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Response of vitamins A, E, hematological and serum biochemical markers in Crucian carp (*Carassius auratus gibelio*) exposed to environmental Pb^{2+} and Cd^{2+}

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Toxicity of Pb^{2+} and Cd^{2+} is a widespread issue in the world; however, few studies have been conducted to understand their effect at environmentally realistic concentration in a mixture. In the present study, Crucian carp was exposed to Pb^{2+} (30 μg·l^{-1}), Cd^{2+} (100 μg·l^{-1}) and their mixture (30+100 μg·l^{-1}) for 96 h and 21 d period to assess changes in the liver and muscle vitamin A and E content, and hematological and serum biochemical parameters. The results indicated significant decline in the level of antioxidant vitamins A, E and alterations in the hematological and serum biochemical indices. The toxicity revealed anemia, impairment of the liver and kidney with evident responses after 21 d exposure due to additive effect of Pb^{2+} and Cd^{2+} in mixture. Moreover, the differential response of vitamins A, E and blood parameters to low levels of waterborne Pb^{2+} and Cd^{2+} in freshwater fish can be used as biomarkers for monitoring contamination of aquatic environment.

Key words: vitamins A and E, hematology, serum chemistry, Crucian carp (*Carassius auratus gibelio)*, Cd^{2+}, Pb^{2+}

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INTRODUCTION

Lead (Pb^{2+}) and cadmium (Cd^{2+}) are the two nonessential divalent metal ions recognized as the most hazardous pollutants to various ecosystems including human and freshwater fish (Zhang *et al.,* 2014). Beside natural occurrence, the anthropogenic sources of discharge into aquatic systems include industrial, agricultural, municipal and residential waste products (Wang *et al.,* 2014). Once entered into the aquatic ecosystem, they remain dissolved in waters or deposited in soil sediments to constitute a source of contamination for aquatic food chain (Thevenod, 2010).

Aquatic organisms can accumulate Pb^{2+} and Cd^{2+} through respiration, adsorption and ingestion and finally reach to the human body by ingestion. Among metals, Pb^{2+} and Cd^{2+} are classified as the potential toxicants due to their adverse effects, even at low concentrations over prolonged period of time (Guardiola *et al.,* 2013). Lead is known to impart a range of physiological, biochemical and behavioral abnormalities in the exposed subjects, including the central and peripheral nervous system, hematopoietic system, reproductive system, cardiovascular system along with liver and kidney dysfunctions (ATSDR, 2007). On the other hand, Cd^{2+} toxicity affects liver, kidney, lungs, reproductive organs, hematopoietic, cardiovascular and nervous systems and may cause hypertension, type 2 diabetes mellitus and cancer under low exposure for prolong time (Colacino *et al.,* 2014; Matović *et al.,* 2011).

Although the importance of Pb^{2+} and Cd^{2+} as environmental health hazards is widely accepted, specific toxicity mechanisms have yet to be fully elucidated. The molecular mechanism behind the biological effects of Pb^{2+} and Cd^{2+} are still unclear (Cannino *et al.,* 2009). It has been suggested that, after absorption, most of Pb^{2+} binds to proteins in erythrocytes and distribute to the soft tissues where it can cause mitochondrial damage by overproduction of ROS (Hambach *et al.,* 2013; Sabath & Robles-Osorio, 2012). On the other hand, Cd^{2+} is transported to the liver bound to blood albumin, where it binds to metallothionein (MT) and is released back into the blood stream as Cd–MT complex (Hambach *et al.,* 2013). To cope with ROS produced by toxic metals, animals have inherent defense mechanisms of enzymatic and non-enzymatic antioxidants. Some non enzymatic antioxidants such as vitamins A and E are consumed under the impact of environmental pollutants and their level may fall below the normal range (Barim & Karatepe, 2010). A disturbed blood profile associated with the toxicity of Cd^{2+} and Pb^{2+} has also been observed in experimental fish models, suggesting alterations in the hematological and biochemical parameters under the absorption of toxicants (Gioda *et al.,* 2007). The response of organisms to contaminants, including metals, can be measured in terms of biochemical changes and could be used as biomarkers. Investigating biomarkers of metal exposure can help in evaluating environmental behavior and impact of metals on fish, which can be used as early warning of pollution (vanderOost *et al.,* 2003).

Pb^{2+} and Cd^{2+} can interact with each other in a complex way and their co-exposure may induce addi-

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; d, days; h, hours; Hb, hemoglobin concentration; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBCs, red blood cells; ROS, reactive oxygen species.

tive, synergistic or new effects that are not observed for single element exposure (Johri *et al.,* 2010; Wang *et al.,* 2014). Previously, majority of animal studies have been conducted using single metal exposure at high concentrations; however, organisms in the contaminated environment receive simultaneous multi-element exposure at low concentrations, indicating the need for experimental work (Spurgeon *et al.,* 2010). Therefore, experimental work with combinations of metals for extrapolation of environmental effects on aquatic animals needs to be conducted. Fish play an important role in balanced and nutritious diet due to its high content of proteins, long chain polyunsaturated fatty acids, fat soluble vitamins and minerals particularly for the low income class. However, fish can get contaminated for human consumption because of bioaccumulation and negative effects of environmental pollutants and may become susceptible to stress and diseases (Belton & Thilsted, 2014; Guardiola *et al.,* 2013).

Crucian carp is an extensively cultured food fish in China and Europe because of its good taste, fast growing and adaptability to mono- and poly-culture in fishponds (Gui & Zhou, 2010). Moreover, Crucian carp has been used as bio-indicator of many persistent organic and inorganic pollutants (Khan *et al.,* 2014; Moon *et al.,*, 2006). Many studies have been conducted on the toxic effects of Pb^{2+} and Cd^{2+} on mammals and fish, suggesting a number of enzymatic, nonenzymatic and biochemical biomarker responses (Eyckmans *et al.,* 2013; Guardiola *et al.,* 2013; Hernandez-Garcia *et al.,* 2014; Wang & Fowler, 2008). However, to the best of our knowledge, there is no information available on the individual and combined effects of Pb^{2+} and Cd^{2+} on vitamins A and E contents and hematological and biochemical markers in Crucian carp. Therefore, the present study elucidated and compared the response of these biomarkers to individual and combined exposure to environmental Pb^{2+} and Cd^{2+} in Crucian carp.

MATERIALS AND METHODS

Reagents. Vitamin A (all-trans-retinol, R2500) and vitamin E (α-tocopherol, T3251) were purchased from Sigma. Lead nitrate and cadmium chloride of purity > 99%, HPLC grade methanol and ethanol, butylated hydroxytoluene, HPLC grade n-hexane, concentrated (glacial) acetic acid,, sodium thiosulphate, ethylenediamine tetraacetic acid and 0.9% sodium chloride solution were purchased from Sinopharm Chemical Reagents Co., Ltd (Beijing, China). Water used for preparation of the reagents was passed through Millipore purification apparatus (Millipore, MA, USA) to a resistance of more than 18.0 MΩ · cm. ICP-Multi-element certified reference materials were obtained from PerkinElmer No.N9300281, Shelton, Connecticut, USA. All other chemicals were of analytical grade and were used without further purification.

Collection of fish and their maintenance. Healthy specimens of Crucian carp (*Carassius auratus gibelio)* with appropriate length (12±2.6 cm) and weight (92±4.2 g) were procured from a freshwater fish breeding farm in Wuhan and transported to the laboratory in plastic buckets filled with pond water. The fish were then acclimated to the laboratory condition for 1 week in a glass tank of 200 l capacity containing dechlorinated tape water with continuous oxygen supply. During the period of acclimatization, the fish were fed with commercial food on daily basis and half of the aquarium water was replaced every day to remove the wastes produced by the fish. Feeding was withheld 24 h prior to the commencement of the exposure assay. The aquarium water was dechlorinated by exposure to light and aeration before use and its physicochemical parameters were as follows: total hardness 148.61±3.57 $mg \cdot l^{-1}$ as $CaCO_3$, temperature 22.41±2.11°C, pH 7.6±0.31, dissolved oxygen 8.26±0.68 $mg \cdot l^{-1}$ as monitored by APHA methods (1992).

Exposure assay. The animals used for the experiments were of both sexes and, after acclimatization, were randomly divided into 4 groups with 5 fish in each group. Group 1 was assigned as the control with similar experimental conditions but without the addition of tested chemicals. Group 2 was exposed to elemental concentration of 30 $\mu g \cdot l^{-1}$ Pb^{2+} in the form of $Pb(NO_3)_2$, Group 3 was exposed to elemental concentration of 100 $\mu g \cdot l^{-1}$ Cd^{2+} in the form of $CdCl_2$, whereas Group 4 was exposed to the combination of the two metals at the same concentrations. The exposure duration for all the groups was 96 h as acute assay and 21 days as chronic assay. Feeding was not administered during the exposure and the aquarium water was monitored by Inductively Coupled Plasma/Optical Emission Spectroscopy to maintain the desired level of Pb^{2+} and Cd^{2+} in the tanks. The respective concentrations of Pb^{2+} and Cd^{2+} were selected on the basis of previous investigations, which indicated significant alterations in oxidative stress biomarkers of the exposed fish and induced a pro-oxidant condition (Khan *et al.,* 2015; Qu *et al.,* 2014). Moreover, these concentrations are somewhat relevant to the contamination level of these metals in rivers and lakes of central China (An *et al.,* 2010; Bing *et al.,* 2013; Li *et al.*,, 2013; Wang *et al.,* 2012; Yang *et al.,* 2009; Zhou *et al.,* 2007). The study was conducted according to the guidelines of Chinese Law for Animal Health Protection and Instructions for Granting Permits for Animal Experimentation for Scientific Purposes (Ethics approval No. SCXK (YU) 2005–0001).

Blood and tissue collection. At completion of 96 h and 21 days exposure periods, blood samples were drawn from the fish caudal vein with the help of disposable syringe. The samples were transferred to heparinized glass tubes and immediately analyzed for hematology, while the blood samples for biochemical parameters were allowed to stand for 30 min in separate tubes. After clotting, the samples were centrifuged at 3000 rpm for 5 min and the decanted serum was stored at –80°C till analysis. Fish from all the groups were then sacrificed to obtain liver and muscle tissues and stored immediately at –80°C till complete analyses were performed.

Determination of tissue vitamins A and E. Tissue levels of vitamins A and E were determined as described earlier (Barim & Karatepe, 2010; Tan *et al.,* 2007) with little modifications. Briefly, 0.5 g tissue was homogenized in a glass homogenizer containing 1 ml cold acetone, 2 ml methanol and 100 µl 250 mM butylated hydroxytoluene in absolute ethanol. The homogenate was then transferred to a polyethylene tube followed by the addition of 0.5 ml n-hexane to extract the vitamins. The tubes were then centrifuged at 10000 rpm for 10 min at 4°C. This step was repeated twice. The residue left after evaporating n-hexane by a stream of nitrogen was dissolved in 250 µl of absolute ethanol and used for HPLC analysis. The chromatogram was monitored at 292 nm simultaneously for both vitamins A and E using the HPLC system (Waters Corporation,

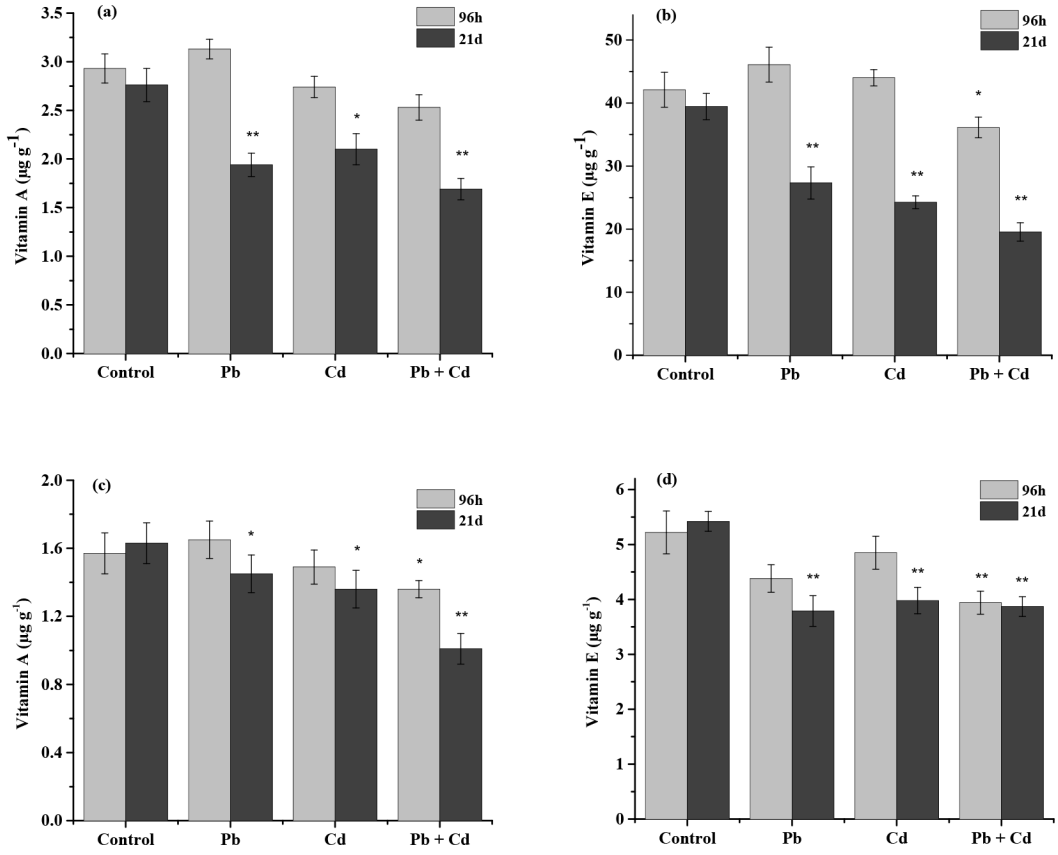

Figure 1. Alterations of vitamins A and E in the liver (a), (b) and muscle (c), (d) of Crucian carp exposed to environmental Pb^{2+} (30 μg · l^{-1}), Cd^{2+} (100 μg · l^{-1}) and Pb^{2+}+Cd^{2+} (30 μg · l^{-1} +100 μg · l^{-1}) for a period of 96 h and 21 days.
The values are presented as mean ± S.D. Significant differences are shown at $P<0.05$ (*) and $P<0.01$ (**) in comparison to their respective control groups.

USA) equipped with Waters e2695 separation module, Waters 2998 photodiode array detector and VP-ODS column (150 mm × 2.0 mm; particle size 5 μm). The injection volume and flow rate were set to 20 μl and 1 ml · min^{-1}, respectively.

Hematological analyses. Hematological measurements were performed by using Coulter HmX Hematology analyzer (Beckman Coulter Inc., Fullerton, CA, USA). The hematological evaluations included red blood cells count (RBCs), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

Biochemical analyses. Serum biochemical investigations were assessed with Synchron Clinical system CX4 (Beckman Coulter, Brea, CA USA) as directed by the manufacturer (Beijing Leadman Biochemistry Technology Co. Ltd., Beijing, China). The parameters included blood glucose level, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).

Statistical analysis. Statistical analyses were performed by SPSS 16.0 Chicago USA. Differences among the control and exposed groups were tested by one way analysis of variance (ANOVA) followed by Duncan Post-hoc test. All the values were expressed as mean ± S.D. The significance levels were defined as $P<0.05$ and $P<0.01$. All the graphs were constructed by ORIGIN 9.0 software (Origin Lab CO., Northampton, MA, USA).

RESULTS

Tissue vitamins A and E

The levels of vitamins A and E in the liver and muscle tissues of Crucian carp after 96 h and 21 days exposure to environmental Pb^{2+} and Cd^{2+} are presented in Fig. 1. In general, the contents of vitamins A and E were found lower in the muscle than in the liver tissue. However, significant differences were observed in both vitamins after treating the fish with Pb^{2+} and Cd^{2+}. As shown in Fig. 1a, significant reduction in the vitamin A content was noticed in the liver under the exposure to Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} for 21 d with a decrease of 30%, 24% and 39%, respectively. Similarly, reduction in vitamin E content was 31%, 38% and 50% in the liver after 21 d exposure to Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+}, respectively, while 96 h exposure caused only 14% decline in vitamin E due to Pb^{2+} + Cd^{2+} exposure (Fig. 1b). In case of muscle, vitamin A content decreased by 13% after 96 h exposure to Pb^{2+} + Cd^{2+}, while exposure to 21 d resulted in a decrease by 11%, 17% and 38% for Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} , respectively (Fig. 1c). On the other hand, changes in muscle vitamin E was only noticed in Pb^{2+} + Cd^{2+} exposed fish after 96 h, but after 21 d exposure a significant reduction was observed in Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} exposed fish with a decrease of 30%, 27% and 29%, respectively (Fig. 1d).

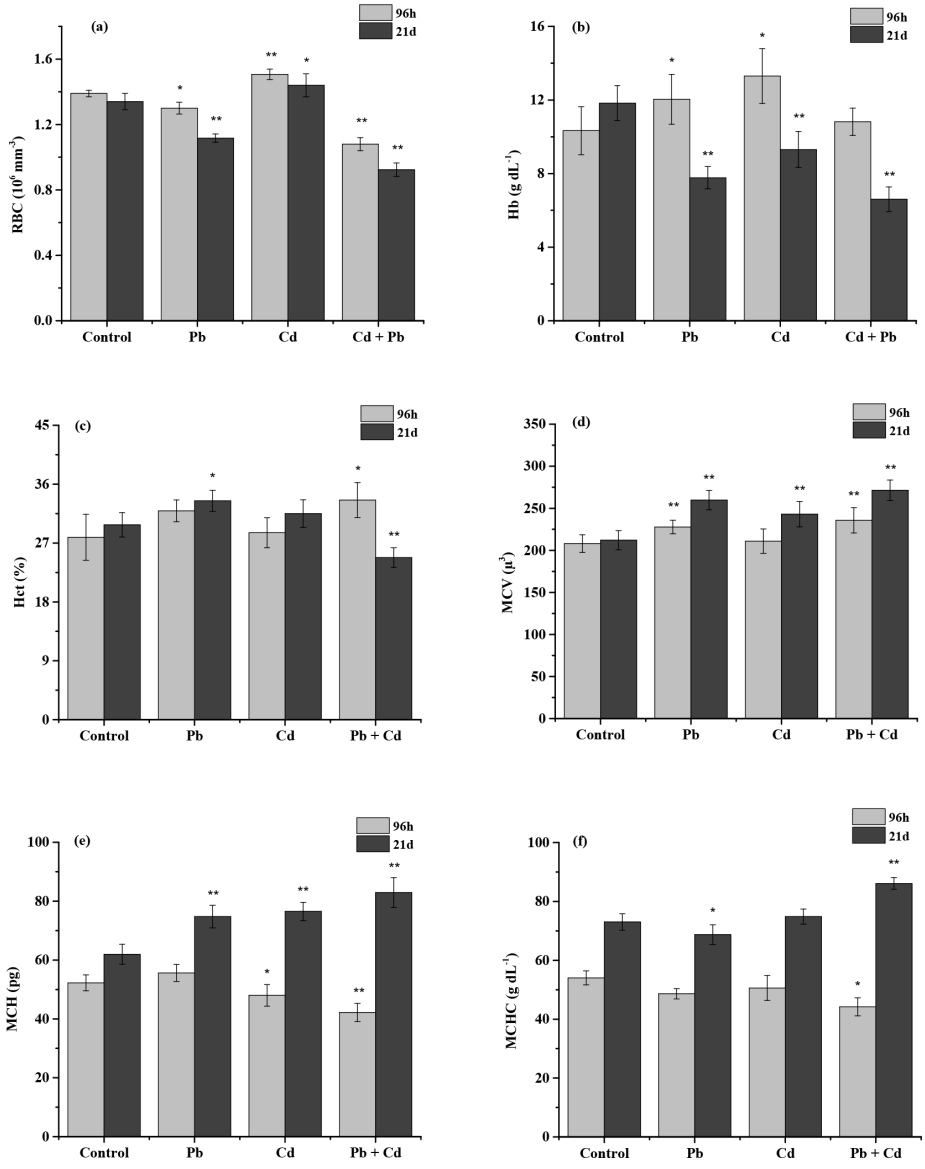

Figure 2. Changes in the hematological parameters of Crucian carp exposed to Pb^{2+} (30 μg · l^{-1}), Cd^{2+} (100 μg · l^{-1}) and Pb^{2+}+Cd^{2+} (30 μg · l^{-1} +100 μg · l^{-1}) for a period of 96 h and 21 days.
(**a**) Red blood cells (RBCs), (**b**) hemoglobin (Hb), (**c**) hematocrit (Hct), (**d**) mean corpuscular volume (MCV), (**e**) mean corpuscular hemoglobin (MCH), (**f**) mean corpuscular hemoglobin concentration (MCHC). The values are presented as mean ±S.D. Significant differences are shown at $P<0.05$ (*) and $P<0.01$ (**) in comparison to their respective control groups.

Hematological parameters

Hematological analysis of fish exposed to various levels of Pb^{2+} and Cd^{2+} revealed significant changes in the selected parameters. The RBCs count decreased in Pb^{2+} and Pb^{2+} + Cd^{2+} groups but showed an increase in Cd^{2+} group under the two exposure regimes (Fig. 2a). The content of Hb was elevated for Pb^{2+} and Cd^{2+} groups after 96 h exposure but declined significantly for all the three treatments as compared to the control group after 21 days exposure (Fig. 2b). The Hct percentage did not change under 96 h exposure, except for Pb^{2+} + Cd^{2+} group, which causes significant increase in the percent Hct, whereas 21 d exposure enhanced the percentage of Hct in Pb^{2+} group, but caused a decline in the Pb^{2+} + Cd^{2+} group (Fig. 2c). A slight increase in the MCV was observed for all groups except for Cd^{2+} group, which did not show any significant change after 96 h exposure (Fig. 2d). The MCH content showed a decrease for all the treated groups after 96 h exposure except of the Pb^{2+} group, but finally increased during 21 d exposure (Fig. 2e). Similarly, MCHC first decreased and then increased significantly after exposure to Pb^{2+} and Cd^{2+} for 96 h and 21 d, respectively (Fig. 2f).

Biochemical parameters

As shown in Fig. 3. fish exposed to Pb^{2+} and Cd^{2+} under different exposure regimes revealed significant changes in the serum biochemical indices. The level of glucose first increased when fish were exposed to Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} for 96 h but subsequently decreased in the groups exposed to Pb^{2+} and Pb^{2+} + Cd^{2+} for 21 d (Fig. 3a). Blood urea nitrogen (BUN) showed a gradual increase to the fish exposed to Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} for 21 d . However, the increase was only

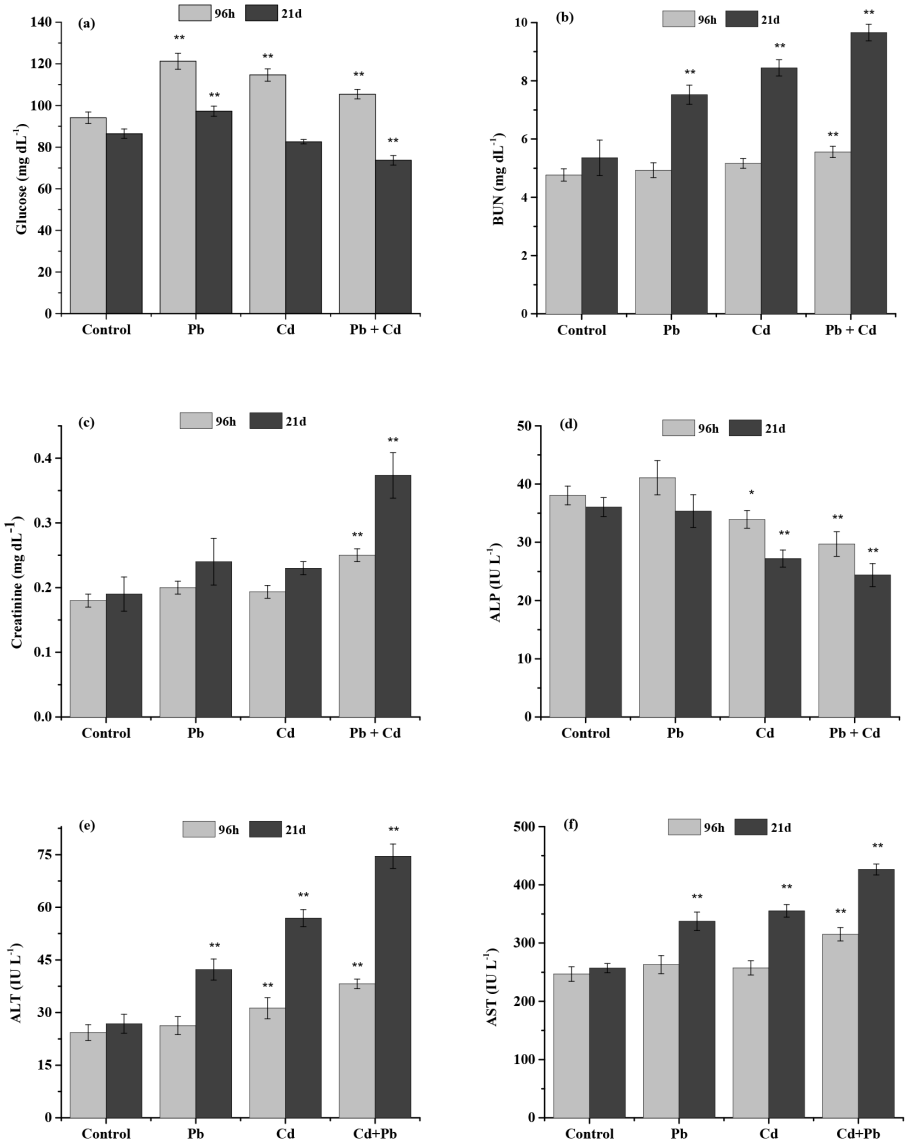

Figure 3. Changes in the serum biochemical parameters of Crucian carp exposed to Pb^{2+} (30 μg · l^{-1}), Cd^{2+} (100 μg · l^{-1}) and Pb^{2+}+Cd^{2+} (30 μg · l^{-1} +100 μg · l^{-1}) for a period of 96 h and 21 days.
(**a**) Glucose, (**b**) blood urea nitrogen (BUN), (**c**) creatinine, (**d**) alkaline phosphatase (ALP), (**e**) alanine aminotransferase (ALT), (**f**) aspartate aminotransferase (AST). The values are presented as mean ± S.D. Significant differences are shown at $P<0.05$ (*) and $P<0.01$ (**) in comparison to their respective control groups.

observed in the Pb^{2+} + Cd^{2+} group after 96h (Fig. 3b). On the other hand, the creatinine level did not change significantly due to individual metal exposure but exposure to the combination of Pb^{2+} and Cd^{2+} for 96 h and 21 days caused noticeable increase in the serum creatinine level (Fig. 3c). Serum ALP level showed a slight to moderate decline when the fish were exposed to Cd^{2+} and Pb^{2+} + Cd^{2+} for 96 h and 21 d but did not show any change in the Pb^{2+} exposed group (Fig. 3d). In contrast, a gradual increase was observed in the serum ALT level of all the treatments, except for fish exposed to Pb^{2+} for 96 h. The highest increase in ALT was caused by the exposure to the combination of both metals (Fig. 3e). Similarly, the serum AST level was also elevated in all the treated fish after 21 d period but no significant change was noted in the Pb^{2+} and Cd^{2+} exposed fish after 96 h (Fig. 3f).

DISCUSSION

Vitamins A and E along with hematological and biochemical profiles of blood can be useful in providing information about the organism's internal environment. Investigation of such parameters under toxic environment is employed both to understand the normal and pathological conditions of the organism as well as to comprehend variable responses of certain parameters as biomarkers of sublethal toxicity (Li *et al.,* 2011; Viarengo *et al.,* 2007).

Among different organs, liver has a well-known role in the metabolism of metals and it also serves as the site of detoxification for toxic substances. In many studies, fish liver has been used as indicator of environmental pollution while fish muscle is an important source of nutritious food for human (Barim & Karatepe, 2010; Sis-

car *et al.,* 2014). Vitamins A and E have an important role in protecting the cells and tissues from the adverse effects of ROS. A decline in the content of these vitamins under different exposures to Pb^{2+}, Cd^{2+} and Pb^{2+} + Cd^{2+} might be due to induction of oxidative stress. Heavy metals, including Pb^{2+} and Cd^{2+}, are known to induce oxidative stress by producing ROS and to interfere in retinoid metabolism (Defo *et al.,* 2014). Different responses of vitamins A and E in the present study might be dependent on the metal, its level and the duration of exposure or on the fish species (Khan *et al.,* 2014). In a previous study, a decline in the non-enzymatic antioxidants including vitamins A and E to environmental pollution was found in the Crayfish, which is in line with the present findings (Barim & Karatepe, 2010).

Hematopoietic system is the most sensitive in revealing pathological conditions under the stress induced by toxicants in both human and animals (Liju *et al.,* 2013). In the present study, a decrease in the RBCs, Hb and Hct by the exposure to Pb^{2+} and Cd^{2+} separately or in combination indicates anemic condition of fish, resulting from the hemolytic effect of these metals. In similar studies, anemic condition was also diagnosed in fish exposed to different metals suggesting a decline of hematological parameters (Ates *et al.,* 2008). Another reason for the anemic condition of fish might be the inhibition of erythropoiesis in the hemopoietic organisms. Reduction in the RBCs count occurs mainly due to internal hemorrhages or abnormal osmoregulation under stress (Kavitha *et al.,* 2010; Ramesh *et al.,* 2014). Inhibition of erythropoiesis by the action of toxic metals may also lead to a decline of RBCs. Generation of ROS by Pb^{2+} and Cd^{2+} and altered membrane permeability for intestinal Fe could be another reason for decreased Hb in the exposed fish. The Hct value declines due to less O_2 in the fish blood because it generally depends on the O_2 carrying capacity of blood (Saravanan *et al.,* 2011). An increase in MCV and MCHC shows swelling of RBCs as a result of sphaerocytosis (Sobecka, 2001). The decrease in the MCH value reveals a compensation for lower O_2 supply due to gill damage (Ates *et al.,* 2008). It can be deduced from the present study that hematological responses are good indicators of fish health under the low dose toxicity of Pb^{2+} and Cd^{2+} exposure. Moreover, metal specific alterations in these parameters can be used as a biomarker in future investigations.

The most obvious effect of toxicants on carbohydrate metabolism includes changes in glucose, glycogen and lactic acid contents. Among these, blood glucose is a potent indicator of disturbed carbohydrate metabolism under toxic metal exposure (Kavitha *et al.,* 2010). The initial hyperglycemic condition may be due to increased utilization of glucose to cope with the metabolic demands of the fish because of stress produced by Pb^{2+} and Cd^{2+}; whereas, the reason for hypoglycemia in fish after prolonged exposure to Cd^{2+} and Pb^{2+} + Cd^{2+} may be due to damages or changes in glycogenic and glycolytic pathways. Variations in the blood glucose level are associated with stress produced by metals and other toxicants, which were also indicated in many other studies (Kavitha *et al.,* 2010; Li *et al.,* 2011; Saravanan *et al.,* 2011). Blood urea nitrogen and serum creatinine are the two important parameters that to some extent reflect the kidney function. The serum creatinine level more precisely reflects the degree of damage to the function of glomerular filtration, because BUN may be influenced by several other factors including gastrointestinal bleeding, dehydration and hypermetabolism (Zhu *et al.,* 2014). Previously it was indicated that Pb^{2+} and Cd^{2+} can induce nephrotoxicity by causing mitochondrial damage, which happens once they bind to the low molecular weight protein and passed freely through glomerular filtration (Sabath & Robles-Osorio, 2012). Alteration in the enzyme activities under stress is another mean to indicate pathological condition in aquatic organisms (Liju *et al.,* 2013; Saravanan *et al.,* 2011). Transaminase enzymes (AST & ALT) play key role in protein and carbohydrate metabolism and their level indicates organ dysfunction in aquatic organisms during stress. An elevation in the level of both AST and ALT represents damage to the liver tissue. The decline in ALP may be due to competitive behavior of Cd for Zn, because ALP has strong dependence on Zn for its activity. Yuan *et al.* (2014) found a similar picture in rates administered orally with a combination of low dose of Pb^{2+} and Cd^{2+}.

CONCLUSION

The results of the present study indicates a significant decline in the content of vitamins A and E in the liver and muscles of Crucian carp after prolonged exposure to environmentally relevant concentrations of Pb^{2+} and Cd^{2+}. Changes in the hematological and serum biochemical indices also reveal disturbed energy metabolism, anemia and impaired kidney and liver functions in the exposed fish. The deleterious effects were more pronounced in the chronic assay and due to co-exposure to Pb^{2+} and Cd^{2+} which had an additive effect. Alterations in the response of the studied parameters to chemical contaminants can be used as a tool to indicate environmental pollution. The findings of the present study also give an insight into the toxicological endpoint of the aquatic pollutants and guide us to ascertain the safe levels of Pb^{2+} and Cd^{2+} in the aquatic environment for protection of aquatic inhabitants.

Conflict of interest

The authors declare that there are no conflicts of interest.

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