statistical processing of the results was done with a One-Way Analysis of Variance (ANOVA) test comparing the values of MDA, total proteins, and glucose among the control group (A) and the group exposed to heavy metals (B), among the group exposed to heavy metals (group B) and groups treated with vitamin C (group C), vitamin E (group D) and vitamins C+E (group E).

RESULTS

The results presented in Tables 1, 2, and 3 show that the acute exposure of fish to heavy metals (Pb, Cd, & Hg) changed the values of all parameters (MDA, total protein, & glucose) investigated in the gills, liver, and muscles. The amount of MDA (Table 1) in gills, liver, and muscles significantly increased ($p<0.01$) in fish exposed to heavy

metals (Table 1, group B) compared to those of the control group (Table 1, group A). The highest amounts of MDA were recorded in the muscles, followed by the liver and gills (muscles>liver>gills). After two weeks of vitamin C, E, and C + E supplementation (Table 1, group C, D, & E) in water and feed of fish treated with heavy metals, the MDA values were close to those of the control group in investigated organs (gills, liver, & muscles). Meanwhile, the difference in the MDA values (gills, liver, & muscles) between groups of fish exposed to heavy metals and those fed with vitamins, is significant (p<0.01; p<0.05).

In the liver of fish that were supplemented only with vitamin C, the total protein level is close to that of the control group (Table 2, group A). A slight increase in the amount of total protein was also observed in the liver supplemented with vitamins C+E, whereas vitamin E was ineffective. As in the gills and liver, also in the muscles, the level of total proteins had a slight increase after the administration of vitamins E and C + E (Table 2, group D & E), but not in the case of the administration of vitamin C. The exposure of fish to heavy metals has caused a significant decrease (p<0.01) in the amount of glucose in gills and muscles, resulting in a significant increase in the liver and plasma (p<0.01) (Table 3, group B) compared to those to the control group.

After heavy metal exposure, the intake of vitamins C, E, and C+E through feed and water has caused the amount of glucose in the gills, liver, and muscles (Table 3, groups C, D, & E) to be close to that of the control group. Thus, in the gills and muscles, there was a significant increase (p<0.01) in the amount of glucose compared to fish treated with heavy metals, while in the liver and plasma, there was a significant decrease (p<0.01) in the level of glucose compared to fish treated with heavy metals.

Table 1. Amount of MDA (MDA µmol $L^{-1)}$ in different organs of *Cyprinus carpio* fish treated with heavy metals (Pb, Cd, & Hg), vitamin C, vitamin E, and vitamins C and E together

Fish groups	Group Comparison	Gills	Liver	Muscles
Control (A)	A	31.50 ± 1.5	26.61 ± 2.1	41.84± 3.4
Exposed to heavy metals (B)	A-B	55.32** ± 2.2	62.77** ± 2.3	68.24** ± 2.8
Vitamin C (C)	B-C	41.38* ± 1.1	39.40* ± 2.3	31.86** ± 3.2
Vitamin E (D)	B-D	27.42** ± 2.4	38.94* ± 4.5	54.28* ± 2.1
Vitamin C +E (E)	B-E	27.10** ± 3.7	23.48* ± 5.2	48.38* ± 3.5

Data present the means ± standard deviation
A-B significant with the control group, **p<0.01
B-C; B-D; B-E; significant with exposed group, **p<0.01; *p<0.05

Table 2. Amount of total protein (Total protein mg $L^{-1)}$ in different organs of *Cyprinus carpio* fish treated with heavy metals (Pb, Cd, & Hg), vitamin C, vitamin E, and vitamins C and E together

Fish groups	Group Comparison	Gills	Liver	Muscles
Control (A)	A	26.27 ± 6.45	25.08 ± 5.85	18.03 ± 2.08
Exposed to heavy metals (B)	A-B	19.30 ± 2.03	15.94 ± 4.74	17.88 ± 2.19
Vitamin C (C)	B-C	17.94 ± 2.07	21.74 ± 3.23	15.40 ± 1.43
Vitamin E (D)	B-D	16.12 ± 2.50	14.18 ± 5.01	25.71 ± 5.27
Vitamin C +E (E)	B-E	15.81 ± 3.05	18.89 ± 2.16	25.67 ± 10.3

Data present the means ± standard deviation
A-B significant with the control group, **p<0.01
B-C; B-D; B-E; significant with exposed group, **p<0.01

Table 3. Amount of glucose (Glucose mg $dL^{-1)}$ in different organs and plasma of *Cyprinus carpio* fish treated with heavy metals (Pb, Cd, & Hg), vitamin C, vitamin E, and vitamins C and E together

Fish groups	Group Comparison	Gills	Liver	Muscles	Plasma
Control (A)	A	62.22 ± 0.97	44.19 ± 2.30	58.30 ± 1.26	39.13 ± 3.67
Exposed to heavy metals (B)	A-B	44.42** ± 1.61	72.04** ± 1.73	39.65** ± 1.57	135.06** ± 6.89
Vitamin C (C)	B-C	55.07** ± 0.94	54.53** ± 1.79	58.60** ± 1.80	31.53** ± 0.40
Vitamin E (D)	B-D	56.11** ± 1.21	46.71** ± 1.15	60.05** ± 1.95	54.09** ± 1.6
Vitamin C +E (E)	B-E	58.25** ± 1.01	41.01** ± 0.60	66.76** ± 3.27	61.55** ± 1.52

Data present the means ± standard deviation
A-B significant with the control group, **p<0.01
B-C; B-D; B-E; significant with exposed group, **p<0.01

DISCUSSION

The harmful impact of heavy metals (Pb, Cd, & Hg) accompanied by oxidative stress and biochemical changes in several organs (liver, gills, & muscles) of fish noticed in this study is consistent with one of the previous investigations (Sahiti *et al*., 2018). In this preliminary research, it was argued that heavy metals (Pb, Cd, & Hg) have caused significant changes in the activity of the aspartate aminotransferase (AST), alkaline phosphatase (ALP), glutathione S-transferase (GST), total protein and MDA. The high utilization of Pb, Cd, and Hg in industry, agriculture, and other spheres of human life has made these metals widely distributed throughout the environment posing a serious risk to humans and wildlife (Wang*et al*., 2004; Flora *et al*., 2006; Hideaki *et al*., 2008).

Several heavy metals are inducers of oxidative stress, which is well documented by the results of many studies done in fish. Therefore, through assessment of oxidative damage and antioxidant defense could be evaluated pollution of the aquatic environment with heavy metals (Livingstone, 2003). In the current study, the results showed notable increases in MDA amount in the homogenate of all tissues included in the research (liver, gills, and muscles) of fish treated with heavy metal mixtures (Pb, Cd, & Hg) compared to the fish of control group. It has been reported by Sevcikova *et al*., (2011) that cadmium, used also in the present study, indirectly causes oxidative stress. By altering glutathione (GSH) levels, cadmium affects the thiol status of the cell thereby promoting the expression of metallothioneins (MTs) in the liver which consequently leads to lipid peroxidation of cell membranes (Sevcikova *et al*., 2011). Glutathione, a three-peptide, intracellular antioxidant that in its composition has the cysteine that has thiol groups, has been shown to have a high affinity for mercury, whose influence was also studied in current research (Santos *et al*., 2018; Ajsuvakova *et al*., 2020). Depletion of GSH as a result of exposure to mercury decreases the antioxidant potential inside the cell, which is why cells are subjected to oxidative stress and cause oxidative damage such as lipid peroxidation (LPO) (Kavitha & Jagadescan, 2006; Sugunavarman *et al*., 2010).

Regarding the effect of lead on oxidative stress, namely lipid peroxidation, data from the literature are contradictory. According to Campana *et al*., (2003), parenteral injection of lead in the toadfish *Halobatrachus didactylus* showed that this metal is not a good inducer of lipid peroxidation. The results of this research showed that MDA decreased in the liver and increased in the kidneys (Campana *et al*., 2003). A study in lead-exposed workers e showed that MDA levels increase with increasing blood lead concentration (Dobrakowski *et al*., 2017).

Heavy metals have also caused changes in the biochemical parameters of the organs involved in the research. The results have shown that the rate of changes in biochemical parameters depends on the metabolic role of these organs (Shahida *et al*., 2021). In this study, a non-significant decrease in total protein in the three investigated organs (liver, gills, & muscles) was observed compared to the fish of the control group. However, a more pronounced decrease has been recorded in the liver and this decrease is because the liver is an organ with a large number of metabolic and detoxifying functions (Kalra *et al*., 2022). After the liver, even gills are sensitive to heavy metal toxicity, with a decrease in total protein higher than in muscles. The surface of the gills which is in contact with the external environment is large; therefore, they are sensitive even to small physicochemical changes in the surrounding environment (Jesus *et al*., 2020).

Contaminated water causes not only morphological changes and damage to the function of gills but also biochemical changes. (Strzyzewska, 2016; Shah *et al*., 2020; Ngo *et al*., 2022)

In this research, the level of proteins in all three organs decreased after exposure to heavy metals. Oxidative stress caused by heavy metals might have led to changes in protein metabolic processes, promoting protein catabolism. Then, the free amino acids can be mobilized to meet the body's additional energy requirements. The findings of this research are in line with the findings of research done on fish by Pretto *et al*., 2014 and Ullah *et al*., 2021; Ngo *et al*., 2022. The level of total proteins shows more pronounced changes in the liver compared to the gills and muscles.

In stressful circumstances, the body mobilizes energy reserves in the liver, which increases plasma glucose (Ko *et al*., 2019). Therefore, glucose is a reliable indicator in stressful conditions, even in cases of exposure to heavy metals (Bartoňková *et al*., 2016). The current research has shown that the exposure of fish to heavy metals has caused changes in the level of glucose in the investigated organs. Glucose has marked a decrease in the amount in gills and muscles, while an increase in the amount in the liver and blood. This decrease in the amount of glucose in the gills and muscles can be justified by the fact that under toxic and oxidative stress conditions the organism needs to consume glucose as a primary energy source (Martínez-Porchas*et al*., 2009). Unlike the muscles and gills that possess carbohydrates - glycogen only for their needs, the liver is the major glycogen store that supply the whole body with glucose (Chang *et al*., 2007). Therefore, this increase in glucose in the liver and plasma may be due to glycogenolysis induced by heavy metal toxicity (Martínez-Porchas *et al*., 2009). Similar findings were also found by some authors in research done with fish exposed to heavy metals (Nanda 2014; Tariang *et al*., 2019). Changes in glucose levels may also be due to the influence of heavy metals on the activity of the enzymes responsible for glucose homeostasis (Tariang *et al*., 2019). The scientific evidence in this regard is the research done by Levesque *et al*. (2002) with *Perca flavescens* chronically exposed to heavy metals on the field. In their research work, they observed changes in the activity of the enzymes that play important roles in carbohydrate metabolism (Levesque *et al*., 2002).

Numerous studies done with various biological models such as broiler chickens, fish, rats, and other animals have shown that taking exogenous antioxidants such as vitamins C and E can improve health against the damage caused by heavy metals (Park *et al*., 2015; Hashem *et al*., 2021; Poli *et al*., 2022). This has been proved also in the current research, where the administration of vitamins C and E has mitigated the damage caused by exposure to heavy metals.

Vitamin C is a powerful antioxidant that is easily absorbed by the tissues. It has an essential role in the body, as it removes free radicals, chelates redox metals, and regenerates other antioxidants (García-Rodríguez *et al*., 2017; Nelson & Cox, 2021). Toxicological studies have also shown that vitamin E is effective against toxicity caused by heavy metals and other pollutants (Azeez & Braimah, 2020). Vitamin E is a fat-soluble vitamin with antioxidant properties and is considered to be a major protector of membranes and lipoproteins from oxidative stress (Böhm, 2018; M. El-Sayed & Izquierdo, 2021).

In this study, the addition of vitamin C in water and vitamin E in food significantly reduced the changes caused by heavy metal exposure in all investigated parameters in all organs, except total proteins. Two weeks after the treatment with these vitamins (C, E, & C+E) the values of MDA and glucose in the gills, liver, and muscles were close to those of the control group of the respective organs, especially when they were administered together (Vitamin C+E). Vitamins C and E together protect cell membranes from lipid peroxidation because vitamin C can recycle oxidized vitamin E to the cell membrane (Zwolak, 2020). Tocopheroxyl radicals are a product of vitamin E, which is created during the reduction of peroxyl radicals. The regeneration of the tocopheroxyl radical is done by vitamin C through reduction (Forman & Zhang, 2021). The findings of our research have also proven the same regarding the action of these two vitamins (C+E) on lipid peroxidation caused by heavy metals in the liver, gills, and muscles.

Proteins are the performers of a large number of vital functions in biological systems, and their function is closely related to their three-dimensional structure (Nelson & Cox, 2021). Heavy metals are known to cause irreversible changes and affect the folding of proteins causing them to misfold and aggregate leading to loss of their function. It is also profoundly clear that heavy metals affect also their homeostasis (Tamás *et al*., 2014; Senthil *et al*., 2020). These might be the reasons why the level of total proteins decreased after the exposure of fish to heavy metals. The administration of vitamins (C, E, & C+E) in water and food after the exposure of fish to heavy metals, has influenced the increase in the level of total proteins in the liver and muscles, which are known as the two main organs in terms of protein metabolism (Wolfe, 2006; Trefts *et al*., 2017). The positive effect of vitamins C and E, separately or together, on protein metabolism have been reported by Sigolo *et al*. 2018.

In contrast to proteins, the administration of vitamins (C, E, & C+E) to fish treated with heavy metals has shown an ameliorative effect in restoring glucose levels to values similar to those of the control group in the liver, gills, muscles, and plasma. Although in the three fish groups (C, D, & E) receiving vitamins (C, E, & C+E) there is an improvement in the amount of glucose; however, in all tissues, it appears that the combination of vitamin C and E showed to be more effective. It has even been reported by other authors that the combination of vitamin E with other antioxidants is more effective than administered alone. A study by Layachi and Kechrid (2012) on the combined protective effect of vitamins C and E on cadmium-induced oxidative liver injury in rats demonstrated that these two vitamins together exert a more synergistic effect on the observed oxidative stress Layachi & Kechrid, 2012.

CONCLUSION

Heavy metals have caused changes in the values of the investigated parameters in the liver, muscles, gills, and plasma. An increase in the amount of MDA (liver, muscles, & gills) and glucose in the liver and plasma has been recorded in the heavy metal expose group (group B). The increase in the amount of glucose in the liver may be a result of the breakdown of glycogen as a consequence of the oxidative stress caused by heavy metals, and consequently, the amount of glucose in the plasma is also increased. The amount of total protein (liver, muscles, and gills) and glucose in gills and muscles have decreased after exposure to heavy metals. Vitamin supplementation (C, E, & C + E) of fish previously exposed to heavy metals has caused recovery of MDA and glucose values in all investigated organs. Regarding the total protein, only vitamin C in the liver has recovered values approximately to those of the control group (group A).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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