

[Research]

Changes in biochemical and physiological responses of common carp, *Cyprinus carpio* L. after long-term exposure to Pb (II)

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ABSTRACT

In this study, the chronic toxic effects of Pb (II) on common carp, *Cyprinus carpio* were investigated using several biomarkers at different levels of biological functions for assessing changes in ecosystem quality. In a bioassay, common carp juveniles were individually exposed to different dose of heavy metal, Pb II (0, 0.45, 0.89, 1.34 mg.l⁻¹) for 28 days. Morphological indices such as hepatosomatic index, condition factor and lipid peroxidation (LPO) level and also the activities of superoxide dismutase (SOD) and catalase (CAT) were measured. These results indicated that there was significant lower condition factor in fish exposed to the highest concentration of Pb (p<0.05). The activity of hepatic antioxidant enzymes (SOD & CAT) and LPO level was significantly elevated after long-term exposure to higher concentration of Pb (p<0.05). Briefly, our findings suggested that common carp exposed to lead, develop oxidative stress, hence long-term exposure to higher concentrations of Pb could seriously affect the health status of fish.

Keywords: *Cyprinus carpio*, Pb, Lipid peroxidation, Oxidative stress, Morphological indices.

INTRODUCTION

The aquatic environment is continuously being contaminated with toxic chemical substances from industrial, agricultural and domestic activities. One of the major families of environmental contaminants is constituted by heavy metals, widely used by industrial and farming activities (Fent *et al.* 2006).

Discharge of heavy metals to aquatic ecosystems have serious effects on the ecological balance of the recipient environment (Canli *et al.* 1998). Among metals, Pb is of special concern since it is considerably toxic to aquatic animals at ecologically relevant concentrations (Zhang *et al.* 2008).

In aquatic animal species, fish are suitable bio-indicators since they are inhabitants of aquatic ecosystems and several species of them respond rapidly to abiotic changes including pollution. Environmental stressors can alter the physiological and biochemical parameters in fish that can be defined as biomarkers, including morphological indices, antioxidant responses and energy metabolic parameters which may be adaptive or may lead to toxicity (Pandey *et al.* 2003).

Biomarkers may be employed as early warning indicators, anticipating possible major disturbances at higher levels of organization.

Hence, they may be of great value in risk assessment and eco-toxicological studies.

They constitute early responses to chemical aggression and may indicate harmful effects. The morphological indices, especially condition factor (CF) and hepatosomatic index (HSI) have been proposed as an "exposure index" to environmental contaminants (Kopecka *et al.* 2008).

CF is an indicator of the overall fish condition, reflects fish shape and energy reserves, and has been used to evaluate fish stress (Lohner *et al.* 2001). Additionally, ratios of organ weight to body weight have been used in various stress-related studies. The most frequently used of these is an HSI.

The oxidative stress is established since the pro-oxidant forces overwhelm the antioxidant defenses.

For antioxidant mechanism, the cellular response includes the increased activity of lipid peroxidation (LPO) and two free radical scavenger enzymes, superoxide dismutase (SOD) (EC 1.15.1.1) and catalase (CAT) (EC 1.11.1.6) (Li *et al.* 2009).

Such antioxidant defense is found virtually in all tissues of vertebrates, but shows in general, high activity in the liver, a major organ for xenobiotic uptake and enzymatic transformation of reactive oxygen species (ROS) (Lemaire *et al.* 1994).

These antioxidant responses can constitute sensitive molecular bio-indicators for contaminant-mediated oxidative stress, and can also indicate the magnitude of response in populations chronically exposed to contaminants, such as metals and other xenobiotics (Woo *et al.* 2006; Firat *et al.* 2009; Li *et al.* 2010a).

Common carp (*Cyprinus carpio* L.) was selected for the present study, because it is a commercially - important fish species around the world. It has an adaptive response in a polluted aquatic environment.

It is well known that some metals can alter the activity of several enzymes by binding to their functional groups or by displacing the metal associated with the enzyme (Viarengo 1993).

Laboratory and field studies published in the last decades indicate that there are effects of some metals, such as lead, on several enzymatic biomarkers (van der Oost *et al.* 2003; Kopecka *et al.* 2008).

The ultimate goal of the present study was to monitor hepatic CAT and SOD enzymatic activities, LPO level and exposure index such as CF, HSI as potential biomarkers of fish exposure to the heavy metal such as Pb in freshwater ecosystems.

MATERIALS AND METHODS

Chemicals

Lead nitrate (II) (99% purity) (CAS no: 467790) was purchased from Sigma-Aldrich Corporation (USA).

All the other chemicals were obtained from Sigma-Aldrich and Merck (Germany).

Fish and acclimatization conditions

Common carp (n = 160), with mean (\pm SE) weight of 30.76 ± 1.05 g and total length of 13.46 ± 0.22 cm, were obtained from a local commercial aquatic breeder.

Fish were transported to the laboratory in aerated water and acclimated to laboratory conditions for 15 days in 500 L tanks filled with filtered and aerated water before being employed in the bioassay.

During this period, fish were fed daily with commercial food and water quality was regularly monitored and changed periodically. The fish were starved for 24 h prior to experimentation to avoid prandial effects during the assay.

Experimental design for toxicity test

Ten common carps were randomly distributed to each of twelve aquaria.

The concentrations of pb used were 0.45, 0.89 & 1.34 mg.l⁻¹, corresponding to the 5, 10, 15% of the 96-h LC₅₀ value.

Another group (n = 10) was used as control exposed to clean freshwater. Each experimental condition was triplicated.

The quality of water during the experiment was monitored at each 24 h by measuring water

dissolved oxygen, pH, total hardness and temperature.

The parameters were: dissolved oxygen: 7.5 ± 2 mg.l⁻¹, temperature: $20 \pm 1.0^\circ\text{C}$, pH 7.4 ± 0.2 and total hardness 241.01 ± 1.4 mg.l⁻¹ and also photoperiod was 12 : 12 h light-dark cycle.

The fish were fed daily with commercial fish pellets at 1% total body weight. 70% of the exposed solution was renewed each day to maintain the appropriate concentration of pb. Three randomly - selected fish from each treatment were sacrificed on days 3, 7, 14 & 28. Morphometric measurements (weight and total and fork length) were collected and liver tissues were quickly dissected, weighed, preserved as fragments in Bouin's fluid and stored at 4°C until required for histological analysis.

The remaining hepatic fragments was quickly removed and then immediately snap-frozen as sub-samples in liquid nitrogen and store at -80°C until biochemical and enzymatic analyses were performed.

Morphological indices

CF and HSI for each fish were calculated on days and 28 according to previous description (White *et al.* 1985):

$$\text{CF} = \text{body weight (g)} / \text{fork length}^3 \text{ (cm)} \times 100$$

$$\text{HSI} = \text{liver weight (g)} / \text{body weight (g)} \times 100$$

Biological material isolation

Frozen tissue samples were weighed (electronic balance, ANDGF-300, Japan, sensitive up to 0.001 g) and liver of each fish was homogenized (homogenizer, SR30 MTOFS, China) in 1:10 (w/v) of phosphate buffer (0.05M, pH 7.0, with 0.1% Triton X-100).

For LPO assay, an aliquot of liver homogenate 200µg.L⁻¹ were put in an eppendorf tube with 4 µg.L⁻¹ of butylated hydroxytoluene, for each sample, while the remaining liver homogenate was centrifuged for 15 min at 15000 g; the supernatant was collected and divided in aliquots for antioxidant enzymes (SOD & CAT) analyses and stored at -80 °C.

Biochemical and enzyme activity analyses

LPO in the fish liver was estimated from the production of MDA, which is one of the final products of lipid peroxidation.

The activity of MDA was measured by quantification of thiobarbituric acid reactive substances (TBARS) at 532 nm according to Ohkawa (1979) & Bird & Draper (1984).

SOD activity was determined in hepatic supernatant according to the method of Flohe & Otting (1984) at 560 nm and also CAT activity was measured according to the method of Aebi (1984) by monitoring residual H₂O₂ absorbance at 240 nm.

All tests were performed in a JENWAY, model 6405 UV/VIS, spectrophotometer.

Enzymatic activities were determined at 25 °C and expressed as activity per mg of protein (second protein determination performed after the enzymatic analysis, as indicated below). LPO was expressed as MDA concentration in nmol of mg protein.

One unit (U) of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of Nitro blue tetrazolium (NBT) by 50%. For CAT, 1 unit was defined as 1 µmol H₂O₂.min⁻¹.

All the protein determinations were done by the Bradford method (Bradford, 1976) with Coomassie brilliant blue, where bovine serum albumin served as a protein standard.

Protein concentration measurements were assayed at 595 nm.

All tests were performed in triplicate.

Statistical analyses

Data were tested for normality by Kolmogorov-Smirnov test and homogeneity of variance was checked using Levene's test.

A Two-way ANOVA was used to identify differences (p<0.05) among the response means in different treatments and among different sampling days.

A Tukey post-hoc comparison was applied to discriminate between the interaction of the mean of treatment groups and time.

Statistical analysis was conducted with Excel version 13.0 and SPSS (New York, USA) version 19.

RESULTS

Antioxidant responses

Hepatic CAT activities of control fish ranged between 51.99 ± 3.34 & 62.5 ± 5.7 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein⁻¹.

Fish exposed to the lowest dose of lead (0.45 mg.L⁻¹) showed CAT values between 55.16 ± 3.46 & 138.18 ± 5.7 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein⁻¹, while for fish exposed to the highest dose (1.34 mg.l⁻¹) of those activities ranged between 73.32 ± 2.27 & 311 ± 24.62 .

For SOD activity, these values varied between 13.97 ± 1.46 U & 15.56 ± 0.19 U.mg protein⁻¹ at control group and also between 15.58 ± 1.7 & 27.24 ± 2.27 at low-dose while for fish exposed to the highest dose, these activities ranged between 16.45 ± 1.27 & 40.12 ± 2.29 .

The level of LPO values in control fish ranged between 5.98 ± 0.03 and 6.28 ± 0.84 nmol.mg protein⁻¹.

Fish exposed to the lowest dose of lead showed LPO values between 6.3 ± 0.75 & 29.91 ± 0.62 nmol.mg protein⁻¹ while for fish exposed to the

highest dose of those activities ranged between 7.07 ± 0.38 & 38.5 ± 1.77 .

As a result, a significant changes in antioxidant enzymes activities (SOD & CAT) and LPO level was found among different treatments ($p < 0.05$) (Fig. 1).

CAT activity was higher in the low, mid and high-dose groups ($58\% \pm 0.4$, $126\% \pm 0.5$ & $259\% \pm 1.7$ respectively) than those in the control (Fig.1B).

The mean SOD activity was approximately $40 \pm 1.02\%$, $82.9 \pm 1.71\%$ & $94.4 \pm 1.83\%$ higher in low, mid and high-dose groups, respectively, than those in control (Fig.1A). The LPO activity was $201 \pm 0.6\%$, $208 \pm 0.5\%$ & $280 \pm 0.71\%$ in low, mid and high-dose groups, respectively, which was higher than in control (Fig.1C).

There was time effect for antioxidant enzymes with increases and decreases over time in lipid peroxide among different treatments (Fig. 1).

Morphological indices

There was a significant lower CF in high-dose group in comparison with control ($p < 0.05$) while there were no values or time differences in HSI between the treatments (Table 1).

Table1. Effect of exposure to Lead on HSI and CF of common carp.

Indices	Exposure day	C (mean \pm SE)	T ₁ (mean \pm SE)	T ₂ (mean \pm SE)	T ₃ (mean \pm SE)
CF	3 day	$1.94 \pm 0.03^*$	1.91 ± 0.1	1.89 ± 0.01	$1.8 \pm 0.1^*$
	28 day	$1.95 \pm 0.03^*$	1.76 ± 0.1	1.72 ± 0.01	$1.63 \pm 0.03^*$
HSI	3 day	2.13 ± 0.14	2.06 ± 0.14	1.86 ± 0.2	1.75 ± 0.12
	28 day	2.1 ± 0.44	1.96 ± 0.2	1.65 ± 0.17	1.38 ± 0.2

The values of control group and experimental concentrations of 0.45, 0.89, 1.34 are shown as C, T₁, T₂ and T₃. * = Significant difference.

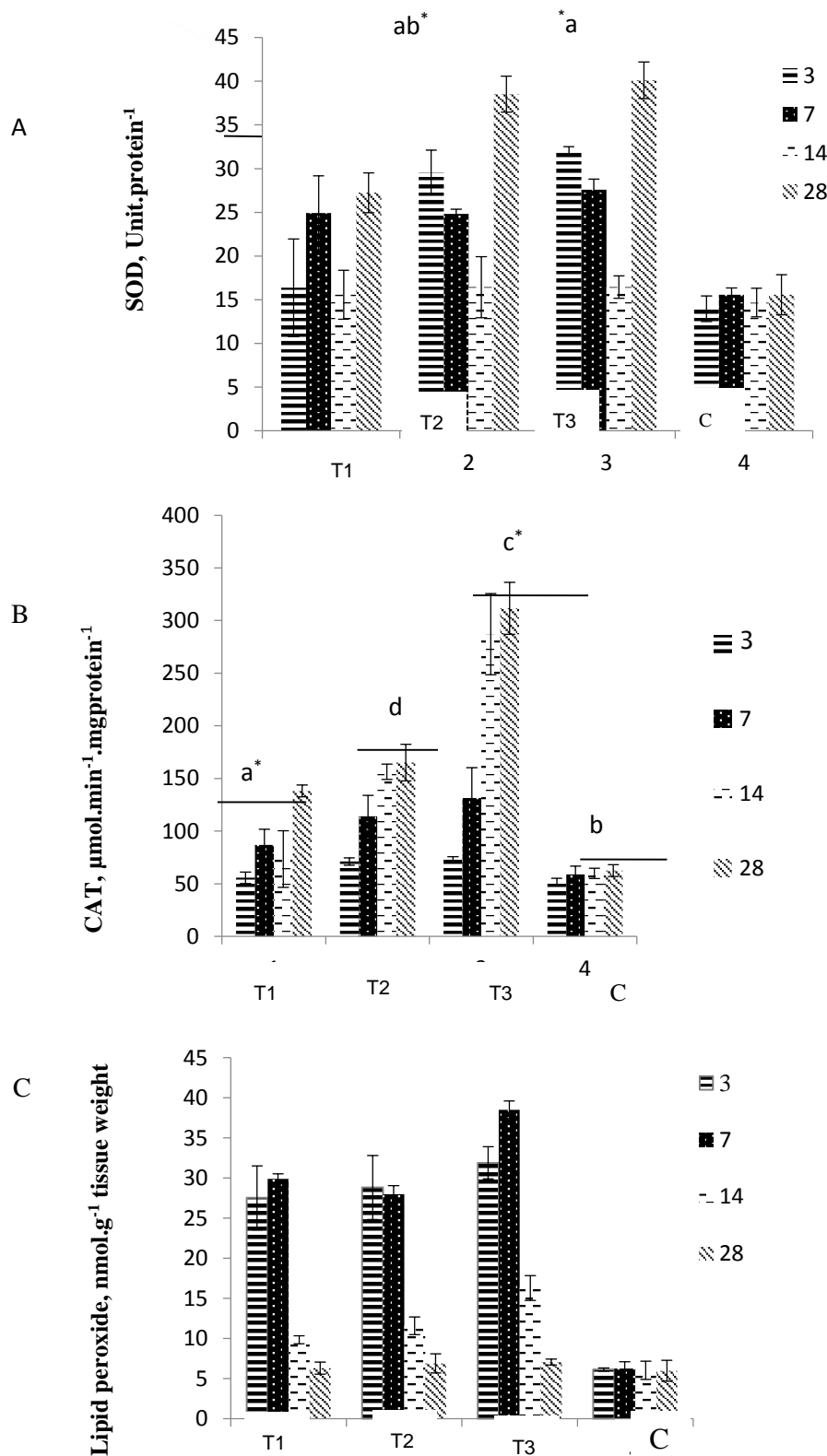


Fig 1. Changes in the mean (A) activities of superoxide dismutase (SOD), (B) activities catalase (CAT) and (C) lipid peroxide concentrations in liver of common carp (*cyprius carpio*) treated with Lead nitrate that did not (control=4) or that did contain Lead nitrate II at 0.45 mg.l⁻¹ (T1) , 0.89 mg.l⁻¹ (T2) and 1.34 mg.l⁻¹ (T3). Control(C) and Lead-treated fish (n = 12) were sampled after 3,7,14 and 28 days of exposure. Error bars indicate 95% confidence intervals. Dissimilar notations indicate a treatment effect (p< 0.05) and the * indicates a time effect (p < 0.05).s compared with control value, * p<0.05.

DISCUSSION

Our present findings demonstrate toxicity-induced alterations in antioxidant response and also changes in liver texture of fish conferring a higher tolerance for oxidative stress (Lemaire *et al.* 1994). Thus, the liver can be considered as target organ for studying effects of heavy metal toxicity on fish. Antioxidant responses in fish livers induced by pollutants exhibit three modes: induction, inhibition and integrated process. The heavy metal toxicity stimulates the oxidative stress and the antioxidant enzymes are induced by binding to their functional groups or by displacing the metal associated with the enzyme (Viarengo 1993). In the present study, antioxidant defenses are typically developed preferentially in liver as a result of the central role of this organ in detoxifying xenobiotics and processing metabolic products for degradation (Li *et al.* 2010b). Our results are in agreement with the previous reports by Yi *et al.* (2007), Viera *et al.* (2008), Ghosh *et al.* (2013) & Grant *et al.* (2011) who reported that hepatic SOD and CAT activities could be elevated in *Carassius auratus*, *Trematomus bernacchii*, *Pomatoschistus microps* respectively after heavy metal exposure such as Pb, Cd, Hg & Cu. One explanation is that CAT activity is more sensitive to these compounds than SOD and the high variability of the SOD data may also inherently limit the significance of changes observed. Oxidative substances in cells may cause an elevation of antioxidant enzymes as a defense mechanism. SOD and CAT enzymes have related functions (Pandey *et al.* 2003). SOD catalyzes the dismutation of the superoxide anion radical to O₂ & H₂O₂ (Li *et al.* 2009). CAT acts as scavengers of H₂O₂. The peroxy radical H₂O₂ was trapped by catalase that primarily occurs in peroxisomes. The target function of catalase is to protect the cells from the accumulation of H₂O₂ by dismuting it to form H₂O & O₂ (Gate *et al.* 1999). SOD and CAT have related functions, and are always considered as the first line of defense against to oxygen toxicity, due to the inhibitory effects on oxyradical formation (van der Oost *et al.* 2003). There were biochemical changes, with effects

on lipid peroxide formation (overall increase), in the low- and high-dose groups.

Hepatic lipid peroxide formation activities of treatment groups showed a decreasing trend after 7 days exposure and continued until 28 days. However, they were still higher than control. This result is in line with those findings reported by Vieira *et al.* (2009), Moreno *et al.* (2011), & Ghosh *et al.* (2013). Lack of increase in MDA content may indicate the accumulation of oxidative radicals, but not enough, to make antioxidant system poisoned (Nogueira *et al.* 2011). Heavy metals especially Pb-binding to the some receptors in liver leading to regulated transcription factors and increased activity in the hepatocyte. There is evidence that increased ROS can lead to increased transcription factors in the liver (Sakurai *et al.* 2008). In the present study, significant lower CF and HSI were observed in the Pb-treated group with the highest concentration (high-dose group), which indicates a decrease in fish growth and overall condition caused by direct metabolic effect of Pb on fish (Li *et al.* 2010c). Some reports have demonstrated that CF and HSI declined in fish exposed to environmental pollutants (Khan 2003; Kopecka *et al.* 2008; Moreno *et al.* 2011). Condition factor, which assumes that heavier fish of a given length is in better condition, is able to indicate fish fitness under stress of pollution as metabolic trade-off is required to deal with detoxification and the energy available for growth may thus be reduced (Fang *et al.* 2009). HSI reflects the relative liver size and is linked to the hepatic enzyme activity for detoxification of compounds which is indicative of exposure to pollutants (Yeom *et al.* 2007).

CONCLUSION

Based on the obtained data, the common carp has enough tolerances to (Pb II) induced changes in surrounding condition such as CF, HSI. With increasing Pb concentration and prolonging the exposure period, the health status of fish was affected seriously. According to results of the present study, this biomarkers such as changes in LPO, SOD, CAT and also CF

and HSI could provide useful information for evaluating the physiological effects on common carp, but the application of these findings will need more detailed laboratory study before they can be established as special indicators for monitoring aquatic environment.

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تغییرات فیزیولوژیک و بیوشیمیایی در ماهی کپور معمولی پس از مواجهه طولانی مدت با سرب

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چکیده

در این مطالعه، اثرات تحت کشندگی فلز سنگین سرب با استفاده از نشانگرهای زیستی در ماهی کپور معمولی برای ارزیابی تغییرات در کیفیت اکوسیستم بررسی قرار گرفت. در این بررسی، ماهیان در غلظت‌های مختلف فلز سرب (۰، ۰/۴۵، ۰/۸۹، ۱/۳۴ میلی گرم بر لیتر) در یک دوره ۲۸ روزه قرار گرفتند. نشانگرهای زیستی مختلفی از جمله شاخص‌های مورفولوژیک (شاخص وزن کبدی، ضریب چاقی)، پراکسیداسیون لیپیدی، فعالیت‌های آنزیم سوپر اکسید دیسموتاز، کاتالاز، و تغییر در ترکیب کبد اندازه‌گیری شد. نتایج نشان داد که میزان ضریب چاقی به طور معنی‌داری در ماهیان با بالاترین غلظت فلز سرب کاهش یافت ($p < 0.05$). فعالیت آنزیم‌های آنتی اکسیدان کبدی و سطح پراکسیداسیون لیپیدی به طور قابل توجهی پس از مواجهه با غلظت‌های بالاتر از سرب تغییر کرد ($p < 0.05$). در مجموع، با توجه به یافته‌های تحقیق حاضر، قرار گرفتن در معرض طولانی مدت غلظت‌های بالای سرب، به طور جدی می‌تواند سلامت ماهی را تحت تاثیر قرار دهد.

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