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Original Research

Metal Accumulation and Effect of Vitamin C and E in Accumulated Heavy Metals in Different Tissues in Common Carp (*Cyprinus carpio*) Treated with Heavy Metals

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Abstract

The aim of our current research was to study the effects of Vitamins C and E separately and jointly in reducing the amount of accumulated heavy metals in different tissues of common carp exposed to heavy metals. After acclimatization the fish were divided into two groups: the control group and the group subjected to heavy metal exposure. The fish were exposed to a mixture of heavy metals containing: lead acetate 0.3 mg/l, cadmium chloride 0.4 mg/l and mercuric chloride 0.001 mg/l. After exposure, there was a significant increase ($p<0.01$) in metal accumulation in fish tissues compared with the control group. Exposed fish were then divided into three groups: one received only vitamin C, the other only vitamin E and last group received a combination of vitamins C and E. Based on the present results, the order of accumulated heavy metals in investigated tissues of all groups is gills> liver> muscles> plasma. Supplementation of vitamins C and E either alone or jointly had significantly decreased ($p<0.01$; $p<0.05$) levels of accumulated heavy metals in investigated tissues compared to the control and exposed groups. Supplementation of vitamin C and E jointly showed more chelating ability compared with the effect of vitamins C and E, separately.

Keywords: *Cyrpinus carpio*, heavy metals, accumulation, vitamin C, vitamin E

Introduction

Heavy metals are naturally occurring elements on the earth's crust and they are also referred to as trace elements [1]. Some of these metals have found application in many areas of human life, such as agriculture, pharmacy, cosmetics, industry, medicine, etc. Human exploitation of these metals has increased the risk of environmental pollution from them. In view of their toxic properties they can be dangerous to all receiving ecosystems [2]. Sources of heavy metals contamination of aquatic ecosystems can be mines, industrial wastewater, fertilizers, pesticides and also deposition and erosion of the geological matrix [3]. Contamination of water with these pollutants can have

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harmful consequences on the living organisms of these ecosystems [4-5]. The consequences of severe metal poisoning depend on the physical chemical factors of water, but also on the amount and duration of the action. Among aquatic organisms, fish are highly sensitive to pollution and as such are quite suitable as bioindicators for water quality monitoring [6].

Research into different types of fish has shown that heavy metals can lead to changes in the physiological activity and biochemical parameters of various tissues of these organisms [2, 7-9]. Heavy metals pose a risk to aquatic ecosystems due to their bioaccumulation. They can be accumulated and biomagnified in water, sediment and the aquatic food chain [10-11]. Since fish are a good source of protein for humans, their poisoning with heavy metals can also endanger human health [12-13]. Studies have shown that antioxidative vitamins such as C and E, which are widespread in many food products, have been shown to have a mitigation effect in heavy metals toxicity [14-16]. These two vitamins have been shown to have chelating abilities, affecting the reduction in the amount of accumulated metals in different tissues to various organisms, including fish [17-18]. The aim of the current work is to investigate in parallel the sensitivity of various tissues to the accumulation of heavy metals, and the chelating effect of tocopherol (vitamin E) and ascorbic acid (vitamin C) either alone or jointly in accumulated heavy metals (Pb, Cd and Hg) in different tissues (liver, gills, muscles and plasma) of common carp (*Cyprinuscarpio*) treated with heavy metals.

Material and Methods

Common carp (*Cyprinus carpio*) was obtained from a small fish pound in the village of Janjevo (central Kosovo) near Prishtina. Their average weight was 150±5 g and average length 20±1 cm. Gender differences in the current research were not taken into consideration. Fish were placed in glass aquariums filled with tap water and acclimatized under laboratory conditions for three weeks. Physicochemical conditions of water during the acclimatization period and experiment were: pH 7.5±0.5 and temperature 17±2ºC. The fish were fed once a day with commercial food while the water was changed every 48 hours. The fish involved in the experiment after the acclimatization period were divided into control and experimental groups. The experimental group (n24) for seven days was exposed to a heavy metal mixture containing lead acetate (Pb $(CH_3COO)_2$) 0.3 mg/l, cadmium chloride ($CdCl_2$) 0.4 mg/l and mercuric chloride (HgCl) 0.001 mg/l. During exposure time, water in aquaria was changed every 24 hours. After heavy metals exposure, six fish were sacrificed and from them we took liver, muscle, gills and blood for heavy metal analysis. The remaining group (n18) was divided into three other groups that have undergone dietary supplements with vitamins for two weeks. Since vitamin C is water-soluble, it has been dissolved with aquarium water in the amount of 5 mg/l. On the other hand, vitamin E is supplemented with food because it is fat-soluble. Food with vitamin E content was prepared in the laboratory. To the 500 g blended commercial food pellets we added 55 ml of soy oil and 500 mg of vitamin E. The pellets formed from such food mixture were dried in the oven for several hours at 100ºC. One group of fish (n6) as supplement received only vitamin C, the other group (n6) only vitamin E, while the third group (n6) received vitamins C and E jointly. After two weeks, the fish of the three groups were sacrificed and the liver, gills, muscle and blood were taken for analysis of heavy metals content.

Heavy metals content (Cd, Pb, Ni and Cu) was determined by atomic absorption spectrophotometry (AAS). Statistical processing of results was done using a one-way analysis of variance (ANOVA) method including least significant difference(LSD) post-hoc test.

Results

The concentrations of heavy metals (Pb, Cd and Hg) in different tissues (liver, muscle, gills and plasma) of the carp (*Cyprinus carpio*) are presented in Tables 1-4 in terms of mean value and standard deviation (±), including LSD values. The control group is compared with the groups treated with heavy metals and the groups treated with heavy metals are compared with the vitamin-treated groups.

Based on results presented in all tables it is noted that the amount of Pb is higher in comparison to Cd and Hg and that gills are tissues where Pb is recorded in the largest quantity (Table 1) compared to other tissues (Tables 2-4). The highest level of cadmium is recorded in the liver of the control group and that of exposed to heavy metals compared to other tissues (Table 1). In liver, exposure to heavy metals caused a significant increase of lead and cadmium accumulation compared to the control group. From results presented in Table 1, it is seen that mercury is an exception because the level of accumulation was increased, but not significantly. Compared to the group exposed to metals, the level of accumulated metals in fish that have received vitamin supplements (C, E and C + E) significantly decreased.In terms of lead and mercury, vitamins C and C+ E jointly have demonstrated a better chelating effect compared to vitamin E separately. Regarding the level of mercury, vitamin C has lowered it below detection level. Meanwhile, with regard to Cd, the best chelating effects have been shown by vitamins C and E separately than these two vitamins jointly. The order of accumulated metals in liver of all groups is: lead>cadmium>mercury.

The results presented in Table 2 show that the highest level of accumulation of lead and mercury has been recorded in gills of all groups of fish and the order of accumulated metals was as well as on the liver (Pb>Cd>Hg). Unlike liver, in gills exposure to heavy metals (Pb, Cd and Hg) caused a significant

Table 1. Bioaccumulation (mg/kg) of Pb, Cd and Hg in liver of carp initially treated with heavy metals and then with vitamin C, E and C + E.

Liver			
Fish groups	Pb	Cd	Hg
Control	1.10±0.01	0.360±0.01	0.07±0.01
Exposed to heavy metals	1.23±0.01*	0.51±0.02*	0.10±0.01
Vitamin C	0.77±0.01[b]	0.15±0.01[a]	ND
Vitamin E	0.86±0.01[c]	0.27±0.01[b]	0.03±0.01[a]
Vitamin C + E	0.67±0.03[a]	0.35±0.02[c]	0.05±0.01[ab]

Data are given in means ± standard deviation
Significant differences based on LSD test:
* significant difference between control and groups exposed to heavy metals
a, b, c significant difference between groups exposed to heavy metals and groups treated with vitamins, a>b>c (differences in results of groups sharing same later are not significant)
ND – not detected

increase of accumulation not only of lead and cadmium but also of mercury (Table 2). As in liver, also in gills, supplementation of vitamins (C, E and C + E) have reacted in the same way, decreasing significantly the amount of accumulated metals compared to the group treated with heavy metals. A comparison of results between fish groups treated with heavy metals and those treated with vitamins by means of the LSD test shows that the most effective chelation ability related to Pb and Cd levels have C + E jointly, while regarding Hg, Vitamin C has demonstrated the highest chelation effect since it was able to reduce the level of Hg under the threshold of detection.

The results for the accumulation of heavy metals in the muscles, as presented in Table 3, shows that the amount of accumulated heavy metals (lead and cadmium) in muscle is lower compared to the liver and gills, while the presence of mercury is not recorded. After exposure, the amount of metal accumulation was increased in groups treated with heavy metals compared with the control group, though not significantly. After the fish group treated with heavy metals underwent diet with vitamins (C, E and C + E), a decrease in the amount of heavy metals could be observed. Regarding Pb, vitamins C + E together showed a higher chelation effect compared to the effect of these vitamins separately. With regard to Cd, it could be noted that vitamin E was more effective in its elimination, while for Hg the highest chelation abilities were related to vitamins C and C + E, which were able to lower Hg levels below the detection threshold.

The results presented in Table 4 show that the lowest levels of accumulated Pb and Cd were recorded in plasma, while the presence of Hg was not recorded at all. Exposure to heavy metals caused an increase in the level of accumulated heavy metals (Pb, Cd and Hg) compared to the control group, but this increase was statistically significant only for Cd. Vitamin supplementation (C, E

Table 2. Bioaccumulation (mg/kg) of Pb, Cd and Hg in gills of carp initially treated with heavy metals and then with vitamin C, E and C + E.

Gills			
Fish groups	Pb	Cd	Hg
Control	1.53±0.02	0.25±0.01	0.14±0.003
Exposed to heavy metals	2.17±0.04*	0.37±0.02*	0.25±0.01*
Vitamin C	1.48±0.02[c]	0.21±0.01[ab]	ND
Vitamin E	1.12±0.02[b]	0.26±0.01[bc]	0.08±0.01[b]
Vitamin C + E	1.02±0.01[a]	0.18±0.003[a]	0.02±0.003[a]

Data are given in means ± standard deviation
Significant differences based on LSD test:
* significant difference between control and groups exposed to heavy metals
a, b, c significant difference between groups exposed to heavy metals and groups treated with vitamins, a>b>c (differences in results of groups sharing same later are not significant)
ND – not detected

Table 3. Bioaccumulation (mg/kg) of Pb, Cd and Hg in muscle of carp initially treated with heavy metals and then with vitamin C, E and C + E.

Muscle			
Fish groups	Pb	Cd	Hg
Control	0.47±0.02	0.05±0.01	ND
Exposed to heavy metals	0.56±0.03	0.09±0.01	0.03±0.003
Vitamin C	0.27±0.02[b]	0.07±0.01	ND
Vitamin E	0.31±0.02[bc]	ND	0.02±0.003
Vitamin C + E	0.14±0.02[a]	0.02 0.01[a]	ND

Data are given in means ± standard deviation
Significant differences based on LSD test:
* significant difference between control and groups exposed to heavy metals
a, b, c significant difference between groups exposed to heavy metals and groups treated with vitamins, a>b>c (differences in results of groups sharing same later are not significant)
ND – not detected

and C + E) reduced the amount of almost all investigated metals (Pb, Cd and Hg) in plasma (Table 4), while a stronger reduction is being noted to Hg, whose amount was lowered below the detection threshold. In relation to Cd, vitamins E and C + E together lowered its level below the detection threshold, while regarding Pb a similar effect was achieved only with vitamin E, which lowered Pb level below the detection threshold.

Discussion

Fish have the ability to uptake and accumulate heavy metals directly from the aquatic environment or even through food. After entering the organism and absorption, heavy metals through blood circulation are transported to various organs, where they are then accumulated [19]. Accumulation of heavy metals in fish can be affected by several factors such as dose and duration of exposure, physicochemical parameters of water, interaction between metals, age of fish and metabolic activity of the fish [20]. In spite of lower levels of metals (Pb, Cd and Hg) to which fish were exposed in the current research, in most cases the level of heavy metals increased in all tissues, and in gills and liver a significant increase of the level of heavy metals could be noted. Based on the results of the current research, it is noted that the order of the metals accumulated in the fish of the control group as well as those of the experimental groups, treated with heavy metals and vitamins for all investigated tissues is: Pb>Cd>Hg. Although the order of accumulated metals even after treatment is the same, there is a difference in the amount of accumulation of heavy metals from different tissues. This shows that different tissues have different affinities for accumulating different heavy metals. The affinity of organs or tissues to accumulate heavy metals can be attributed to their high metabolic activity but also to their morphology. Results of current research show a greater accumulation of heavy metals

Table 4. Bioaccumulation (mg/kg) of Pb, Cd and Hg in plasma of carp initially treated with heavy metals and then with vitamin C, E and C + E.

Plasma			
Fish groups	Pb	Cd	Hg
Control	0.05±0.01	0.002±0.0006	ND
Exposed to heavy metals	0.09±0.01	0.006±0.003*	0.001±0.003
Vitamin C	0.01±0.003[a]	0.001±0.003[a]	ND
Vitamin E	ND	ND	ND
Vitamin C + E	0.03±0.006[ab]	ND	ND

Data are given in means ± standard deviation
Significant differences based on LSD test:
* significant difference between control and groups exposed to heavy metals
a, b, c significant difference between groups exposed to heavy metals and groups treated with vitamins, a>b>c (differences in results of groups sharing same later are not significant)
ND – not detected

(Pb and Hg) in gills compared to liver, and the other two tissues (muscle and blood). As regards Cd in liver, it is at higher accumulation levels than in the gills and other tissues. One of the main reasons that gills are the main site of metal accumulation, as has been estimated in the current study, may be attributed to its direct contact with the outside environment as well as their structural organization. Due to the highly branched structure of the gills, their surface results to be greatly increased and together with the large volume of water passing through the surface of the gills, make the gills a major place for the accumulation of metals [21]. Research related to the accumulation of metals in different tissues and organs from different authors has also shown the sensitivity of gills in the accumulation of heavy metals [22-25].

Despite the fact that the liver is not in direct contact with water, it is a place for metal accumulation. This has also been argued by the findings within the current research. However, the level of metals (Pb and Hg) accumulated in the liver is lower than the level of metals accumulated in gills, with the exception of Cd, which has the highest value in the liver. According to Kent (1998), high metabolic activity and the detoxifying role makes the liver the site of collection of heavy metals that arrives there form other organs, including gills and muscles through the bloodstream, in order to eliminate them later [26]. Another reason why gills and liver are metals accumulation sites may be metallothionein production [27]. In addition, these tissues are also rich in the metal binding-SH groups that have the tendency to bind to soft elements/ions such as mercury, lead, or cadmium [28].

In the present study, the level of heavy metal concentration in muscle is lower compared to gills and liver. Research on the accumulation of heavy metals in various tissues, including muscle tissue, has shown that this tissue is not a place for the accumulation of these metals [22, 24-25]. From this point of view, muscles are covered outside with the skin, which prevents direct contact with the outside environment. Another reason why muscles are not an active place for metal accumulation may be that muscles do not have roles in the detoxification process. The lowest level of metal accumulation in all groups is recorded in blood plasma. In fact, the blood serves as a carrier of heavy metals in other tissues and organs.

The purpose of our current research is also to investigate the effects of vitamins C and E separately, as well as jointly in decreasing the level of accumulated heavy metals in investigated tissues (liver, gills, muscle and plasma) of common carp (*Cyprinus carpio*). It is known that seafood is a good protein source for humans and the accumulation of heavy metals in tissue of aquatic animals can pose a risk to human health. A very effective therapeutic way commonly used for heavy metal poisoning is chelation therapy, which promotes the excretion of metals. Natural antioxidant compounds such as vitamins C and E which, besides possessing antioxidant activity, are also good chelators used in metal toxicity management. Viewed from this perspective, the result of the present research also confirms the chelating role of vitamins C and E in reducing metal content in investigated tissues in common carp. From the results presented in Tables 1-4 we can see that vitamins C and E separately, but also jointly, have reduced the level of accumulated metals in the investigated tissues in the groups that have taken supplements of vitamins (C, E and C + E) compared to the group of fish treated with metals. However, these vitamins have shown that they have different chelating abilities, and based on the LSD test a comparison of groups treated with metals with groups that after metal exposure have been subjected to diet with vitamins (C, E and C + E), a differentiation of vitamins with higher chelating ability could be done. In groups that have been subjected to diet with vitamins, the level of metal accumulation in most cases is significantly lower compared with the heavy metals-treated groups and in cases where the concentration of metals has been low these vitamins either separately or jointly have lowered the concentration of metals under the detection threshold.

In fish groups in which vitamins C and E are applied separately, both of these vitamins caused a significant reduction in the amount of accumulated metals compared to the heavy metal group, but the effect of these two vitamins in relation to each other with regard to the chelation ability in a few cases turned out to be more or less the same. However, in the liver and partially in the gills and muscles, vitamin C has been shown to be more effective in eliminating metals in these tissues compared to vitamin E. In a study related to the effect of vitamins C and E on some biochemical parameters and in accumulation of Cd in some tissues of the Ross broiler chicken treated with Cd, Cinar et al. (2011) also found that vitamin C was more effective in reducing the Cd level in the liver (1, 05 kg^{-1}) than vitamin E (3.23 kg^{-1}) [29]. In general, based on results of many studies, vitamin C supplementation in animals exposed to heavy metals not only affects the reduction of oxidative stress, but also significantly reduces the levels of these metals in different tissues such as blood, liver, kidney, muscle, gills, brain, etc. [14, 17, 30-31].

The combination of vitamin E with other antioxidants, especially with vitamin C, in many studies has shown higher efficacy in antioxidant protection. Vitamins E and C jointly protect lipid structures against per oxidation [32]. Tezcan et al. (2012) have reported that vitamin E and vitamin C together may prevent cytotoxic damage of erythrocytes at low and moderate Cd concentrations [33].

The results of the current research have proven that in general the combination of vitamins C and E has been shown to be more effective in reducing the level of accumulation of heavy metals in the tissues (except liver) than when these vitamins acted separately. The combination of these two vitamins has proven to be beneficial also against the toxicity of heavy metals, which has been proven by the results of much research. Vitamin E and C jointly create protection against lipid per oxidation. Vitamin C is able to recycle oxidized vitamin E, recovering the antioxidant ability of vitamin E [32, 34]. Ebuehi et al. (2012) indicate that oral administration of vitamins C and E significantly reduced the blood lead concentration, ameliorates the hepatic damage and significantly reduced the oxidative stress in the brain of rats [35]. A study by Rendón-Ramírez et al. (2014) with employees exposed to Pb (73 μg Pb / dL blood), also argued the synergistic effect of vitamins C and E. A year after oral vitamin C and E supplementation (1 g daily vitamin C and 400 IU daily vitamin E), lipid peroxidation in erythrocytes was reduced to between 47.1% and 69.4%, which were no longer statistically different from those of non-Pb exposed workers. The total antioxidant capacity in erythrocytes also returned to values between 58.9% and 67.7% in workers exposed to Pb after treatment, a level similar to those in exposed non-Pb workers [36].

Results of the present work indicate that the intake of vitamins (C, E and C + E) has significantly decreased the amount of accumulated heavy metals in all investigated tissues.

Conclusions

The observations of our data regarding the accumulation of heavy metals showed that gills and liver are sites of accumulation of heavy metals compared with muscles and blood (gills>liver>muscles>plasma), and the order of metal level in all investigated tissues is Pb>Cd>Hg.

Antioxidants (vitamins C, Eand C + E), either separately or jointly, have been shown to have a good chelating effect on reducing the level of accumulated heavy metals in tissues involved in the research. However, their cheating ability was different.

From this point of view, it can be seen that the use of vitamins C and E separately has been shown to have a similar effect on reducing the amount of heavy metals accumulated in the tissues involved in the research. However, based on the results we obtained, it can be concluded that supplementation of vitamins (C + E) jointly is much more effective than in case these vitamins are separately applied. This may be due to the synergistic effect that produces the combination of vitamins C and E together.

Conflict of Interest

The authors declare no conflict of interest.

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