Garlic, Cilantro and Chlorella's Effect on Kidney Histoarchitecture Changes in Cd-intoxicated Prussian carp (*Carassius gibelio*)

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Abstract

Natural chelators from some natural sources have been shown their detox heavy metals ability in human and animals. So the present study was carried out to histological compare the aspect of kidney tissue of Prussian carp's 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 as $CdCl_2$ x ½ H_2O), 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).

The potential protective effect of the three lyophilized products against the impact of cadmium toxicity was evaluated in terms of hystopathological characteristics. For this purpose, fragments of kidney were removed and routinely processed at the end of experimental period and analyzed in light microscopy. A specific QuickPHOTO Micro 2.2 software has been used for the histological study.

Tissue alterations were assessed using the histopathological score ranging from - to +++ depending on the degree and extend of lesions: (-) none, (+) mild occurrence, (++) moderate occurrence, (+++) severe occurrence.

Cd contamination has definitely affected the kidney, inducing severe damage in its structure as: swelling and hypertrophy of tubules with nuclear deterioration, pyknosis and cariorrexis, nucleus and cytoplasm degeneration, capillary ectasia and congestions.

Active compounds from garlic and cilantro powder have shown the most chelating and antioxidant potential, leading to the evident recovery of kidney architecture, while the response at chlorella treatment was less effective than E2 group and without significant difference compared with E3 group.

Keywords: cadmium toxicity, fish, kidney histopathology, lyophilization, natural chelators

1. Introduction

Toxic metals comprise a group of minerals that have no known function in the body and, in fact, are very harmful to living organisms. They are also persistent and cumulative. Cadmium is among the most toxic of the heavy metals due to its ubiquity, toxicity, and long half-life [1]. 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 [2].

Chelation—pulling heavy metals from cells with synthetic or natural agents-is the standard treatment for heavy metal toxicity.

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Beside of advantages of synthetic chelating agents such as DMPS, DMSA, EDTA, etc. (their strong ability to capture and removing poisonous metals from the body), there are disadvantages to using them. Even when this treatment is given in low doses are themselves reported to have a number of different safety and efficacy concerns, including: random and erratic removal of vital minerals along with the toxic ones [3-5]; unbalancing the body; toxicity of many chelating agents; removal of toxic metals is not thorough enough; chelation does not remove a dozen toxic metals very well; chelation does not address some deeper causes of illness, and indeed often makes body chemistry more unbalanced; chelation removes toxic metals in an unnatural order; cost and necessity, no matter what anyone claims; chelation therapy is an allopathic, or drug approach to healing [5].

But natural chelators from some vegetal sources, if used properly have proved to be highly effective at removing heavy metals from the body [6-16].

Fish has now been considered as the model organism for conducting different experimental studies, including those of pharmacology and toxicology [17].

Histopathology is the microscopic evaluation of disease processes. Histopathological indices have been largely used as biomarkers in the monitoring of fish health status during exposure to pollutants such as heavy metals, both in the experimental and environmental studies [18-21].

Kidney is the principle target organ of cadmium toxicity and chronic cadmium exposure in almost all animal species. Cadmium toxic effect is characterized by varying degree of renal damage [22-24].

This study aimed to determine firstly the histopathological effects induced by sub-lethal concentration of cadmium on renal histoarchitecture and secondly to highlight the garlic, cilantro and chlorella's powder abilities in reduction of kidney alterations resulting from cadmium toxicity.

2. Materials and methods

Biological material

Prussian carp fingerlings *(Carassius gibelio* Bloch) were chosen as test species on the basis of accessibility and ecologically representativeness. Individuals belonging to this species are easily acquired and adapted to captivity. Fresh fish samples were caught from a local farm and gravimetrically selected. Thus, individuals of 10-12 g weight were acclimated for two weeks under laboratory conditions prior to experiment, removing those with suspicions on their health. Then, 150 Prussian carp's fingerlings were transferred in 5 separate glass aquariums equipped with aeration system and a 60 L capacity at a stocking density of 30 fishes per aquarium. The control and test organisms were randomly distributed in test solutions according to doses of cadmium, garlic, cilantro, chlorella and their combinations as follows: the control ones were maintained in Cd-free freshwater, the others four groups receiving 10 ppm Cd into the water as $CdCl_2$ x ½ H_2O (E1), 10 ppm Cd into water+2% lyophilized garlic in feed (E2), (10 ppm Cd into the water+2% lyophilized cilantro in feed (E3), 10 ppm Cd into the water+2% lyophilized chlorella in feed (E4) respectively.

Chemicals

Fish in groups E1, E2, E3 and E4 were exposed for 21 days to chronic cadmium intoxication as $CdCl_2$ x ½H_2O in concentration of 10 ppm from a stock solution. The sub lethal treatment was calculated from percentage mortalities of fish as described by Veena et al. [25].

Fish nutrition

Fish were fed twice a day with commercial dry pellets. Freeze-dried garlic, cilantro and chlorella's incorporation in fish's feed has involved the following steps:

- bringing the dry pellets to fine size particles;
- 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 Oprea and Georgescu [26];
- wetting and homogenization of the mixture obtained;
- regranulation and drying of the final pallets (Figure 3).

Chlorella pyrenoidosa powder was produced by Bucharest Medical Laboratories; garlic (Figure 1) and coriander (Figure 2) powders were obtained by lyophilization to 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 lyophlization efficiency was 38.07% for garlic and 10.55% for cilantro respectively.

Figure 1. Lyophilized garlic transformed into fine powder

Figure 2. Lyophilized cilantro and transformed into fine powder

Figure 3. Final regranulated pallets designed to carps' feeding

Monitoring of physical-chemical parameters of the water in the aquariums

Physical-chemical indicators of water were daily measured throughout the exposure: water temperature and dissolved oxygen with a movable oxygen-meter with water resisting microprocessor Hanna HI 9145; pH, NO_2^-, NO_3^-, pH, hardness of water with a photoFlex colorimeter and a Germany termatest kit.

The controlling conditions included (T: 19-21°C; pH: 7.44-8; total hardness: 120 mg/l; DO: 7.5-8 mg O_2 L^{-1}; nitrites: <0.3 mg/l; nitrates: <12.5 mg/l).

The medium was weekly renewed by adding $CdCl_2$ from a stock solution to maintain the constant concentration of Cd and a 12 h light/12 h dark photoperiod was assured.

Histopathological examination

Kidney samples were removed at the end of a 21 days experimental period after fish euthanasia with an overdose of the anesthetic tricaine methanosulfonate (MS-222) (>250 mg/L) [27]. The material was subjected to histological analysis, being fixed in a 10% formalin solution, dehydrated, clarified, impregnated and embedded in histological paraffin, according to standard routine methods in the Histology laboratory [28]. Five-micrometer-thick sections were obtained and stained by Mallory's trichrome method. Kidney 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 histopatological study.

Tissue alterations were assessed using the histopathological score [29] ranging from – to +++ depending on the degree and extend of lesions: (-) none, (+) mild occurrence, (++) moderate occurrence, (+++) severe occurrence.

Statistical Analysis

Statistical analysis was performed using SPPS IBM 22 software. Data were reported as a significant level of $p<0.05$. Testing differences between means was realized by ANOVA completed with post-hoc Tukey test.

3. Results and discussion

Histopathological study of the kidney (group C)

In the structure of the renal parenchyma, the study of histological sections from control samples points out the normal aspect of nephrons. They consist of renal corpuscles and renal tubules (Figure 4).

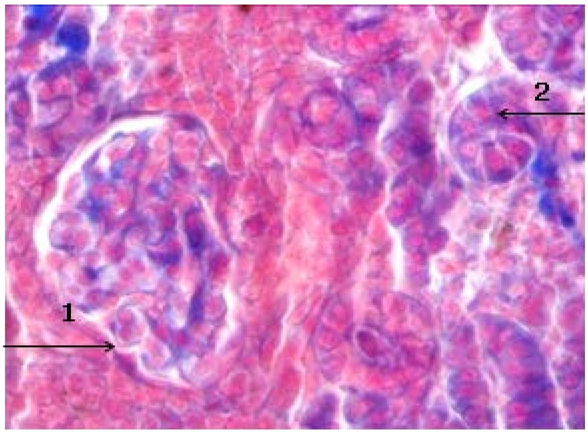

Figure 4. Kidney – C
1: renal glomerulus; 2: renal tubules
(trichrome Mallory staining, 1000x)

Renal corpuscles include renal glomerulus and Bowman'n capsule with double walled. The walls of renal tubules are lined with nephrocyte cubic or prismatic epithelium provided with obvious brush border to the apical pole. Nephrocytes have a spherical heterochromatic nucleus, and a basophil and abundant cytoplasm (Figure 5). Capillary vascular network composed of peritubular capillaries with small lumen is noticed.

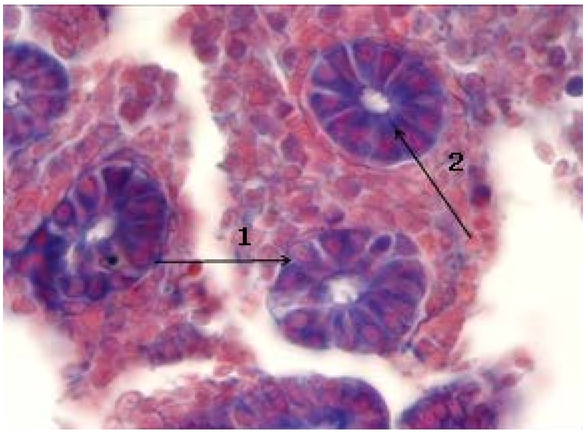

Figure 5. Kidney – C
1: renal tubules; 2: brush border
(trichrome Mallory staining, 1000x)

Histopathological study of the kidney (group E1)
Microscopic study of sections made through the kidneys belonging to Cd contaminated fish shows a number of changes in the renal parenchyma, but also in the stroma. Thus, glomerular and peritubular capillary hypertrophy is an issue frequently raised (Figure 6-7), having the appearance of some small bleeding "lakes" in certain territories.

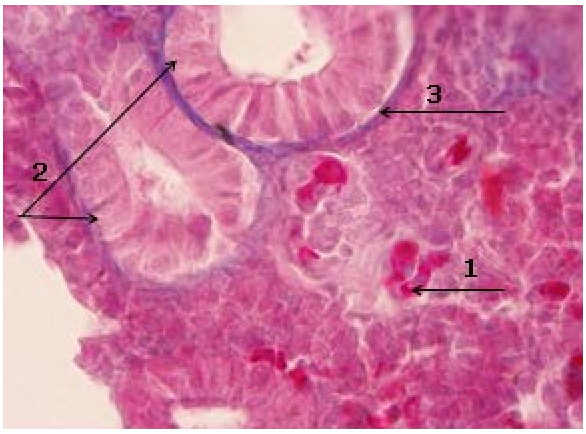

Figure 6. Kidney – E1
1: capillary hypertrophy; 2: clear cytoplasm;
3: fibrotic processes
(trichrome Mallory staining, 1000x)

At the uriniferous tubules on relatively large areas, nephrocyte epithelium has a disorganized looks, cells are increased in volume, having clear cytoplasm and peritubular edema are formed (Figure 7). In the stroma, local, peritubular and interstitial fibrotic processes and leukocyte infiltrations are reported.

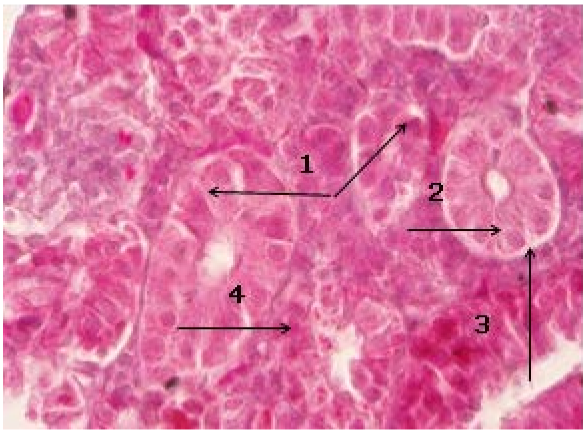

Figure 7. Kidney – E1
1: disorganized looks of nephrocyte epithelium
2: clear cytoplasm; 3: peritubular edema;
4: leukocyte infiltrations
(trichrome Mallory staining, 400x)

On small areas, nephrocyte epithelial cells have hypertrophic appearance with clear cytoplasm and pyknotic nucleus, and their basal plasmalemma separates from subepithelial basement membrane.
Loss of the contact cells with the basal membrane causes peritubular edema (Figure 8).

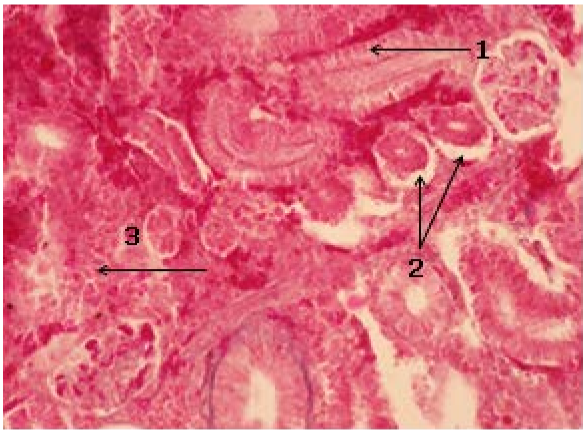

Figure 8. Kidney – E1
1: clear cytoplasm; 2: peritubular edema;
3: leukocyte infiltrations
(trichrome Mallory staining, 400x)

Arteriolar peritubular capillary network is hypertrophic (Figure 9) and vascular congestions are signaled on large areas.

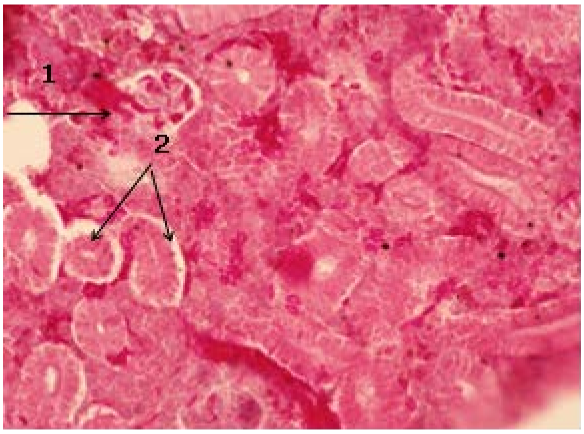

Figure 9. Kidney – E1
1: bleeding "lakes"; 2: peritubular edema
(trichrome Mallory staining, 400x)

Several other researchers were also recorded more or less similar results. Thus, degeneration and hypertrophy of epithelial cells of renal tubules were observed by Zeinab et al. [30], Imam et al. [31], Hussein et al. [32], Farzad et al. [33], Bhuyan et al. [34], Asia Al-Mansoori et al. [35] in kidney of fish exposed to cadmium and other water- or foodborne pollutants. Peritubular capillary network hypertrophy, massive vascular congestions [36] and even hemorrhages [31, 33, 37, 38] in renal parenchyma were histopathological changes presented in kidney samples from fish specimens in experimental studies on the toxic effect of cadmium chloride. Glomerular edemas were noticed in experimental kidney sections from fish exposed to cadmium sulphate or cadmium chloride as well [39, 40, 41].

Similarly to our findings, nephrocyte cytoplasmic vacuolization, pyknotic nucleus [31, 42, 43, 44], peritubular fibrosis [45], leukocyte infiltration between renal tubules and glomeruli [35], are other lesions occurred in previous papers.

Congestion and hemorrhage in kidney may be attributed to the decline in the hematological parameters as Kaoud et al. [46] and Zaki et al. [47] observed, since the hemoglobin contents are insufficient for the respiration of the tissue. Rothstein (1959) cited by Hadi [48] argued that metals affect permeability of cell membranes and resulting in inhibition of the ion-transporting systems causes disturbances in fluid transport from and into cells. Hadi and Alwachi, (1995) [49] explain incidence of congestion and hemorrhage in catfish, *Clarias lazera*, kidney under aluminum exposure in that the metal integrate with cell membranes causing a large amount of fluid filtrate, as well as disperse of serum albumin and red blood cells in the interstitial substance due to destruction of capillaries endothelium.

Weber et al. (2003) [50] showed that pyknotic and fragmented nuclei, as indicators of apoptotic and necrotic cell death, were mostly observed in the epithelial cells of proximal and distal convoluted tubules and rarely associated with other renal cells. Moreover, they added that the occurrence of dilated tubules appears to be a consequence of dead and dying epithelial cells, while a thickening of Bowman's capsule can arise as a result of fibrosis.

Leukocytes infiltration in kidney show immunological defense of the fish as response to heavy metals stress manifested by of cell irritability, inflammation and hypersensitivity [51-53].

Cadmium is known to produce free radicals and oxidative stress which is one of the mechanisms of its toxicity [16] as it combines with thiol groups of enzymes involved in antioxidant mechanisms and inactivates them.

Kidney tissue contains a thiol-rich protein named metallothionein; MTs, a group of stress response proteins induced at a high level by reactive oxygen species or heavy metals, is generally considered to be a critical defense mechanism in several organisms [54]. Exposure to toxic metals triggers the production of this protein which binds tightly to the metal, retaining it in the kidney tissue in a relatively harmless form [55]. But when kidney's capacity for production of metallothionein is overwhelmed (acute high doses of metal, or an increase in the chronic dose level), it can readily precipitate renal failure with out its mechanism toxicity to be clearly understood [56].

Histopathological study of the kidney (group E2)

Light microscopic study of the kidney sample from fish exposed to Cd and garlic powder supplemented diet (group E2) showed some changes. Renal corpuscles are congested (Figure 10) and slightly hypertrophic on small areas, due to the glomerular capillary hypertrophy (Figure 11). Easy hypertrophic capillary vessel network is disposed around the renal tubules, she becoming congestive on narrow area.

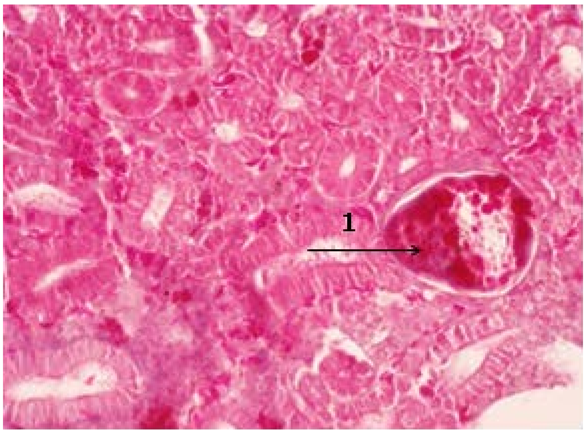

Figure 10. Kidney – E2
1: congested renal corpuscle
(trichrome Mallory staining, 400x)

Also, peritubular edema occurs as a result of nephrocyte epithelial cells detachment from their basal membrane and only on small areas (Figure 11).

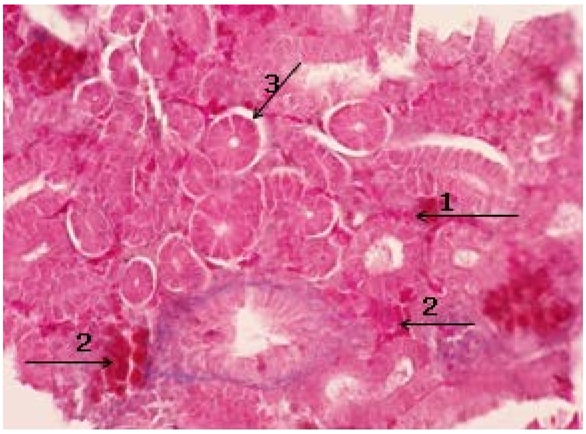

Figure 11. Kidney – E2
1: glomerular capillary hypertrophy;
2: congestive capillaries; 3: peritubular edema
(trichrome Mallory staining, 400x)

Simultaneous treatment of fishes exposed to cadmium chloride with garlic extract showed significant less severity in comparison to exclusively cadmium chloride exposed groups. The histopathological observations for both the control and those samples exposed to sublethal cadmium were summarized in Table 1.

The efficiency of garlic was perhaps due to the presence of sulfur-containing amino acids and compounds having free carboxyl (C=O) and amino (NH_2) groups in their structures. These biologically active compounds might have chelated cadmium and enhanced its excretion from the body, resulting in reduced cadmium accumulation in tissues. Bilal and Kour [57] consider that the mechanism of *Allium sativum* mediated chelation of cadmium might include formation of ionic bonds between sulfur containing compounds and cadmium. Garlic's richness in sulphur-containing compounds derived from cysteine, essential elements and vitamins C and E may also protect from metal-catalyzed oxidative damage [58].

Histopathological study of the kidney (group E3)

Malpighi corpuscles of kidney samples from group E3 (Cd+cilantro), are heterogeneous in terms of their size and appearance. Some of them are hypertrophic with congested glomeruli and capsular space minimized; others have highly compressed glomeruli and reduced in volume. An obvious aspect is the hypertrophy of the peritubular capillary network, a process accompanied by numerous congestions (Figure 12).

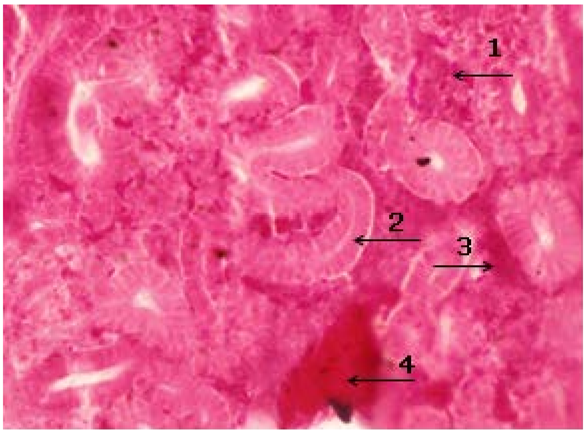

Figure 12. Kidney – E3
1: compressed glomerulus; 2: vacuolization; 3: peritubular capillary hypertrophy; 4. congestion
(trichrome Mallory staining, 400x)

It was shown that coriander extracts contain phenolic compounds and flavonoids, suggesting that these compounds contribute to the antioxidative activity. Phenolic substances such as flavonoids, cumarins, cinnamic acid and caffeic acids are believed to have antioxidant properties, which play an important role against degeneration [59]. In the same time cilantro is considered a natural chelator. In animals, it decreased lead absorption into bone and inhibition of the deltaaminolevulinic acid dehydratase (ALAD) enzyme (AGA & al. [60]). In the study, of Chinchore and Mahajan [61] performed on the *Bellamya bengalensis*, the rate of O_2 consumption was observed to be decreased in chronic concentration of $PbNO_3$ as compared to the control and $LC_{50}/_{10}$ with 5 ml/lit of aqueous extract of *Coriandrum sativum.* Instead dried cilantro does not work in Mercola and Klinghardt's [55] experiences, which suggests that the active substance is in the volatile fat-soluble portion of the plant probably an aromatic substance.

Table 1. List of severity of kidney's lesions in Prussian carps experimental specimens

Kidney's lesions	Control group	E1 10 ppm $CdCl_2$	E2 10 ppm $CdCl_2$ +2% garlic	E3 10 ppm $CdCl_2$ +2% cilantro	E4 10 ppm $CdCl_2$ +2% chlorella
Capillary hypertrophy	-	+++	+	++	+
Nephrocyte hypertrophy	-	+++	+	++	++
Peritubular edema	-	+++	+	+	+
Vascular congestive processes	-	+++	+	++	+
Cytoplasm vacuolization		+++	+	+	++
Fibrosis of renal tissue	-	+++	+	-	++
Pyknotic nucleus	-	+++	-	-	-
Leucocytes infiltrates	-	+++	-	-	-

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

Histopathological study of the kidney (group E4)

Kidney samples from group E4 (Cd+chlorella) have relatively uniform renal corpuscles in their size and appearance (Figure 13, 14). Among these corpuscles with normal aspect, corpuscles with congested glomerular capillaries there are. Nephrocyte epithelial cells are hypertrophic, with vacuolated cytoplasm with or without nucleus on small areas, (Figure 14). Arterial capillary network is hypertrophic, in certain territories.

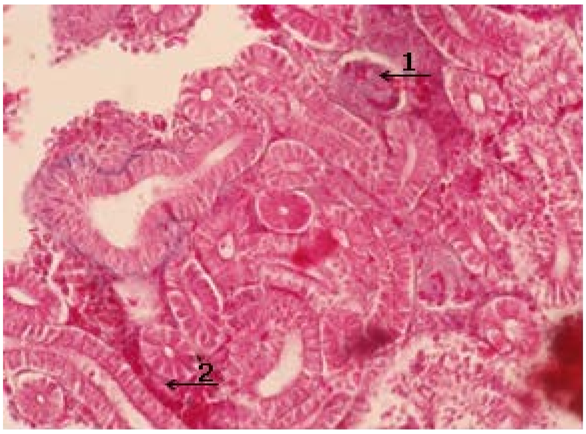

Figure 13. Kidney – E4
1: slightly congested glomerulus, 2: congested glomerular capillaries
(trichrome Mallory staining, 400x)

Simultaneous addition of chlorella powder has been found to diminish the cadmium chloride induced histopathological disorders in kidney. The lesions were less severe in the kidney when compared to the fishes exposed to exclusively cadmium chloride but more frequent when compared with those of group E2 and without significant difference compared with E3 group (Table 1).

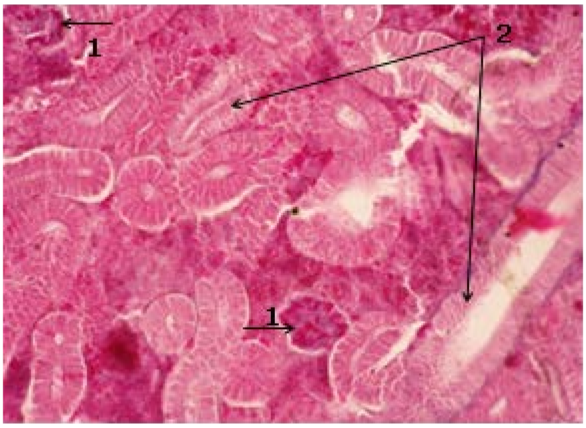

Figure 14. Kidney – E4
1: slightly congested glomerulus;
2: vacuolizations
(trichrome Mallory staining, 400x)

Chlorella si considered a mild chelator of heavy metals. Its heavy metals detox potential is based on its morphological characteristics. The mucopolysaccharides in chlorella's cell wall absorb rather large amounts of toxic metals similar to an ion exchange resin. Laboratory studies showed that there were two active absorbing substances - sporopollenin (a naturally occurring carotene like polymer which is resistant to degradation) and a microfibrillar, cellulase digestible layer deposited between the trilaminar component and the plasma membrane [66].

4. Conclusions

The severe histological changes observed in the Prussian carp's kidney after exposure, were characteristic of direct damage by cadmium and the secondary effects caused by a stress response.
Chronic cadmium intoxication was characterized by swelling and hypertrophy of renal tubules, nuclear deterioration (pyknosis and cariorrexis), cytoplasm degeneration (vacuolization), capillary ectasia and congestions.
Active compounds from garlic and cilantro powder have shown the most chelating and antioxidant potential, leading to the evident recovery of kidney architecture, while the response at chlorella treatment was less effective than E2 group and without significant difference compared with E3 group.

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