Garlic, cilantro and chlorella’s effect on liver histoarchitecture changes in Cd-intoxicated Prussian carp (Carassius gibelio)

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Abstract

Bioactive compounds from natural sources can act as oxygen free radical scavengers or metal chelators, which enables them to be used as natural antagonists to heavy metals toxicity. So the purpose of this study was to compare histologically the aspect of liver tissue of Prussian carps’ specimens, subjected to chronic Cd intoxication with and without garlic, cilantro and chlorella dietary supplementation. 150 Prussian carps, with weight of 10-12 g, were divided according to the following treatments for 21 days: C (without treatment), E1 (10 ppm Cd into water), E2 (10 ppm Cd into water+2% lyophilized garlic in feed), E3 (10 ppm Cd into water+2% lyophilized cilantro in feed), E4 (10 ppm Cd into water+2% lyophilized chlorella in feed). Fragments of liver were removed at the end of experimental period and analyzed in light microscopy and a specific QuickPHOTO Micro 2.2 software has been used for the histological study. Mentioned epithelium suffered evident histomorphological alterations under the action of Cd while the active principles from garlic, cilantro and chlorella powder have showed chelating and antioxidant potential, cilantro and garlic being the most effective.

Key word: cadmium (Cd) toxicity, fish, liver, histopathology, garlic, cilantro, chlorella

1. Introduction

The metals are considered as major toxic pollutants that entering into biogeochemical cycles they accumulate in natural and artificial aquatic ecosystems. Terrestrial sources that generate heavy metals are natural - volcanic eruptions, natural weathering leading to rocks’ erosion and anthropogenic - wastewater treatment plants, industries, mining and agriculture [1]. Non-essential metals (lead, arsenic, mercury, cadmium) are highly toxic, even at very low levels [2], especially if they accumulate in metabolically active sites. Among them, cadmium is considered one of the most toxic environmental substances due to its ubiquity, toxicity and long half-life [3]. Cadmium not having an efficient excretory mechanism, its half-life in the liver is between 4 and 19 years and, in the kidneys is between 6 and 38 years [4]. There are three major routes to uptake heavy metals by aquatic organisms: respiratory system (gills), digestive system and overall body surface (skin) [5]. In benthic aquatic ecosystems, where pollutants are associated with sediments, feed is an important source of their uptake [6]. Thus, uptake of the contaminants leads to their concentration into aquatic organisms’ tissues, including fish. Fish are ideal indicators of heavy metal contamination in aquatic ecosystems.
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[6]. The contaminant can be sequestered and then neutralized, and/or may have specific interactions with endogenous molecules (inhibition of enzyme) and/or can be metabolized by enzymes of the biotransformation system. All these interactions may result in: long-term storage (neutralized fraction); direct or indirect toxic effects (after biotransformation); excretion of contaminants or their metabolites [7]. The cell membrane is the first target structure when metals penetrate the cell. It has been shown that metals can bind to membrane proteins and phospholipids altering their structure and functions [8]. Heavy metals can stimulate lipid peroxidation processes, a complex sequence of biochemical reactions, defined as "polyunsaturated lipid oxidative deterioration". The entire process results in production of highly toxic compounds for the cell, because of high reactivity compared to other cell components (membrane proteins, DNA). Toxic metals with high affinity to the active groups of proteins may affect the structure and function of these molecules and finally, the cell physiology [9]. Chelation therapy is the most commonly used therapeutic strategy for heavy metal poisoning [10]. Synthetic chelating agents, such as DMPS, DMSA, EDTA and others, bind poisonous metals rendering them inert and promoting their excretion from the body without further interaction. There are advantages and disadvantages to using these ones. One advantage is the power of their mobilizing activity –they are quick to mobilize and eliminate certain metals in the body, but this may place a huge burden on the body’s detoxification systems [11]. But natural chelators from some vegetal sources as chlorella [12], garlic [13] and coriander [14] have been shown their detox ability in human and animals. Garlic contains many active sulfur compounds derived from cysteine with metal-chelating potential properties; these garlic constituents may also protect from metal-catalyzed oxidative damage [15]). Garlic increases antioxidant action by scavenging reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes and increasing glutathione in the cells [16]. Cilantro (Coriandrum sativum) can bind and immobilize cadmium chloride from liver and kidney [17] and decrease toxic tissue cadmium accumulation in cultured rainbow trout Oncorhynchus mykiss [18]. One of its principal constituents is linalool. Several animal studies provide evidence that coriander seeds can promote the hepatic antioxidant system [19]. Chlorella, a unicellular green algae with the ability to bind cadmium (in animal models) [20], has been used to detoxify wastewater of metal contaminants as well [21]). The aim of the present study was first to highlight severity of histological liver damages and secondary to assess comparatively the chelating and antioxidant property of chlorella, coriander and garlic compounds in Prussian carp’s cadmium-induced intoxication.

2. Material and Methods

Biological material - 150 Prussian carp fingerlings (Carassius gibelio Bloch) were chosen as test species on the basis of accessibility and ecologically representation. Individuals belonging to this species are easily acquired and adapt to captivity. Fresh fish samples were collected from a local pond and were gravimetrically selected. Thus, individuals of 10-12 g weight were acclimated for two weeks under laboratory conditions prior to experiment, removing those ones with suspicions on their health. Then, Prussian carp fingerlings were transferred in 5 glass aquariums equipped with aeration system and 60 L capacity at a stocking density of 30 fishes per aquarium. The control and test organisms were randomly distributed in test solutions as follows: the control ones were maintained in Cd-free freshwater, the others four groups receiving: E1 (10 ppm Cd into water), E2 (10 ppm Cd into water+2% lyophilized garlic in feed), E3 (10 ppm Cd into water+2% lyophilized cilantro in feed), E4 (10 ppm Cd into the water+2% lyophilized chlorella in feed), respectively.
Chemicals - Fish in groups E1, E2, E3 and E4 were exposed for 21 days to chronic cadmium intoxication in concentration of 10 ppm from a CdCl$_2$ x ½H$_2$O stock solution. The sub lethal treatment was calculated from percentage mortalities of fish as described by B. VEENA & al. [22].

Fish nutrition - Fish were fed twice a day with commercial dry pellets. Freeze-dried garlic, cilantro and chlorella’s incorporation in fish feed has involved the following steps: 1) bringing the dry pellets to fine size particles; 2) adding the lyophilized material in the proportion determined by the experimental protocol setting the daily feed amount needed by a specimen, according to the calculation model in fish proposed by L. OPREA & R. GEORGESCU [23]; 3) wetting and homogenization of the obtained mixture; 4) regranulation and drying of the final pellets. Chlorella pyrenoidosa powder was produced by Bucharest Medical Laboratories; garlic (figure 1) and coriander (figure 2) powders were obtained by lyophilization from Interdisciplinary Training and Research Platform "Sustainable Ecological Agriculture, and Food Safety" from USAMVB Timisoara, Romania. Lyophilization was carried out at a temperature of -53°C and a pressure of 0.05 mTorr and lyophilization efficiency was 38.07% for garlic and 10.55% for cilantro, respectively.

Monitoring of physical-chemical parameters of the water in aquariums - Physical-chemical indicators of water-dissolved oxygen, temperature, NO$_2^-$, NO$_3^-$, pH, hardness were daily measured (water temperature and dissolved oxygen with a movable oxygen-meter with water resisting microprocessor Hanna HI 9145; pH, NO$_2^-$, NO$_3^-$, pH, toughness of water with a Germany termatest kit).

Histopathological examination - Fragments of liver were removed at the end of a 21 days experimental period after fish euthanasia with an overdose of anesthetic tricaine methanosulfonate (MS-222) (>250 mg/L). The material was subjected to histological analysis, being fixed in a formalin solution 10%, dehydrated, clarified, impregnated and embedded in histological paraffin, according to standard routine methods in the Histology laboratory. Five-micrometer-thick sections were obtained and stained by Mallory’s trichrome method. Liver was estimated for 10 specimens from each population. Microscopic examination was performed using a CX41 Olympus light microscope equipped with a digital camera and specific QuickPHOTO Micro 2.2 software has been used for the histopathological study. Tissue alterations were assessed using the histopathological score [24] ranging from – to +++ depending on the degree and extend of lesions: (-) none, (+) mild occurrence, (++) moderate occurrence, (+++) severe occurrence. Statistical analysis was performed using SPPS IBM 22 software. Data were reported as a significant level of p<0.05. Test of differences between means was realized by ANOVA completed with post-hoc Tukey test.

3. Results and Discussion

Histopathological study of the liver

Histopathological alterations can be used as indicators for the effects of various anthropogenic pollutants on aquatic biota and they are a reflection of the overall health of entire population in the ecosystem [25]. The investigation of histological changes in organs of fish is an accurate way to assess the effects of xenobiotics compounds in experimental studies [26]. The liver performs many essential functions related to digestion, metabolism, immunity, and the storage of nutrients within the body. At the same time, the liver is associated with detoxification and biotransformation processes, and due to these functions combined with its location and access to the blood supply, it is one of the organs most affected by water contaminants [25]. But, liver ability to degrade toxic compounds can be overwhelmed by
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Elevated concentrations of these compounds, and could subsequently result in structural damage [27]. The microscopic examination of the liver sections of control group revealed that liver parenchyma consists of liver cells arranged in uniform cords (figure 1). Hepatocytes have a polygonal appearance, the membrane is well defined and nucleus is spherical, centrally or slightly eccentrically located with obvious nucleolus. Binucleated cells are also seen, they being common among hepatocytes and they can account for as much as 25% of the hepatocytes population [28]. Sinusoidal capillaries with normal appearance are arranged between hepatic cell cords (figure 2). Connective tissue from stroma structure is less obvious.

Liver belonging to cadmium-poisoned fish loses its normal architecture and frequently sinusoidal capillaries hypertrophy and congestive processes are manifested (figures 3 and 4). Interlobular spaces are wide. Venulare and arteriolar vascular endings are frequently congested and increased in volume into interportal spaces. The hepatocytes are arranged in cords, they have a polygonal form, and clear cytoplasm due to the accumulation of numerous microlipid droplets (fatty degeneration). Intense hypertrophic processes, nuclear chromatin condensation, karyolysis processes are reported on wider territories, resulting in cell death. Unlike the control group, in the individuals of this one, intralobular connective stroma becomes visible. Consequently, interstitial fibrosis processes occur in liver of these specimens (figure 5).

Light microscopic study of the liver sample from fish exposed to Cd and garlic powder supplemented diet showed several changes: hepatocytes have polygonal form, clear and central, slightly eccentric nucleus, or eccentric. Frequently, in the liver parenchyma cell hypertrophy occurs. Cells are increased in volume, cytoplasm is clear, and a large number of cells have no nucleus. Mild perisinusoidal lymphocytic infiltrates are present in the connective tissue. Hypertrophic sinusoids capillary, containing erythrocytes, are arranged between hepatocytes. Interlobular spaces are filled with fine blades of connective tissue, containing fine capillaries (figure 6) and slightly hypertrophic vascular ramifications also can be found in interportal spaces. Histological changes in liver specimens exposed for 21 days to Cd intoxication and feed with an incorporated coriander diet were hepatic parenchyma consists of dense cords of hepatocytes with a polygonal appearance, a spherical central, slightly eccentric or totally eccentric nucleus, with obvious nucleolus; the cytoplasm is clear, but has fine granulations diffusely spread; hypertrophic hepatocytes with pyknotic nucleus or without nucleus appear on smaller areas (figure 7). Hypertrophic sinusoidal capillary network with rich erythrocytes content are distributed among the hepatocyte cords. Hypertrophic
processes appear in the peri- and interlobular venules and arterioles, vessels in which is also viewing erythrocyte content. Lymphocytic infiltrates (figure 8) are reported surrounding the connective tissue of the blood vessels. Histopathological alterations observed after sublethal Cd exposure in liver of fish fed with a chlorella incorporated diet were: hepatocytes arranged in cords, have clear cytoplasm with central, slightly eccentric or totally eccentric spherical nucleus; hepatocytes in various stages of atrophy, increased in volume, with a single block condensed chromatin, or showing karyolysis are observed on large areas (figure 9).

Sinusoidal capillaries have wide lumen containing abundant red cells. Also, the terminals veins are hypertrophic and have content. The liver is frequently identified as a target organ in toxicity studies [29], partly attributable to its anatomical location and functional complexity. If accumulation of toxins is faster than the liver metabolizing ability, hepatic damage may occur [30]. Most of the hepatotoxic agents, including cadmium, damage liver cells mainly by inducing lipid peroxidation and by generation of reactive oxidative intermediates in liver [30]. Hepatic tissues of the fish exposed to cadmium chloride showed high degree of congestion (table 1) that may be attributed to the decline in hematological parameters as H. A. KAOUĐ & al. [31] observed since the hemoglobin contents are insufficient for the respiration of the tissue. Congestion of the hepatic central vein and sinusoids was also reported by H. A. KAOUĐ & al. [31]; Z. A. EL-GREISY & A. H. A. EL-GAMAL [32]. In our study, the abundance of intrahepatic lymphocytes (table 1) found in cadmium treated fish and fed with an incorporated garlic (E2) and cilantro (E3) diet, indicate that liver is severely affected by the toxic chemical. This action on the immunological defense of the fish was confirmed in many works as response to heavy metals stress [33], showing evidence of cell irritability, inflammation and hypersensitivity to Cd. The fibrosis is other liver damage caused by cadmium poisoning in the present work (table 1) and confirmed in a lot of studies [34]. Although hepatic parenchymal fibrosis is rare, J. C. WOLF & M. J. WOLFE [35] affirms that is not unusual to observe a scirrhous reaction in the fish liver that is located around the bile ducts. The fibrogenic effect of oxidative stress induced by Cd [36] is characterized by excessive connective tissue deposition in extracellular matrix. Lipocytes (hepatic stellate cells (HSC)) may be the cell type responsible for periductal fibrosis [37]. In a healthy liver, HSCs are quiescent cells and contain numerous A vitamin lipid droplets, constituting the largest reservoir of A vitamin in the rats body [38]. When the liver is injured due to viral infection or any hepatic toxins, HSCs receive signals secreted by damaged hepatocytes and immune cells, causing them to trans-differentiate from a resting A vitamin-rich cell into active, proliferating, fibrogenic, and contractile cell leading to hepatic fibrosis.
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Oxidative stress represents an imbalance between oxidant and antioxidant agents [39]. Cytoplasmic vacuolation and fatty degeneration associated with lipid accumulation was the pathological features observed by us in all experimental groups (table 1).

Table 1. List of severity of liver lesions in Prussian carps specimens

<table>
<thead>
<tr>
<th>Liver lesions</th>
<th>Control group</th>
<th>E1 10 ppm CdCl₂</th>
<th>E2 10 ppm CdCl₂ +2% garlic</th>
<th>E3 10 ppm CdCl₂ +2% cilantro</th>
<th>E4 10 ppm CdCl₂ +2% chlorella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive processes</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lymphocytic infiltrates</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibrosis of hepatic tissue</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vacuolization</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Necrosis</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Pyknotic nucleus</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Karyolysis</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
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</tr>
</tbody>
</table>

(−) none, (+) mild, (++) moderate, (+++) severe occurrence.

Liver fat accumulation in fish may be due to a various causes such as: greater capacity of wild or captive fish species to store large amounts of lipid (triglyceride) in their livers compared to mammals (G. J. ROSSOUW (1987), cited by J. C. WOLF & M. J. WOLFE [35]), overfeeding of captive fish with an excessively energy-rich diet [40], lipid peroxidation, caused by the feeding with diets high in polyunsaturated fats coupled with a lack of adaptive peroxisomal proliferation, the suppression of vitamin [41] or toxicant-induced [42]. In our study, this lipid vacuolization has led to an increase in the size of hepatocytes. But there are authors who found that beside the toxicological causes of hepatocites’ hypertrophy there are physiological ones such can be seen in reproductively active female fish [43] or in male fish which have been exposed to exogenous estrogenic compounds, due to the ability of estrogens to boost hepatocyte metabolism for vitellogenin (yolk lipoprotein) production [44]. Vacuolation of hepatocytes is a cellular defense mechanism against injurious substances and it is associated with inhibited protein synthesis, energy depletion, disaggregation of microtubules, or shifts in substrate utilization, as described by E. K. AJANI & B. U. J. AKPOILIH [45]. A. ANNABI & al. [46] previously noted the cytoplasmic vacuolization with lipid droplets and glycogen accumulation in the liver tissue of Mosquito fish (Gambusia affinis) tissues following acute and chronic cadmium exposure as well. Nuclear pyknosis observed in the four experimental groups (table 1) suggests that the manifestation of apoptotic cell death and karyolysis are followed by hepatocyte necrosis. This pathologic response of the fish liver to Cd is in agreement with those of S. A. OMER & al. [47]. The results listed above highlights the ameliorative effect of garlic, cilantro and chlorella powder on the fish liver histopathological changes. This could be due to their antioxidant properties in combating the free radical-induced oxidative stress and liver injury resulting from cadmium chloride exposure. In the same time, these plants produce chelating compound involved in heavy metal detoxification. Oxidative stress as a crucial factor in liver diseases, (H. CICHOŻ-LACH & A. MICHALAK [39]), affecting the major cellular components: proteins, lipids and DNA as a result of excessive production of reactive oxygen species (ROS) [48] such as superoxide ions, hydroxyl radicals, and hydrogen peroxide. Protective actions against ROS are performed by several enzymes (e.g., superoxide dismutase-SOD, catalase and glutathione peroxidase), as well as non-enzymatic compounds (e.g., tocopherol, E vitamin beta-carotene, ascorbate and glutathione-GSH) [49] many of them can be found in vegetables and microalgae. Thus, carotenoids play an important role in quenching reactive oxygen species (ROS) generated during photosynthesis, especially singled oxygen [50]. Green leafy vegetables are rich in carotenoids, which have several proven and postulated health benefits. Carotenoids mostly consumed in general diets are α-carotene, β-carotene, lutein, zeaxanthin, neoxanthin, violaxanthin, and lycopene. Among these carotenoids, β-carotene is the most important.
provitamin [51]. There are studies showing that carotenoids contribute significantly to the total antioxidant capacity of microalgae [52]. L. C. WU & al. [53], has demonstrated that antioxidants from spirulina and chlorella water extracts are able to inhibit the proliferation of HSCs. A potential treatment for liver fibrosis is to inhibit activated hepatic stellate cell (HSC) proliferation and, subsequently, to induce HSC apoptosis. The phenolic compounds salicylic, trans cinnamic, synaptic, chlorogenic, chimic and caffeic acids found in the methanolic chlorella extract may contribute to its higher antioxidant activity [54]. F. J. KAO & al. [55] analyzed the total carotenoid content (TCC; i.e., lutein, zeaxanthin and β-carotene) in 25 fresh vegetables commonly consumed in Taiwan, and found cilantro, Thai basil leaves, sweet potato leaves, and choy sum contain the highest amount of TCC carotenoid-rich vegetables. Garlic increases antioxidant action by scavenging reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes and increasing glutathione in the cells [16]. M. ABDEL-TAWWAB & al. [56] reported that garlic contains numerous sulphur components, including the most valuable sulphhydryl groups, which oxidize mercury, copper, cadmium and lead, make these metals water-soluble, and facilitate the body excretion of these substances. On the other hand, chelators have the effect of mobilizing metals from tissues and maintaining the chelate moiety during circulation to the kidneys for excretion in urine, and to the liver for excretion in the bile [57]. Not only animals, but also plants produce chelating compounds (R. PAL and J. P. N. RAI, [58] and metallothionein content of foods may affect bioavailability as well as metabolism of toxic metals such as cadmium [59]. Thus, chlorella is a mild chelator. Its detoxification capacity is due to its unique cell wall extremely resistant to breakage and the material associated with it, sporopollenin (a naturally occurring carotene like polymer, which is resistant to degradation) [60]. Cilantro is also considered as a natural chelator. In animals, it decreased lead absorption into bone and inhibition of the delta-aminolevulinic acid dehydratase (ALAD) enzyme [61]. Garlic is a good source of N-Acetyl-L-Cysteine (NAC). N-acetyl-cysteine (NAC), contained in garlic is a chelator of toxic elements and may stimulate glutathione synthesis, and production.

4. Conclusions
The current result indicates that Cd contamination definitely affects the liver, inducing pathological lesions, which can be summarized as follow: cytoplasmic vacuolization, blood congestions, lymphocytic infiltrates, fibrosis and necrosis. Concomitant treatment with Cd+cilantro, Cd+garlic and Cd+chlorella powder showed evident recovery and an almost normal liver architecture with mild residual degeneration. Among the three lyophilized products, cilantro powder proved to have the most chelating and antioxidant potential (liver samples showed few lipid droplets and little focal necrotic area), while the response at chlorella treatment was less effective.

References
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